**Bile Acid Deconjugations, Transformations, and (Re)conjugations
by Diverse Human Gut Bacteria**

By

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Dedication

I dedicate this body of work to my parents, Carol and Mike Lucas.

Abstract

Bile acids (BAs) play a crucial role in human physiology by facilitating the absorption of lipophilic nutrients, interacting with hormone receptors throughout the body, and modulating gut microbiota through their antimicrobial activity. Our current knowledge regarding microbial BA transformations comes primarily from biochemical studies on a relatively small number of species or from bioinformatic predictions that rely on homology to known BA-transforming enzyme sequences. Therefore, much remains to be learned regarding the variety of BA transformations and their representation across gut microbial species.

In chapter 2, we systematically assessed bacterial BA transformations of the unconjugated human BAs: cholic acid (CA), chenodeoxycholic acid (CDCA), and deoxycholic acid (DCA). In chapter 3, we assess bacterial deconjugation of the five most prevalent conjugated BAs in humans: glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), and taurodeoxycholic acid (TDCA). All gut bacteria were grown in pure culture and endpoint sampled, providing a snapshot of BA deconjugation and transformation ability. Selected strains were further analyzed in time-course coculture experiments to reveal BA transformation dynamics, and sequential transformations throughout growth. In addition, we performed a bioinformatic analysis to investigate hydroxysteroid dehydrogenase (HSDs), the *bai* operon, and amino acid motifs associated with BSH specificity.

Our systematic analyses revealed the widespread ability of bacteria to deconjugate and/or transform BAs. We identified bacterial production of 7-oxoBAs by hydroxysteroid dehydrogenases (HSDs) to be the most prevalent transformation, we expanded upon known genera to possess 7α-dehydroxylation activity, observed a human gut bacterium produce a mouse primary BA, and identified 44 novel microbially conjugated BAs (MCBAs). In addition, we provide support that BSH is a rate-limiting step for diversification of the BA pool, and we confirm

that gut bacteria possessing unique enzymes can perform sequential transformations on BAs. Finally, we provide further evidence for BSH amino acid motifs associated with taurine or glycine specificity. Altogether, these data provide a foundational understanding of gut bacterial BA deconjugation and transformation, which will facilitate the engineering of synthetic microbial communities with predictable effects on BA composition in *in vivo* systems. A new suite of questions can be asked about how bacteria act by themselves or in concert with others to diversify the BA pool.

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Chapter 1: Background and rationale

1.1 Human gut microbiome

The human gut microbiota and its collective genomes, also referred to as the 'microbiome', has gained scientific interest in the last few decades for its essentiality in maintaining human health. The gut microbiome plays an important role in host health and disease through nutrient metabolism, drug metabolism, intestinal health, immune response, mental health, and protection against pathogens (1-3). Gut microbes enable humans to extract otherwise inaccessible nutrients from food and they also train our immune systems to recognize harmful invaders and stimulate our bodies to produce compounds that fight disease-causing microbes. On the other hand, we also know that changes to the composition of an individual's gut microbiome are associated with disorders such as allergies, asthma, anxiety and depression, cardiovascular disease, inflammatory bowel disease, irritable bowel syndrome (IBS), coeliac disease, type 2 diabetes, and more (4,5). With so much variability between hosts, associating specific bacteria and their metabolism with specific health outcomes has proved to be challenging.

1.2 Bile acids impact host physiology

BAs have emerged as a new target for understanding the connection between diet, the gut microbiome, and host health. BAs are amphipathic molecules synthesized from cholesterol in the human liver. At high concentrations in the gut, BAs form micelles that facilitate the absorption of lipophilic nutrients from the host diet. BAs found at lower concentrations, however, are still biologically relevant, due to their ability to act as agonists or antagonists for membrane and nuclear hormone receptors found throughout the body. Through interaction with these

receptors, BAs can modulate their own synthesis, glucose and lipid metabolism, and even

impact the host immune system and nervous system (Fig 1.1) (6). Finally, BAs have varying

antimicrobial effects, depending on the BA and bacteria in question (7).
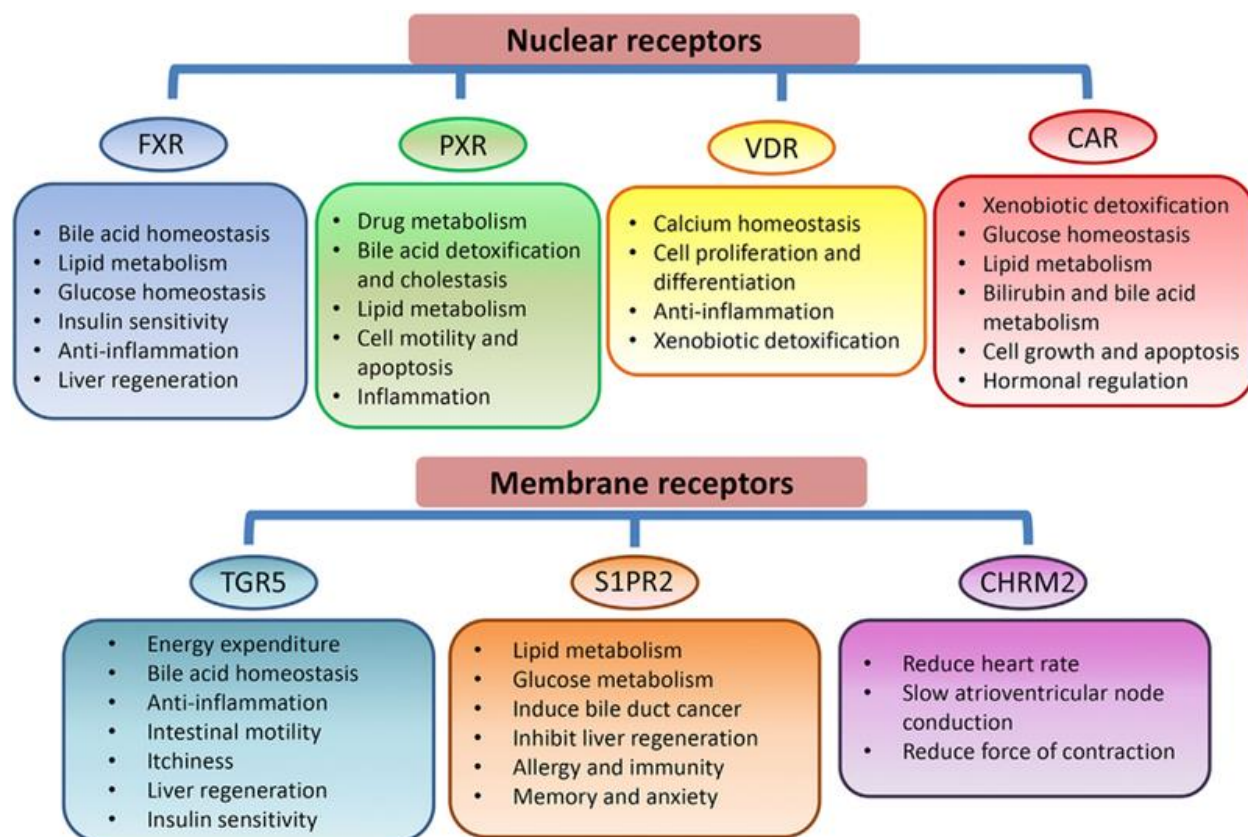
Wan, Y. Y., & Sheng, L. (2018). Regulation of BA receptor activity. *Liver research*, *2*(4), 180–185. https://doi.org/10.1016/j.livres.2018.09.008

Fig 1.1 Schematic overview of the functions of bile acid receptors. The key functions of BA receptors are summarized in the figure. Abbreviations: FXR, farnesoid X receptor; PXR, pregnane X receptor; VDR, vitamin D receptor; CAR, constitutive androstane receptor; TGR5, Takeda G protein receptor 5; S1PR2, sphingosine-1-phosphate receptor 2; CHRM2, cholinergic receptor muscarinic 2 (32).

Humans *de novo* synthesize two 'primary' BAs named cholic acid (CA) and

chenodeoxycholic acid (CDCA). These two BAs are conjugated to either glycine or taurine by a

host enzyme, BA-CoA:amino acid N-acyltransferase (BAAT), and stored along with

phospholipids, cholesterol, bilirubin, electrolytes, and water as bile in the gallbladder (Fig 1.2). In

humans, conjugated BAs (CBAs) are found in a ratio of 3:1, glycine- to taurine-conjugated BAs, respectively, with higher levels of glycine-conjugated BAs in vegetarians (8). After ingesting a meal, bile is secreted from the gallbladder through the common bile duct and into the duodenum. BA concentrations in bile are highest upon initial release and diminish by orders of magnitude by the time they reach the large intestine (9). CBAs travel through the acidic small intestine where some BA transformations occur, but most are reabsorbed and returned to the liver to circulate in a process known as enterohepatic circulation (Fig 1.2) (15). The portion of CBAs that reach the large intestine are subject to deconjugation and subsequent transformation (8,10-12), or the recently discovered microbial bile acid conjugation, by gut bacteria (13,14).
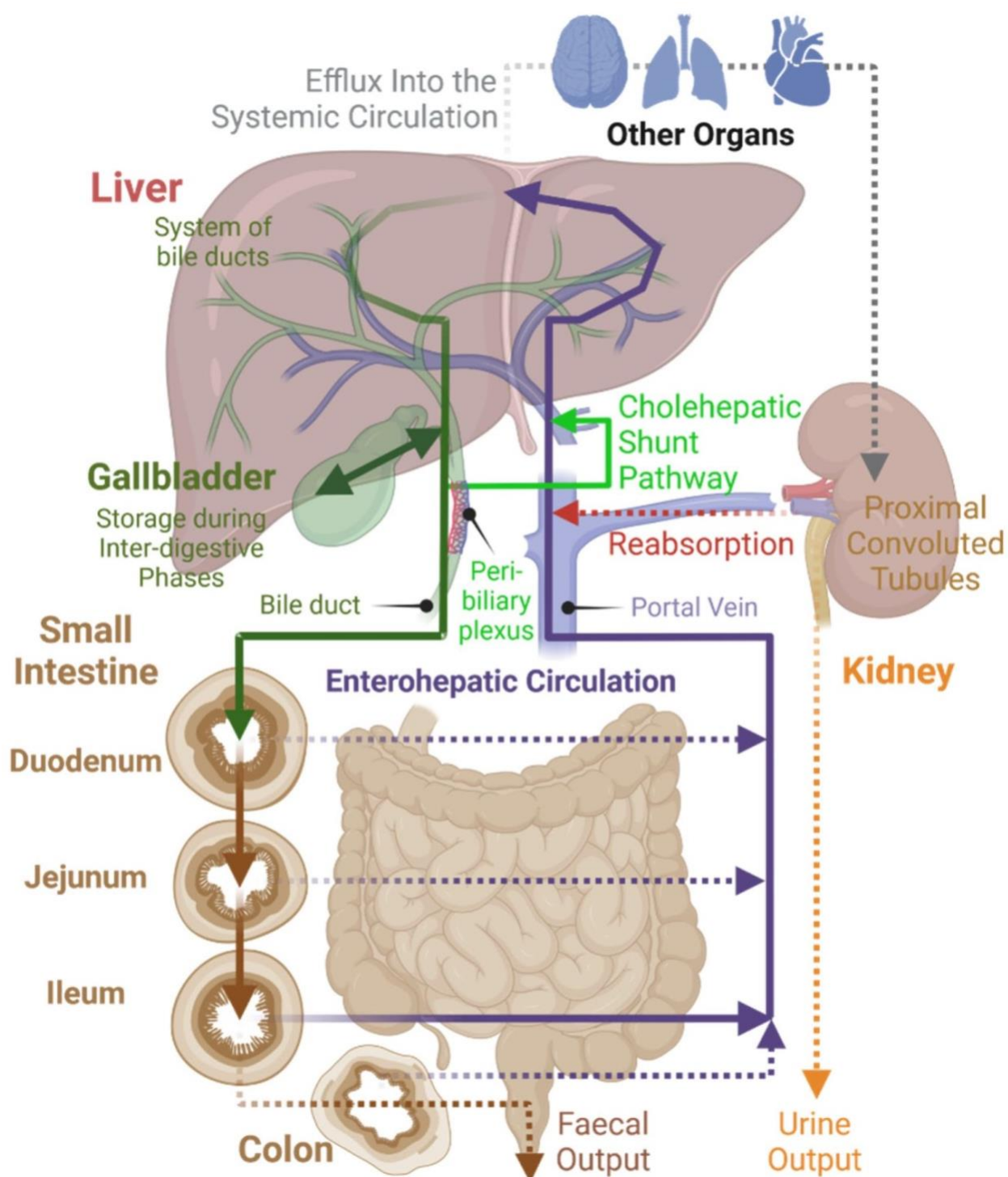
Figure 1.2 Schematic overview of enterohepatic circulation (15).

The four CBAs are deconjugated by the gut microbiota to release the two primary BAs, CA and CDCA. These two primary BAs can be subsequently transformed through dehydrogenation, 7α-dehydroxylation, epimerization, sulfation, or microbial BA conjugation to produce a potential pool containing hundreds of chemically unique BAs (16). BAs that are reabsorbed through active transport and passive diffusion are circulated throughout the body, reaching tissues, organelles, and cells that are connected to the vascular system. Receptors for BA steroid hormones can be found throughout the body in tissues such as the brain, adipose tissue, gallbladder, liver, and intestines (Fig 1.1 and 1.2) (17).

Through interaction with receptors, BAs mediate crosstalk between the host and the gut microbiota, and directly impact host physiology. For these reasons, many BA-gut-axes have been proposed (18-22). Therefore, any intervention of the gut microbiota should consider the potential role of BAs in that system. Continued research to discover all BAs and their modifications found in humans is needed. More studies should compare patients in diseased and healthy states to continue to uncover associations between BAs, the gut microbiota, and health status. Understanding contributions by individual gut microbes to positive and negative health outcomes will launch the scientific community into a new phase of medical treatments.

1.3 Bile acid structure influences its effect

BA structure impacts its rate of secretion and absorption by the liver and the gut, the agonistic or antagonistic potential for BA hormone receptors, and the level of toxicity it has to other microorganisms living in the gut (23). The role that BA structure plays in uptake informs our understanding of BA homeostasis and enterohepatic circulation. Knowledge on hormone receptor affinity of conjugated and unconjugated primary and secondary BAs will allow for associations between BA structure and host physiology to be made. Finally, the discernment of

BA toxicity on diverse microbes will inform our ability to predict microbiome composition as a function of the BA pool.

BAs interact with many different transporters as they circulate throughout the body. These interactions play a critical role in regulating bile flow and adapting to disease states, and impaired receptor activity can result in liver or intestinal disease (24-26). BAs are taken up from portal blood into the liver via the sodium taurocholate co-transporting polypeptide (NTCP) and the human organic anion-transporter (OATP) and secreted across the canalicular membrane into bile by the bile salt export pump (BSEP) (24-27). NTCP and OATP mediate uptake of both conjugated and unconjugated BAs but have the highest affinity for conjugated di- and trihydroxy BAs, especially taurocholic acid (TCA) (27, 28). BSEP has poor affinity for unconjugated BAs, but high affinity for conjugated BAs, favoring TCDCA > TCA >TDCA > GCA (29). The apical sodium bile salt transporter (ASBT) mediates absorption of BAs from the intestinal lumen into epithelial cells, while the organic solute transporter α/β (OSTα/β) heterodimer mediates BAs transport across the basolateral membrane into the portal blood via facilitated diffusion (24). Human ASBT prefers conjugated BAs to unconjugated and has an inconsistent higher affinity for dihydroxy over trihydroxy BAs (30). The facilitated diffusion mechanism of OSTα/β depends on substrate concentration and electrochemical gradient. Understanding the relationship between BA structure and uptake will allow researchers to influence BA circulation and homeostasis mediated by bacterial BA transformation.

In addition to transporters, BAs interact with nuclear and membrane hormone receptors to promote physiological change on the host. BA structure dictates their agonistic or antagonistic receptor potential. Unconjugated primary and secondary BAs (CDCA > DCA > LCA > CA) are known to interact with farnesoid x receptor (FXR), which upon activation leads to the regulation of genes whose function is to decrease the concentrations of BAs (31,32). FXR activation reduces BA synthesis and increases BSEP activity, stimulating the excretion of BAs

from canalicular transporters (33). G protein-coupled receptor TGR5 is a membrane receptor expressed in the gut, liver, gallbladder, adipocytes, endocrine glands, muscles, and immune organs. TGR5, which regulates metabolism and inflammation, as well as cell proliferation, muscle relaxation, and itchiness, has the endogenous BA ligand TLCA, as well as LCA > DCA > CDCA > CA > UDCA (32). CBAs were found to interact with the G protein-coupled receptor sphingosine-1-phosphate (S1P), which regulates proliferation, immunity, cell trafficking, and inflammation (34). PXR, which acts as a sensor for BA dysregulation, has affinity for LCA > DCA > CA (31). Finally, secondary oxo-BAs are known to interact with vitamin D receptor (VDR), which plays a role in insulin secretion and secondary BA detoxification (31). Regulation of host physiology by BAs is heavily influenced by their structure and conjugation status. Through interactions with BA receptors, BAs can exert their effects at low concentrations and in distant organs, providing yet another layer of dynamic crosstalk between the gut microbiota and the host.

Due to the amphipathic nature of BAs, much research on BAs as antimicrobials has focused on their use as an antimicrobial delivery system to disrupt bacterial membranes (7). However, BAs themselves also possess antimicrobial activity, which is especially relevant in today's world with the rise of antibiotic resistance. CBAs, due to their hydrophilic nature, require transporters to pass through bacterial membranes, and are known to be less toxic than their unconjugated BA counterparts (23). In addition, taurine-conjugated BAs are consistently less toxic to bacteria than glycine-conjugated BAs (12, 35). Arguably, BSH activity should produce the more toxic free BAs, but while some strains exhibited decreased growth and colonization with mutated *bsh*, others have increased growth and colonization when *bsh* is knocked out (36). In general, BAs with fewer hydroxyl groups are more hydrophobic and therefore more toxic, implicating 7α-dehydroxylating bacteria in increasing BA toxicity. Interestingly, some BA toxicity appears to be species specific. For example, CA has a minimum inhibitory concentration of 20

mM against *Staphylococcus aureus* (7) and DCA and CDCA reduced viability of *Neisseria gonorrhoeae* more than 100,000-fold after 1 min (37). Further research is needed to expand our current knowledge on BA affinity for host transporters and receptors and BA toxicity to host cells and the gut microbiota. A comprehensive understanding of BA diversity, toxicity, and transport will improve our ability to promote physiological change mediated by gut microbial BA metabolism.

1.4 Role of BAs in human health and disease

Recent BA research reinforces the idea that BA metabolism can be leveraged to develop gut related interventions to treat a variety of diseases. BAs play a critical role in the onset of liver diseases, atherosclerosis, Alzheimer's, inflammatory bowel disease, depression, diabetes, and obesity to name a few (38-42). Many probiotics geared towards augmenting BSH activity have been proposed (36, 43, 44). Fecal microbiota transplants (FMT), which have been used to treat *Clostridium difficile* infection, have been demonstrated as potential treatments for metabolic disorders as well through their effect on BA metabolism (45). It has also been proposed that BAs and their derivatives can be used to design prodrugs capable of exploiting the BA transport system (46).

Probiotic BA bacteria are proposed to modulate host physiology through BSH activity and its relationship with cholesterol and lipid metabolism. Several studies have investigated the potential for BSH-active probiotic bacteria that can lower cholesterol levels in users (36, 44, 47-49). Increased BA deconjugation decreases cholesterol absorption by enterocytes and enhances its excretion in feces (50). In addition, CBAs activate FXR, which increases metabolism and insulin sensitivity, which impact weight gain (32, 50, 51). Finally, free BA production as a result of high BSH activity has been suggested to facilitate intestinal motility and metabolism in pregnant women to improve quality of life (52). Valid concerns about secondary

BA production, and their association with gallstone formation, should be considered when identifying potential BA probiotic strains (36). Accumulation of hydrophobic BAs has been shown to promote inflammation, injury, cirrhosis, and the development of cancer (32).

In addition to effects on lipid and cholesterol metabolism, BA activity by gut bacteria appears to confer resistance against pathogens in some cases. It is known that taurine-conjugated BAs, such as TCDCA, are less toxic to *Clostridium difficile* than their unconjugated and glycine-conjugated counterparts (53, 54). Therefore, it has been suggested that a probiotic organism with taurine specific *bsh* could be taken orally to combat *C. difficile* infection. Additionally, through disruption of the membrane and proton motive force, both unconjugated and conjugated BAs inhibited the growth of *Staphylococcus aureus* (55). BAs have also been identified to play a role in excystation upon parasitic infection by *Cryptosporidium* species (56), *Eimeria* species (57), *Clonorchis sinensis* (58), and *Entamoeba invadens* (59). Administration of deoxycholic acid (DCA) and CDCA reduced the cystic pool of *Giardia duodenalis*, while their conjugated counterparts were found to be inert (50). Research on bacterial and parasitic infections should continue to survey BA associations to determine any causative mechanisms or preventative treatment strategies.

Through interaction with host receptors, BAs have also been implicated in immune responses, both innate and adaptive (55, 60, 61). Through interaction with TGR5 and FXR on macrophages, BAs induce an upregulation of IL-10 and a downregulation of the pro-inflammatory cytokines IL-6 and INF-γ (55). In dendritic cells, BAs down-regulate the production of TNF-α and IL-12, resulting in a reduced inflammatory response (55). A dysregulated BA metabolism has been associated with inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, both of which are immune regulated (21, 50). In mice, it was found that administration of 3-oxoLCA and isoalloLCA reduced TH17 cell differentiation and increased Treg cell differentiation in the intestinal lamina propria (60). These examples demonstrate an

anti-inflammatory and immunomodulatory role of BAs in health and disease conditions.
Continued understanding of BA diversity and BA metabolizing species will be critical for
improving our control of the influence of gut microbiota mediated strategies for treating human
health and disease.

1.5 Objectives of this work

To provide a foundational understanding of BA activity by human gut bacteria, we
systematically investigated diverse gut bacteria for their ability to deconjugate and subsequently
transform BA *in vitro*. We employed HPLC-MS/MS to quantify known BAs and identify novel
BAs. Our goal was to expand current knowledge on which gut bacterial species can carry out
which BA transformations. We investigated Bsh as a rate-limiting enzyme and examined its
specificity across phyla. Finally, we grew bacteria in monoculture and coculture to understand
the role of BA deconjugation and transformation dynamics over time. Altogether, we contribute
to the scientific understanding of BA metabolism by gut bacteria and our work provides a basis
for hypothesis testing to tease apart the functional roles of the ever-expanding global BA pool
and the bacteria that produce them.

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Chapter 2: Dominant Bacterial Phyla from the Human Gut Show Widespread Ability to Transform and Conjugate Bile Acids

Authors' contributions: LNL wrote the manuscript. All authors edited the manuscript and provided feedback. FER, DAN, and KB designed experiments. KB, RLK, LEC, and LNL performed systematic analysis of gut bacteria. LNL and DMS performed the LC-MS measurements and data analysis. QZ performed bioinformatic analyses.

2.1 Abstract

Gut bacteria influence human physiology by chemically modifying host-synthesized primary bile acids (BAs). These modified BAs, known as secondary BAs, can act as signaling molecules that modulate host lipid, glucose, and energy metabolism and affect gut microbiota composition via selective antimicrobial properties. However, knowledge regarding the BA-transforming capabilities of individual gut microbes remains limited. To help address this knowledge gap, we screened 72 bacterial isolates, spanning seven major phyla commonly found in the human gut, for their ability to chemically modify unconjugated BAs. We found that 43 isolates, representing 41 species, were capable of *in vitro* modification of one or more of the three most abundant unconjugated BAs in humans: cholic acid, chenodeoxycholic acid, and deoxycholic acid. Of these, 32 species have not been previously described as BA transformers. The most prevalent BA transformations detected were oxidation of 3α-, 7α-, or 12α-hydroxyl groups on the steroid core, a reaction catalyzed by hydroxysteroid dehydrogenases. In addition, we found 7α-dehydroxylation activity to be distributed across various bacterial genera, and we observed several other

complex BA transformations. Finally, our screen revealed widespread bacterial conjugation of primary and secondary BAs to glycine, a process that was thought to only occur in the liver, and to 15 other amino acids, resulting in the discovery of 44 novel microbially conjugated bile acids (MCBAs).

2.1.1 Importance

Our current knowledge regarding microbial BA transformations comes primarily from biochemical studies on a relatively small number of species or from bioinformatic predictions that rely on homology to known BA-transforming enzyme sequences. Therefore, much remains to be learned regarding the variety of BA transformations and their representation across gut microbial species. By carrying out a systematic investigation of bacterial species commonly found in the human intestinal tract, this study helps better define the gut bacteria that impact composition of the BA pool, which has implications in the context of metabolic disorders and cancers of the digestive tract. Our results greatly expand upon the list of bacterial species known to perform different types of BA transformations. This knowledge will be vital for assessing the causal connections between the microbiome, BA pool composition, and human health.

2.2 Introduction

Within the last decade, the central role that the gut microbiota plays in human health has become widely recognized. A fundamental way by which the gut microbiota affects human physiology is via a wide variety of microbially encoded biochemical activities such as vitamin synthesis, production of small organic acids, and modification of BAs (1). While each of these has a significant impact on human health, chemical modification of BAs by gut microbes is of

particular interest due to the fact that bacterially modified BAs can act as signaling molecules (i.e., hormones) within the host (2–7) and can also directly modulate gut microbiota composition (4, 5, 8).

The human liver produces two BAs, termed primary BAs, from cholesterol: cholic acid (CA) and chenodeoxycholic acid (CDCA) (Fig. 2.1) (9). After synthesis, primary BAs in the liver are conjugated to taurine or glycine, which enhances their solubility and facilitates their circulation within the body (10). Conjugated BAs are then shuttled through the gallbladder into the small intestine, where they act as detergents to help emulsify and solubilize fats and facilitate the transport of lipids and lipid soluble vitamins (Fig. 2.1). As BAs circulate, the majority (~95%) are actively absorbed across the distal small intestine wall (Fig. 2.1) (8). The reabsorbed BAs are returned to the liver and gallbladder and continue through this cycle, called enterohepatic circulation. However, a small amount (~5%) escape ileal bile salt transport and enter the large intestine. In the large intestine primarily, but also in the small intestine, BAs are subjected to chemical modification by bacteria (Fig. 2.1) (11). Primary BAs that have been modified by bacteria are termed secondary BAs; more than 50 secondary BAs have so far been documented in human feces (12–15). Secondary BAs are passively reabsorbed through the large intestine wall and into the bloodstream, where they join other BAs undergoing enterohepatic circulation (11–14).
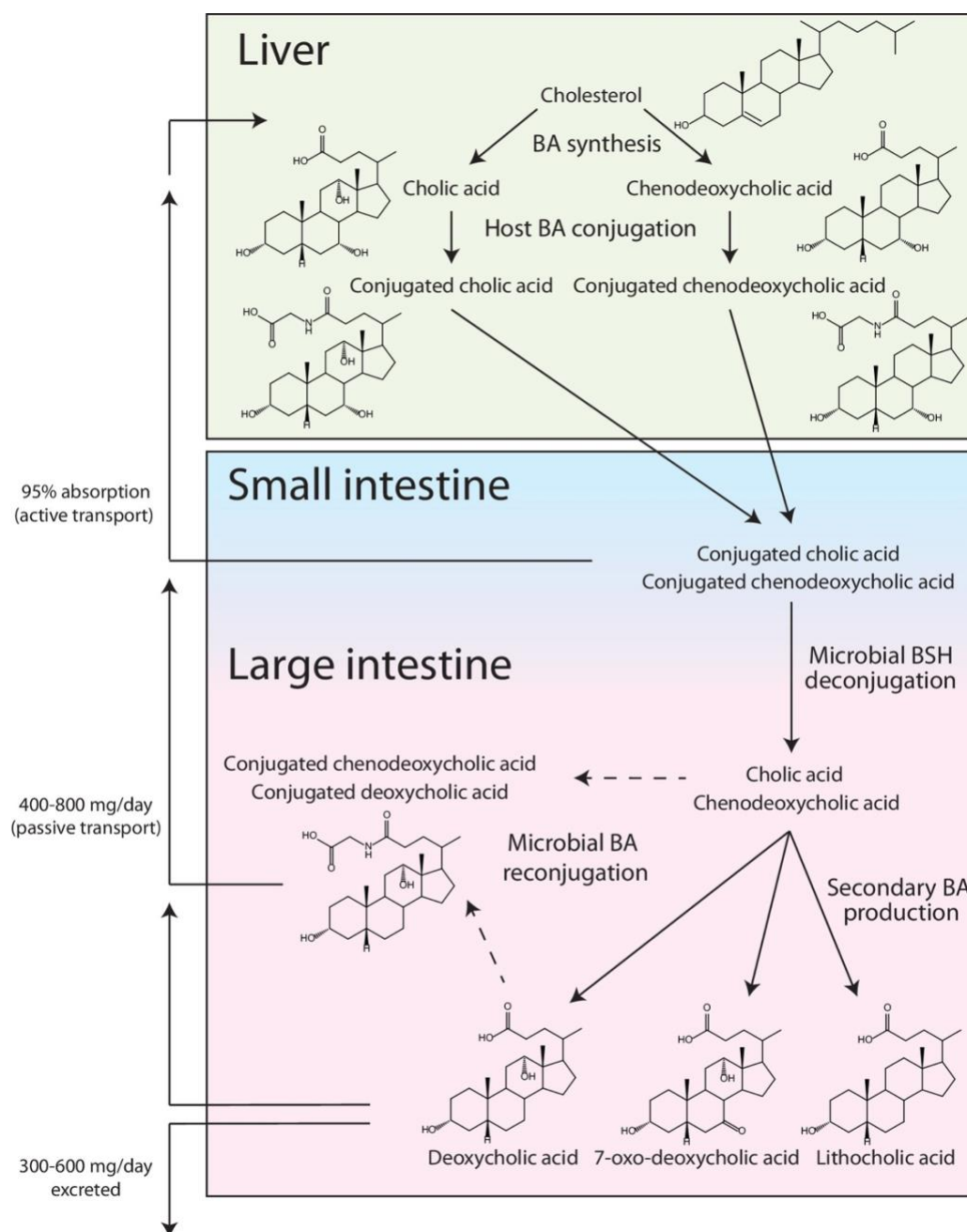
FIG 2.1 Schematic of bile acid production and enterohepatic circulation. Conjugated BAs are synthesized from cholesterol in the liver and stored in bile in the gallbladder. After being released from the gallbladder into the duodenum, BAs travel through the jejunum and into the ileum. Ninety-five percent of BAs are actively absorbed from the small intestine and returned to the liver via enterohepatic circulation. The remaining 5% that reach the large intestine can be passively reabsorbed or deconjugated and transformed into secondary BAs by the gut microbiota. Bacterial transformation of BAs can also occur in the small intestine to a lesser extent. Secondary BAs can be excreted, or they can be absorbed in the large intestine to join enterohepatic circulation. This figure shows primary BAs conjugated to glycine moieties.

