



# *Lactobacillus* bile salt hydrolase substrate specificity governs bacterial fitness and host colonization

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**Primary bile acids (BAs) are a collection of host-synthesized metabolites that shape physiology and metabolism. BAs transit the gastrointestinal tract and are subjected to a variety of chemical transformations encoded by indigenous bacteria. The resulting microbiota-derived BA pool is a mediator of host–microbiota interactions. Bacterial bile salt hydrolases (BSHs) cleave the conjugated glycine or taurine from BAs, an essential upstream step for the production of deconjugated and secondary BAs. Probiotic lactobacilli harbor a considerable number and diversity of BSHs; however, their contribution to *Lactobacillus* fitness and colonization remains poorly understood. Here, we define and compare the functions of multiple BSHs encoded by