One of the most widespread BA transformations carried out by gut bacteria is deconjugation (i.e., removal of the glycine or taurine moieties) of glycine- and taurine-conjugated primary BAs (Fig. 2.1) (2, 14, 16, 17). The hydrolysis of the amide bond connecting taurine or glycine to the BA steroid core is performed by bacterial bile salt hydrolases (BSH) (10, 18, 19). BSH activity appears to be common in gut microbes, though less widespread in Gram-negative than in Gram-positive bacteria (11, 16, 20–23). The efficiency by which BAs are recirculated throughout the body is dependent on their structure; glycine- and taurine-conjugated BAs are more readily removed from the gastrointestinal (GI) tract by active transport and are also more readily taken up by the liver during systemic circulation (2, 5, 24, 25). In humans, CA, CDCA, deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), and lithocholic acid (LCA) can be reconjugated to glycine or taurine by host enzymes in the liver (25).

Following deconjugation, bacterial production of secondary BAs can involve several categories of reactions, including dehydroxylation, dehydrogenation, esterification, epimerization, and oxidation (9). These chemical transformations may be carried out by a single species harboring multiple enzymes or may require the sequential action of different bacterial species, each possessing distinct capabilities (11). One of the most common enzyme classes in BA transformations is hydroxysteroid dehydrogenases (HSDs) (26, 27). HSDs catalyze the reversible oxidation of hydroxyl groups on the C-3, C-7, and C-12 carbon positions of the BA steroid core (Fig. 2.2A to 2.2E). HSDs have stereospecificity; for example, a 3α-HSD can oxidize an α-OH group at the C-3 position, whereas a 3β-HSD can oxidize a β-OH group at the C-3 carbon. Thus, the sequential action by both α- and β-HSDs can result in epimerization of an -OH group on the BA steroid core (28–30). Another well-recognized transformation is the dehydroxylation of the primary BAs CA and CDCA at the α-oriented hydroxyl group on C-7, which results in the two most common secondary BA species, DCA and LCA (Fig. 2.2F) (30).
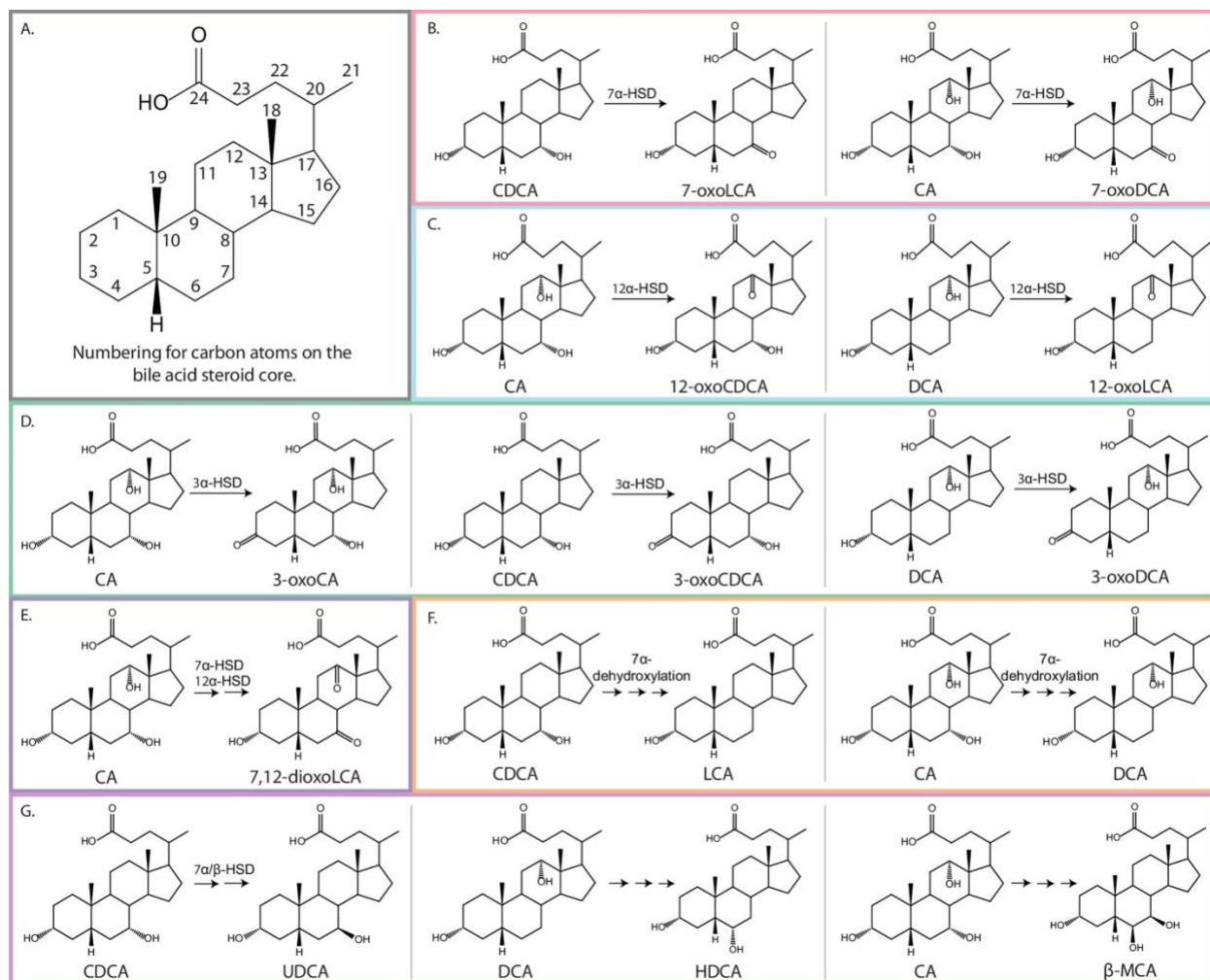
FIG 2.2 Secondary bile acid transformations observed *in vitro*. (A) Numbering of carbon atoms on the BA steroid core. (B) 7α-dehydrogenation of CDCA and CA. (C) 12α-dehydrogenation of CA and DCA. (D) 3α-dehydrogenation of CA, CDCA, and DCA. (E) Transformation of CA into 7,12-dioxoLCA. (F) 7α-dehydroxylation of CDCA and CA. (G) Other transformations. CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; β-MCA, β-muricholic acid; 3-oxoCA, 3-oxocholic acid; 3-oxoCDCA, 3-oxochenodeoxycholic acid; 3-oxoDCA, 3-oxodeoxycholic acid; 7-oxoDCA, 7-oxodeoxycholic acid; 7-oxoLCA, 7-oxolithocholic acid; 12-oxoCDCA, 12-oxochenodeoxycholic acid; 12-oxoLCA, 12-oxolithocholic acid; 7,12-dioxoLCA, 7,12-dioxolithocholic acid. A complete list of BAs and abbreviations used in this study can be found in Table S2.2.

Variations in gut microbial community makeup can lead to variable composition of an individual's BA pool (5, 12, 33, 34), with differential effects on both the host and gut microbiota.

BAs can bind and modulate the activity of nuclear hormone receptors (NHRs) in the liver, including farnesoid X receptor (FXR), vitamin D receptor (VDR), and the pregnane-activated receptor (PXR), which are involved in the regulation BA homeostasis, xenobiotic metabolism, triglyceride metabolism, and glucose metabolism ([2–4], [7]). BAs can also modulate activity of G protein-coupled receptors (GPCRs) in the intestine, which participate in the regulation of BA metabolism, energy expenditure, and glucose metabolism ([2–4], [6]). By interacting with these receptors in the liver and intestine, BAs can elicit broad effects on host physiology ([1–8]). Deviations from typical BA levels have been associated with illnesses such as cholesterol gallstone disease ([35]), recurrent GI tract bacterial infections ([36]), and liver and colon cancers ([37], [38]). There is growing evidence that BAs produced by HSD activity are involved in the pathogeneses of hormone-dependent cancer, hypertension, and obesity ([26]), and high levels of hyodeoxycholic acid (HDCA), a ligand for liver X receptors and GPCRs, have been associated with cholestatic liver disease or intestinal malabsorption ([39], [40]). In contrast to the association of BAs with disease, UDCA is used as a therapeutic for cholesterol gallstones, primary biliary cirrhosis, primary sclerosing cholangitis, and recurrent pancreatitis ([27]).

The full variety of BA transformations and their representation across gut microbial species are still unknown; current knowledge is primarily based on biochemical studies from a relatively small number of species or bioinformatic searches that rely on known sequences of genes involved in BA transformation to make predictions ([18], [28], [35], [41–47]). Because of this, our current knowledge regarding microbial BA transformations is not sufficient to predict how a particular microbe impacts the BA pool and, thus, host physiology. To shed light on these questions, we performed a systematic *in vitro* investigation of the BA-transforming capabilities of 72 bacterial species commonly found in the human intestinal tract. Our results significantly expand upon the lists of bacterial species known to perform BA transformations, and we also

identify novel conjugated secondary BAs. The results of this study will help scientists better understand how the gut microbiome shapes the composition of the BA pool.

2.3 Results and Discussion

2.3.1 In vitro screen for bile acid-transforming activity in gut bacteria.

Seventy-two bacterial isolates, representing 70 species across seven phyla found in the human gut, were assessed for their *in vitro* ability to chemically transform the primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA) and the prominent secondary BA deoxycholic acid (DCA) (Table S2.1). All strains used in this study, with the exception of *Clostridium scindens*, were previously sequenced, and their genomes are publicly available (Table S2.1). The *C. scindens* strain was newly isolated from a human fecal sample (see Materials and Methods). Bacterial isolates were incubated with each of these three BAs separately (i.e., CA, DCA, and CDCA) at 100 µM concentrations, which are within physiological ranges (48, 49). In addition, we incubated each isolate with all three BAs combined, totaling 300 µM. Samples were collected at 24 and 48 h post-inoculation for quantitation of BAs (see Materials and Methods). We used a non-targeted high-pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method to screen for the presence of transformed BAs (39 of which were directly matched to standards), including conjugated BAs (see Table S2.2).

We observed that BA-transforming activity was highly prevalent among the gut microbes analyzed and was not phylogenetically constrained (Fig. S2.1). Of the 72 strains screened, we found that 43 (representing 41 species) performed at least one BA modification on one or more of the BAs tested. Of the 43 strains that showed BA-transforming capabilities, 32 species were novel BA transformers. Additionally, of the 9 species already known to transform BAs, all showed additional capabilities not previously recognized. The 43 bacterial strains exhibited a range of BA transformations: 42 possessed hydroxysteroid dehydrogenase (HSD) activity, 5

showed 7α-dehydroxylation activity, and 3 performed other less-well-studied transformations on the BA core (Fig. 2.2, 2.3, and S2.1). Interestingly, we also found that 28 strains, representing 27 species, were capable of conjugating BAs to amino acids. Most strains with BA-transforming capabilities were found in the *Actinobacteria*, *Firmicutes*, or *Bacteroidetes* phyla, which were our most heavily sampled phyla.
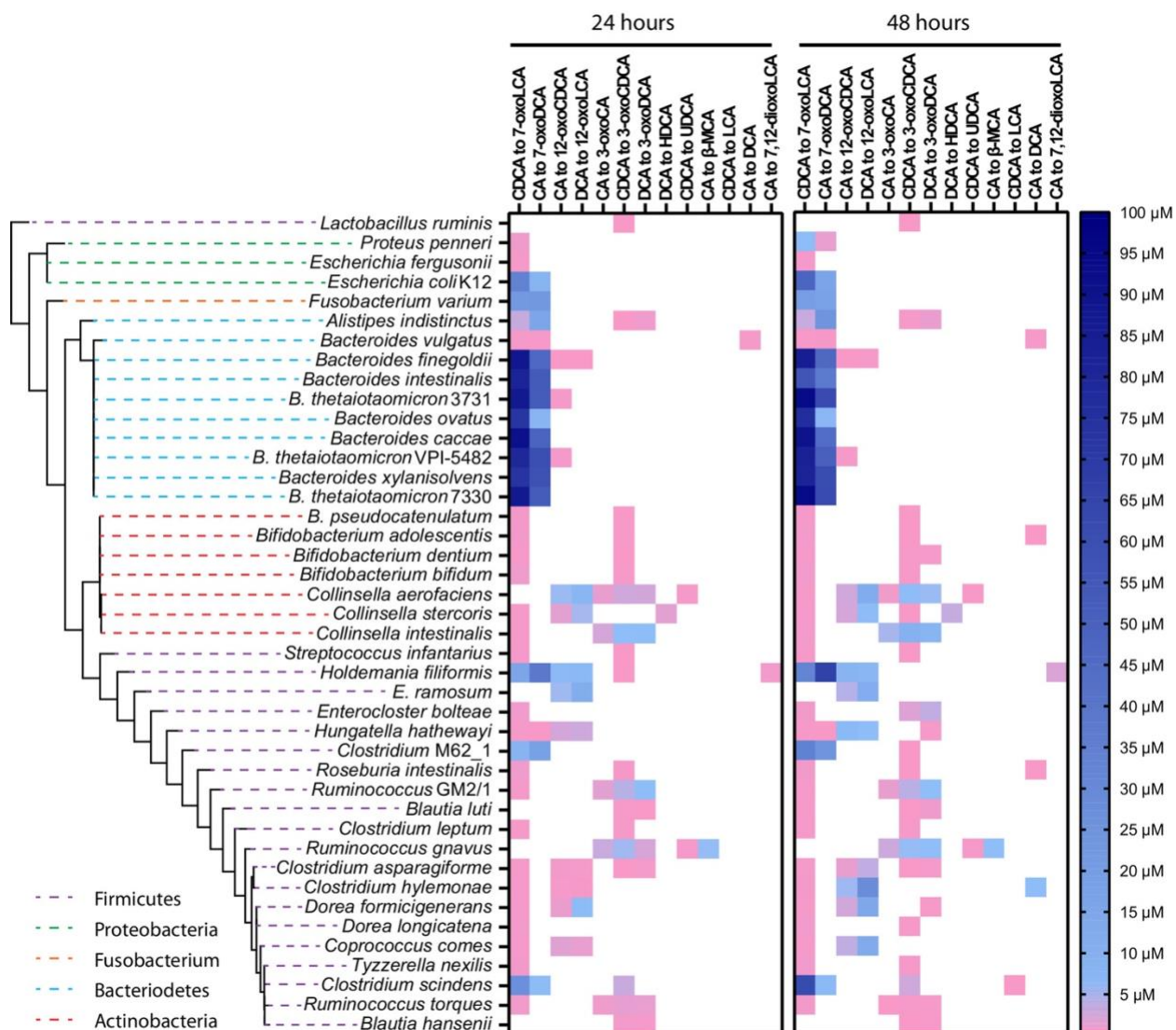
FIG 2.3 Secondary bile acid production across bacterial strains. The heat map shows the BA steroid core transformations carried out by each isolate. The color scale denotes amounts of secondary BA produced at 24 (left) and 48 h (right). Bacteria were provided with 100 μM cholic acid (CA), chenodeoxycholic acid (CDCA), or deoxycholic acid (DCA). The detection limit was below 0.05 μM for all BAs. Phyla information is indicated by color-coded dashed lines in the

phylogenetic tree. Heat maps for BA production when CA, CDCA, and DCA were added in combination are shown in Fig. S2.2.

Our *in vitro* analysis also revealed a wide range in the efficiency by which different bacterial isolates transform BAs; for example, some microbes transformed >90% of the added BA, while others transformed 5% or less. Most BA transformations occurred within 24 h of growth, but several strains displayed a measurable increase in BA production from 24 to 48 h (Fig. 2.3 and S2.2). Finally, we observed no significant differences ($P > 0.05$, two-tailed paired *t* test) in BA transformations when BA substrates were added individually or in combination.

2.3.2 3α-, 7α-, and 12α-hydroxysteroid dehydrogenase activity.

The most common BA transformation that we encountered in our screen was oxidation of 3α-, 7α-, or 12α-hydroxyl groups on the steroid core (Fig. 2.2A to 2.2E, 2.3, S2.1, and S2.2). 7α-HSD activity was present in 38 strains and yielded the highest concentrations of secondary BAs in our screen (Fig. 2.2B, 2.3, S2.1, and S2.2). On average, the strains that possessed 7α-HSD activity transformed ~30.8 µM CA (ranging from 0.12 to 66.4 µM) and ~22.8 µM CDCA (ranging from 0.16 to 93.9 µM) by 48 h (Fig. 2.3 and Table S2.3). Members of the *Bacteroidaceae* family, with the exception of *Bacteroides vulgatus*, exhibited the highest production of secondary BAs, transforming 46.1 ± 18.5 µM or 82.4 ± 12.2 µM added CA or CDCA, respectively, into 7-oxo BA intermediates by 48 h (Fig. 2.3 and Table S2.3). Several strains of *Bacteroides thetaiotaomicron* have been shown to exhibit 7α-HSD activity, but the *B. thetaiotaomicron* 3731, VPI-5482, and 7330 strains, all of which displayed high BA-transforming activity, were not previously described (Fig. 2.3) (50). Additionally, we corroborated that *Bacteroides intestinalis* exhibited 7α-HSD activity on both CA and CDCA (44). Also consistent with previous

findings, we found that *Escherichia coli* K-12 substrain MG1655 possessed 7α-HSD activity, acting on both CA and CDCA (9, 11, 44, 51–53).

We observed 3α-HSD activity in 25 isolates, 16 of which were *Firmicutes*, mostly in the *Lachnospiraceae* family (Fig. 2.2D, 2.3, and S2.2). On average, these strains displayed much lower activity than those possessing 7α-HSD activity (Fig. 2.4A). By 48 h, the average concentrations of secondary BAs produced by 3α-HSD activity were ~0.93 µM (ranging from 0.12 to 2.29 µM) for CA, ~0.97 µM (ranging from 0.10 to 9.35 µM) for CDCA, and ~1.54 µM (ranging from 0.16 to 7.23 µM) for DCA (Fig. 2.3 and Table S2.3). Most strains that exhibited 3α-HSD activity only acted on one or two of the BA substrates, but two of three *Collinsella* strains and all three *Ruminococcus* strains tested were able to transform all three added BAs, CA, CDCA, and DCA (Fig. 2.3). Furthermore, these five strains produced the highest concentrations of 3-oxo BAs (Fig. 2.3 and Table S2.3). *Collinsella aerofaciens* has only been shown to produce 3-oxoCA from CA; thus, production of 3-oxoDCA and 3-oxoCDCA expand upon its previously known BA-transforming capabilities (16). Our lab isolate of *C. scindens* produced 3-oxoCDCA from CDCA and 3-oxoCA from CA but did not produce 3-oxoDCA from DCA (Fig. 2.3 and S2.2), which is consistent with a previous study reporting that both *C. scindens* VPI 12708 and *C. scindens* ATCC 35704 produce 3-oxo BAs from both CA and CDCA (45).
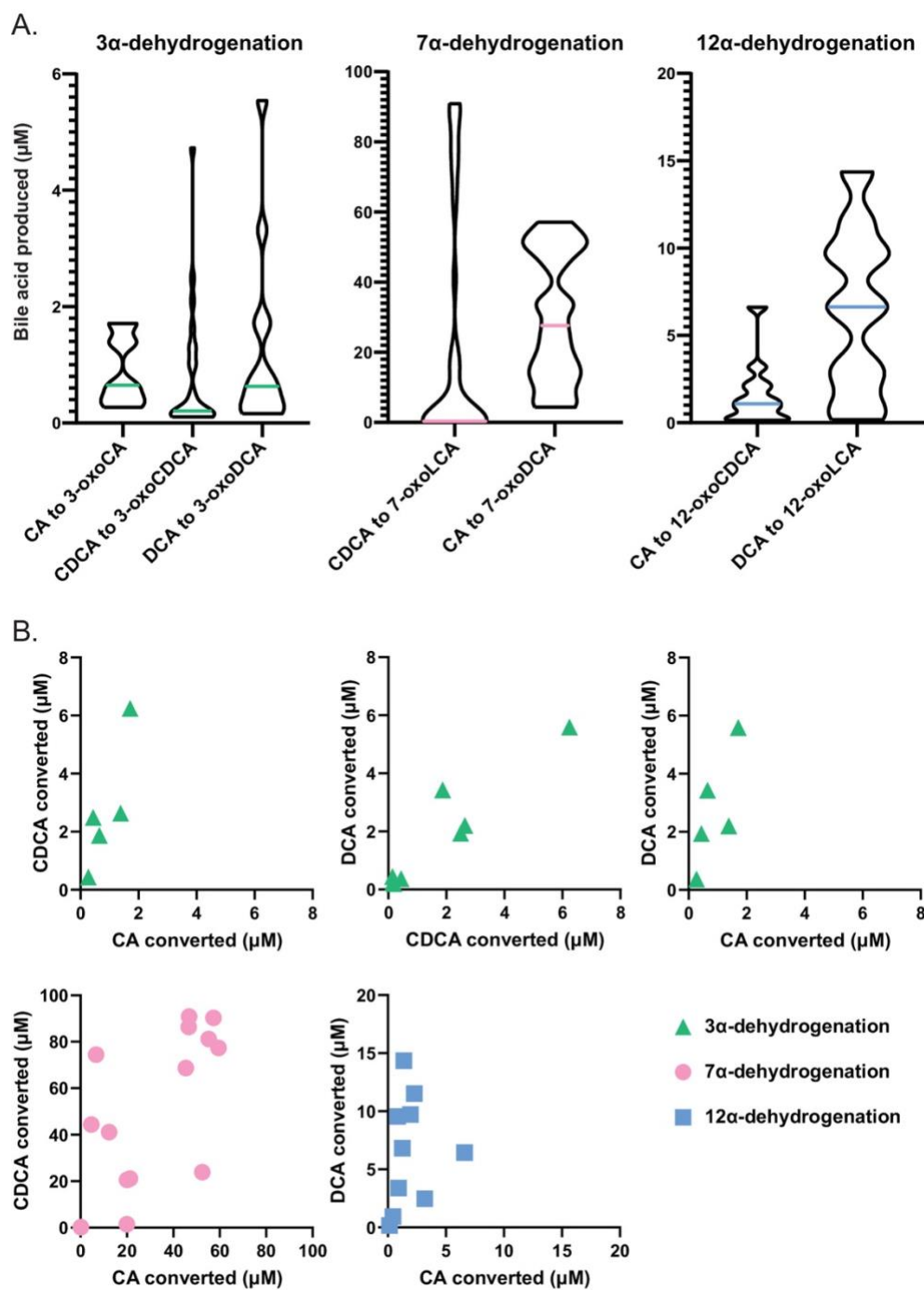
FIG 2.4 Relative activity and specificity of α-dehydrogenase activity. (A) 3α-, 7α-, and 12α-dehydrogenation activity when BAs were administered separately. The median production of oxo BAs is denoted by a solid-colored line. Data shown are the average of 24 and 48 h measurements. (B) For each isolate capable of 3α-dehydrogenation, the amounts of CDCA, CA, and DCA transformed were plotted against each other. For each isolate capable of 7α-dehydrogenation, the amounts of CA and CDCA transformed were plotted against each other. For each isolate capable of 12α-dehydrogenation, the amounts of CA and DCA transformed were plotted against each other. Data shown are the averages of 24 and 48 h measurements.

12α-HSD activity was detected in 12 strains (Fig. 2.2C, 2.3, S2.1, and S2.2). 12α-HSD activity was previously detected primarily in the genus *Clostridium*, but our results show it to be much more widespread (Fig. 2.3 and S2.1) (12, 27). Members of the *Firmicutes* and *Actinobacteria* phyla produced higher concentrations of 12-oxo BAs than members of the *Bacteriodetes* (Fig. 2.3 and S2.1). On average, strains that exhibited 12α-HSD activity transformed ~2.0 μM CA (ranging from 0.09 to 7.92 μM) and ~9.74 μM DCA (ranging from 0.19 to 28.6 μM) at 48 h, a level of activity that is intermediate between the 7α-HSD and 3α-HSD activities discussed above (Fig. 2.3 and 2.4A; Table S2.3). In several instances, the 12-oxo BA concentration increased significantly between 24 and 48 h ($P < 0.005$, two-tailed paired t test) (Fig. 2.3 and S2.2).

12α-HSD activity on BAs was demonstrated previously in 7α-dehydroxylating bacteria *Clostridium leptum*, *C. scindens*, and *Clostridium hylemonae* as well as in the gut commensal *C. aerofaciens* (16, 27). We observed that *C. hylemonae* produced 12-oxo BAs from CA and DCA, but we did not observe 12α-HSD activity in *C. leptum* and *C. scindens* (Fig. 2.3) (11). Consistent with previous observations (16), we found that *C. aerofaciens* transformed CA and CDCA into 12-oxoCDCA and 12-oxoLCA, respectively.

Lastly, we found that *Holdemania filiformis* produced a small amount of 7, 12-dioxoLCA from CA by 48 h (Fig. 2.3 and S2.2, and Table S2.3). The transformation of CA to 7, 12-dioxoLCA requires both 7α-dehydrogenation and 12α-dehydrogenation on the same molecule; while we identified 5 strains capable of performing both dehydrogenations on CA, only *H. filiformis* produced the doubly modified BA (Fig. 2.2E).

2.3.3 Specificity of α-hydroxysteroid dehydrogenase activity.

A comparison between the amounts of secondary BAs produced by HSD activity at the 3α-, 7α-, and 12α-positions within each strain revealed interesting trends (Fig. 2.4B). For example, in nearly all isolates, 3α-HSD and 12α-HSD activity exhibited preference toward DCA and/or CDCA over CA (Fig. 2.4B). For 7α-HSD activity, there was a slight preference toward CDCA over CA for most microbes. However, some isolates, such as *H. filiformis* and *Alistipes indistinctus*, showed a clear preference for CA over CDCA, while *C. scindens* and *B. ovatus* exhibited a marked preference for CDCA over CA (Fig. 2.3 and S2.2; Table S2.3).

Bacterial isolates also showed some specificity regarding the types of HSD activity they displayed. That is, of the 42 strains that possessed HSDs, 14 showed only one type of HSD activity (3 species were able to act at C-3, 10 species at C-7, and 1 species at C-12), 21 displayed two types of HSD activity, and only 5 isolates possessed all three types of HSD activity (Fig. 2.3 and S2.1).

2.3.4 7α-dehydroxylation activity.

BA 7α-dehydroxylation (e.g., transformation of CA into DCA, and CDCA into LCA) (Fig. 2.2F) is a multistep pathway performed by the BA inducible (*bai*) operon, which encodes seven enzymes and a transporter (11, 54, 55). This pathway is reported to be highly conserved and has primarily been observed in *Clostridia* species (36, 47, 56), but our results suggest it to be more widespread (Fig. 2.3 and S2.1).

In our screen, 7α-dehydroxylation of CA to DCA was more prevalent than 7α-dehydroxylation of CDCA to LCA (Fig. 2.3). We found 5 strains capable of 7α-dehydroxylation: *Bacteroides vulgatus*, *Bifidobacterium adolescentis*, and *Roseburia intestinalis* dehydroxylated CA, *C. scindens* dehydroxylated CDCA, and *C. hylemonae* dehydroxylated both CA and CDCA.

Three previously reported 7α-dehydroxylating strains are *C. hylemonae* DSM 15053, *C. scindens* ATCC 35704, and *C. leptum* ATCC 29065. *C. leptum* ATCC 29065 and *C. scindens* ATCC 35704 have been shown to dehydroxylate both CA and CDCA, while *C. hylemonae* DSM 15053 has been shown to only dehydroxylate CA (27, 42, 57, 58). We were unable to reproduce 7α-dehydroxylation of both CA and CDCA in *C. leptum* ATCC 29065 but observed that *C. hylemonae* DSM 15053 dehydroxylated CA to DCA as previously reported (27, 58). Our *C. scindens* isolate showed inconsistent low-level production of LCA from CDCA and DCA from CA (Fig. 2.3 and Table S2.3).

Of all five strains we found to perform 7α-dehydroxylation, only *C. hylemonae* possessed the canonical *bai* operon, encoding BaiA2, BaiB, BaiCD, BaiE, BaiF, BaiG, and BaiH but not BaiI (see Materials and Methods). Interestingly, our lab isolate of *C. scindens*, which displayed only low levels of the expected 7α-dehydroxylation activity, also possessed the canonical *bai* operon without *baiI*. However, when comparing our *C. scindens* isolate to the previously sequenced *C. scindens* VPI 12708, we identified one mismatch in the protein sequences for BaiB (amino acid substitution H214L) and BaiH (amino acid substitution V526A) and two mismatches in BaiE (amino acid substitutions T74S and A95E). It is possible that these alterations were enough to decrease its 7α-dehydroxylation activity, but further analyses will need to be performed to support this hypothesis. Finally, *R. intestinalis*, *B. vulgatus*, and *B. adolescentis* were all predicted to possess only four or five genes of the canonical eight-gene operon. This suggests that divergent or nonhomologous genes may encode the 7α-dehydroxylation activity in the newly identified strains.

2.3.5 Other bile acid transformations: hydroxyl group conversions at C-6 and C-7.

Consistent with previous reports, we observed that both *C. aerofaciens* and *Ruminococcus gnavus* produced small amounts of UDCA from CDCA (Fig. 2.3 and S2.2) (28, 29). The

transformation of CDCA to UDCA requires the combined action of 7α- and 7β-HSDs to epimerize the C-7 hydroxyl group on the steroid core (Fig. 2.2A and 2.2G) ([11](#)). This BA transformation is of particular interest, because UDCA is used therapeutically to treat gastrointestinal tract diseases ([29](#), [59](#)).

Another BA transformation of interest is conversion of DCA to HDCA (Fig. 2.2G) ([60](#), [61](#)). *Collinsella stercoris* produced 1.55 µM HDCA from DCA at 48 h (Fig. 2.3 and S2.2). While the enzymes responsible for this transformation are not known, it is almost certainly a multistep mechanism involving α-dehydroxylation of the C-12 hydroxyl group followed by a hydroxylation on C-6.

To the best of our knowledge, production of β-muricholic acid (β-MCA) by human gut isolates has not been previously reported. Here, we found that *R. gnavus* can transform CA to β-MCA (Fig. 2.2G, 2.3, and S2.2). This transformation requires α-dehydroxylation at C-12, epimerization of the C-7 hydroxyl group of CA, and addition of a hydroxyl group at the C-6 position. Interestingly, bacterial 12α-dehydroxylation of BAs appears to be a rare transformation ([62](#), [63](#)).

2.3.6 Amino acid conjugation of bile acids by gut bacteria.

Our *in vitro* screen revealed that 25 strains, representing 24 species, were capable of conjugating DCA, CDCA, or CA to glycine *in vitro* (Fig. 2.5A and Table S2.4). Twenty-three strains produced up to 1.02 µM (averaging ~0.236 µM at 48 h) of glycodeoxycholic acid (GDCA) from DCA, 16 strains produced up to 0.70 µM (averaging ~0.145 µM at 48 h) of glycochenodeoxycholic acid (GCDCA) from CDCA, and 7 strains produced up to 0.098 µM (averaging 0.036 µM at 48 h) of glycocholic acid (GCA) from CA (Fig. 2.5A and Table S2.4). To our knowledge, this is the first report of bacterially mediated reconjugation of BAs to glycine.

FIG 2.5 Production of amino acid-conjugated bile acids across bacterial strains. (A) The heat map shows the BA-to-amino acid conjugations carried out by each isolate at 24 h. The data are presented as raw signal intensities normalized by Z-score, which is denoted by the color scale. Bacteria were provided with 100 µM cholic acid (CA), chenodeoxycholic acid (CDCA), or deoxycholic acid (DCA). Phyla information is indicated by the color-coded dashed lines in the phylogenetic tree on the left. The heat map for conjugated BA production at 48 h is shown in Fig. S2.3. (B) MS/MS spectra of selected novel microbially conjugated BAs. Parent ion structure, mass, and retention time are listed along with the structure and exact masses for

three identifying fragments: the major sterol fragment, the fragment resulting from amino acid loss, and the amino acid fragment.

In addition to the microbial conjugation of CDCA, DCA, and CA to glycine, we also observed glycine conjugation to the most prominent secondary BA produced in our screen, 7-oxolithocholic acid (7-oxoLCA) (Fig. 2.5, S2.3, and Table S2.4). All strains that could perform this conjugation were members of the *Bacteroidaceae* and produced detectable amounts of glycol-7-oxolithocholic (G-7-oxoLCA) acid when provided with CDCA as a substrate.

A recent study reported that the mouse microbiome is capable of conjugating CA to the amino acids phenylalanine, tyrosine, and leucine and showed that *Enterocloster* (formerly *Clostridium*) *bolteae* can carry out CA conjugations to phenylalanine and tyrosine *in vitro* (15). Guided by these data, we searched our data for the presence of BAs conjugated to amino acids. We found that 28 isolates, representing 27 species, were capable of conjugating CDCA, DCA, or CA to one or more of the following amino acids: glutamate, glutamine, aspartate, asparagine, methionine, histidine, lysine, serine, tryptophan, valine, alanine, and arginine; they were also able to conjugate to the previously described phenylalanine, leucine/isoleucine, or tyrosine (Fig. 2.5 and S2.3). In total, we identified 44 novel amino acid-BA conjugates. The amino acids most frequently conjugated to BAs were glycine and phenylalanine (Fig. 2.5 and S2.3). CDCA and DCA were conjugated to amino acids much more frequently than CA. Of these 28 isolates, 25 overlapped with strains that possessed the ability to conjugate BAs to glycine (Fig. 2.5, S2.3, and Table S2.4). To corroborate the identification of conjugated BAs, we used tandem mass spectrometry (MS/MS) to generate high-resolution spectra for each conjugated BA of interest (Fig. 2.5B); these spectra displayed the expected fragmentation patterns (Table S2.5) (15).

Interestingly, in some bacterial strains, we noticed double chromatographic peaks corresponding to the exact masses and with the expected characteristic MS/MS spectra for BAs

conjugated to alanine or valine (Fig. S2.4, Table S2.5). Enantiomers, which have the same mass, can have different retention times; thus, this observation suggests that some bacterial strains are conjugating both D- and L-alanine or valine to CDCA, DCA, or CA (Fig. S2.4 and Table S2.5).

Our results indicate that the ability to conjugate amino acids to primary and secondary BAs is widespread among gut microbes (Fig. 2.5 and S2.3; Table S2.4). BA conjugation to taurine was not observed in our screen, either because organisms lack the necessary enzymes or because the growth medium did not supply sufficient amounts of the compound. Further investigation is required to determine the possibility of microbial taurine conjugation to BAs. The enzymes responsible for amino acid conjugation are currently unknown, and while it is plausible that microbial BSH may participate in glycine conjugation (64), it is unclear what enzymes would be responsible for BA conjugation to all other amino acids. Interestingly, we saw most conjugation ability in the *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*, which are the same three phyla reported to possess glycine reconjugation activity and BSH activity. In addition, most species fall into three families: *Bifidobacteriaceae*, *Lachnospiraceae*, and *Bacteroidaceae* (Fig. 2.5 and S2.1) (5). The physiological effects of amino acid-conjugated BA—other than those conjugated to glycine or taurine—are unknown, although it was recently reported that cholic acid conjugated to phenylalanine, leucine, and tyrosine can act as FXR agonists and are enriched in patients with inflammatory bowel disease and cystic fibrosis (15). In addition, it is unknown whether BA conjugation to amino acids other than glycine or taurine enhances their intestinal absorption and enterohepatic circulation. Further studies are required to investigate these important questions.

2.3.7 Comparison of bioinformatically predicted and observed HSD activity.

A previous study by Kisiela et al. included a bioinformatic search for 3α-, 7α-, and 12α-HSD homologs in all sequenced bacteria and archaea (at the time of analysis) and identified putative HSDs across numerous genera (26). The study, which evaluated 69 of our 72 bacterial strains, used known protein sequences to identify HSD homologues. They predicted 22 of the 40 bacterial species that exhibited HSD activity in this *in vitro* study. We were able to confirm all predicted HSD activities (3α-, 7α-, and 12α-HSD) in all species except two (*C. hylemonae* and *C. scindens*). We confirmed 7 of 8 species predicted to possess 3α-HSD activity, 6 of which were an exact strain match, 10 of 10 species predicted to possess 7α-HSD activity, 8 of which were an exact strain match, and 9 of 10 species predicted to possess 12α-HSD activity, 9 of which were an exact strain match (Fig. S2.1 and Table S2.6). Although *C. hylemonae* was predicted to possess both 3α- and 12α-HSD activity, we observed 7α- and 12α-HSD activity in our screen and only trace 3α-HSD activity (i.e., production of 3-oxoCDCA from CDCA of less than 0.1 μM). Interestingly, prior *in vitro* studies on *C. hylemonae* showed only a functional 12α-HSD but not a 3α- or 7α-HSD (27). In addition, *C. scindens* was predicted to possess 3α-, 7α-, and 12α-HSD activity, but in our lab isolate, under the specified experimental conditions, we only observed 3α- and 7α-HSD. Of the 69 sequenced species that overlap between our *in vitro* study and the Kisiela et al. bioinformatic analysis (26), we found many species that displayed HSD activity that were not predicted by their analysis (see Table S2.6). We identified 13 additional species (13 strains) with 3α-HSD activity, 24 additional species (26 strains) with 7α-HSD activity, and 2 additional species (3 strains) with 12α-HSD activity (Fig. S2.1 and Table S2.6). This suggests that there are yet to be identified HSDs with low or no homology to currently known enzymes or that some low-level transformations may represent nonspecific enzyme catalysis.

A different study by Doden et al. used a bioinformatics approach to identify bacteria with previously unreported 12α-HSD activity (27). They predicted 12α-HSDs to be widespread

among the phyla *Firmicutes* and *Actinobacteria*. Our results confirmed the predicted 12α-HSD

activity in 11 species identified by their analysis (Fig. S2.1 and Table S2.6). Interestingly, their

study predicted 12α-HSD activity in 10 species belonging to the phylum *Bacteroidetes* that we

analyzed in our *in vitro* screen, but we only observed 12α-HSD activity in two of them. The

reason for this discrepancy is unknown, but it is plausible that *Bacteroidetes* species only induce

12α-HSD activity under specific growth conditions, and the specific medium used in this study

(see Materials and Methods) may have suppressed the expression of these class of enzymes. It

is worth pointing out that the study by Kisiela et al. (26) only identified two *Bacteroidetes* strains

with 12-HSD activity, *Bacteroides pectinophilus* and *Bacteroides fragilis* (neither of which were

assessed in our study), while Doden et al. identified a large number of *Bacteroidetes* strains

with this activity, including both aforementioned strains and 12 of the species analyzed in this

study (Table S2.6) (27). The discrepancies between these two studies are likely a reflection of

the distinct methodologies used and the specific selection of seed gene sequences, but they

also highlight the difficulty in accurately predicting HSD activity from gene sequence homology.

    To provide further insight into this issue, we performed a bioinformatic search to predict HSD

proteins in our strains based on known sequences (Table S2.6) (see Materials and Methods).

As summarized in Table S2.6, the predictions of our own bioinformatic analysis showed fair

overlap with the Kisiela et al. (26) and Doden et al. (27) studies but still failed to predict the

observed *in vitro* BA-transforming HSD activity of many species. Overall, these results suggest

that nonhomologous genes may be responsible for the observed *in vitro* HSD activity, but

further research is required to determine what combination of mutations, deletions, gene

duplications, or gene regulation might be responsible for causing discrepancies between

observed and predicted HSD activity. In addition, BA-transforming activity may be influenced by

the gut environment and interspecies interactions *in vivo.*

2.4 Conclusion

Our results greatly expand upon the lists of organisms known to perform different types of BA transformations. The high prevalence of BA transformations, with few discernible patterns based on phylogeny, illustrates the widespread distribution of BA-transforming capabilities across human gut bacteria. We anticipate that knowledge generated by this study will facilitate engineering of synthetic microbial communities with predictable effects on BA composition in *in vivo* systems, which will be vital for assessing the physiological effects of microbially transformed BAs. A new suite of questions can now be asked about how bacteria act by themselves or in concert with others to transform BAs and generate a diverse BA pool.

2.5 Materials and methods

2.5.1 Strains and media.

All strains are listed in Table S2.1 in the supplemental material. All strains were grown on Mega medium, which was filter sterilized and stored in a Coy anaerobic chamber (5% H_2, 20% CO_2, and 75% N_2) at least 24 h prior to use. Mega medium contains (per liter tap distilled water): 100 ml (1 M, pH 7.2) potassium phosphate buffer, 10 g tryptone peptone, 5 g yeast extract, 5 g meat extract, 4 ml (25 mg/100 ml) resazurin, 1.8 g D-glucose, 0.9 g D-maltose, 0.86 g D-cellobiose, 0.46 g D-fructose, 1 g sodium acetate trihydrate, 0.02 g $MgSO_4 \cdot 7H_2O$, 2.1 g $NaHCO_3$, 0.08 g NaCl, 1 ml (0.8 g/100 ml) $CaCl_2$, 1 ml (1 mg/ml in 100% ethanol) vitamin K_3 (menadione), 1 ml (1.2 mg hematin/ml in 0.2 M histidine, pH 8.0) histidine hematin, 2 ml (25% [vol/vol]) Tween 80, 10 ml ATCC MD-VS vitamin mix, 10 ml ATCC MD-TMS trace mineral mix, 1 ml (40 mg/100 ml) $FeSO_4 \cdot 7H_2O$, and 0.5 g L-cysteine HCl. For cultures of *Akkermansia muciniphila*, the medium was amended with 0.5 mg/ml mucin. This specific medium was designed to allow growth of all species in this study. *Clostridium scindens* was isolated from a quercetin-degrading anaerobic enrichment inoculated with a human fecal sample (65). The *C.*

scindens genome was sequenced by the Microbial Genome Sequencing Center (MiGS, Pittsburgh PA). Genome hybrid assembly with Illumina and Oxford Nanopore Technologies (ONT) reads was performed using he Unicycler v.0.4.8 pipeline; 1.083 Gbp Illumina reads and 1.004 Gbp ONT reads were assembled into a single contig of 3,941,835 bp (530× coverage) with 47.61% GC content. Assembly annotation was performed using Prokka v.1.14.5. The genome sequence data of the *C. scindens* strain isolated in this study can be accessed with the NCBI reference sequence NZ_CP080442.1.

2.5.2 Sample handling and growth conditions.

Strains were grown at 37°C in an anaerobic chamber with an atmosphere of 75% N_2, 20% CO_2, and 5% H_2. Starting from freezer stocks, strains were first grown overnight to a high density (optical density at 600 nm [OD_{600}] range of 0.349 to 1.9, measured directly in the tube) in Hungate tubes containing Mega medium. These cultures were then used to inoculate (1:15 dilution) 3 ml of Mega medium in 5-ml polypropylene tubes, amended with BAs. There were 5 sets of conditions for the quantitative screen of BA-transforming activity. Under the first three conditions, medium contained one of each of the three BAs, cholic acid (CA), chenodeoxycholic acid (CDCA), or deoxycholic acid (DCA), at 100 µM each. Under the fourth condition, all three BAs were combined, totaling 300 µM. Under the fifth condition (control), no BAs were provided. In addition, we tested for spontaneous BA degradation or transformation in uninoculated controls containing BAs. At 24 and 48 h after inoculation, 1 ml of culture was collected and spun down at room temperature for 10 min at 10,000 × *g*, and the supernatant was transferred to a fresh tube. The supernatant was diluted to 1:100 using ultrahigh-pressure liquid chromatography (uHPLC)-grade H_2O, and 100 µl was transferred to an HPLC vial for analysis. In addition to the quantitative screen, we also carried out an initial qualitative screen in which strains were grown in 96-well plates (200-µl cultures) for 24 h with BAs mixed totaling 300 µM.

In combination, 5 independent measurements of BA-transforming activity were carried out for each bacterial strain in this study: four quantitative measurements and an initial qualitative assessment.

2.5.3 uHPLC-MS/MS measurements.

Samples were analyzed using an ultrahigh-pressure liquid chromatography-tandem mass spectrometry (uHPLC-MS/MS) system consisting of a Thermo Scientific Vanquish uHPLC system coupled to a heated electrospray ionization (HESI; using negative polarity) and hybrid quadrupole high-resolution mass spectrometer (Q Exactive Orbitrap; Thermo Scientific). Settings for the ion source were as follows: auxiliary gas flow rate of 10, sheath gas flow rate of 30, sweep gas flowrate of 1, 2.5-kV spray voltage, 320°C capillary temperature, 300°C heater temperature, and S-lens radiofrequency (RF) level of 50%. Nitrogen was used as nebulizing gas by the ion trap source. Liquid chromatography (LC) separation was achieved using a Waters Acquity UPLC BEH C_{18} column with 1.7-μm particle size, 2.1 by 100 mm in length. Solvent A was water with 10 mM ammonium acetate adjusted to pH 6.0 with acetic acid. Solvent B was 100% methanol. The total run time was 31.5 min with the following gradient: a 0- to 24-min gradient from 30% solvent B (initial condition) to 100% solvent B; hold 5 min at 100% solvent B; drop to 30% solvent B for 2.5-min reequilibration to initial condition. The flow rate was 200 μl/min throughout. Other LC parameters were as follows: autosampler temperature, 4°C; injection volume, 10 μl; column temperature, 50°C. The MS method performed a full MS1 full scan (290 to 1,000 *m/z*) together with a series of parallel reaction monitoring (PRM) scans. These MS2 scans (all-ion fragmentation) were centered at *m/z* values of 370, 408, 446, 484, and 522; each using an isolation width of 40.0 *m/z*. Fragmentations were performed at 60 normalized collision energy (NCE). All scans used a resolution value of 70,000, an automatic gain control (ACG) target value of 1E6, and a maximum injection time (IT) of 40 ms.

Experimental MS data were converted to the mzXML format and used for BA identification. BA peaks were identified using MAVEN (metabolomics analysis and visualization engine) ([66], [67]). For tandem mass spectrometry of conjugated BAs, MS2 scans (selected ion fragmentation) were performed at 20, 30, and 40 NCE (normalized collision energy). All scans used a resolution value of 17,500, an automatic gain control (ACG) target value of 1E6, and a maximum injection time (IT) of 40 ms.

2.5.4 Determination of BA concentrations.

BA quantitation was achieved using standard concentrations of each BA ranging from 0.01 µg/ml (or 0.019 µM to 0.027 µM depending on the BA) to 1 µg/ml (or 1.94 µM to 2.65 µM depending on the BA) to generate five-point standard curves. The detection limit was below 0.05 µM for all BAs. The threshold for reported core BA transformations was 0.1 µM. Standards were purchased from Avanti Polar Lipids and dissolved and stored in methanol at −80°C. See Table S2.2 for BA standard names and structural features. For BAs conjugated to amino acids, compounds were identified by their exact mass (mass error of less than 2 ppm) and predicted retention times but could not be quantified, since standards are not commercially available (Table S2.5). The only exceptions were BAs conjugated to glycine (i.e., GDCA and CDCA), for which standards were available.

2.5.5 BLASTP search for the *bai* operon.

Bacterial genomes were downloaded from NCBI ftp site using GenBank assemblies (GCA) or RefSeq assemblies (GCF). Eight amino acid sequences from *Clostridium scindens* VPI 12708 were downloaded from UniProt and used as reference sequences: BaiB ([P19409]), BaiCD ([P19410]), BaiE ([P19412]), BaiA2 ([P19413]), BaiF ([P19413]), BaiG ([P32369]), BaiH ([P32370]), and BaiI ([P32371]). Each of these eight Bai proteins were searched against all protein

coding genes (CDS) from the downloaded bacterial genomes, with an E value threshold of 1E−10. Gene coordinates (start position, end position, strand) in the genome were obtained from the GFF file on the NCBI ftp site. A *bai* operon was considered highly homologous if gene queries yielded eight *bai* operon genes within a close genome region (<100,000 bp).

2.5.6 Hidden Markov model search for the HSD gene.

Bacterial genomes were downloaded from the NCBI ftp site using GenBank assemblies (GCA) or RefSeq assemblies (GCF). To annotate 3α-, 7α-, 12α-HSD genes, hidden Markov model (HMM) searches were performed using custom HMM profiles against all protein coding genes (CDS) from the downloaded bacterial genomes. To generate 3α-, 7α-, 12α-HSD HMM profiles, reference sequences were downloaded from UniProt, and only reviewed (Swiss-Prot) sequences or sequences validated by experiments in the literature were used: for 3α-HSD, reference sequences included Q59718 (*Pseudomonas* sp. B-0831), P80702 (*Pseudomonas testosteroni*), A7B3K3 (*Ruminococcus gnavus* strain ATCC 29149/VPI C7-9), C8WMP0 (*Eggerthella lenta*), P19337 (*Clostridium scindens* strain JCM 10418/VPI 12708), and P07914 (*Clostridium scindens* strain JCM 10418/VPI 12708). For 7α-HSD, reference sequences included P0AET8 (*Escherichia coli* strain K-12), Q8YIN7 (*Brucella melitensis* biotype 1 strain 16M/ATCC 23456/NCTC 10094), Q5LA59 (*Bacteroides fragilis* strain ATCC 25285/DSM 2151/JCM 11019/NCTC 9343), G9FRD7 (*Clostridium absonum*), and P50200 (*Clostridium sordellii*). For 12α-HSD, reference sequences validated by Doden et al. (27) were used, including C0BWQ2 (*Clostridium hylemonae* DSM 15053), P21215 (*Clostridium* sp. strain ATCC 29733/VPI C48-50), R7AM69 (*Eggerthella* sp. CAG:298), C8WLK7 (*Eubacterium lentum* strain ATCC 25559/DSM 2243/JCM 9979/NCTC 11813/VPI 0255), B0NG52 (*Clostridium scindens* strain ATCC 35704/DSM 5676/VPI 13733/19), and B6FYX7 (*Clostridium hiranonis* strain DSM 13275/JCM 10541/KCTC 15199/TO-931). Reference sequences from each HSD

were aligned using MUSCLE (68) version 3.8.31, and the HMM profile was constructed using

hmmbuild3 (69). All CDS in bacterial genomes were searched using HMM search version 3.2.1.

The identified cutoff in each profile HMM was determined by a minimal bit score to maximize the

F measure. In each 3α-, 7α-, and 12α-HSD profile HMM, the positive group was defined as its

reference sequences, and the negative group was defined as a combination of the other two

references. For example, the positive group for the 3α-HSD profile HMM was six 3α-HSD

reference sequences, and the negative group for the 3α-HSD profile HMM was the combined

7α- and 12α-HSD reference sequences. The F measure score was defined as follows: $F = 2/(recall^{-1} + precision^{-1})$, where "recall" is the true positive/(true positive + false negative) and

"precision" is the true positive/(true positive + false positive). By this procedure, the cutoff for 3α-

HSD was 146.1, the cutoff for 7α-HSD was 220.6, and the cutoff for 3α-HSD was 120.2.

All identified HSD genes together with HMM reference sequences were aligned with

MUSCLE (68) version 3.8.31. Conserved motif sequences were visualized by WebLogo3 (70).

2.5.7 Phylogenetic tree construction.

We used publicly available full genomes and constructed the tree using the Genome

Clustering tool using the Joint Genome Institute's IMG/MER, which is based on hierarchical

clustering of the taxonomy of selected genera (Fig. S2.1; Table S2.1) (71, 72).

2.5.8 Data availability.

The HMM search file is publicly available at https://github.com/qijunz/Lucas_BA_paper. *C. scindens* genome sequence data have been deposited in NCBI under reference

sequence NZ_CP080442.1.

2.6 Supplemental files

FIG S2.1 Phylogenetic tree transformation summary of assessed bacterial isolates.
Strains are listed along with the types of BA transformations that they can perform. For strains listed with conjugation abilities, all types of amino acid conjugation were grouped together, i.e., each marked strain can conjugate a BA to either glycine or other amino acids (Table S2.4). Previous bioinformatic predictions of BA transformation capabilities (26, 27) are also indicated. Phylum information is color coded on the tree. The tree was constructed using publicly available genomes, see Materials and Methods.

FIG S2.2 Bile acid transformations across bacterial strains. The heat map shows BA transformations carried out by each isolate. The color scale denotes amounts of secondary BA produced at 24 (left) and 48 h (right). Bacteria were provided with 100 µM cholic acid (CA), chenodeoxycholic acid (CDCA), and deoxycholic acid (DCA), totaling 300 µM combined BAs. The detection limit was below 0.05 µM for all BAs. DCA was one of the added BAs; therefore, its production was not quantified in this experiment. Phyla information is indicated by the color-coded dashed lines in the phylogenetic tree on the left.
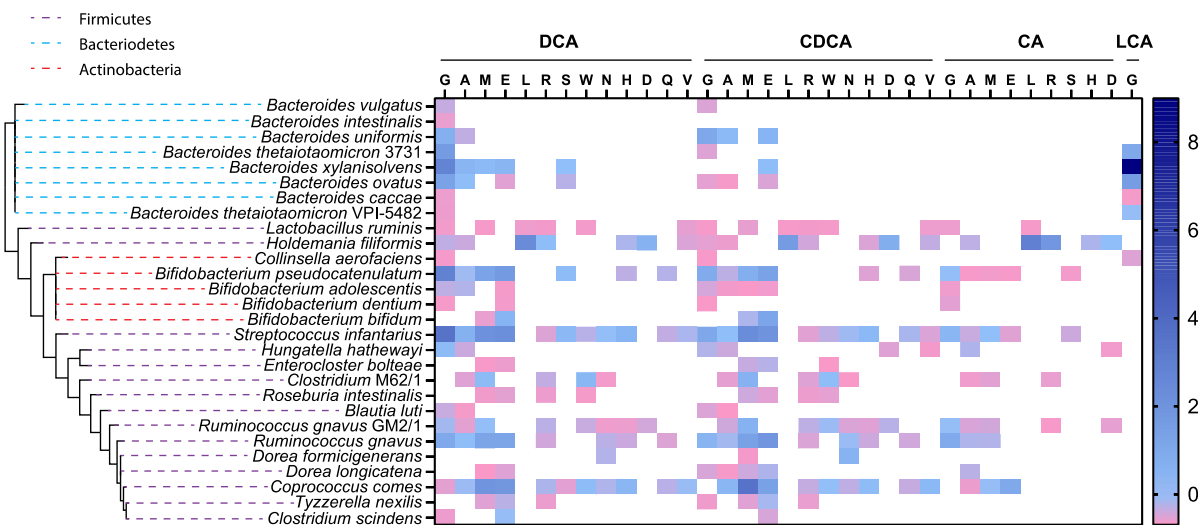
FIG S2.3 Production of amino acid-conjugated bile acids across bacterial strains. The heat map shows the BA-to-amino acid conjugations carried out by each isolate at 48 h. The data are presented as raw signal intensity normalized by Z-score, which is denoted by the color scale. Bacteria were provided with 100 μM cholic acid (CA), chenodeoxycholic acid (CDCA), or deoxycholic acid (DCA). Phyla information is indicated by the color-coded dashed lines in the phylogenetic tree on the left.
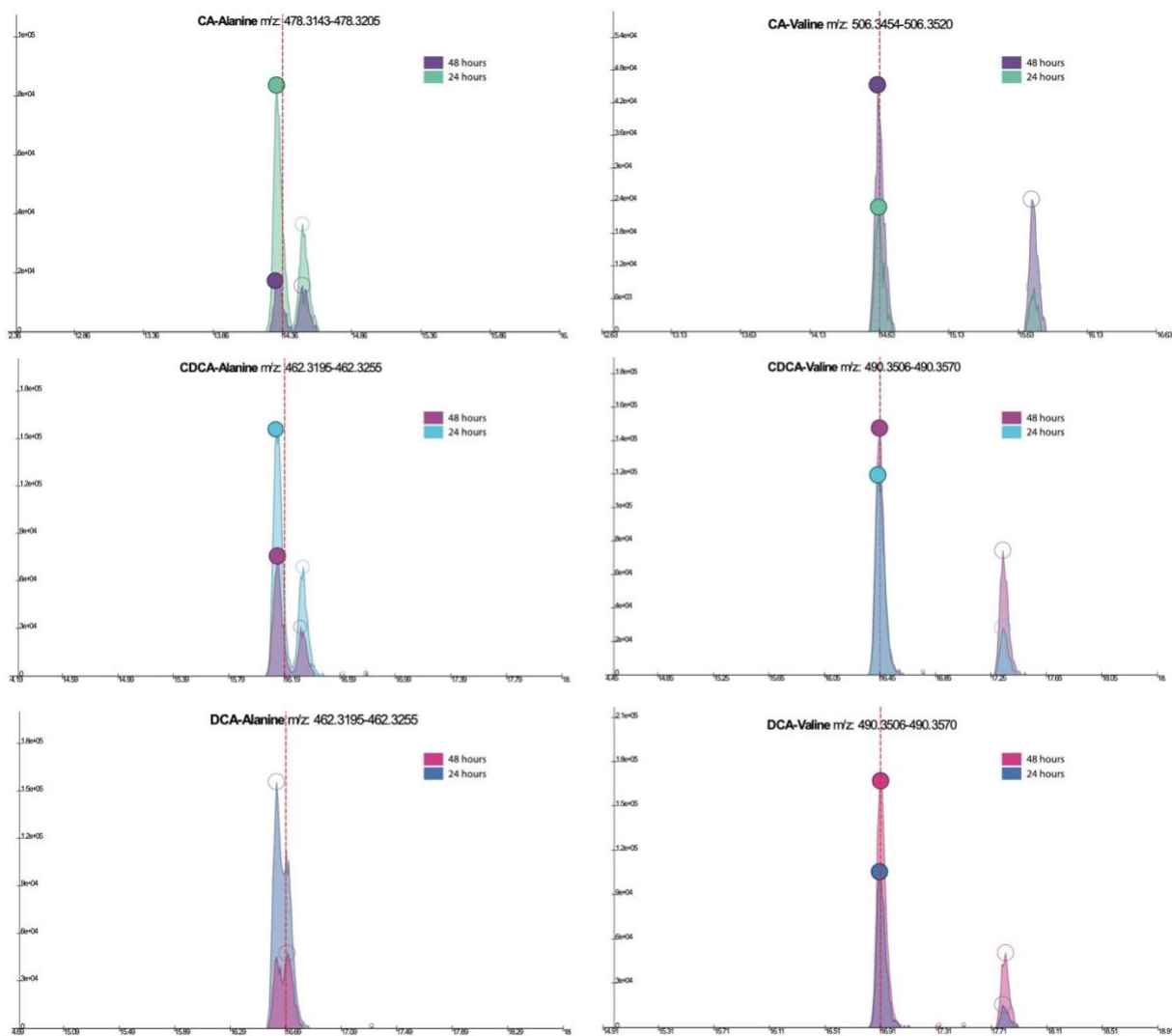
FIG S2.4 Extracted MAVEN files showing double peaks for amino acid conjugated bile acids. Raw files were exported to show peaks for suspected L- and D-enantiomers of alanine and valine conjugated to BAs.

2.7 Supplemental tables

Supplemental tables can be made available upon request to LLucas3@wisc.edu.

Table S2.1 – Bacterial strain names, ID number, and accession numbers.
Table S2.2 – Bile acid names and structure description for standards used for LC-MS/MS analysis.
Table S2.3 – Bile acid steroid core transformation data.
Table S2.4 – Amino acid conjugation data.
Table S2.5 – Retention time data and MS/MS fragmentation data for conjugated bile acids.
Table S2.6 – Comparison of observed bile acid transformation in this study and bioinformatic predictions of HSD activity.

2.8 Acknowledgements

2.9 References

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Chapter 3: Bile salt hydrolase activity from diverse human gut bacteria in monoculture and coculture

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3.1 Abstract

The human gut microbiota influences human physiology by chemically modifying bile acid (BA) steroid hormones. Conjugated BAs (CBA) and free BAs interact with human nuclear hormone receptors and G-protein coupled receptors in host tissues such as the liver and gastrointestinal tract, modulating lipid and glucose metabolism, energy expenditure, and drug metabolism. Knowledge regarding the breadth of BA transformations and BA transforming species is growing, but we still lack systematic analyses on large cohorts of gut bacteria. To complement our previous study on primary BA transformations, we investigated bile salt hydrolase (BSH) activity on CBAs by diverse human gut bacteria. BSH activity is widespread and at high levels: 51 of the 76 analyzed bacteria were able to deconjugate over 90% of at least one of the provided CBAs. Time series analyses revealed that some BSH are active during exponential phase while others are active during stationary phase. Coculture experiments demonstrate that BA transformations can be carried out sequentially by multiple species harboring unique enzymes and that BAs must be deconjugated before they can be further transformed, representing an ecological bottleneck. Our data support the idea that BSH active species may be ecological keystone species in the gut environment. This foundational

knowledge will strengthen our ability to build synthetic gut communities with predictive outputs, and understand causal connections between the microbiome, BA pool composition, and human health.

3.2 Introduction

The human gut microbiota plays a crucial role in human health and disease. One avenue by which gut bacteria mediate host physiology is via their ability to chemically transform bile acids (BAs), which has gained scientific interest in the last two decades due to their role in mediating host-microbiome interactions. BAs are amphipathic steroid hormones produced from cholesterol in the host liver that aid in fat and lipid absorption. BAs also influence host cholesterol and glucose levels, energy metabolism, and inflammation by interacting with various hormone receptors throughout the body (1). In addition, through BA toxicity, chemical transformation of BAs by bacteria represents a way that the gut microbiota can directly modulate its own composition.

Two primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized by the human liver and conjugated to either glycine or taurine in a two-step reaction by BA-CoA synthetase (BACS) and BA:CoA *N*-acyl transferase (BAAT) (2). Conjugated BAs (CBA) are stored in bile in the gallbladder (Fig 3.1). After a meal, bile is secreted into the common bile duct and the duodenum. CBAs travel through the acidic small intestine where some BA transformations occur, but most are reabsorbed and returned to the liver to circulate in a process known as enterohepatic circulation. The portion of CBAs that reach the large intestine are subject to deconjugation by BSHs and subsequent transformations by a variety of bacterial enzymes (Fig 3.1) (3, 4). In addition, BAs may undergo the recently discovered microbial conjugation of free BAs to glycine and other amino acids to produce microbially conjugated BAs,

or MCBAs (2, 5-7). The discovery that gut bacteria can conjugate BAs to glycine upends

historical BA dogma that considered glycine-conjugated BAs to be solely host-derived (6).
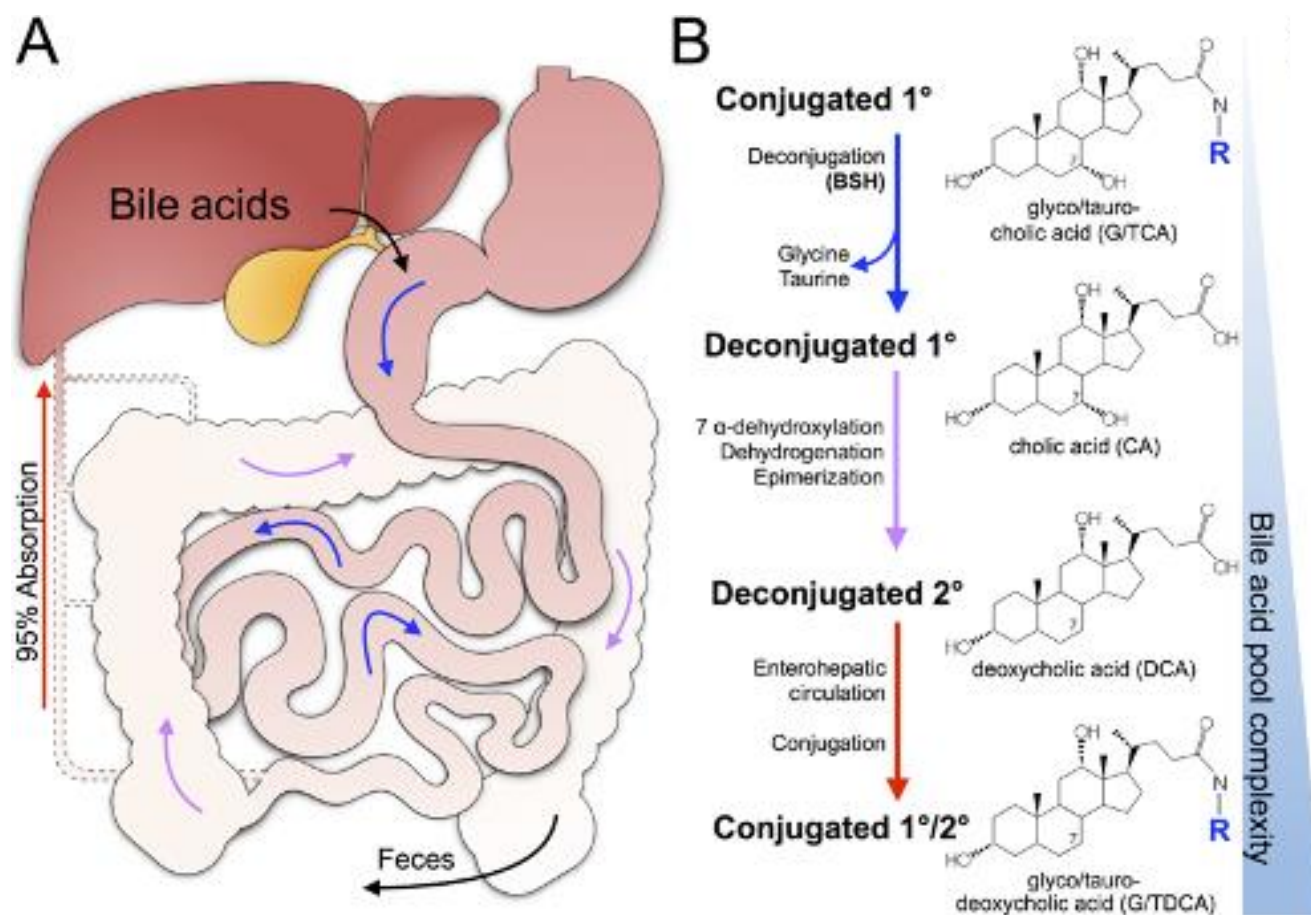
Fig 3.1 Bile acid deconjugation, transformation, and circulation. (A) BAs are synthesized in the liver and stored in the gallbladder before entering the small intestine through the duodenum where they reach millimolar concentrations. BAs are reabsorbed in the ileum and recirculated to the liver through the portal vein. The remaining are excreted in feces. Recirculating BAs access host tissues outside the intestines to impart systemic effects on host physiology. (B) BSHs cleave the amide bond in conjugated BAs to open up the BA pool to increased complexity. Conjugated primary BAs are deconjugated to primary BAs, which can be further transformed into secondary BAs, and then conjugated or not to be circulated or excreted (4).

Through bacterial BA modification, the four conjugated primary human BAs can be

transformed into a pool of hundreds of different BAs (Fig 3.1). BA modifications are important

because BA structure relates to its rate of reabsorption, affinity for hormone receptors, and toxicity to other microbes. For example, glycine- and taurine-conjugated BAs are more hydrophilic than their hydrophobic unconjugated BA counterparts, and thus are less toxic to gut bacteria than unconjugated BAs, more readily removed from the gastrointestinal (GI) tract by active transport, and more readily taken up by the liver during systemic circulation (8-10). As for hormone receptor affinity, CBAs, secondary BAs, and primary BAs all have differential agonist potencies for nuclear receptors, such as farnesoid X receptor (FXR), pregnane X receptor (PXR), and aryl hydrocarbon receptor (AHR) (2, 11). Thus, there is a relationship between an individual's gut microbiota, their BA pool, and their physiology.

Various explanations have been proposed for why bacteria can transform BAs. Primary and secondary 'free BAs' cause damage to cell membranes and chromosomal DNA, whereas CBAs require concentrations 10-200 times higher or more to exhibit the same antimicrobial effect (12). Microbial BA conjugation has also been suggested as a means of BA detoxification; however, when MCBAs were administered to several bacterial species, antimicrobial efficacy varied (7). It is plausible that gut microbiota members with auxotrophies and BSH activity can utilize released glycine and taurine residues as carbon, nitrogen, and sulfur sources (13). Ultimately, the role of BSH within the context of a gut microbial community remains poorly understood.

Bile salt hydrolases (BSHs) have been implicated in both positive and negative health outcomes in both humans and mice (1). BSH-*active* bacteria are reported to fight hypercholesterolemia (14) and non-alcoholic fatty liver disease (15). In addition, high levels of primary BAs due to high BSH activity, has been shown to stimulate the liver's accumulation of hepatic NKT cells and antitumor immunity in a mouse model (16). BSH activity has also been associated with colonization resistance to *Clostridium difficile* infection (17). However, in mice BSH inhibition has been reported to limit host weight gain on a high-fat diet and favor lipid

utilization instead of carbohydrates for energy (18). Therefore, inhibiting bacterial BSHs has been proposed as a method to treat metabolic diseases. Low BSH can also be a detriment though. Increased levels of CBAs, resulting from decreased BSH activity, are associated with inflammatory bowel disease (IBD) (19), Type 2 diabetes (20), and the development of cholangiocarcinoma (CCA), an often-fatal cancer of the biliary tract (21), and colorectal cancer (22). With such varied outcomes it's clear we need a broader understanding of gut bacterial BSH activity.

To provide a systematic understanding of BSH activity in human gut bacteria, we screened 76 species spanning 7 phyla for their ability to deconjugate, transform, and (re)conjugate the human conjugated BAs: taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA), and taurodeoxycholic acid (TDCA). Bacteria were provided with a cocktail of CBAs at two concentrations, each CBA at 100 μM or each CBA at 500 μM. We then compared selectivity for glycine and taurine conjugated primary BAs, which revealed phylogenetic and biochemical based patterns. For selected bacteria, we further analyzed BA deconjugation and transformation dynamics throughout their growth in monoculture and coculture, providing the first *in vitro* demonstration that gut bacteria are capable of consecutive BA transformations, which informs our understanding of BA dynamics in a community setting. In addition, we identify putative BSHs in all our sequenced gut microbes to compare with known motifs that confer specificity for glycine or taurine conjugated CBAs. Understanding the bacterial potential for BA deconjugation and transformation will provide a foundation for forming testable hypotheses to elucidate causal connections between the microbiome, BA pool composition, and human health.

3.3 Results

3.3.1 Bile salt hydrolase activity is widespread and variable

Seventy-six human gut bacteria from seven different phyla were tested for their ability to deconjugate a pool of the five of the most prevalent conjugated BAs (CBA) in humans, GCA, GCDCA, TCA, TCDCA, and TDCA. Each species was grown anaerobically, in duplicate, in colossal mega medium (CMM) until stationary phase with the pool at 100 µM and 500 µM of each CBA (Fig. 3.2).
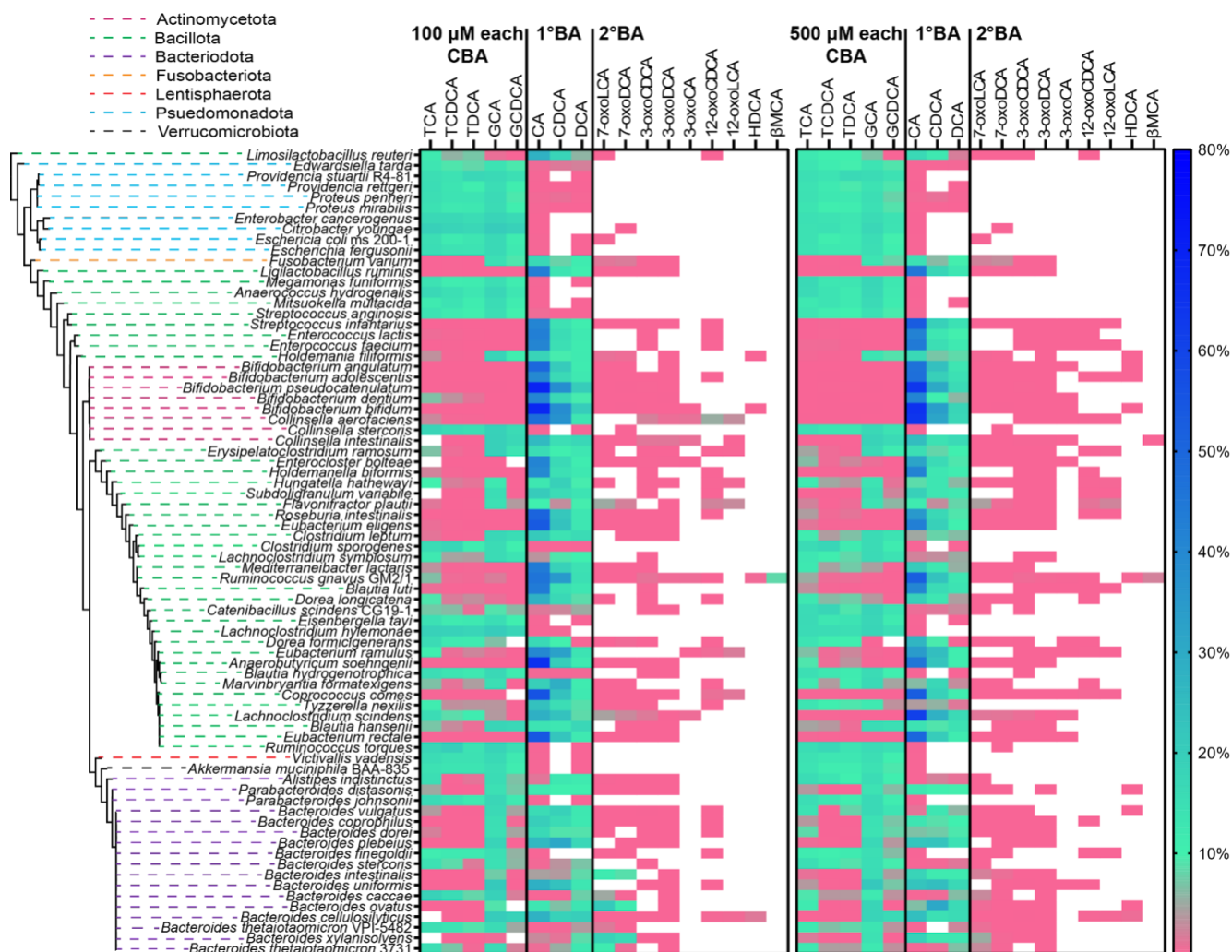
Fig 3.2 Deconjugation and transformation of conjugated bile acids when bacteria are supplied with 100 µM or 500 µM of each CBA. The heat map shows percent of BAs as it relates to the starting CBA concentration. Bacterial cultures were grown in duplicate in anaerobic Hungate tubes until stationary phase before they were sampled. Color scale depicts undetectable BAs in white, .008% in pink, 10% in green, and 80% as dark blue. Phyla information is indicated by color-coded dashed lines in the phylogenetic tree.

Consistent with previous findings, the highest levels of deconjugation were observed in the Actinomycetota, Bacillota, and Bacteroidota, and the lowest levels in the Pseudomonadota (Fig 3.2) (23-25). *Bifidobacterium* and *Enterococcus* species are widely known to possess the *bsh* gene, and as expected, exhibited high levels of deconjugating activity at both concentrations of CBA administered (2, 23, 25-31). *Fusobacterium varium* exhibited deconjugating activity on taurine-conjugated CBA, which was consistent with bioinformatic analyses that identified a BSH in the organism (23, 25). Unsurprisingly, the probiotic genera *Lactobacilli* had BSH activity, *L. ruminis* having deconjugated all CBAs, while *L. reuteri* appeared to prefer glycine-conjugated BAs (23, 25, 32, 33). We observed genus level variation in BSH activity amongst the *Bacteroides*, *Collinsella*, and *Streptococcus*, consistent with prior experimental and bioinformatic analyses (23-25, 34, 35). In addition, members of the Bacillota exhibit variation in BSH activity, with some genera having deconjugated all CBAs, while others did not deconjugate any CBAs (Fig 3.2). The highest levels of activity were in *Enterocloster bolteae* (5), *Roseburia intestinalis*, *Eubacterium eligens*, *Eubacterium rectale*, *Ruminoccocus* sp. GM2/1, *Dorea longicatena*, *Anaerobutryricym soehngenii* (36), *Coproccous comes* (37), and *Tyzzerella nexilis* (23, 25). Due to the important role of BSH in the gut, systematic analyses on BSH activity are invaluable to our understanding of the probiotic potential of gut bacteria. Further research is needed to understand what regulates BSH activity levels and specificity across diverse gut bacteria.

BSH are claimed to be gatekeepers of BA metabolism, initiating the rate-limiting step for subsequent BA transformations. However, only one study has experimentally assessed the potential for a single species to perform secondary transformations on conjugated and unconjugated BAs. They found that side chain conjugation prevented 7α-dehydroxylation, while free BAs could be dehydroxylated (3). Consistent with those findings, we see measurable secondary BAs produced only if a species can deconjugate CBAs to produce free CA, CDCA,

and/or DCA. The Actinomycetota, Bacillota, and Bacteroidota have both the highest levels of deconjugation and highest production of secondary BAs. More specifically, the Bacteroidota were the only phylum to produce high levels of secondary BAs, generating ~10% of the total provided CBAs into 7-oxoBAs (Fig 3.2). Interestingly, there is high variability in BSH activity within the Bacteroidota, and if BSH were not rate-limiting, based on prior studies, we would expect to see higher levels and higher frequencies of 7-oxo BA production, regardless of CBA deconjugation (6).

Bacteria were assessed for BA deconjugating and transforming activity at two concentrations to capture a range of potential BA concentrations bacteria would encounter in the gut environment. We did not anticipate that regardless of the initial CBA concentrations, bacteria would deconjugate and transform roughly the same percent of total provided CBAs (Fig. 3.2). For example, if a species can deconjugate 80 µM of TCA when provided 100 µM CBAs, one might assume that it would also deconjugate 80 µM of TCA when provided more substrate. Instead, we see that bacterial species are capable of deconjugating a percentage of total available BAs, regardless of initial concentration. At this point, we don't fully understand the mechanism regulating this phenomenon, but find the observation to be interesting, nonetheless.

Interestingly, not all administered BAs are recovered by our analysis. When we add up conjugated and unconjugated, primary and secondary supernatant BAs, we see that the Actinomycetota and Bacillota have high recovery rates, while the Bacteroidota have highly variable recovery rates (Fig. S3.1). Surprisingly, the Actinomycetota and Bacillota are the phyla that also produce MCBAs, whereas the Bacteroidota, which have lower recovery rates, do not produce relatively high levels of MCBAs. The fate of unrecovered BAs is unclear but could be due to BA loss to the cellular cytoplasm or bacterial membrane (38). Further studies should investigate the phylogenetic origin and mechanisms resulting in variations in BA recovery.

3.3.2 Bacterial conjugation to produce microbially conjugated bile acids

Recent studies have identified another mechanism for diversifying the BA pool, bacterial BA conjugation to produce microbially conjugated BAs (MCBAs) (5, 6). Using previously determined retention times, we identified a broad spectrum of amino acids to be conjugated to CA and DCA, with the most abundant conjugations to CDCA (Fig 3.3) (6). Glycine and taurine MCBAs could not be measured in this study because they were provided as a substrate for BA deconjugation and transformation. Although BSH have been shown to possess the aminoacyltransferase activity required to generate CBAs, not all BSH possessing strains produce MCBAs (Fig 3.3).

Fig 3.3 (Re)conjugation of bile acids at 100 µM. The heat map shows relative levels of MCBAs produced as denoted by z-score. Phyla information is indicated by color-coded dashed lines in the phylogenetic tree.

The Bacillota and Actinomycetota had the highest levels of MCBA production, while Bacteroidota produced fewer and less diverse MCBAs (Fig 3.3). Surprisingly, we observed some MCBA production in the Pseudomonadota, a phylum previously unidentified to perform (re)conjugation. The species with the highest levels and most broad conjugating capabilities

were *Streptococcus infantarius, Bifidobacterium bifidum, Enterocloster bolteae, Rumminococcus* sp. GM2/1, *Coproccocus comes*, and *Eubacterium rectale.* Our findings are supported by other studies on MCBA production (6, 39). *Ruminoccocus* species and *Bifidobacterium dentium* were also identified as robust conjugators in previous studies (40). Consistent with prior observations, *Akkermansia muciniphila* (Verrucomicrobiota), was not able to microbially conjugate BAs to amino acids.

MCBAs are known to interact with the host hormone receptors FXR, VDR, CAR, PXR, and AHR (2) and they may play a role in resistance against colonization by *C. difficile* (17). Further studies are needed to understand the role of MCBAs for both bacteria and the host in the context of the gut.

3.3.3 Bile salt hydrolase specificity

There is growing appreciation for the role that BSH substrate specificity can play for its potential influence on disease outcomes (17, 18, 41). However, few studies have attempted to delineate BSH specificity in bacterial culture (32, 33, 41). To this end, we plotted the amount of GCA that was converted against the amount of TCA that was converted at both concentrations (Fig 3.4). The same analysis was performed on GCDCA and TCDCA. Deoxycholic acids were left out of this analysis because we did not provide the less biologically relevant GDCA in our experiments.
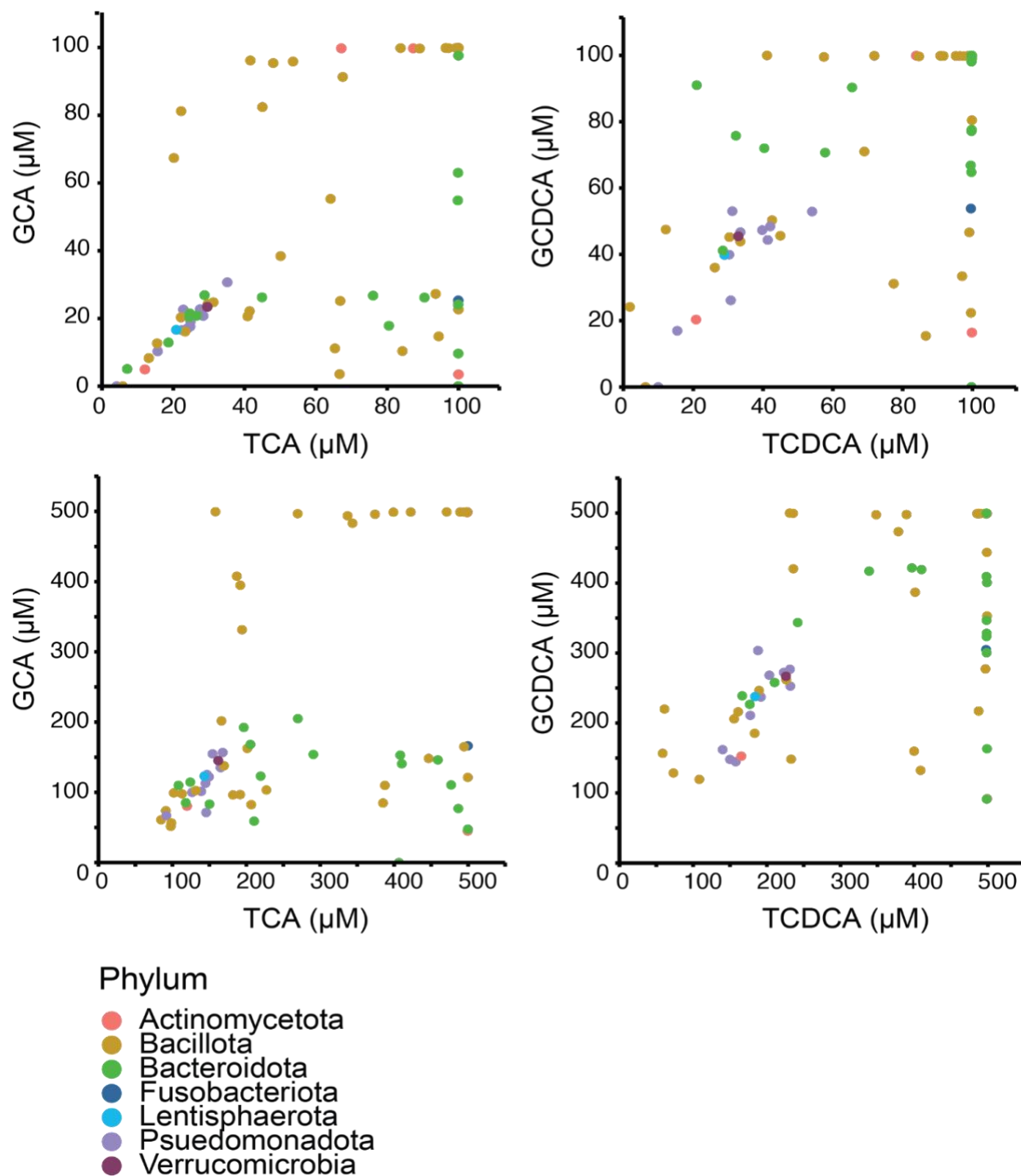
Fig 3.4 Bacterial specificity for glycine- compared to taurine-conjugated bile acids. On the left, GCA and TCA are plotted against each other for each strain at both concentrations. On the right, GCDCA and TCDCA are plotted against each other for each strain at both concentrations. Individual dots represent individual species and their ability to deconjugate CBAs.

Our comparison of BSH activity revealed several interesting trends, both phylogenetically and biochemically. The Actinomycetota are mostly located in the top right corner at (100 μM, 100 μM) and (500 μM, 500 μM) of each graph, due to their high levels of activity on both glycine- and taurine-conjugated BAs (Fig 3.4). Of the Bifidobacteriaceae, *Bifidobacterium dentium* is the only exception, and it exhibits G-BSH preference under 100 μM conditions. *Collinsella intestinalis* also favors taurine-conjugated BAs, while *Collinsella stercoris* has a less active BSH overall. At both concentrations, the Bacteriodota prefer cholic acids conjugated to taurine, and showed more variable preference for conjugated CDCA. This observation is consistent with findings of taurine-specific paralogues in *Parabacteroides distasonis*, *Bacteroides vulgatus*, and *Bacteroides uniformis* (34). Bacillota can be found throughout each grid, clustering differently for cholic acids than chenodeoxycholic acids, suggesting the role of steroid core structure in specificity (Fig 3.4). Finally, *Fusobacterium varium* consistently deconjugates taurine-conjugated BAs completely, while leaving between 20-65% of glycine-conjugated BAs intact.

Interestingly, for species with lower levels of transformation, BSH activity appears to be less specific. The Pseudomonadota, Lentisphaerota, and Verrucomicrobia do not exhibit specificity for CBAs. In addition, visualizing the data in this way allows us to better visualize the interesting and surprising observation that the patterns in ratios of BAs deconjugated were consistent across concentration. At this point, we don't fully understand the mechanism for this regulation, but we find it interesting nonetheless because BSH may experience a wide range of BA concentrations along the gut axis or at different times of the day. Further research should be performed to understand what governs BSH specificity, whether it be related to environmental conditions, enzymatic activity, or another form of regulation. Altogether these findings support the idea that specificity may be based on the amino acid conjugate or core BA structure (18, 42).

3.3.4 Bioinformatic search of bile salt hydrolase reveals motifs associated with specificity

To continue our analysis of *bsh*, we compiled genomes for all 76 species in this study and identified their Bsh protein sequences from the Refseq NCBI database using the keywords "bile salt hydrolase" and "cholylglycine hydrolase". For lab isolates, we used genomes from the most closely related species (Table S3.1). Bsh proteins were identified in screened bacterial genomes by BLASTP using the genus specific cutoff of sequence identity of greater than 30%, which was calculated based on pairwise sequence identity between and within genera. To increase BSH identification accuracy, the number of amino acids in each protein sequence was limited to 300 to 400 (PMID: 30674356). To retain conserved domain features in BSH gene sequences, the BLASTp hit genes were mapped to Bsh HMM profiles, built using HMMER3 (PMID: 22039361). To build our HMM, we also used 18 Lactobacillaceae BSHs that were recently associated with specificity (17). We then searched for BSH substrate preferences for either glycine or taurine-conjugated BAs in the identified BSH gene (PMID: 36914755) (Fig 3.5).

Fig 3.5. Amino acid motifs associated with BSH specificity. Alignment of BSH genes and their specificity motifs identified by the HMM. BSHs motifs for taurine specificity are listed in light blue and motifs for glycine specificity are listed in red.

Our analysis revealed that Bsh specificity could be accurately predicted in many phyla, except for the Actinomycetota (Table 3.1). Of our 76 species, 38 were predicted to have Bsh. Thirty-two species had one Bsh, while six species had two (Fig 3.5). We found specificity could be predicted based on amino acid motifs. There were 21 proteins predicted as taurine-preferring BSH with the motif "G-X-G" (X=A/H/Q) and 22 proteins predicted as glycine-preferring BSH with the motif "S-R-X" (X=G). Of these, approximately half of the species exhibited their predicted specificities. A prior analysis on the Lactobacillaceae observed taurine-preferring BSH to have the motif "G-X-G" but with X=T/V and glycine-preferring enzymes to have "S-R-X" (X=G/S) (41).

These findings are particularly interesting because the Foley et al. paper used only BSH from

the Lactobacillaceae to perform their analysis, but the motifs seem to be conserved across

phyla. Our evidence suggests that specificity motifs are highly conserved across evolutionarily

related, but phylogenetically distinct BSH.

Table 3.1 Agreement between *in silico* analysis and *in vitro* observations. BSH specificity
was determined by finding the difference in BAs deconjugation for cholic acid and
chenodeoxycholic acids. The average difference in BA deconjugation was determined and for
species deconjugations beyond one standard deviation away from the average are highlighted
in red for taurine specificity and in green for glycine specificity. The motifs identified by our
analysis are in column 6 and agreement is listed in column 7.

Strain name	Difference in BA deconjugation				Motif	Agreement?
Limosilactobacillus reuteri	-54.6	-28.0	-137.4	-264.0	SRG	yes
Providencia rettgeri	0.2	-13.2	-0.4	-64.9	DSG	yes
Proteus penneri	5.7	-21.8	21.5	-115.6	DSG/DSG	yes
Proteus mirabilis	7.7	-7.5	30.0	-45.4	DSG	yes
Fusobacterium varium	74.6	45.7	333.3	192.7	GAG	yes
Ligilactobacillus ruminis	0.1	-0.4	0.3	-2.4	TRG	yes
Streptococcus infantarius	0.1	-0.2	-0.5	-3.5	SRG	no
Enterococcus lactis	-2.7	-1.0	-0.5	-3.4	SRG	no
Enterococcus faecium	0.0	-0.5	-9.9	-10.4	SRG/SRG	no
Holdemania filiformis	73.9	52.5	329.5	219.4	n/a	no
Bifidobacterium angulatum	0.1	-0.1	0.2	-0.7	GAG	no
Bifidobacterium adolescentis	0.1	-0.1	0.2	-1.2	GAG	no
Bifidobacterium pseudocatenulatum	0.2	-0.1	0.3	-1.1	GAG	no
Bifidobacterium dentium	-32.6	-16.1	0.0	-1.1	GAG	no
Bifidobacterium bifidum	0.1	-0.1	0.2	-0.7	GAG	no
Collinsella aerofaciens	-12.5	-1.2	-0.6	-0.7	GAG	no
Collinsella intestinalis	96.5	83.4	454.4	407.0	n/a	no
Erysipelatoclostridium ramosum	63.1	63.6	300.1	270.3	n/a	no
Lachnoclostridium bolteae	0.1	-0.2	-139.6	-12.3	GQG/SRG	yes

Holdemanella biformis	-10.6	-0.7	-0.4	-2.5	SRG	no
Hungatella hathewayi	11.7	-9.1	38.8	-95.0	GQG	no
Subdoligranulum variabile	77.4	0.2	378.3	54.8	GQG	yes
Flavonifractor plautii	66.3	19.4	298.3	145.8	FSG	yes
Roseburia intestinalis	0.0	-0.3	-100.0	-9.1	SRG/SRG	yes
Eubacterium eligens	-3.4	-0.7	-28.3	-3.1	SRG/SRG	no
Clostridium leptum	79.7	77.2	123.9	240.1	FSG	yes
Clostridium sporogenes	1.8	-35.3	2.8	-158.9	SQG	yes
Mediterraneibacter lactaris	-16.1	-0.5	-202.9	-108.4	SRG	yes
Ruminococcus gnavus GM2/1	-23.8	-2.4	-121.9	-12.4	SRG	yes
Blautia luti	-3.4	-0.4	-76.7	-3.7	SRG/SRG	no
Dorea longicatena	-42.3	-8.1	-220.6	-149.2	SRG	yes
Catenibacillus scindens CG19-1	8.8	-2.0	46.1	-98.1	AQG	yes
Dorea formicigenerans	-47.2	-58.8	-341.4	-269.0	n/a	no
Eubacterium ramulus	-47.4	-3.4	-156.9	-11.7	SRG	yes
Anaerobutyricum soehngenii	-0.4	-0.2	-227.3	-14.0	SRG/SRG	yes
Blautia hydrogenotrophica	7.2	-10.4	29.2	-57.1	GQG/FSG	no
Marvinbryantia formatexigens	41.6	-4.6	124.2	14.4	SRG	no
Coprococcus comes	-0.6	-0.2	-1.4	-1.8	SRG	no
Tyzzerella nexilis	-37.4	-15.0	-35.4	-184.5	SRG	yes
Lachnoclostridium scindens	-59.0	-42.1	-3.0	-3.7	SRG	yes
Blautia hansenii	54.2	71.2	277.5	276.2	n/a	no
Eubacterium rectale	0.0	-0.1	-4.5	-2.2	SRG	no
Alistipes indistinctus	3.1	32.7	-1.7	-72.3	GHG/GAG	no
Parabacteroides distasonis	49.3	34.9	255.4	151.5	GAG	yes
Bacteroides vulgatus	62.6	22.2	66.9	335.6	GAG	yes
Bacteroides coprophilus	75.8	1.6	269.6	170.2	GAG	yes
Bacteroides dorei	64.3	22.6	136.7	197.9	GAG	yes
Bacteroides plebeius	90.3	-0.1	409.8	88.7	GAG	yes

Bacteroides intestinalis	36.8	0.7	366.4	98.1	GAG	yes
Bacteroides uniformis	99.9	99.8	406.5	174.5	GSG	yes
Bacteroides ovatus	45.0	-0.2	151.6	-1.3	GGN	yes
Bacteroides cellulosilyticus	100.0	99.7	452.0	407.1	n/a	no
Bacteroides xylanisolvens	2.4	-0.2	312.9	-1.4	GGN	yes

In general, Fusobacteriota, Bacteroidota, and Actinomycetota have only taurine-preferring BSH, while Bacillota have glycine-preferring BSH (Fig 3.5). *Lachnoclostridium bolteae* has both taurine- and glycine-preferring BSH (Fig 3.5). Consistent with our predictions, *Fusobacterium varium*, *Parabacteroides distasonis*, and *Subdolingranulum variable* have preference for taurine-conjugated BAs, and *Limosilactobacillus reuteri*, *Clostridium sporogenes*, *Dorea longicatena*, *Eubacterium ramulus*, *Tyzzerella nexilis*, and *Lachnoclostridium scindens* exhibit high preference for glycine conjugated BAs (Fig 3.2, Table 3.1). *Bacteroides* species largely prefer taurine conjugated BAs, as predicted by the *in silico* analysis and observed *in vitro*. In addition to the G-A-G motif associated with taurine specificity, we observe G-S-G in *Bacteroides uniformis* to also confer taurine specificity. Interestingly, Bsh from *Bacteroides finegoldii*, *Bacteroides caccae*, and *Bacteroides thetaiotaomicron* VPI-5482, which have slight preference for GCDCA over TCDCA, were not identified by this analysis, and *Bacteroides ovatus* and *Bacteroides xylanisolvens*, with motifs G-G-N, did not exhibit strong specificity.

Inconsistent with the predictions, *Bifidobacteria* did not exhibit preference for taurine conjugated BAs, and deconjugated all provided BAs at both concentrations. In addition, *Streptococcus infantarius*, *Holdemanella biformis*, *Eubacterium eligens*, *Blautia luti*, and *Tyzzerella nexilis* were predicted to have glycine preference, but have high levels of indiscriminate deconjugating activity. Finally, *Holdemania filiformis*, *Collinsella intestinalis*, and *Erysipelatoclostridium ramosum* possess BSH that are highly taurine specific and were

unidentified by this analysis, suggesting a different evolutionary lineage of those Bsh enzymes (Table 3.1).

Overall, this analysis predicted glycine and taurine specificity very well. Some discrepancies between *in vitro* and *in silico* analyses are to be expected due to limitations of homologous protein searches and HMMs based on known protein sequences. It is likely that some species possess unidentified Bsh or other proteins capable of deconjugation. Further research should use experimentally validated BSH to guide bioinformatic analyses on glycine and taurine specificity.

3.3.5 Coculture experiments reveal bile salt hydrolase impact on bile acid pool

BA dogma states that consecutive transformations may be carried out by multiple species harboring different BA transforming enzymes (13, 23). However, to our knowledge, that claim has not been validated experimentally. Our goal was to understand the potential role of BSH in a more ecologically relevant setting. To do so, we identified two species with varying levels of BSH activity, *Bifidobacterium angulatum* and *Clostridium symbiosum*, and we identified a species with limited BSH activity, but high secondary BA production *Bacteroides thetaiotaomicron* VPI-5482. By pairing these organisms together, we were able to visualize and compare BA deconjugation and subsequent transformation dynamics when only BSH activity levels are altered (Fig 3.6).

A.
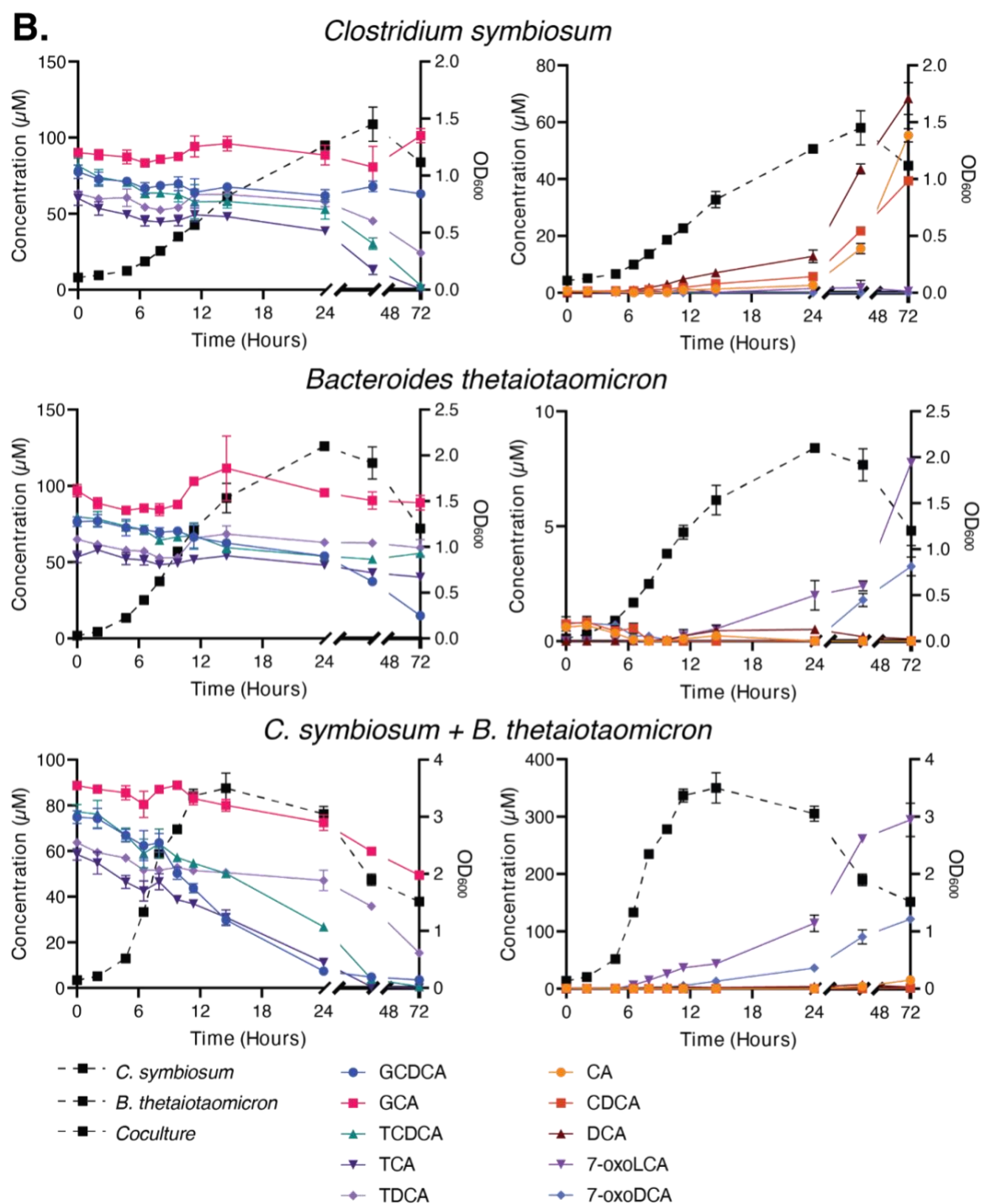
Fig 3.6 Bile salt hydrolase dynamics in coculture. Strains with differing BA transforming capabilities were grown individually and in coculture and sampled over the course of 72 hours. A) *B. angulatum*, a highly active BSH strain, and *B. thetaiotaomicron*, which has limited BSH and high secondary BA production, were grown in monoculture and coculture. B) *C. symbiosum*, a moderately active BSH strain, and *B. thetaiotaomicron*, which has limited BSH and high secondary BA production, were grown in monoculture and coculture.

While our systematic assessment of BSH activity in gut bacteria provides abundant information about numerous strains, with only a single time point of sampling, it is impossible to draw conclusions about how these bacteria behave in their natural environment. Through time-course analyses in monoculture and coculture, we reveal differences in deconjugation activity, primary BA production, and secondary BA production when only BSH activity is modulated.

When *B. angulatum* is grown alone, during exponential phase, it rapidly deconjugates all provided CBAs and produces measurable amounts of the three freed BAs in our study, CA, CDCA, and DCA (Fig 3.6A). This observation is in agreement with a prior study on the growth phase dependence of BSH activity in *Bifidobacterium longum* (43). In contrast, when *C. symbiosum* is grown alone, it deconjugates taurine-conjugated BAs, and only after 24 hours, when it's close to stationary phase (Fig 3.6B). Due to its late deconjugation, free BAs are not released until after 24 hours as well. Under our conditions, multiple time-course analyses of *B. thetaiotaomicron* revealed that BSH activity was limited to deconjugation of GCDCA after 24 hours, or once in stationary phase (Fig 3.6). Because *B. thetaiotaomicron* has a very active hydroxysteroid dehydrogenase (HSD), it quickly oxidizes any free BAs into 7-oxoBAs (Fig 3.6). Stationary phase expression of BSH has been reported in *Bacteroides fragilis*, but has not been previously reported in the *Clostridium* (13).

By coculturing these species, we were able to delineate the impact of BSH activity on secondary BA production. When *B. thetaiotaomicron* is present, it produces 7-oxo BAs from any free BAs, whether that be during exponential growth or in stationary phase (Fig 3.6). Therefore, the pool of secondary BAs that can be produced is limited by the BSH capacity of the deconjugating organism. With *B. angulatum*, an exponential phase, high activity BSH possessing strain, an appreciable amount of CA and CDCA are observed, whereas with *C. symboisum*, the rate at which free BAs are produced is overwhelmed by the rate at which 7-oxo BAs are produced. Interestingly, when *C. symbiosum* and *B. thetaiotaomicron* are grown in

coculture, they reach stationary phase more quickly, and also deconjugate CBAs more quickly than either strain grown in monoculture. Further analyses will need to be performed to understand how growth patterns of these strains were altered when grown in coculture.

Our coculture experiments also revealed interesting dynamics for MCBAs (Fig S3.2). When *B. thetaiotaomicron* is grown in monoculture, it produces measurable levels of glycine-conjugated 7-oxoBAs after 24 hours, but when it's grown with *B. angulatum* MCBAs are not detected throughout growth. In contrast, when *B. thetaiotaomicron* is grown in coculture with *C. symbiosum*, glycine and taurine conjugated 7-oxoBAs are measured throughout the time series (Fig S3.2). These observations suggest that BSH are responsible for deconjugation of MCBAs as well as for their production.

Altogether, these data show that BA deconjugation and transformation may be carried out by multiple species harboring unique enzymatic capabilities. In addition, we observed differences in rates of BSH activity, suggesting that each BSH may respond to different cues in their environments. For example, bacteria may respond to types of BA pools or different lengths of exposure to BAs, which could ensure maximal survival under dynamic environmental conditions (42). We also demonstrate data for the first time that suggests BSH are responsible for deconjugation of MCBAs.

3.4 Conclusion

In this study, we showcase BSH/T activity across diverse phyla of human gut bacteria, we elucidate patterns in BSH specificity, both *in vitro* and *in silico*, and we demonstrate the impact of variations in BSH activity on the BA pool in bacterial coculture. In addition, we continued our survey of bacterial conjugation of BAs to amino acids to produce MCBAs. Altogether, our findings contribute to scientific understanding of how the gut microbiota impact host physiology through BA transformation. Due to the prominent role of BA metabolism in

human health and disease, continued research to discover all BAs and their modifications found in humans is needed. Continued understanding of BA metabolism will aid our ability to develop BA-mediated health interventions.

3.5 Materials and methods

3.5.1 Strains.

All strains are listed in Table S3.1. Most strains are previously sequenced and come from the Human Microbiome Project. Further information for strains isolated in our lab: Strain "1RE7" was isolated from an anerobic enrichment in medium supplemented with rutin, inoculated with a human fecal sample. The strain consumes both rutin and quercetin. The sequence of the full-length 16S gene is 96% identical to that of *Catenibacillus scindens* CG19-1. Strain "J02" was isolated from an anerobic enrichment in medium supplemented with rutin, inoculated with a human fecal sample (WLS #82). The sequence of the 16S gene is >99% identical to that of *Eisenbergella tayi* strain B086562 (783/784 bases match). Strain "K01" was isolated from an anerobic enrichment in medium supplemented with rutin, inoculated with a human fecal sample. The sequence of the 16S gene is >99% identical to that of *Enterococcus durans* JCM8725 (900/901 bases match) and similarly matches many *Enterococcus faecium* strains (902/903 bases match). A full-length 16S gene sequence might be more definitive. Strain "J01" was isolated from an anerobic enrichment in medium supplemented with quercetin, inoculated with a human fecal sample. The sequence of the 16S gene is >99% identical to that of several *Enterococcus* species (*lactis, durans, faecium)* all with 870/871 bases matching. A full-length 16S gene sequence might be more definitive. Strain "K02" was isolated from an anerobic enrichment in medium supplemented with rutin, inoculated with a human fecal sample (WLS #10). The sequence of the 16S gene is >100% identical to multiple *Proteus mirabilis* strains (823/823 bases match). Strain "L02" was isolated from an anerobic enrichment in medium supplemented with

quercetin, inoculated with a human fecal sample. The sequence of the 16S gene is >99% identical to that of several *Streptococcus anginosis* strains (854/856 bases match).

3.5.2 Media.

For the systematic BSH analysis, all strains were grown on Colossal Mega Medium, which was filter-sterilized and stored in a Coy anaerobic chamber (5% H_2, 20% CO_2, and 75% N_2) at least 24 hours prior to use. Colossal Mega Medium contains (per liter tap distilled water): 100 mL (1M, pH 7.2) potassium phosphate buffer, 10 g tryptone peptone, 5 g yeast extract, 5 g meat extract, 4 mL (25 mg/100 mL) Resazurin, 1.8 g D-glucose, 0.9 g D-maltose, 0.86 g D-cellobiose, 0.46 g D-fructose, 2 g sodium acetate trihydrate, 0.02 g $MgSO_4 \cdot 7\ H_2O$, 2.1 g $NaHCO_3$, 0.08 g NaCl, 1 mL (0.8g/100mL) $CaCl_2$, 1 mL (1 mg/mL in 100% ethanol) vitamin K_3 (menadione), 1 mL (1.2mg hematin/mL in .2M histidine, pH 8.0) histidine hematin, 2 mL (25% vol/vol) Tween 80, 10 mL ATCC MD-VS vitamin mix, 10 mL ATCC MD-TMS trace mineral mix, 1 mL (40mg/100mL) $FeSO_4 \cdot 7\ H_2O$, and 0.5 g L-cysteine·HCL. This specific medium was designed to allow growth for all species in this study. Additions and modifications for specific strains were as follows: For cultures of *Akkermansia muciniphila* the medium was amended with 1 mg/ml mucin. For cultures of *Clostridium orbiscindens* the medium was amended with lysine.

For time-course cocultures, all strains were grown on Low Yeast Extract Medium, which was made anaerobic using a triple-vacuumed pressure bottle before being brought into a Coy anaerobic chamber (5% H_2, 20% CO_2, and 75% N_2), and then filter sterilized. Low yeast medium contains (500 ml Milli-Q water): 50 ml (1 M, pH 7.0) potassium phosphate buffer, 0.36 g tricine, 2.0 ml (0.025%) resazurin, 1 g yeast extract, 0.5 ml (25% [vol/vol]) tween 80, 3.4 g sodium acetate trihydrate (FW 136), 0.55 g sodium succinate hexahydrate (FW 270), 1.46 g sodium chloride (FW 58.44), 0.54 g ammonium Chloride (FW 53.49), 3.6 g d-glucose (FW 180.16), 1.8 g d-maltose

(FW 360.3), 1.0 ml (0.5 M) potassium sulfate, 1.0 ml (1.0 M) magnesium chloride hexahydrate (MgCl$_2$. 6H$_2$O), 0.2 ml (1.0M) calcium chloride dihydrate (CaCl$_2$.2H$_2$O), 1.68 g sodium bicarbonate (FW 84.0), 0.5 ml (1.2 mg hematin/ml in 0.2 M histidine, pH 8.0) histidine hematin solution, 0.125ml vitamin K1+ K3 solution (used "2x" stock), 10 ml ATCC MD-VS vitamin mix, 5 ml 50x trace mineral mix solution [0.29 ml (30 µM) MnCl2.4H$_2$O, 0.06 ml (10 µM) ZnCl$_2$, 0.047 ml (4 µM) CoCl2.6H$_2$O, 0.012 ml (1 µM) Na$_2$MoO4.2H$_2$O, 0.008 ml (1 µM) Na$_2$SeO$_3$, 0.059 ml (5 µM) NiCl$_2$.6H$_2$O, 0.016 ml (1 µM) Na$_2$WO$_4$.2H$_2$O, adjust volume to 1 L, store under N$_2$, refrigerated], 1 ml ferrous sulfate heptahydrate (FeSO4·7H2O), and 0.25 g l-cysteine HCl. Adjust pH to ~7.3-7.1.

3.5.3 Sample handling and growth conditions.

For BSH systematic analysis, strains were grown at 37° C in an anaerobic chamber with an atmosphere of 75% N$_2$, 20% CO$_2$, 5% H$_2$. Starting from freezer stocks, strains were first grown overnight to a high density (O.D. 600 range of .349-1.9, measured directly in the tube) in Hungate tubes containing Colossal Mega Medium. These cultures were then used to inoculate (1:15 dilution) 3mL of Colossal Mega Medium in Hungate tubes amended with BAs. There were 2 sets of conditions; media contained 100 µM or 500 µM of each of the five conjugated BAs, glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), and deoxycholic acid (DCA). In addition, we tested for spontaneous BA degradation or transformation in uninoculated controls containing BAs. Once cultures reached stationary phase, 1 mL of culture was collected, spun down at room temperature for 10 minutes at 10,000 g, and the supernatant transferred to a fresh tube. Using uHPLC-grade H$_2$O, the supernatants were diluted 1:200 or 1:1000 for the 100 µM or 500 µM conditions, respectively. After dilution, 100 µL were transferred to an HPLC vial for analysis.

For the monoculture and coculture time course analyses, individual freezer stocks were inoculated into Colossal Mega Medium (CMM) and grown overnight to a high bacterial density (OD_{600} > 2). The following day, multiple dilutions were made for each culture in LYE medium containing 0.1% yeast extract and allowed to grow overnight. Cultures in exponential phase were then used as inoculum for monoculture and coculture experiments. Growth curves were performed in Hungate tubes containing 10 mL of 0.1% LYE medium amended with 100 μM each of GCA, GCDCA, TCA, TCDCA, and TDCA. Monoculture starting ODs were 0.05 and cocultures were started with an equal proportion of both monocultures (total OD being ~ 0.1). For *B. thetaiotaomicron* and *C. symbiosum*, monocultures starting ODs were 0.03 and 0.12, respectively, with the coculture OD at ~ 0.15. Uninoculated controls containing BAs were used to assess spontaneous BA degradation and transformation. All experiments were performed in triplicates. Samples were drawn at multiple timepoints based on growth pattern: rigorous sampling was done during exponential phase (7-8 timepoints) and 4-5 timepoints were included in stationary phase. 0.3 mL of culture was collected at each timepoint and spun down, and the supernatant was diluted at 1:100 using HPLC-grade H_2O for analysis by HPLC-MS.

3.5.4 uHPLC-MS/MS measurements.

Samples were analyzed using an ultra-high pressure liquid chromatography-tandem mass spectrometry (uHPLC-MS/MS) system consisting of a ThermoScientific Vanquish uHPLC system coupled to a heated electrospray ionization (HESI; using negative polarity) and hybrid quadrupole high resolution mass spectrometer (Q Exactive Orbitrap; Thermo Scientific). Settings for the ion source were: auxiliary gas flow rate of 10, sheath gas flow rate of 30, sweep gas flow rate of 1, 2.5 kV spray voltage, 320°C capillary temperature, 300°C heater temperature, and S-lens RF level of 50. Nitrogen was used as nebulizing gas by the ion trap source. Liquid chromatography

(LC) separation was achieved using a Waters Acquity UPLC BEH C18 column with 1.7 μm particle size, 2.1 x 100 mm in length. Solvent A was water with 10 mM ammonium acetate adjusted to pH 6.0 with acetic acid. Solvent B was 100% methanol. The total run time was 31.5 min with the following gradient: a 0 to 24 min gradient from 30% solvent B (initial condition) to 100% solvent B; held 5 min at 100% solvent B; dropped to 30% solvent B for 2.5 min re-equilibration to initial condition. The flow rate was 200 μL/min throughout. Other LC parameters were as follows: autosampler temperature, 4°C; injection volume, 10 μL; column temperature 50°C. The MS method performed a full MS1 full-scan (290 to 1000 m/z) together with a series of PRM (parallel reaction monitoring) scans.

3.5.5 Determination of bile acid concentrations.

BA quantitation was achieved using standard concentrations of each BA ranging from .0625 to 2 μM to generate six-point external standard curves. The detection limit was below 0.01 μM for all BAs. The threshold for reported core BA transformations was 0.1 μM. Standards were purchased from Avanti Polar Lipids and dissolved and stored in methanol at -80 °C. For BAs conjugated to amino acids, compounds were identified by their exact mass (mass error of less than 2 parts per million) and previously determined retention times but could not be quantified since standards are not commercially available. Production of BAs conjugated to glycine or taurine (i.e., G/T-CA, G/T-CDCA and TDCA) could not be quantified because they were administered to all microbes in the media.

3.5.6 *In silico* analysis.

Access the genome sequence in NCBI for 76 bacteria strains. Get all CDS genes from the 76 available genomes (amino acid sequences). Use curated 84 BSH genes including 18 Lactobacillaceae BSHs in Foley et al. paper (PMID: 36914755) as BSH reference database for BLASTp. Use the same 84 BSH genes to build the HMM (Hidden Markov Model) to reserve BSH conserved domains when predicting BSH genes. Use the following criteria to determine the BSH from all CDS: gene length between 300 to 400 bp; has at least one BLASTp to BSH reference genes (identity > 25%); has hit to BSH HMM (full sequence score > 100). Clustal Omega was used for multiple alignment of predicted BSH genes, taurine- or glycine- preferring BSH was predicted by a 3-residue selectivity loop: taurine-preferring BSH contain 'G-X-G' motif and glycine-preferring BSH contain 'S-R-X' motif.

3.6 Supplemental figures
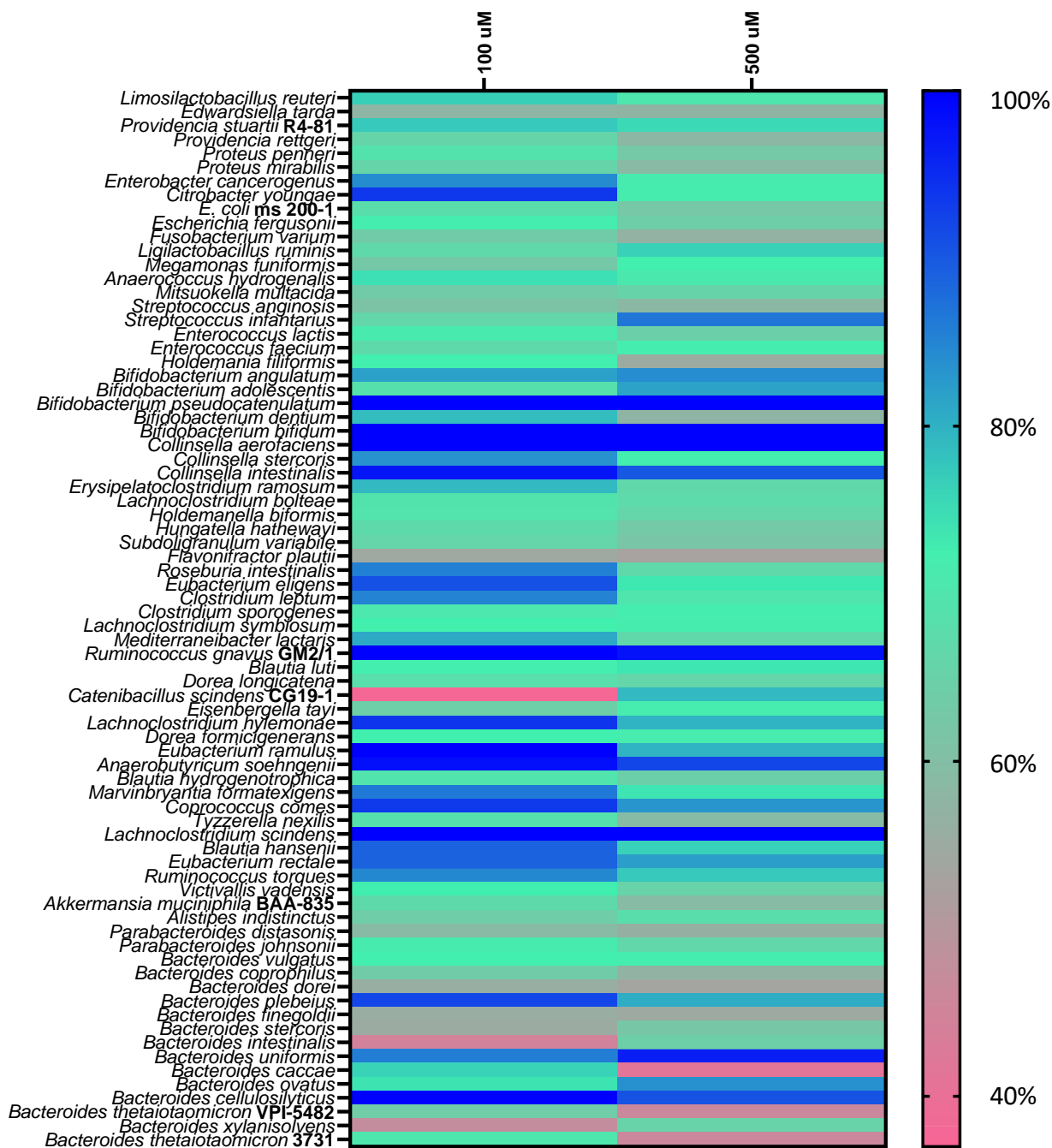
FIG S3.1 Bile acid recovery rates from systematic analysis. BAs were summed for each bacterial species. Pink color indicates ~40% recovery whereas blue indicates closer to 100% recovery of BAs.

A.

B.
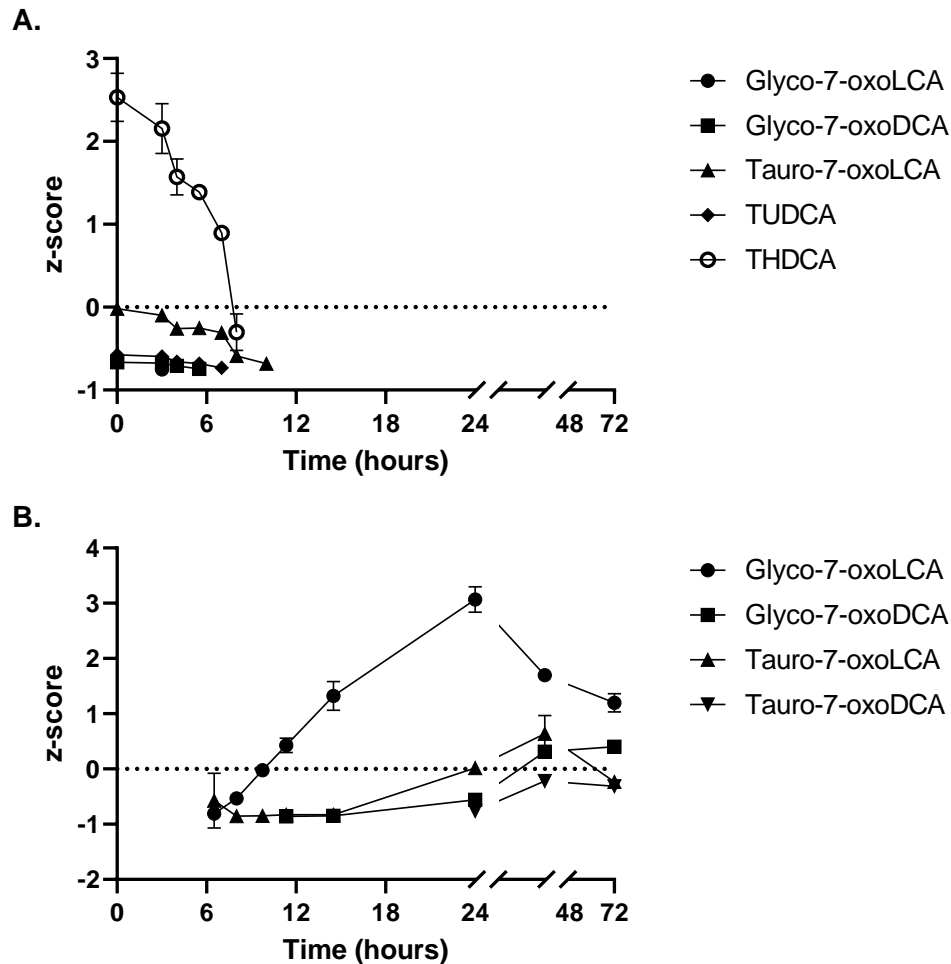
FIG S3.2 Microbially conjugated bile acid coculture measurements. MCBAs are listed using a z-score for relative abundance over the time-course analysis. A) Pair 1: *B. angulatum* + *B. thetaiotaomicron*. B) Pair 2: *C. symbiosum* + *B. thetaiotaomicron.*

3.7 Supplemental tables

Supplemental tables can be made available upon request to LLucas3@wisc.edu.

Table S3.1 – Bacterial strain names, ID number, and accession numbers.

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Chapter 4: Conclusions and future directions

4.1 Contributions to the field

4.1.1 Microbial bile acid conjugation to glycine and other amino acids

The work described in this thesis advances the field of BA metabolism. One major contribution from this thesis was the discovery that gut bacteria can conjugate both primary and secondary BAs to glycine, a process that was thought to only occur in the host liver (1). The discovery of microbial glycine conjugation suggests the possibility that bacteria are contributing more greatly to the BA pool than previously thought. In support of this possibility, data show that germ-free mice have lower levels of all glycine conjugated BAs than colonized mice (2). In addition to BA conjugation to glycine, our *in vitro* screen revealed microbial BA conjugation to 15 other amino acids, resulting in our discovery of 44 novel BAs, significantly increasing the known diversity of BAs (1). These unconventional BAs are referred to as microbially conjugated BAs (MCBAs), and were most commonly produced by the Actinobacteria, Firmicutes, and Bacteroidetes (3). While we were not the first to identify MCBAs, our work highlights their diversity and prevalence. MCBAs have since been shown to interact with host receptors (4), and to have varying antimicrobial potential (5). Because MCBAs interact with host receptors, they are suggested to play a role in signaling as a form of host-microbe crosstalk. It is also likely that BSH/T activity to produce MCBAs may be a form of nutrient sequestration or 'chemical warfare' on susceptible species. Finally, following the discovery of MCBAs, a new role of bile salt hydrolase (BSH) as an aminoacyl transferase, has been identified, reinvigorating a field that was previously thought to be saturated (4, 5).

Ultimately, the discovery of MCBAs, an entire new class of BAs, has opened the door for an entire new area of research in BA metabolism. It should be expected that many more types of microbial conjugations to BAs are discovered as any 'unconventional' attachments to the

terminal carboxylic acid or the steroid core multiplies the number of known secondary BA transformations. In addition, BSHs may be less specific than we thought, hydrolyzing or transamidating a diversity of MCBAs.

4.1.2 Bile acid transforming species are widespread

We provide the first systematic analyses on BA metabolism, starting with either conjugated BAs or deconjugated BAs. We observed that BA-transforming activity was highly prevalent among the gut microbes analyzed and was not phylogenetically constrained. More than half of the strains tested could perform at least one BA modification and roughly 75% of those species were novel BA transformers. Additionally, of the species already known to transform BAs, all showed additional capabilities that were previously unrecognized. We found that BSH prevalence to be similar. When administered CBAs, 67% of species could deconjugate over 90% of at least one of the provided CBAs. Most strains with BA-transforming capabilities were found in the Actinobacteria, Firmicutes, or Bacteroidetes.

Our *in vitro* analyses on BA metabolism in pure culture have proven to be effective and informative. We contribute greatly to the scientific awareness of BA metabolism by human gut bacteria, and we were able to confirm bioinformatic predictions on HSD activity (6, 7) and BSH activity (8, 9).

4.1.3 Cocultures reveal patterns in bile acid transformation dynamics

Our coculture experiments provided evidence for widely accepted concepts in BA metabolism. First, we show that secondary BA production is dependent on BSH activity in both monoculture and coculture. Next, we show that multiple microbes harboring unique enzymes can act sequentially to transform and diversify the BA pool. And we provide preliminary

evidence that bacterial interactions influence both bacterial growth and BA transforming capacity.

Our tests of bacterial BA metabolism on first unconjugated and then conjugated BAs allowed us to make inferences about BSH activity. Because we knew which bacteria can transform primary BAs into secondary BAs, we can deduce that BA conjugation prevented secondary BA production in species lacking an active Bsh. In monoculture experiments, bacteria were able to perform secondary BA transformations only if they could also deconjugate CBAs. We further substantiated this observation in coculture experiments. We showed that the rate at which a BA pool can be diversified is heavily dependent on the BSH activity of the community. In cocultures, when BSH activity occurred during exponential growth, the secondary BA transformer produced 7-oxo BAs during exponential growth and when BSH activity occurred during stationary phase, secondary BA production was also delayed to stationary phase. We provide some of the first experimental evidence that BSH performs the rate-limiting reaction before subsequent BA transformations can take place.

Another commonly stated concept is the idea that bacteria can perform sequential transformations on BAs. We experimentally validated this claim by growing bacterial species with unique capabilities in coculture. Only when a species with BSH and a species with secondary BA metabolism were grown together did the BA pool contain secondary BAs. When the BSH possessing strain deconjugated BAs slowly, secondary BA production was also slowed. When the BSH possessing strain deconjugated BAs quickly, a brief spike in primary BAs was observed, followed by a dramatic increase in secondary BAs. With the gut environment rapidly changing, it makes sense ecologically that BSH may be active during different phases of growth or in response to different environmental cues. The slow acting BSH coculture never accumulated measurable primary BAs, suggesting that limited deconjugation activity could drastically alter, or reduce, the diversity of the BA pool.

The coculture experiments revealed interesting trends concerning MCBAs. In all scenarios with *B. angulatum* present, MCBAs disappear. But when *C. symbiosum* was grown with *B. thetaiotaomicron*, MCBAs could be detected throughout the time series. These observations provide the first evidence that BSH/T are deconjugating MCBAs. And they provide the first evidence that BSH activity plays a role in shaping the MCBA pool.

Interestingly, it appears that bacterial interactions may influence bacterial growth rates, which impacts their BA transformation rates. Our coculture of *C. symbiosum* and *B. thetaiotaomicron* suggests bacterial interactions change BA dynamics. These two strains grew synergistically together, reaching stationary phase much faster than either species could alone. In turn, their BA transformations occurred earlier. CBAs were deconjugated at a faster rate and secondary BA production also increased. Coculture experiments like these provide invaluable information regarding species growth and its relationship to BA transformations.

BSH is a clear regulator of community dynamics in the gut. A limited number of species possess BSH activity, and many of those BSH are specific to certain CBAs. Therefore, BSH has direct control over BA pool diversity, which in turn through receptor activity, mediates host enzymes that maintain the BA pool. We provide further evidence that BSH is required for the release of primary BAs to be transformed into secondary BAs, we validate the claim that multiple species can sequentially transform BAs, and we show that bacterial interactions play a role in both secondary BA production and MCBA production. Altogether, we shed light on the ecological rules that govern bacterial BA metabolism.

4.1.4 Implications of this research

BA homeostasis is crucial for host health. BAs have been identified to play a role in countless healthy and diseased states. Probiotic species are designed to enhance BSH activity, while other species are known to inhibit colonization by *C. difficile*. And it's likely that these

outcomes are impacted by bacterial interactions as well as bacterial exposure to a diversity of BAs in the gut. We must continue to understand which microbes contribute which BA metabolisms and under what conditions if we are to truly maximize the potential of BAs for human health. From antibiotics, to probiotics, to FMTs, our data support the idea that any intervention of the gut microbiota should also consider the potential role of BA metabolism in its outcome.

4.2 Future Directions

Ultimately, our goal is to predict how interactions between bacteria and BAs shape the gut microbiome composition and function. To do so, we will need to identify all remaining BAs and the bacteria that transform them, we will need a comprehensive understanding of bacterial susceptibility to BAs, and we will need to use computational methods to test and learn how varying inputs produce different outcomes in the gut environment. Continued anaerobic culturing work, systems biology, computational models, and germ-free mouse models will allow us to determine causal relationships between the BA pool and gut microbiome community structure. These future directions will allow us to generate a mechanistic understanding of how BAs act as major modulators of gut microbiota composition and thus, host physiology.

4.2.1 Bacterial BA diversity

Metabolomic experiments provide rich data sets that can be continuously mined as new hypotheses are generated. With recent discoveries of BA diversity, I will create new compound lists to identify non-canonical BAs, such as those with novel conjugations. I will use this new, expanded compound list to identify novel BAs in our entire gut bacterial strain collection. I will confirm BA identifications by using predicted parent ion m/z and signature fragment peaks in

mass spectra. Ideally, in the near future more diverse BA standards will become commercially available to aid quantitation of these very highly anticipated BAs.

4.2.2 Bacterial susceptibility to BAs

I plan to explore bacterial susceptibility by growing each strain with CBAs, primary BAs, and secondary BAs at 10 mM, 1 mM, 100 µM and with diluted mouse bile (total mouse bile BAs at 10 mM and 1 mM) from conventionally raised mice. In addition, I will vary the pH of the media to measure how pH affects BA toxicity, which is important because the pH varies throughout the length of the gut. Bacterial sensitivity to pH and BAs may inform our understanding of bacterial localization along the gastrointestinal tract. These experiments will be performed in 96-well plates using an anaerobic plate reader so that sensitivity can be easily tracked over time.

In addition to our analyses on pH, systems-level time course analyses will be performed on bacterial strains to further our understanding of BSH and subsequent transformation dynamics. Using metabolomics, we will determine rates of BSH activity across phyla and identify associations between bacterial growth, BA toxicity, and BA transformations. In conjunction with metabolomics, we will perform lipidomics to understand the relationship between BA toxicity and its effect on bacterial membranes. We hypothesize that highly sensitive bacteria will have highly disrupted membranes. In addition, transcriptomics, proteomics, and microscopy can be performed to provide further information. We anticipate that some patterns in bacterial susceptibility to BAs will be delineated by phylogeny, and some associations will relate to % BA recovery described in Chapter 3. We also hope to shed light on contradicting statements about conjugated and unconjugated BA toxicity. Altogether, these *in vitro* experiments will contribute to our understanding of how BAs impact bacterial membranes and metabolism.

4.2.3 Modeling of gut microbiome structure and function

Ultimately, our goal is to be able to predict how the gut microbiome will respond to a variety of environmental factors including the BA pool. To do so, we will need more information regarding bacterial transformations and BA susceptibility listed above. In addition, we will need to learn more about bacterial interactions in a community setting and how those interactions affect BA metabolism. We will also need a more in depth understanding of *in vivo* BA metabolism if we hope to eventually model it. The Daniel Amador-Noguez lab will collaborate with the Federico Rey lab for all mouse related work and with the Ophelia Venturelli lab for all modeling related work.

4.3 References

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Appendix A. Scientific Teaching at the Undergraduate Level

A1. Introduction

In my time at UW-Madison I had the wonderful opportunity to engage in many teaching trainings and courses to enhance my education and stimulate my career goals. I participated in several courses offered by the Delta program, which promotes the development of future STEM faculty through dissemination of effective teaching practices for diverse student audiences. In one class I had the opportunity to learn in a community of TAs, in another, I learned how to be a more effective research mentor. In addition, I was lucky enough to take a course called "Inclusive Practices in the University Classroom" where we learned how to make learning more accessible to all students. In that class I learned to address charged topics such as racism, sexism, and transphobia through readings, activities, and discussions. The instructor for that course would have 'meta moments' after each activity to discuss the modality in which it was given and how it was designed to facilitate our learning. Those 'meta moments' were what I consider to be 'transparent' teaching, which has heavily influenced my teaching philosophy. In addition to courses, I attended trainings that were hosted by the Center for Integration of Research, Teaching and Learning, or CIRTL Network, which is a cross-campus network committed to advancements in high education learning. I learned about a variety of topics ranging from implicit bias and stereotype threat to fostering belonging and equity-oriented inclusive teaching. I had the opportunity to partake in a two-day workshop for instructors called, Learning Environments and Pedagogics, where we learned primarily about backward design, assessments, and active learning, but also about the importance of an effective syllabus, rubric, and timely feedback.

In 2020, I was accepted to Scientific Teaching Fellows program, hosted by the Wisconsin Institute for Science Education and Community Engagement, or WISCIENCE. In the year-long Teaching Fellows program, I gained practical teaching experience in a collaborative

and innovative environment. During my first semester with the Teaching Fellows cohort, the COVID-19 pandemic hit, and everything was moved online. While this upheaval disrupted many plans, it also provided me the opportunity to virtually teach a class of 16 students for an entire semester, rather than the previously planned 3 weeks. My involvement in these programs has helped me become a better teacher, a better writer, a better mentor, and generally, a better person. The concepts of inclusivity and universal design will serve me throughout my career, whether that be in biotech, academic research, or academic teaching.

As a Teaching Fellow, I created a lesson plan for an activity called "Microbe World". In a bonus fourth semester with the Teaching Fellows instructor, I had the opportunity to write my lesson plans into publications in the only peer-reviewed higher education lesson plan journal, Course Source. The Lesson Plan is included in this appendix in section A3.

A2. Teaching Philosophy

My interest in teaching microbiology began with mentoring students in my research lab. I tried to find students who were in their junior or senior years and still needed lab experience to bolster their applications to graduate school. This method for choosing undergraduate volunteers allowed me to help the students who were most in need achieve their goals and became the cornerstone of my teaching philosophy. My goal as an educator is to create equity for underprivileged students by increasing access to learning. I believe that by fostering student internal motivation and student preparedness for a future in STEM, I can help students feel confident in their ability to learn, which will encourage their pursuit of STEM degrees. To foster internal motivation, I am transparent with my reasoning and expectations for assigned activities and I create an inclusive classroom environment for my students. To prepare students for their futures in STEM, I provide tools for effective group work and I teach critical thinking skills.

Fostering Internal Motivation: Transparency and Inclusivity

One of the biggest challenges instructors face in the teaching-learning process is student internal motivation. Scientific teaching and research on evidence-based practices tells us that external motivations, i.e. grades and GPAs, are not factors that increase student investment in learning. By being a transparent and inclusive educator, I hope to increase access to learning, which will help my students develop their confidence and internal motivation.

I believe that students are the most invested in their learning when they understand why they are doing what's being asked and when they believe they have something to gain from doing so. I am transparent with my students by providing clear, concise instructions, and by giving the context for the activity, the goals they will accomplish, and the rubric that will be used for grading. For example, in a three-week activity called "Microbe World" that I created, students

can read the learning objectives in the instructions; after the first part of the activity, they should be able to identify microbes based on their metabolism, synthesize information from primary literature sources, and explain the role of evolution in microbial diversity. Because these goals are clearly stated, students can measure their learning as they work through the activity, and they can clearly identify the most important skills to gain. In addition, providing context for students can increase their internal motivation. For the same assignment, I tell students that they will gain practice in doing online research and in understanding the three domains of life, both of which will be important for their success in future classes and careers in STEM. However, without feedback from students, even the best efforts to remain transparent can fall short. In conjunction with my efforts to remain inclusive, I must provide frequent opportunities for my students to tell me I'm being unclear. For students, transparency and opportunities for feedback allows students to see the long-term goals of their learning, which increases student buy-in and internal motivation.

I believe that an inclusive environment and inclusive activities are critical to student internal motivation, and thus student learning and success. I create an inclusive environment by creating accessible activities, by including diverse examples of scientists in my material, and by addressing students by their correct names and pronouns. To create accessible activities, I use the concept of universal design; many means of engagement, many means of representation, and many means of expression. For example, in the "Microbe World" project mentioned above, the activities and assessments are varied. Students are asked to engage in many different forms of active learning: design a microbial environment on their own using google slides, evaluate their drawings against a rubric with a peer, and answer challenging short answer questions in a small group. Not only is the activity multi-modal, but the instructions are written out, as well as demonstrated in a recorded video for students to watch, listen, and take notes on. In addition, the background material was delivered via a recorded PowerPoint lecture,

including a transcript, as well as through provided primary literature sources. All materials are created to meet accessibility guidelines and no single aspect of this activity was graded at significantly greater weight than any other aspect, allowing each student to showcase their strengths, without being heavily penalized in areas where they are being challenged. Student feedback tells me that students are varied in which aspects of the activity they appreciate the most, but there is always something for everyone. By creating an environment where everyone feels seen, students are able to perform their best, feel confident, and remain internally motivated to achieve their goals.

Fostering Preparedness for Futures in STEM: Group Work and Critical Thinking

Success in STEM has often been boiled down to how well students can memorize inordinate amounts of information. I want students to understand that this belief is outdated and that their ability to collaborate effectively in a group and think critically to solve problems will serve their futures much better than the skill of rote memorization.

I believe that students learn best when they work together because they can solidify their knowledge through teaching their peers and they can expose misconceptions in their logic and gaps in their understanding through conversation. In addition, science is collaborative by nature, and the sooner students learn how to work in a group effectively, the more often they can practice their skills, and the more confident they will become in their ability to work with diverse groups. One activity that I designed asks students to do pre- and post-group work reflections. First, before engaging in group work, I ask my students to reflect on their constructive and destructive behaviors and to think about what would be helpful for their group to know about themselves. When they work together in a group for the first time, I ask the students to be vulnerable, to share their reflections with the group, and then altogether, use that information to designate roles for the activity. Finally, after students work in their groups a couple of times, I

ask them to reflect on how they benefitted from working in a group, how it helped them learn, and how they might approach group work differently next time. Students tell me that they previously were nervous about group work because it has been unsuccessful in their past, but now they feel confident in their ability to present themselves, set standards for the group, and to assume the role that best fits their personality. By providing a process for how students can effectively work together, I believe students achieve autonomy, and through self-reflection, they leave my classroom with successful collaboration skills for their futures.

In addition to being an effective collaborator, I believe that students in STEM must hone their critical thinking and comprehension skills. An ability to think critically and to comprehend material allows students to apply their existing knowledge to new situations in the future. To practice critical thinking skills, I have students read about a signaling pathway and then predict what will happen when levels of a signaling molecule change. To remain transparent, I explicitly tell the students that they are being asked challenging questions that require critical thinking and comprehension of the material. I explicitly state that the goal is to think logically, rather than to report the "right" answer, a value that's reflected in my rubrics. I find that students start to ask new questions, worry less about their grades, and become more internally motivated to learn when they know that their thought process is valued. Success in applying knowledge helps students feel confident in their ability to learn, which increases their internal motivation to remain in STEM.

Ultimately, my goals are to increase student internal motivation, which I achieve through fostering effective collaboration, garnering trust and effort through transparency, and providing space for all students to achieve their full potential when it comes to critical thinking and collaboration. My experiences as an instructor have taught me that investing time into the creation of inclusive and concise activities is challenging and time consuming, but benefits

student learning in measurable ways. I must remain adaptable as an instructor, taking in what I learn from my students year to year and applying it to my philosophy to help increase access to learning to all students.

A3. Microbe World: An Online Exploration of Microbial Ecosystems to Understand Core Concepts in Biology

By Lauren N. Lucas, Fátima Sanchezanieto, and Cara H. Theisen (to be submitted to Course Source Journal).

A3.1 Scientific Teaching Context

Learning Goal(s)

Students will:

1. Understand how the core concept of Evolution plays a role in microbial community composition.

2. Value how Pathways and Transformations of Energy and Matter (PTEM) play a role in microbial community metabolism.

3. Apply the core concept Information Flow, Exchange, and Storage (IFES) to understand microbial signaling in an ecosystem.

Society Learning Goal(s)

From the Microbiology Learning Framework:

1. Students identify microbial domains: "List the three Domains of the phylogenetic tree of life. State a unique characteristic of each Domain" under Evolution.

2. Questions about evolutionary relatedness pertain to: "Draw inferences about evolutionary relatedness of organisms based on phylogenetic trees" under Evolution.

3. Students identify chemical reactions by microbes in an environment where some products are reactants for other reactions: "How are the interactions of microorganisms

among themselves and with their environment determined by their metabolic abilities?': Give an example where the waste product of one microorganism serves as an important substrate for another organism (e.g., ammonium-oxidizing bacteria and nitrite-oxidizing bacteria, hydrogen producers and methanogens, sulfur oxidizers and sulfur reducers, etc.)" under Metabolism.

4. Students are asked to predict how removal or addition of specific microbes impacts the environment: "How do microorganisms interact with their environment and modify each other?': Choose a perturbation to a novel environment, and predict the change to the resident microbial community" under Systems.

5. Students are asked infer the relationship between metabolite levels in an environment and protein expression: "How is the regulation of gene expression is influenced by external and internal molecular cues and/or signals?: Give examples of how an external chemical signal can control gene expression./ Predict the growth behavior of microbes based on their growth conditions, e.g., temperature, available nutrient, aeration level, etc" under Information Flow and Genetics.

Learning Objective(s)

Students will be able to:

1. Identify microbes, their metabolisms, and their domains by performing research.

2. Determine evolutionary relatedness of microbes based on a phylogenetic tree.

3. Explain why microbes can evolve quickly.

4. Identify reactants and products for chemical reactions and understand that enzymes facilitate chemical reactions.

5. Demonstrate understanding that products from one chemical reaction may be reactants for another.

6. Distinguish between energy and matter and explain how both are conserved in chemical reactions.

7. Predict how environmental signals relate to protein or enzyme expression and how this response affects the microbial environment.

8. Predict how removal of one species alters the environment.

A3.2 Introduction

Microbial ecosystems exist in and on our bodies (1), in our foods (2), in the soil (3) and oceans (4), and even in the clouds (5). Microorganisms, also called 'microbes', play a role in human health and disease, environmental nutrient cycling, and in the production of many types of foods. Earth is a microbial world! This lesson, called "Microbe World", makes use of remote instruction to expose students to three of the core concepts of biology as outlined in *Vision and Change* (6), Evolution, Pathways and Transformations of Energy and Matter (PTEM), and Information Flow, Exchange, and Storage (IFES), through the lens of microbial ecosystems. Using the gut microbiome and fermenting kombucha as semi-familiar and relatable example microbial ecosystems, "Microbe World" engages students with the core concepts through a series of online activities.

"Microbe World" piques student interest in the biological subdiscipline of microbiology, while simultaneously providing the core concepts as a framework for understanding *all* of biology. The core concepts (6), which serve as guidelines for topics that should be taught in any biology course (7, 8), regardless of subdiscipline, will help students build a knowledge structure, or framework, in which to integrate subsequent information (9). Each core concept represents an individual element of biology, but all are related to each other in important ways. The design of "Microbe World" directly reflects the knowledge structure we hope students will build; core concepts are presented as individual elements each week, yet they are also interconnected, which is why each component of the activity builds upon the last. Knowledge structures aid deep student learning and retention by helping to students connect concepts and ideas in a logical manner (9). Thus, students who participate in "Microbe World" will be better prepared for the remainder of their undergraduate biology careers and beyond.

Research shows that students enter their undergraduate degrees already holding many misconceptions when it comes to the topics of Evolution and PTEM (10). Students consistently have misconceptions about evolution, whether they be due to student misunderstanding of ideas during their education, or from misconceptions due to misrepresentations of evolution in the media (11). In addition, students often have trouble understanding microbial metabolism, which is most apparent

when students use the terms 'matter' and 'energy' interchangeably

(https://evolution.berkeley.edu/evolibrary/misconceptions_teacherfaq.php) (12). In addition, most

students are not familiar with the core concept IFES. They are not aware that information can flow in

an interkingdom manner, which can result in drastic changes to the environments in which microbes

(and people!) live (13, 14). There are several types of published instructional activities geared towards

addressing the core concepts: microbial evolution, microbial metabolism (PTEM), or microbial

genetics, signaling, and communication (IFES). These activities include visualizations of how

antibiotics perturb a gut microbiome using pasta (15), multi-week labs on isolating microbes from

yogurt (16), lessons on PTEM at the organismal and ecosystem scales (17, 18), and lessons on

signaling in eukaryotic organisms (19). However, to our knowledge, no single lesson layers multiple

core concepts to clarify and build knowledge structure, nor are any current lessons specifically

designed for remote instruction. The "Microbe World" activity provides an opportunity for instructors to

address these misconceptions and knowledge gaps early, clearly, and iteratively in their courses to

set students up with a solid foundation in biology.

  "Microbe World" is designed for a remote or fully online, introductory bioscience course. In this

lesson, students move through a series of activities, divided into three sessions. Each of the three

sessions follows a similar pattern, but focuses on a different topic; Session A is on Evolution, Session

B on PTEM, and Session C on IFES. Another, perhaps more tangible, way to think about each

session is as the "who" (Evolution), "what" (PTEM), and "how" (IFES) of microbial ecosystems. Each

session consists of two parts: a synchronous meeting followed by a period in which students work

asynchronously to complete the activity. In the synchronous portion, the instructor introduces the

activity and answers questions/discusses misconceptions. In Sessions A and B of the asynchronous

portion, students first watch a tutorial video, then apply what they learned to illustrate the fermenting

kombucha microbial ecosystem. Afterwards, students exchange and evaluate their drawings using a

rubric, and last, students work in small groups to answer challenging critical thinking questions, which

they share with their classmates using a virtual discussion board. In Session C of the asynchronous portion, students work on their own to tackle logic-based questions about microbial signaling. While remote classes often suffer from a lack of instructor-student and student-student interactions, "Microbe World" purposefully builds instructor-student dialogue into the learning management system (LMS) and activity descriptions. In addition, the use of both synchronous group work and asynchronous discussion boards fosters student-student interactions consistently throughout the lesson. Overall, "Microbe World" provides students with many opportunities for interaction to build relationships with their instructor and their peers and it provides multiple microbial ecosystems that display the themes of Evolution, PTEM, and IFES to serve as a framework for which students can organize their knowledge to facilitate future STEM learning.

Intended Audience

"Microbe World" is most appropriate for students interested in pursuing a biology, microbiology, or related, major. It is appropriate for early undergraduate STEM students, advanced high school students, and community college students who would benefit from having foundational knowledge of biological core concepts and microbiological themes. In addition, minor changes to the lesson plan would allow "Microbe World" to be appropriate for upper-level undergraduate and early career graduate students.

Required Learning Time

In its current implementation, it will take approximately 5-5.5 hours to complete this activity; Sessions A, B, and C are each made up of a synchronous session lasting 10-15 minutes, followed by an asynchronous session lasting 1.5 hours. "Microbe World" was first implemented in a remote instruction course but can be adapted for in-person or blended courses, which may alter the required learning time.

Prerequisite Student Knowledge

Session A, Evolution, "Who": Students would benefit from having used Google Slides, but should not need extensive prior knowledge on microbiology as the provided resources are sufficient for students to complete the Microbe World Drawing. Students should know that different microbes come from different domains of life, and they should have a basic understanding of cellular division, DNA replication, and mutation to answer the group questions. Session B, PTEM, "What": Students should have some experience writing chemical reactions. It would be helpful if students had prior exposure to laws of conservation of mass and energy. Session C, IFES, "How": Students should be aware that microbes can respond to environmental signals. A brief overview of how the microbes in your system interact would be beneficial for answering questions related to protein levels and metabolic responses by microbes.

Prerequisite Teacher Knowledge

"Microbe World" was taught by both microbiologists and non-microbiologists alike. It would benefit instructors to have in depth knowledge on biological processes, with a general breadth of knowledge in microbiology. Instructors need to understand the core concepts Evolution, PTEM, and IFES, and how they relate to microbiological processes. Instructors should have a general understanding of microbial evolution in the context of genetic mutation and evolutionary relatedness for the microbes they've chosen to depict. Instructors should be able to identify microbial metabolisms and the chemical reactions that demonstrate those metabolisms. Finally, instructors should understand how microbes in the chosen environment respond to environmental signals, as well as how removal or addition of a species affects metabolic activity. Because this lesson was taught via remote instruction, familiarity with online learning management systems would also benefit the instructor.

A3.3 Scientific Teaching Themes

Active Learning

Throughout this lesson, students engage in all three types of interaction that lead to effective online learning. Students engage actively with the material asynchronously (learner-content) by drawing a microbial ecosystem and then evaluating their own work against a rubric. Students then engage with each other asynchronously (learner – learner interaction) when they evaluate a peer's drawing against a rubric and synchronously when they engage in small group discussions to answer short critical thinking questions. Students engage with the instructor (learner – instructor) when the instructor synchronously addresses common misconceptions found in some of the drawings and responses that were turned in, giving them an opportunity to ask clarifying questions, share insights, and then return to correct these misconceptions in their own drawings and discussion posts iteratively throughout the duration of the assignment.

Assessment

Rubrics are used by both instructors and students to formatively assess a student's understanding of the material. As part of the assignment, students are asked to grade their own drawing and a peer's using the same rubric that instructors will use. Students were asked to provide scores and justification for both participants, which the instructor can then use to inform final grades and adjudicate when discrepancies occur between the self and peer scores. For short answer group questions, students were assessed on the completeness and accuracy, as well as inclusion of all group participants in their answers.

During a follow-up synchronous session, the instructor would address common misconceptions found in student answers and drawings, allowing students an opportunity to clarify and learn from these misconceptions. Students are instructed return to their work and address misconceptions as part of their grade. In this way, the formative assessment serves as an iterative

tool that allows students and instructors to assess understanding, as well as give an opportunity for students to correct their misconceptions and bridge their gaps in learning.

Inclusive Teaching

The "Microbe World" activity exemplifies inclusive teaching themes in both content and design. First, we critically engaged difference by following the principles of universal design to make sure all our materials were accessible. To do so, we added closed captioning to all video recordings and checked that all documents could be read by screen readers for folks with impaired hearing or vision. We also checked our formatting to have high contrast words and images, punctuation at the end of sentences and phrases, and formatted numbering and bullet points when lists were used. In addition, for all activities, we delivered instructions in multiple modes; oral instructions were given during the demonstration videos, written instructions were provided on activity sheets, and expectations for student work were transparently outlined in rubrics. Rubrics provide standards for consistent and equitable grading practices and serve as a way for instructors to communicate their expectations to students. Besides multiple modes of instruction, we also included multiple modes for assessment. Throughout the "Microbe World" activity, students were assessed on their drawings, evaluations, group answers, and revisions of those answers, providing students with a diversity of opportunities to demonstrate their learning.

The background material included the topics of health disparities and cultural awareness when discussing healthy gut microbiomes and fermented foods. Connecting content to student interests, lived experiences, and local community concerns, increases student investment in learning and retention of the material. Students were instructed to assign roles such as facilitator, timekeeper, and recorder when working on group questions. These roles switched between sessions as the students worked on different parts of the project.

A3.4 Lesson Plan

Supporting Table 1 – Overview of supporting files

Supporting File Names*	Session A - Evolution	Session B - PTEM	Session C - IFES
[1] Slides^ (S)	[1-A]	[1-B]	[1-C]
[2] Demo Video^ (A)	[2-A]	[2-B]	[2-C]
[3] Drawing (A)	[3-A]	[3-B]	n/a
[4] Evaluations (A)	[4-A]	[4-B]	n/a
[5] Questions (A)	[5-A]	[5-B]	[5-C]
[6] Review/Revise (A)	n/a	[6-B]	[6-C]

* Supporting files are referenced using brackets. For example, the slides for all sessions are referred to as [1] and the slides specifically for Session A are referred to as [1-A] throughout the text.
^ Files should be recreated by instructor.
(S) – synchronous
(A) – asynchronous

Lesson Overview:

In three, two-part sessions of short synchronous meetings followed by longer asynchronous activities, students will engage in a series of activities, altogether referred to as "Microbe World", that will improve their understanding of microbial ecosystems and core concepts of biology. Students will learn about microbial ecosystems through demonstrations about the gut microbiome and they will apply that knowledge to work through activities based on the kombucha microbial ecosystem. The gut microbiome and fermenting kombucha ecosystems are chosen as the model ecosystems because both demonstrate the core concepts effectively and both may be familiar to students. With a variety of low stakes activities, "Microbe World" is designed to engage students of different strengths and varying interests. "Microbe World" can easily be adapted to accommodate a range of schooling levels and modalities, whether they be fully online, fully in-person, or a blend of the two.

Overall Workflow:

Each session (A, B, and C) follows a similar pattern in activities and is motivated by a different learning goal associated with three of the five core concepts outlined in Vision and Change

(6) (See Supporting Table 1 – Overview of Supporting files). Session A focuses on what microbes are present in the ecosystem through the lens of Evolution, Session B on microbial metabolism through the lens of Pathways and Transformations of Energy and Matter (PTEM), and Session C on microbial responses to their environments, which demonstrates the core concept Information Flow, Exchange, and Storage (IFES). In other words, who's there (Session A), what are they doing (Session B), and how (Session C)? In general, the instructor will host a synchronous session where they introduce the upcoming activities using Slides [1] and review misconceptions from the previous session's activities. Following the synchronous session, students work asynchronously on activities [2-6] as outlined in Supporting Table 1 – Overview of Supporting files. These activities typically go as follows: students watch a demonstration video created by the instructor [2], they use that information to illustrate a microbial ecosystem [3]; students then evaluate their drawing and a peer's drawing based on a rubric [4], and answer questions related to that week's content [5], and finally, students review and revise their previous answers based on feedback from the instructor [6]. Because each session follows a similar format, the few key differences between each are addressed in detail below.

Instructor Preparation:

Organize the LMS: This lesson relies on the use of an LMS to distribute class materials, receive student work submissions, and grade and provide feedback for each activity. Because the students need to navigate each lesson on their own, it is important that the instructor creates an organized and intuitive LMS. Outline each session's activities into separate modules, with separate components for each activity within the module. For example, in the first module, label a Text Header 'Session A – Evolution', followed by a "Page" for 'Session [1-A] Slides', followed by a "Page" for the 'Demonstration Video'. "Pages" are used to convey information, "Assignments" are used for graded activities, and "Graded Discussion Boards" are used to facilitate online interaction. Upload all files that students will need throughout the activity to the LMS (examples for each activity sheet can be found in Supporting

Files). To ease the transition from one module component to the next and to provide context for the upcoming activity, the instructor should include brief descriptions, or summaries, for each "Page", "Assignment", or "Discussion Board". These descriptions serve as "dialogue" between the instructor and students in remote classes (examples included in sections below).

Gather resources to share with students: Provide students with literature sources about the kombucha microbial ecosystem (see Supporting Table 2 – Teaching Timeline for full citations). Remove extraneous information so that students can easily identify relevant information, while still gaining experience in scientific reading comprehension.

LMS Page Description example:

These research papers have been pared down and curated by [insert instructor name] to highlight the most important information. We hope this will help you to complete the Microbe World Activity effectively and efficiently. These resources will help you to complete activities associated with Microbe World Session A - Evolution and Microbe World Session B - PTEM (coming next week). For this Page, after you've downloaded the research papers, you can click 'Mark as done' in the upper right corner, which will allow you to move forward in this module. Then you can refer to the primary literature sources as you work through the Microbe World Session A and B Individual Drawings.

Make a shareable drawing template (Google slides): After organizing the LMS, the instructor should up a shared file drive, such as a Google Drive or Box folder, with the Microbe World Drawing Template Slides for their students (See Supporting Files [3-A], [3-B], and Supporting Table 2 – Teaching Timeline for details). The template provides students with all the clip art and word boxes they will use to create their Session A drawing [3-A] and all of the clip art, reactants and products for

the chemical reactions for their Session B drawing [3-B]. Providing clip art allows students to focus on learning about the microbes rather than drawing the microbes. The instructor may choose to recreate these templates using their own drawings of microbes, but should remain conscious of color choice for students with color blindness.

Make demonstration videos: The instructor should understand the instructions, the learning objectives, and steps of the activity in detail before making the demonstration, or tutorial, videos. These demonstration videos serve to outline the process for completing the Microbe World Individual Drawing and may not contain sufficient background information for students to complete the lesson. In these videos, the instructor should go over how to access and download the drawing template, how to edit/copy the Google Slides, and how to do each activity. Explain the instructions and learning objectives for Microbe World Session A – Evolution – Individual Drawing [3-A], using the gut microbiome as an example. Talk out loud as you work through identifying which microbes do what, what their scientific names are, and which Domain they belong to for Session A. For Session B, make a similar video that shows how to incorporate chemical reactions into the drawing as in the supporting file Microbe World Session B – PTEM – Individual Drawing [3-B]. The instructor may choose to include important information about microbial ecosystems and biological scales in the video. The instructor should make a plan for how they would like to incorporate background information into this activity, potentially in the demonstration videos or in the synchronous session presentations. Be sure to include transcripts of your demonstration videos in the LMS; students benefit from being able to revisit the written information after watching the video.

LMS Page Description example:

Please be sure to read the "Microbe World Session A – Evolution [linked]" activity sheet before watching this video. In this video, [insert instructor name] walks you through how

to complete your Microbe World Session A - Evolution individual drawing. They talk out loud as they create their drawing and label the correct information.

Make student groups: Lastly, the instructor puts students into groups of 4, with 2 pairs in each group (groups of 3 will still evaluate each other's drawings, just not in pairs). Students will remain in these groups throughout the activity. Student groups should intentionally be made equitable, i.e., students who are less familiar with the material should be grouped with students who are more familiar with the material.

Session A – Evolution:

Instructor preparation: Instructor prepares slides for the synchronous session (See Supporting Table 2 – Teaching Timeline Table for details).

Synchronous:

Slideshow presentation: Session A slides should include: the overarching lesson learning goals, an overview of the flow of activities throughout all sessions, a screenshot of the LMS so students know where to find everything, and the learning objectives students should achieve after completing Session A (below). The overarching learning goals help students know broadly what they will gain from participating in this activity: a greater understanding of the role of evolution in development of microbial communities, the role of microbial metabolism in microbial ecosystems, and the role of environmental signaling in microbial ecosystems. An overview of the activities helps students plan their time. And the learning objectives allow students to see specifically what they will be able to do by the end of the lesson. In addition, by presenting an overview of "Microbe World", instructors can explain that each session's learning will build on top of itself and that students should use this organization as a knowledge structure. The instructor should let all students know that the LMS was

designed to be moved through sequentially because each step builds on the last. Tell students what groups they will be working in for the duration of the activity and encourage students to start planning their group meetings as soon as possible. Finally, because most of this lesson takes place asynchronously, it is important that the instructor be very clear about student expectations and channels for communication should issues arise.

Session A Learning Objective(s)

Students will be able to:

1. Identify microbes, their metabolisms, and their domains by performing research.

2. Determine evolutionary relatedness of microbes based on a phylogenetic tree.

3. Explain why microbes can evolve quickly.

Asynchronous:

Demonstration Video: In the first part of the module, students will listen to and watch the first demonstration video called Microbe World Session A – Evolution – Demonstration Video. They will follow along as the instructor demonstrates how to work through the assignment. Because the video references the activity, it is crucial that students read the activity sheet before watching the demonstration video. Make this clear to students in the video description, the activity instructions, and in the synchronous session. After watching the demonstration video and clicking "Mark as done", students should be able to access the provided Primary Literature Sources.

Primary Literature Sources: After watching the demonstration video, students will download pared down research papers to assist with their Microbe World Session A - Evolution – Individual Drawings. These materials contain information necessary for students to complete Session A and Session B of "Microbe World". Along with four primary literature articles, provide a graphic showing

relative sizes of microbes, cells, and cellular components. Students should click "Mark as done" to move onto the next part of the module.

Individual Drawing: After students have read the activity sheet and watched the demonstration video about the gut microbiome, they will create their kombucha microbial ecosystem drawing using the provided template. The activity sheet describes the instructions for the drawing, starting with an explanation of the demonstration video as it correlates to the activity sheet, followed by learning objectives that students should achieve by the end of the activity, and explicit instructions for completing each step of the activity. To complete their drawings, students should save a copy of the template, use their primary literature sources to identify organisms capable of performing each metabolism, and label the domain for each organism. To simplify the process, we provided students with microbial names that they can match to each metabolic process. For example, *Schizosaccharomyces pombe, Saccharomycodes ludwigii, Saccharomyces cerevisiae,* and *Zygosaccharomyces bailii* are all listed in one box together. Another box has these microbe names listed: *Acetobacter xylinoides, Bacterium gluconicum, Komagataeibacter kombuchae,* and *Gluconobacter oxydans*. A student might choose *Saccharomyces cerevisiae* from the first box and then search for that microbe's metabolism in the pared down primary literature sources, where they would find that it's an ethanol fermentation microbe. From the next box they might choose *Acetobacter xylinoides,* which is identified as an acetic acid producing bacteria in the literature sources. Some microbes perform multiple types of metabolism and it should be made clear to students that they have not made a mistake if a single organism can be used more than once in their drawings. After matching each metabolism to a microbe and labeling the cellulose strands students should write each microbe name in proper scientific notation and label it with its appropriate domain. The domain for each organism will help students answer questions about relative sizes of organisms and the biological scales at play. In this lesson, the gut microbiome example contains organisms from

each of the three domains, Bacteria, Archaea, and Eukarya, but for the kombucha ecosystem only Bacteria and Eukarya are present. After students have matched a microbe to each metabolism and labeled its domain, they will take a screenshot of their drawing and submit to the LMS. After submitting on the assignment page, students will move on to evaluate their drawings and their partner's drawing against a rubric.

Evaluations: After submitting screenshots of their drawings, students gain access to a Page outlining the self- and peer-evaluation instructions (see supporting file Evaluations [4-A]). Students are instructed on how to use a rubric to grade their drawings and what the benefits of rubrics are for both instructors and students. In our implementation of the lesson, students were assessed on the correctness of the labeling of microbial names, metabolisms, and domains, and on the accuracy of relative biological scales in their drawings (see supporting file Evaluations [4-A]). Students were provided an answer key for comparison and were instructed to submit their evaluations as a text entry that included point values and accompanying explanations for both the self and peer grades. As a final note, students are asked to update their drawings if any inaccuracies were found before moving on to Session B.

Group Questions: After students have completed their individual drawing and subsequently used rubrics and answer keys to evaluate and update their drawing in pairs, they then move on to answer stimulating conceptual questions as a group. For group questions, students are instructed to arrange a time to meet virtually using whichever platform they prefer. Once students are together, they are instructed to introduce themselves, choose a facilitator, 2 recorders, and a timekeeper (groups of 3 will not have a timekeeper and the facilitator can help with this role) before working together to answer the questions. One recorder will scribe and post answers to group questions on 'Biological

Scales' on an LMS discussion board and the other recorder will post answers to group questions on 'Evolution' on a separate discussion board (see supporting file Questions [5-A]).

Biological Scales questions ask for examples of drawing components that belong to three orders of biological scales: molecular/cellular, organismal, and ecosystem. Students may answer that the cellulose strands are cellular, the ethanol fermenting yeast is organismal, and the entire kombucha drink is the ecosystem. This organization for biology becomes a little trickier when we think about microbiology because an organism can also be a single cell! The questions about biological scale are intended to guide your students towards the realization that biological scales are relative and do not correlate to absolute measurements of size.

The Evolution questions are based directly on the core concepts of biology in addition to the more specific "conceptual elements" that characterize a core concept (8). In this activity, the Evolution questions ask students to use a simplified phylogenetic tree to determine the evolutionary relatedness of each microbe in their ecosystem drawing. The goal here is that students realize bacteria and archaea are more closely related to each other than either are to fungi. In addition, students are asked to relate the concept of co-evolution in an ecosystem to fitness, and they are asked why microbes can evolve so quickly. Thirty minutes is the suggested time students should spend answering group questions.

Session B – PTEM

Key Differences: The key difference between the sequence of activities in Session A compared to Session B is that in Session B students have the additional task to review and revise their answers to the group questions based on instructor feedback (see supporting file Review/Revise [6-B]). On the Discussion boards from Session A, students can either respond to their own group's discussion post, correcting a misconception or clarifying a point, or they can comment on another group's response,

asking questions or elaborating on ideas. Even though each group had one recorder, all students are required to "Revisit the Discussion Boards from Session A".

Instructor Preparation: Because the instructor set up the LMS in advance of starting the activity, preparing for Session B can be focused on grading and providing student feedback. The instructor should review discussion boards for common errors or misconceptions and incorporate any feedback they have into the synchronous session slide presentation. From our classroom, many students had misconceptions about evolution, believing that evolution can occur in an individual rather than on populations over the course of generations.

Synchronous:

Slideshow presentation: Using slides, the instructor goes over Evolution and Biological Scales misconceptions from the Session A group question answers on the discussion boards and introduces Microbe World Session B, which is built around the core concept of pathways and transformations of energy and matter, or PTEM. The instructor should go over the learning objectives for Session B and should outline the differences between Session A and B. Plan time for students to ask questions.

Session B Learning Objective(s)

Students will be able to:

1. Identify reactants and products for chemical reactions and understand that enzymes facilitate chemical reactions.

2. Demonstrate understanding that products from one chemical reaction may be reactants for another.

3. Distinguish between energy and matter and explain how both are conserved in chemical reactions.

Asynchronous:

Demonstration video: In Session B on PTEM, students will watch a demonstration video explaining the instructions and expectations for the drawing. Similar to the Session A demo video, students follow along while the instructor works through the exercise for the gut microbiome. This time, instead of identifying microbes based on their metabolisms, the instructor is adding the reactant and products for the chemical reactions that define each metabolism. Students will gain the most from the demonstration video if they have already read the activity sheet for the drawing.

Individual drawing: Students will now individually work on the second part of their Microbe World drawings (see supporting file Drawing [3-B]). Using their drawings from the first session, students will add in the substrates and products for each microbial metabolism in the fermenting kombucha ecosystem. In the activity sheet, students are provided a table with the reactants, products, and enzymes for chemical reactions that take place in fermenting kombucha, along with their definitions, and simple cartoons of each component. Students are told that each of the four microbes will have a chemical reaction associated with it, and one microbe has a reaction catalyzed by an enzyme that is also listed in the word table. In addition, students should be told that some of the reactants or products will be used more than once. Finally, students should bold the reactants and underline the products for each reaction. Submissions are in the form of screenshots, which will unlock the next component of the module, self- and peer-evaluations.

Evaluation: After completing their drawings, students will work in the same pairs as in Session A to swap their drawings and evaluate them against a rubric (see supporting file Evaluations [4-B]). They will submit the grades and justification through a text entry box on the LMS. For Session B on PTEM, students were evaluated on their ability to correctly match microbial metabolisms to a chemical

reaction's reactants and products. Students should correctly distinguish between reactants and products. And finally, students are assessed on whether they followed instructions and updated their Session A drawing to be accurate based on the evaluation and answer key. As in Session A, students were provided an answer key for comparison and were instructed to submit their evaluations as a text entry that included point values and accompanying explanations for both the self and peer grades.

Group questions: Students will meet virtually in groups to answer challenging questions about PTEM (see supporting file Questions [5-B]). Students will assign timekeeper, facilitator, and recorder roles again, but should assume different roles than they did in Session A. In Session B, there is only one discussion board; the first recorder is in charge of posting answers to questions 1 and 2 and the second recorder will post answers to questions 3 and 4.

In Session B, student groups will tackle questions related to the core concept Pathways and Transformations of Energy and Matter, or PTEM. Students are instructed to refer to their drawings and identify which molecules are being broken down, via catabolic reactions, to provide energy for the microbes. Students are then asked the converse; which microbe performs an anabolic, or building, reaction in kombucha? In addition, they are asked where energy is stored, which is in the molecular bonds. Student should identify the cellulose producing microbes as the ones performing anabolic reactions. Interestingly, cellulose can be found in both the kombucha and the gut environments. Cellulose is a final product in kombucha, but a reactant in the gut. We hope they make the connection that kombucha is ingested and transformed by the microbes in the gut on their own, and the instructor should also be sure to point it out in the following synchronous session. Finally, students are asked to relate their answers to questions #2 and #3 to the core concept of PTEM in 3-5 sentences. They should include at least one conceptual element in their explanations. Students should work on the Session B group questions for 30-45 minutes.

Revisit Session A discussion boards: Students are asked to respond, revise, or clarify their answers on the discussion boards from Session A based on what the instructor covers in the synchronous session (see supporting file Review/Revise [6-B]).

Example LMS Description:

Now that you have had a chance to work on Microbe World Session B - PTEM, and we have had a chance to address some common areas for improvement on your group answers to Microbe World Session A - Evolution, every person from each group should go back and respond to their own group's discussion posts, correcting one misconception or clarifying one of your answers to better fit the response's relation to the Core Concepts. Alternatively, you are welcome to comment on other groups' responses; asking questions, elaborating on their ideas further, or bringing up new ideas are all ways to achieve credit for this activity. Click on both discussion board links to make a comment.

The goal of this activity is to ensure that all students have interacted with both the content, their classmates, and their instructors. Allowing students an opportunity to revise their work based on feedback can reinforce a growth mindset as well as reinforce accurate material that will serve as the foundation for future student learning. Students received full credit for contributing new ideas, asking questions, or clarifying statements on both Evolution and Biological Scales discussion boards.

Session C – IFES

Key Differences: Session C covers the core concept of Information Flow, Exchange, and Storage, or IFES, and is the most streamlined of the three sessions. In Session C, students are presented background information on microbial signaling in the kombucha environment, and then asked to answer logic-based problem-solving questions individually. Similar to Session B, however, students

will also revisit their answers to group questions on PTEM based on instructor feedback during the synchronous session.

Instructor Preparation: Similar to Session B, Session C instructor preparation can be focused on grading and providing student feedback. The instructor should review the PTEM discussion board for common errors or misconceptions and either comment directly in the discussion board or incorporate any feedback they have into the synchronous session slide presentation. From our classroom, many students had misconceptions about matter and energy, often using the words interchangeably. Students also struggled with the concept of an enzyme and how it is neither a reactant nor product.

Synchronous:

Slideshow presentation: Using slides, the instructor goes over PTEM misconceptions from the Session B discussion board and introduces Microbe World Session C. The instructor should go over the learning objectives for Session C and should outline the differences between this and previous session's activities. Plan time for students to ask questions.

Session C Learning Objectives

Students will be able to:

1. Predict how environmental signals relate to protein or enzymes expression and how this response affects the microbial environment.

2. Predict how removal of one species alters the environment.

Asynchronous:

Background information video: Students will watch an introduction video explaining the core concept of Information Flow, Exchange, and Storage (IFES) before answering the final set of

questions in this activity pertaining to the kombucha fermentation ecosystem individually. This video contains general information about central dogma and protein expression regulation, but also specific information about invertase levels as it relates to glucose levels in the kombucha environment. Students should be able to make logical jumps from the information given in the background video to answer the follow-up IFES questions.

Individual questions: For the individual questions, students are provided a figure and citation from a paper about kombucha signaling, May et al., 2019 (see supporting file Questions [5-C]). Based on the background video, the figure, and their drawings, students should be able to answer why protein or enzyme regulation is important for the yeast in this system. In this activity, students are also asked to predict outcomes for a few different scenarios: What happens to the levels of invertase if all the bacteria suddenly die? What if the bacteria suddenly double? Students are also asked to infer when during fermentation is invertase the most active and how this relates to glucose levels throughout the production of kombucha. Finally, students are asked to relate what they've learned about the system to what they've learned about the core concept IFES.

Revisit Session B discussion boards: When students revisit discussion boards this time, they will still make comments, ask questions, elaborate on ideas, but on the topic of PTEM rather than Evolution. All students, not just the recorder, should participate in this activity for full points (see supporting file Review/Revise [6-C]).

A3.5 Supporting Table 2 – Teaching Timeline

Activity	Description	Est. Time	Notes
Unit Preparation (Instructor only)			

| Set up LMS homepage | Set up each activity in 'Modules' on Canvas. Put each component into its own page.

Modules should be locked until students have completed the last part because the "Drawing Evaluation" page will contain an answer key and rubric for the drawing. | 30 min | Session A can be set up as follows:
Text Header: Microbe World Session A – Evolution
Text Header: Synchronous Session Kickoff Meeting on XX/XX/XX
Page: Meeting Slides
Quiz: Pre-Unit Background Knowledge Probe
Page: Demonstration Video
Page: Primary Literature Sources
Assignment: Individual Drawing
Assignment: Drawing Evaluation
Page: Group Questions
Discussion Board: Group questions on Biological Scales
Discussion Board: Group questions on Evolution

Session B can be set up as follows:
Text Header: Microbe World Session B – PTEM
Text Header: Synchronous Session Kickoff Meeting on XX/XX/XX
Page: Meeting Slides
Page: Demonstration Video
Page: Primary Literature Sources
Assignment: Individual Drawing
Assignment: Drawing Evaluation
Page: Group Questions
Discussion Board: PTEM Group questions
Assignment: Revisit Microbe World Session A Discussion Boards

Session C can be set up as follows:
Text Header: Microbe World Session C – PTEM
Text Header: Synchronous Session Kickoff Meeting on XX/XX/XX
Page: Meeting Slides
Page: Background Information Video
Assignment: IFES Questions
Assignment: Revisit Microbe World Session B Discussion Boards |
| Set up graded discussion boards | Discussion Boards for Session A on Biological Scales of Organization and Evolution.

Discussion Boards for Session B on PTEM. | 5 min | Copy/paste the questions from each activity sheet into the LMS discussion boards.

At the beginning of the module, discussion boards are set so that **only people who post answers can see responses**. On the day of the sync session (after everything had been |

	Discussion Boards for Session C on IFES.		turned in) change the setting so that everyone can see the responses. **See Supporting Files under 5. Questions, [5-A], [5-B], [5-C]**
Create Microbe World Template	Create a template for students to complete their microbe world drawings.		The Session A template should contain word boxes: • Cellulose strands • Invertase producing microbe • Acetic acid producing microbe • Cellulose producing microbe • Ethanol fermentation microbes And Clip art: • Cellulose strands • 2 different colors/sizes of a budding yeast • 3 different colors/sizes of bacteria The Session B template should contain word boxes and clip art for each of the following items: • Sucrose • Glucose • Fructose • Ethanol • Cellulose • Acetic Acid • CO_2 • Invertase **See Supporting Files under 3. Drawing, [3-A] and [3-B]**
Create demonstration videos – Session A and Session B	In these videos, the instructor walks the students through how to complete the microbe world drawing by using a the gut microbiome as a demonstration tool.		Use the gut microbiome as an example microbial ecosystem to walk students through how to: Session A: • Find the drawing template • Make a copy of the drawing template • Modify shapes and sizes of words/art • Perform literature research to determine which microbes perform which metabolisms • Label microbes to metabolisms • Label microbes to their domains • Adjust components to their relative sizes Session B: • Match microbes to their metabolisms by using information from the literature as well as deductive reasoning

			• Bold reactants and underline products Be sure to provide captions in the video as well as a transcript on the video page.
Create background information video – Session C, IFES	In this video, students are provided background information on the core concept of IFES, or Information Flow, Exchange, and Storage.		The instructor should provide an overview of: • how proteins themselves can react to changes in the environment, • which then feeds information back to the organisms that produce those proteins, • signaling the organism to produce more or less of the protein in response to changing environmental conditions. • how invertase relates to glucose levels in kombucha and • how glucose levels may change as metabolic activity continues. In this video, the instructor can specifically talk about the enzyme invertase which was in the

Session A - Evolution

Preparation for session (Instructor only)

Make sure all students have access to Microbe World Drawing Template.	Share using google slides or email template to students.	5 min	If sharing using a google drive, double check the template once or twice each day in the beginning to make sure students aren't accidentally working on the master template. **See Supporting Files under 3. Drawing, [3-A] and [3-B]**
Put students into groups of 4, with 2 pairs, for Microbe World.	Email students their group member's names and contact information. Encourage students to connect sooner rather than later. Let them know there will be group work.	10 min	-Students should stay in these groups for all three weeks. -Students can be grouped based on their background knowledge of microbiology and fermentation. -Students can be grouped based on who works equitably together. -Student groups of 3 will a 3-way swap with each other.

Synchronous session (Students and Instructor)

Session A Slides - Introduce "Microbe World"	Provide overview of the Unit, including learning goals and organization. Provide overview of Session A activities.	10 min	The instructor presents these slides synchronously to the whole class. Slide 1: List overarching learning goals Slide 2: Overview of 3-week lesson Slide 3: Screenshot of Canvas Module 1 Slide 4: Learning objectives for Session A

Asynchronous Session (Students only)			
Microbe World Session A – Evolution - Demonstration Video (DI) (SP)	Students watch the demo video.	20 min	Demo video is 12 mins long, but students may want to watch some parts twice.
Download Primary Literature Sources	These sources contain information needed to complete the activity.	3 min	Citations: Villarreal-Soto et al. *Understanding Kombucha Tea Fermentation: A Review*. J. of Food Science. 2018; 83(3): 580-588 https://doi.org/10.1111/1750-3841.14068Links to an external site. Knoller et al. *Fast-Growing bacterial cellulose with outstanding mechanical properties via cross-linking by multivalent ions.* Materials. 2020; 13(12):2838 https://doi.org/10.3390/ma1312283 8Links to an external site. May et al. *Kombucha: a novel model system for cooperation and conflict in a complex multi-species microbial ecosystem*. PeerJ. 2019; 7:e7565 https://doi.org/10.7717/peerj.7565Link s to an external site. Mohite and Patil. *A novel biomaterial: bacterial cellulose and its new era applications.* Biotechnology and Applied Biochem. 2014; 61(2): 101-110 https://doi.org/10.1002/bab.1148Links to an external site.
Microbe World Session A – Evolution - Individual Drawing (GI)(A)	Students draw a microbial ecosystem using the provided template outline above.	30 min	This should be done individually. Students will submit a screenshot upon completion. **See Supporting Files under 3. Drawing, [3-A].**
Microbe World Session A – Evolution – Drawing Rubric (GI)(A)	Students will evaluate their drawings against an answer key and rubric. They will also swap with their assigned group mate and evaluate their partner's drawing.	10 min	Student's drawings are assessed on the accuracy of scale used to depict each microbe, the correctness of each metabolism matched to microbes, and on the correct identification of microbial domains. **See Supporting Files under 4. Evaluations, [4-A].**
Microbe World Session A –	Students will answer questions on the biological	30 min	If meeting in a virtual group will not be possible for some students due to technology

Evolution - Group Questions (GI)(A)	scales of organization and on evolution. Answers are posted to discussion boards.		limitations or for any other reason, instructor should have alternative options. Only students who have posted answers should be able to see other responses at this point in time. **See Supporting Files under 5. Questions, [5-A].**
Session B – Pathways and transformation of energy and matter (PTEM)			
Preparation for session (Instructor only)			
Review/grade discussion boards for common errors. Incorporate feedback into synchronous session.	As the instructor grades group responses to the Session A questions, they should make notes of common errors or misconceptions. These should be added to the **Session B Slides**. Instructor can grade using the **Microbe World Drawing Answer Key** in supporting file Evaluations [4].	30 min	For the **Revisit Session A Discussion Boards** activity later, Instructor can comment on student responses and then later ask them to respond to the comments, or instructor can forgo comments and simply ask students to clarify their answers later. At this point, instructor can change the visibility of the discussion boards so that all students can see it. **See Supporting Files Evaluations [4] and Review/Revise [6-B].**
Grade Microbe World Session A drawing.	Instructor takes into account self and peer grades when making the final decision on student drawing grades.	20 min	When there are discrepancies between self and peer grades, double check the student's work against the rubric and grade accordingly.
Synchronous Session (Students and Instructor)			
Session B Slides	Using slides, go over evolution misconceptions and wrap up evolution. Introduce Microbe World Session B, new topic of PTEM.	10 mins	The instructor presents these slides synchronously to the whole class. Slide 1: Evolution and Biological scales of organization misconceptions. (Content will depend on student responses) Slide 2: Final Evolution wrap-up (Content will depend on student responses. What concepts do need to be reinforced? Descent with modification? Populations evolve, not individuals?) Slide 3: Screenshot of Canvas Module 2, details listed above. Slide 4: Learning objectives for Session B
Asynchronous Session (Students only)			
Microbe World Session B –	Students watch the demo video.	20 min	Demo video is 11 mins long, but students may want to watch some parts twice.

PTEM - Demonstration Video (DI) (SP)			
Microbe World Session B – PTEM - Individual Drawing (GI)(A)	Students write chemical reactions for the microbes they illustrated in Session A.	30 min	This activity should be done individually and students will submit a screenshot upon completion. **See Supporting Files under 3. Drawing, [3-B].**
Microbe World Session B – PTEM - Drawing rubric	Students will evaluate their drawings against an answer key and rubric. They will also swap with their assigned group mate and evaluate their partners drawing.	10 min	Student's drawings are assessed on the accuracy of chemical reactions as they relates to microbial metabolism, on the distinction of reactants vs products, and on the correctness of their Session A components of the drawing. **See Supporting Files under 4. Evaluations, [4-B].**
Microbe World Session B – Group questions	Students will answer questions on PTEM as it relates to the kombucha microbial environment. Answers are posted to discussion boards in the LMS.	35 min	If meeting in a virtual group will not be possible for some students due to technology limitations or for any other reason, instructor should have alternative options. Only students who have posted answers should be able to see other responses at this point in time. **See Supporting Files under 5. Questions, [5-B].**
Revisit Microbe World Session A Discussion Boards (SP)(A)	The instructor will have commented on student responses at this point. Students have the option to respond to the comments or questions from instructors. Alternatively, students can clarify or expand upon their answers with new information or new questions.	10 min	All students should have visibility of the discussion boards. All students should participate in this activity, regardless of their role when answering the group questions initially. **See Supporting Files under 6. Review/revise, [6-B].**
Session C – Information Flow, Exchange, and Storage (IFES)			
Preparation for the session (Instructor only)			
Review discussion boards for common errors. Incorporate feedback into	As the instructor grades group responses to the Session B questions, they should make notes of common errors or misconceptions. These should be added to	30 min	For the **Revisit Session B Discussion Boards** activity later, Instructor can comment on student responses and then later ask them to respond to the comments, or instructor can forgo comments and simply ask students to clarify their answers later.

synchronous session.	the **Session C Slides**. Instructor can grade using the **Microbe World Drawing Answer Key** in supporting file Evaluations [4].		At this point, instructor can change the visibility of the discussion boards so that all students can see it.
Grade Microbe World Session B drawing.	Instructor takes into account self and peer grades when making the final decision on student drawing grades.	20 min	When there are discrepancies between self and peer grades, double check the student's work against the rubric and grade accordingly.
Synchronous Session (Students and Instructor)			
Session C Slides	Using slides, go over PTEM misconceptions. Introduce Microbe World Session C, new topic of IFES.	10 mins	The instructor presents these slides synchronously to the whole class. Slide 1: Warm-up Slide 2: Misconceptions about PTEM. (Confusion with products, reactants, enzymes. Confusion with the difference between matter and energy.) Slide 3: Screenshot of Canvas Module 3 Slide 4: Learning objectives for Session C
Asynchronous Session (Students only)			
Microbe World Session C Introduction Video (DI)(SP)	Students watch this video to gather background information about the core concept IFES as it relates to the kombucha microbial ecosystem.	10 min	This video is 4.5 mins long, but students may choose to watch some or all of it more than once. Instructors may choose to present this material in a different way.
Microbe World Session C - Individual questions (GI)(A)	Students will answer questions on IFES as it relates to the kombucha microbial environment; how do microbes respond to signals from their environments? How does removal or addition of key species affect the environment?	30 min	These questions will be answered individually and submitted through the LMS. **See Supporting Files under 5. Questions, [5-C].**
Revisit Microbe World Session B Discussion Boards (SP)(A)	The instructor will have commented on student responses at this point. Students have the option to respond to the comments or questions from instructors. Alternatively, students can clarify or expand upon their	10 min	All students should have visibility of the discussion boards. All students should participate in this activity, regardless of their role when answering the group questions initially. **See Supporting Files under 6. Review/revise, [6-C].**

	answers with new information or new questions.		

A3.6 Teaching Discussion

The "Microbe World" Lesson exposes students to 3 core concepts of biology (Evolution, PTEM, and IFES) in a sequential activity that builds upon itself after each session. Students learn the who, what, and how of microbial ecosystems such as the gut microbial environment, or the fermenting kombucha environment. By performing literature searches, illustrating a microbial ecosystem, answering challenging critical thinking questions, and evaluating each other's work, students get a mix of formative and summative assessments to showcase their learning.

<u>Lesson Effectiveness</u>

For the Evolution session, students were generally successful in matching microbes to their respective metabolisms listed in the provided word boxes. In addition, students mostly understood that microbes evolve quickly due to their asexual reproduction and their fast rates of reproduction, together increasing the potential for rapid accumulation of mutations. Students struggled to identify evolutionary relatedness of microbes from their drawings, but this problem could likely be overcome by writing the question more clearly or by providing more background information.

For the PTEM session, students were great at writing out the appropriate chemical reaction to match their metabolism, and they were consistently able to identify reactants and products of chemical reactions. However, students struggled to identify invertase as an enzyme that facilitates one of the chemical reactions in their drawing. In addition, student's poor understanding of "matter" and "energy" made it difficult for them to explain why their networks of chemical reactions demonstrated the core concept PTEM. Instructors should explain the concepts of "energy" and "matter", and their distinctions, early and often to ensure student success.

For the IFES session, student answers to critical thinking questions showed their understanding of how signaling occurs in the kombucha microbial environment. Students understood that protein levels in the environment can cause changes in microbial protein expression levels. They also understood that removing a key species would disrupt the levels of reactants of products of certain chemical reactions, which in turn affect metabolic activity of microbes in the ecosystem. Students only struggled with this session if they misread the scenarios outlined in the short-answer questions.

Student reactions to the Lesson:

Roughly 2/3 of students across two implementations of "Microbe World" reported that the lesson helped them understand the core concepts of Evolution, PTEM, and IFES as they apply to microbial ecosystems. Specifically, students identified group work and meeting synchronously to be the most helpful for their learning. Some students appreciated the active learning approach to "Microbe World", while others struggled with learning on their own citing that the activity was more challenging because they could not ask questions in real time. Consistently, students wished that there was more synchronous time to allow for asking questions and reviewing common misconceptions. Students appreciated videos containing background material and especially valued the written transcripts for recalling information quickly from videos. Finally, students were grateful for detailed feedback on discussion posts, although this varied by section because each instructor had a slightly different approach.

Adaptations:

Modality: The "Microbe World" Lesson can be adapted by in-person format by introducing background information and demonstrations into a lecture format. Students should be provided guided notes that they can fill out as they follow along to ensure active engagement and increased retention of material.

In addition, group questions and follow-up discussion board posts can be replaced with in-person group discussions, while still maintaining that each group have a facilitator, timekeeper, and recorder. The "Microbe World" illustrations may be completed using colored cutouts and paper, or if the classroom is fitted with student laptops, the instructor may still choose to have students use Google Slides. If the instructor decides to have the students create analog illustrations, be sure to limit the amount of "art" they will need to do, so that the focus can remain on demonstrating biological core concepts. In addition, if primary literature research is to be performed in class, to remain inclusive, students should be provided computers with internet access, or printed out copies of relevant papers.

Intended audience: Because this lesson was created for first-year undergraduate students with a general interest in biology, there are many examples of tasks that could be made more challenging. For example, students were provided with word boxes containing the potential microbes and microbial metabolisms that exist in the fermenting kombucha environment. In addition, students were provided with reactants and products for chemical reactions in the second session of the activity. To expand the number of skills and concepts students gain, as well as increase student ownership from this activity, the instructor could have students build their microbial ecosystems from scratch. Students can choose their own microbial ecosystems, choose four (or more) microbes present in that ecosystem, and then add in the chemical reactions for the metabolism performed by each of their chosen microbes. Some examples of microbial ecosystems can be found in foods (cheese rinds, pickled foods, and other fermented foods and drinks), in the environment (local rivers or lakes, hydrothermal vents, and soils), or on the body (the oral cavity, skin, or nose).

A3.7 Supporting Files

1. Slides: See supporting Table 2 – Teaching Timeline for detailed information on slide content for [1-A], [1-B], and [1-C].

2. Demo Video: should be recreated by the instructor.

3. Drawing:

[3-A]: Activity Sheet and Drawing Template

Microbe World Activity Sheet – Exploring the Gut Microbial Environment and Fermenting Kombucha

Microbe World Session A – Evolution – Individual Drawing Activity Sheet:

Instructions: Please read this document before watching the demonstration video.

In this video, Lauren will demonstrate how to draw a microbial ecosystem using the cartoon shapes we've provided you in Google Slides. She will use the gut microbiome as her microbial environment and you will use fermenting kombucha! She is doing this as a guide to model how you should do the assignment. You will notice that this activity sheet has a word list for both the gut microbiome and kombucha. This is just for you to see that the assignment Lauren did is in parallel to yours.

By the end of this assignment, you will be able to:

1. Identify microbes across the three domains: Bacteria, Archaea, and Eukarya.
2. Identify what microbes are involved in the fermentation of kombucha based on their metabolism.
3. Synthesize relevant information from the provided primary literature resources.
4. Explain the role of Evolution in the development of diverse microbes and microbial ecosystems.
5. Visualize how biology interacts at the molecular/cellular, organismal, and ecosystem scales.

First, make a copy of this Google Slides Template and save it to your Google Drive.

For your drawing, be sure to include all elements listed in the table below. Your drawing should be roughly to scale as you saw in the Introduction to Microbe World Session A - Evolution Demonstration Video. Your drawing does not need to be perfect; we would just like to get you thinking about relative sizes of different parts of an ecosystem.

By the end of this assignment, you will have added many components to your drawing and it may start to look a little crowded. But don't worry! Your drawing does not need to be the work of Maria Peñil Cobo! The rubric evaluates the content of your drawing and not its appearance.

As in the demonstration video, it will be easiest to do this assignment if you complete each part in this order:

1. **Add all elements from Table 1 below** – In Google Slides, feel free to copy/paste each shape to duplicate them. You can also rotate the shapes and adjust the sizes!
2. **Add a microbial scientific name using Table 2 below** – Use the list of microbes we've provided. They are grouped by function, but we purposefully did not tell you which function each microbe is capable of performing. You will have to search the provided resources for that type of information. Scientific names are written as *Genus species*. Both words are italicized, the genus is capitalized, and the species name is all lowercase. It is important that you understand how scientific names of organisms are formatted so that you can read and write

scientific literature correctly. (Depending on what articles you've chosen, this may be important for your Discovery Poster Project!)

3. **Label what domain each microbe belongs to** – Once you've learned what microbes can perform which functions, you will be able to designate a domain towards them. Don't forget, fungi are Eukarya! And as a reminder, "Eukarya" is a domain of life, while "eukaryote" refers to an organism whose cells contain a nucleus.

Listed below are the components for Session A. This information is also provided in your template:

Table 1.	
The Gut Microbiome (we do)	**Kombucha (you do)**

• Epithelial cells
• Microbes:
   a. Fermentative, cellulolytic microbe
   b. Microbe that performs cellular respiration
   c. Syntrophic methanogen
   d. Probiotic microbe
	• Cellulose strands (biofilm)
	• Microbes:
	   a. Invertase producing microbe
	   b. Microbe that produces acetic acid
	   c. Cellulose producing microbe
	   d. Microbe that performs ethanol fermentation

Microbe Scientific Name List:

Please note that the gut microbiome functions do not align to the kombucha categories. I.e. ***Bacteroides cellulosilyticus*** does not do the same thing as ***Acetobacter xylinoides*** (both in bold below).

Table 2.	
The Gut Microbiome (we do)	**Kombucha (you do)**
Bacteroides cellulosilyticus *Ruminococcus albus* *Fibrobacter succinogenes* *Enterococcus faecalis*	***Acetobacter xylinoides*** *Bacterium gluconicum* *Komagataeibacter kombuchae* *Gluconobacter oxydans*
Escherichia coli *Salmonella enterica* *Gordonibacter pamelaeae*	*Acetobacter xylinum/Komagataeibacter xylinus** *Acetobacter pasteurianus* *Gluconobacter hansenii* *Agrobacterium tumefaciens*

Saccharomyces boulardii *Lactobacillus acidophilus* *Lactobacillus reuteri* *Bifidobacterium lactis*	*Schizosaccharomyces pombe* *Saccharomycodes ludwigii* *Saccharomyces cerevisiae* *Zygosaccharomyces bailii*
Methanobrevibacter smithii *Methanosphaera stadtmanae* *Methanomassiliicoccus luminyensis*	For your kombucha drawing, one of these categories will be used twice!

*Sometimes bacteria are renamed or reclassified. The first name is an old name that has now been updated to be the second name.

When you've completed you're drawing, please take a screen shot and turn it in through Canvas. The rubric is located in the next part of the module. It will unlock after you submit this part of your assignment.

Session A drawing template:
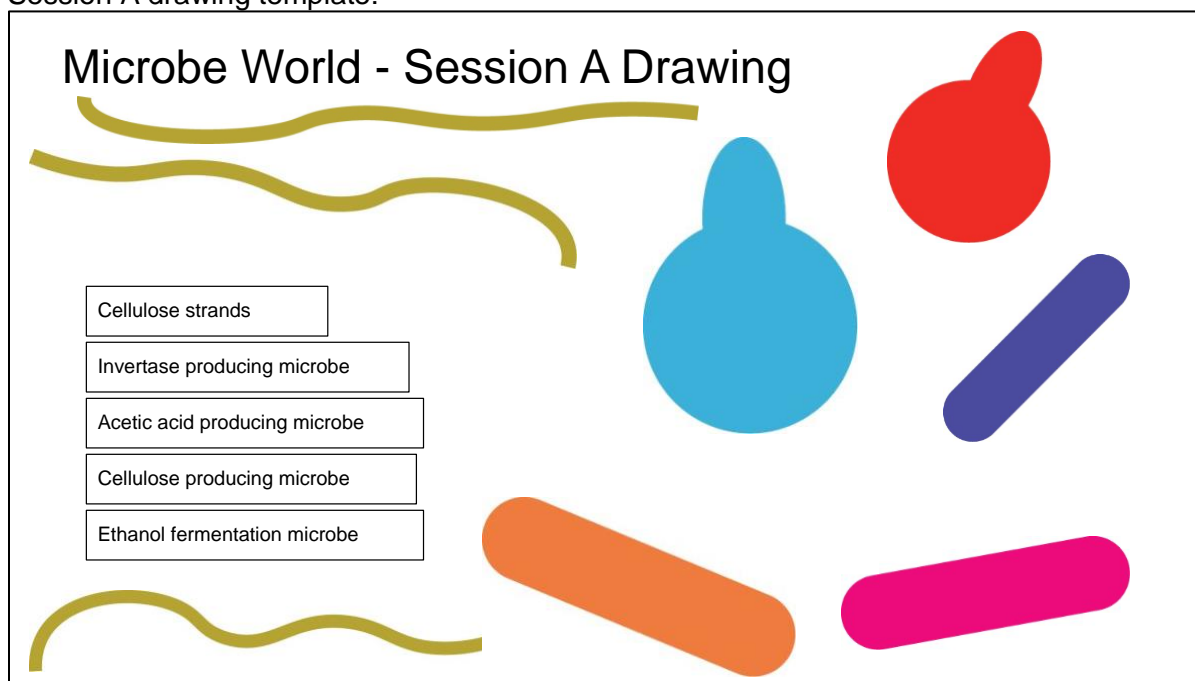
[3-B]: Activity Sheet and Drawing Template

Microbe World Activity – Exploring the Gut Microbial Environment and Fermenting Kombucha Instructions
Microbe World Session B – PTEM - Individual Drawing:

Using your map from last week, you will continue to add components of the microbial ecosystem that is fermenting kombucha. **The purpose of this activity is to improve your understanding of PTEM in microbial ecosystems while learning about the science behind kombucha brewing.** Remember you will be working on the drawing by yourself, as you did in Session A. Be sure to submit a screen shot of your drawing on this page and then exchange your drawing with your assigned peer for your self- and peer-evaluations. Third, you will come together as a group to answer the follow-up questions listed at the bottom of this page.
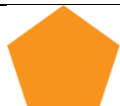
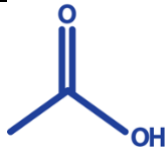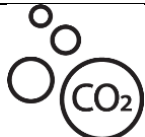By the end of this activity, students will be able to:
1. Define the substrates and products for microbes, both fermenting and non-fermenting, in the kombucha microbial ecosystem.
2. Explain how Pathways of Transformation of Energy and Matter (PTEM) are utilized to create a mixture of molecules in a kombucha microbial community.
3. Use a rubric to appropriately evaluate the work of your peers.

Note to students: This week we have the questions listed at the bottom of this activity page. You will still be answering these questions as a group and the recorders will still post the group response in a discussion board. Like you did last week, please choose a facilitator, a timekeeper, and two recorders. Make sure to switch roles so that nobody has the same role twice (groups of 3 may have repeat recorders). **One recorder will be in charge of posting the answers to questions 1 and 2 and the other will post the answers to questions 3 and 4 as a separate discussion post.** Recorders can choose to type the group answers into the discussion board as the group is meeting, which might save some time.

Instructions (this part should take you 20-40 min to complete):

1. Read through the definitions in the table below. Add in reactants/products for each type of microbe. You DO NOT need to draw molecular structures! Feel free to use the cartoons we have provided or their written names.
   a. You can use the icons below by copy and pasting them from this document into your Google Slide. Or you can find the cartoon shapes in the same Google Drive folder for your section from last week.
   b. Use the vocabulary terms listed below and described in the background material. It may help to revisit some of the Session A videos. If you think it useful, you can do additional research on these terms, though you should be able to complete this activity with the information from primary sources we will be providing you.
   c. **Note that each microbe will have a reaction associated to it and some of the molecules will be used more than once.**
2. In your drawing, make sure you **bold your reactants** and underline your products.

Products, Reactants, and Enzymes		Definitions
Sucrose		Table sugar, a 12-carbon polysaccharide. A sugar made up of one glucose and one fructose molecule.
Glucose		A simple sugar. It can make up more complex sugars and is used by organisms as an energy source.

Fructose		A simple sugar. It can make up more complex sugars and is used by organisms as an energy source.
Ethanol		The alcohol most commonly found in alcoholic beverages that is safe to consume at low quantities. **Note:** alcoholic fermentation produces ethanol. Though people say "fermenting kombucha" to refer to the entire brewing process, not all reactions by the microbes in kombucha are fermentation reactions.
Acetic Acid		This is acid is the main component of vinegar, and gives kombucha its vinegary taste. **Cool bio fact:** a group of insects commonly known as vinegaroons produce this acid as part of their defense mechanism when threatened.
CO_2		Colorless gas, byproduct of cellular respiration and fermentation. If the fermentation is sealed, this makes kombucha fizzy!
Invertase		This enzyme takes sucrose and breaks it up into its simple components: fructose and glucose. Because this is an enzyme, it is considered neither a product nor a reactant. Place it on top of the arrow for the reaction which it facilitates. **Hint:** the organism that produces this in kombucha releases it into its environment, where the reaction will take place.
Cellulose Fibers		A molecule consisting of hundreds – sometimes even thousands – of carbon atoms (in addition to hydrogen and oxygen). It makes up cell walls in plants, and humans can't digest it, though it does provide fiber. Some microbes can also produce cellulose

Submit a screen shot of your drawing on Canvas!

Session B Drawing Template:

Microbe World - Session B Drawing

Glucose
Fructose
Ethanol
Cellulose
Acetic acid
CO2
Invertase

4. Evaluations:

[4-A, 4-B]: Activity Sheet, Answer key, and Rubric for Individual Drawing Evaluations

Instructions apply to both Session A and Session B Evaluations:

Now that you've submitted a screen shot of your Microbe World drawing, be sure to also send your assigned groupmate a copy. This component of the activity ensures you have feedback, but also allows you to see how someone else might have created an entirely different drawing than you! Using the provided rubric, we would like you to evaluate your drawing **and** your peer's. Submit your score and your peer's score along with your reasoning.

The rubric has criteria listed on the left and point values for different levels of completion. Each criterion for Session A is worth up to 4 points, totaling 12 points, and up to 5 points for Session B, totaling 15 pts. Read each description to determine how many points are awarded. After completing your evaluation and receiving feedback, correct and clarify your drawings.

Rubrics provide standards for consistent and equitable grading practices. It is also a way for us as instructors to communicate our expectations to you, the students. It can help you to understand what is important in this assignment and how your work will be graded.

Some things to think about when you are evaluating: What were some things from another student's drawing that surprised you? Was there anything you did not consider? - You do not need to turn in answers to these questions, they are just to get you thinking about your approach to the assignment.

For full points on this assignment, you should submit:

1. A self-evaluation of your drawing based on the rubric.
2. An explanation of your grade based on the rubric.
3. A peer-evaluation of another student's drawing based on the rubric.

4. An explanation of the grade you gave based on the rubric.

Answer key for student evaluations of Session A and B drawing:

Cellulose strands	Invertase

| Glucose | Ethanol | Sucrose | Glucose | Ethanol | Acetic acid |
| Fructose | CO2 | | Fructose | CO2 | |

| Glucose | Cellulose |

Cellulose producing microbe:
Any of these (bacteria):
Aerobacter xylinum/
Komagataeibacter
xylinus
Agrobacterium tumefaciens
Azotobacter
Rhizobium

Ethanol fermentation microbe:
Any of these (Eukarya):
Schizosaccharomyces pombe
Saccharomycodes ludwigii
Saccharomyces cerevisiae
Zygosaccharomyces bailii

Invertase producing microbe:
Any of these (Eukarya):
Schizosaccharomyces pombe
Saccharomycodes ludwigii
Saccharomyces cerevisiae
Zygosaccharomyces bailii

Acetic acid producing microbe:
Any of these (bacteria):
Acetobacter xylinoides
Bacterium gluconicum
Komagataeibacter
kombuchae
Gluconobacter oxydans

[4-A] Rubric for student evaluations of Session A drawing

Microbe World Session A – Individual Drawing Rubric – 12 pts total			
Criteria	4 pts - Complete	2 pts - Satisfactory	0 pts – Incomplete
Scale	All microbes are depicted to scale with their environmental components. Bacteria and archaea are roughly 1/3-1/5 the size of yeast. And the cellulose strands are much longer than both.	Some, but not all, scale relationships are correct.	All microbe sizes are incorrect or the assignment was not turned in or turned in late.
Labels	All microbes and environmental components are correctly labeled (2 points) AND Scientific names are written in correct format (2 points).	Some microbes and environmental components, but not all, are correctly labeled (1 point). Some microbe names are written in the correct scientific format (1 point).	Microbes are incorrectly labeled and names are not in the correct format or the assignment was not turned in or turned in late.
Microbial Domains	All microbial domains are identified correctly.	Some microbial domains are identified correctly.	Microbial domains are incorrectly labeled or the assignment was not

			turned in or turned in late.

[4-B] Rubric for student evaluations of Session B drawing

Microbe World Session B – Individual Drawing Rubric – 15 pts total			
Criteria	5 pts - Complete	2.5 pts - Satisfactory	0 pts – Incomplete
Diagrams Matching microbes to reactants and products.	All substrates and products are correctly labeled for each microbe. See answer key.	Only some products and substrates are correctly labeled for microbes.	No products and substrates are correctly associated with the corresponding microbe; work is turned in late or not at all.
Products and Reactants Correct identification of products, reactants, and reaction direction.	Reaction arrows are pointing in the correct direction. Products are in underlined and reactants in bold.	Only some reactions have arrows in the correct directions OR Arrows are in the correct direction but products and reactants are not correctly identified.	Arrows are pointing in the incorrect direction and products and reactants are not correctly identified ; work is turned in late or not at all.
Session A drawing is accurate Drawings were updated based on what was learned in the Session A evaluations.	Microbes are labeled with the correct metabolisms and domains based on the Session A Microbe World Drawing Rubric.	n/a	Errors from Session A remain in this drawing.

5. Questions:

[5-A]: Activity Sheet and Group Questions

Microbe World Activity Sheet – Exploring the Gut Microbial Environment and Fermenting

Kombucha Microbe World Session A – Evolution – Group Questions Activity Sheet:

Instructions:

When you meet in your group for the first time, be sure to introduce yourselves to one another. Make sure to share your name and pronouns (if you are comfortable), as well as your hometown (however you choose to define it), and your favorite fermented food or beverage! If you can't think of a fermented food or beverage that you love, ask your group mates for recommendations!
In your groups, take turns sharing your answers to your reflection questions about working in a group effectively. Each student should take 1-2 minutes sharing what they've written down.

In your group, be sure to designate roles for answering and turning in these questions. As mentioned in this week's kickoff meeting, your reflection on your constructive and destructive behaviors can help inform what role may fit you best. Remember what role you have this week because next week we will ask you to fulfill a different role. Groups of 4 will have one person without a role, but that does not mean they don't contribute. Every member of the group should contribute to the question answers. The reason we assign roles is to help keep your group working efficiently and effectively.

Facilitator – This person makes sure that your group stays on task, works efficiently, and most importantly, ensures that each group member has an opportunity to contribute in an equitable manner.

Recorder – The recorder makes sure to note what people are saying and ask for clarification as needed. This person will also be responsible for turning in the group's responses to Canvas. Feel free to post answers in written or video format! If you have any questions that are not already addressed on the Technology Canvas page in Module 0, please contact your instructor.

NOTE: For this assignment, we would like you to have **two recorders**. One person will record the group answers for Microbe World questions related to the Biological Scales of Organization and one person will record the group answers for Microbe World questions related to Evolution.

Timekeeper – The timekeeper ensures that the group moves through the work at a timely pace. For example, the timekeeper may ensure that the group spends no more than 10 minutes on each question today.

<u>In your groups, answer these questions:</u>

If you are the recorder, post the group answers to the discussion boards on Canvas by the [insert due date]. Feel free to make your post a video post or a voice recording if you're feeling adventurous! Even though only one student from your group is posting answers, everyone should check the discussion pages to see the responses other groups came up with.

One discussion board is called Microbe World Session A – Evolution – Group Questions on Biological Scales:
1. Give an example from either system (microbiome and fermentation) that fits into each of these three scales: o Molecular/cellular: o Organismal: o Ecosystem:
2. Think back to the Unit Introduction Video when Lauren talked about elephants as organisms and bacteria as organisms. In a few sentences, explain why or why not you think the biological scales of organization directly correlate to size. We have included the figure for your reference below.

Biological Scales of Organization

Raven/Berg, Environment, 3/e
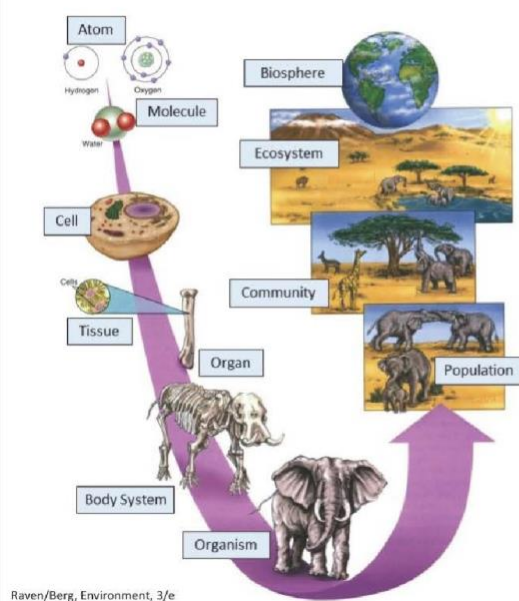
Atom	The basic unit of a chemical element
Molecule	Groups of atoms, smallest unit of most chemical compounds
Cell	Smallest functional unit of life
Tissue	The material of living things, consisting of specialized cells and their products
Organ	Groups of tissues with similar functions
Body system	Collection of organs that work together for a common purpose
Organism	Individual living thing
Population	Group of organisms of one type that live in the same area
Community	Populations that live together in a defined area
Ecosystem	Community, and its nonliving surroundings
Biosphere	The part of Earth that contains all ecosystems

The other discussion board is called Microbe World Session A – Evolution – Group Questions on Evolution:

3. Using what you have learned about microbes, microbial communities, and the core concept Evolution, answer the questions below. These conceptual elements are taken from the Evolution Core Concept List E1-E9.
    o   E1: All living organisms share common ancestors at some time in the past.
        ▪   Pick one group member's drawing to refer to. Which microbes in that drawing are most closely related to each other? The least? Rank each relationship in order of most closely related to least.
            1.
            2.
            3.
            4.
        ▪   Hint: remember that phylogenetic trees show evolutionary distance between organisms with the length of the lines between two organisms. You may find it helpful to refer to the figure from Brock's Biology of Microorganisms 13th Edition below for your answer.
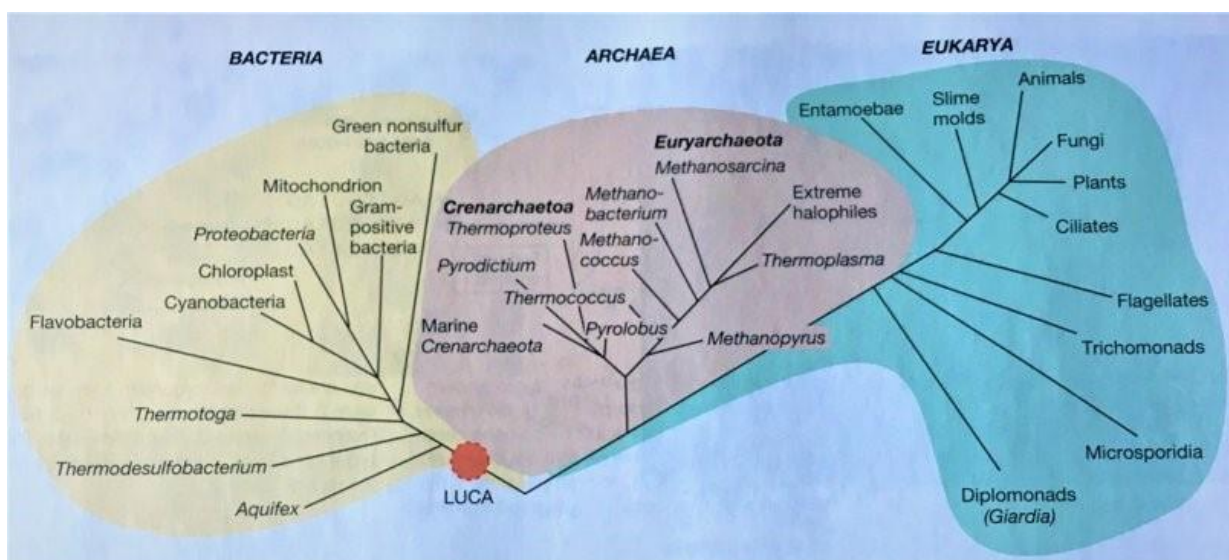
- o E5: Organisms have greater fitness if they have a phenotype that increases their ability to survive and reproduce in a particular environment.
    - How does this idea relate to the co-evolution of humans and their gut microbial community? How does it relate to microbes in the gut ecosystem?
- o E8: The rate of evolutionary change varies and is influenced by many factors, including mutation rate, generation time, and environmental variation.
    - In a few sentences, answer the question: Why are microbes capable of evolving so quickly?

[5-B]: Activity Sheet and Group Questions

Microbe World Activity Sheet – Exploring the Gut Microbial Environment and Fermenting

Kombucha Microbe World Session B – PTEM – Group Questions Activity Sheet:

Group Questions Instructions:
These questions are to be answered in the Canvas Discussion Board called 'Microbe World Session B – Group Questions', but are listed here for your reference. As a group, answer the following questions (30-45 min):

One recorder will be in charge of posting the answers to questions 1 and 2 and the other will post the answers to questions 3 and 4 as a separate post.

PTEM questions for both ecosystems:
1. In each of these systems, which molecules are being broken down to provide energy to the microbes? Where in the molecules is this energy stored?
   - o Gut microbiome:
   - o Kombucha:
   - o Energy is stored in the _____.
2. In kombucha, there is one example of a microbial metabolism that stores, rather than releases, energy. Which microbe performs that metabolism? Can any of the other microbes in your drawing break down that product?

3. Using Lauren and JP's gut microbiome drawing as a reference (embedded in the discussion board page), which microbe in your gut would break down the product from your answer to question #2?
4. In 3-5 sentences, using both your own drawing, the reference drawing below, and the conceptual elements, explain how your answers to the previous two questions exemplify the core concept of PTEM. Include at least one conceptual element in your explanation.

Post your question answers to Canvas in either written, audio, or video format. As mentioned last week, if you have any Canvas tech questions that are not addressed in Module 0, please reach out to your instructor(s).

[5-C] Activity Sheet and Individual Questions

Microbe World Activity – Exploring the Gut Microbial Environment and Fermenting Kombucha

Instructions
Microbe World Session C:

In Session B you explored how yeast and bacteria in kombucha use glucose and fructose to get energy and produce all of the products listed in this figure below in the beaker on the right. Some of your drawings may have components that look similar to the one below by May et al., 2019. The figure below will help you complete this activity.
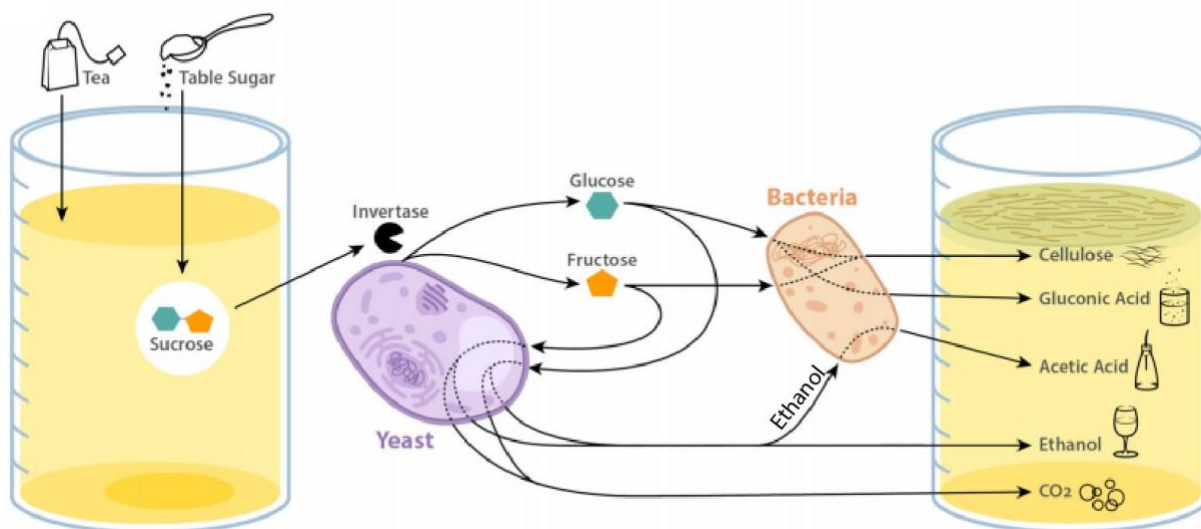

Table sugar (sucrose) is what is originally added to the kombucha mix to feed the yeast and bacteria. But yeast and bacteria cannot use sucrose (green + orange above), instead needing the simple sugars, glucose (green) and fructose (orange). Invertase (pacman looking shape in drawing) is an enzyme that yeast produces that breaks down sucrose into glucose and fructose for use by the microbes in the kombucha. Information from the environment regulates the amount and effectiveness of invertase so that the yeast does not go without glucose, but also so that it is not spending energy making invertase that is not needed. Answer the following the questions that illustrate how information from the environment regulates the activity of this enzyme. Keep in mind that we are more interested in how you work through these questions rather than whether you get them right or wrong. Try to do

this with the information provided in the introductory video and in the figure, without doing additional research.

1. As a kombucha brew master, you do some research and find that the gene which controls invertase production is turned on when there are low levels of glucose (low glucose --> more invertase).
   a. How might this be beneficial to the yeast?
   b. Predict what would happen to the amount of invertase produced by the yeast in the kombucha if all of the bacteria suddenly died? What if instead of dying, all of the bacteria suddenly doubled? **Hint**: remember that there is a limited amount of sucrose in the environment. Be sure to support your answers by explaining your reasoning.
   c. How does this scenario demonstrate the core concept of IFES?

2. Interested in the flavor of your kombucha and wanting to avoid it tasting like vinegar, you do more research and find out that invertase is most active (works best) in environments that are somewhat, but not too, *acidic*.
   a. Knowing this, predict when the invertase would be most active: *early in fermentation, halfway through fermentation, at the end of fermentation (ie. A long time later)*? Be sure to support your answer as to why this would be when it is most active.
   b. At the stage you just described in (a), what is happening to the glucose levels in the kombucha? How are these glucose levels then affecting the production of new invertase by the yeast? To simplify the system, you can ignore the bacteria and yeast metabolizing the glucose and focus solely on the effects of invertase on the glucose levels and vice versa.
   c. How does this scenario demonstrate the core concept of IFES?

6. Review/Revise

[6-B] Instructions:
Now that you have had a chance to work on Microbe World Session B - PTEM, and we have had a chance to address some common areas for improvement on your group answers to Microbe World Session A - Evolution, **every person from each group** should go back and respond to their own group's discussion post, correcting one misconception or clarifying one of your answers to better fit the response's relation to the Core Concepts. Alternatively, you are welcome to comment on other groups' responses; asking questions, elaborating on their ideas further, or bringing up new ideas are all ways to achieve credit for this activity. Click on both of the discussion board links to make a comment.

Every student must make a comment in each of the discussion boards to receive full points for this activity (5 points per response/discussion board).

[6-C] Instructions:
Now that you've finished Microbe World Session C - IFES and we have had a chance to review the answers to the group questions, make sure to revisit the discussion board like you did last week and 'reply' to your answers or another group's answers. Your reply should update or add to what was already posted for at least one of the three questions to receive full credit. Your reply might ask

questions, elaborate on ideas further, or bring up new ideas. These are all ways to achieve credit for this activity.

Every student must make a comment in each of the discussion boards to receive full points for this activity (5 points per response/discussion board).

A3.8 References

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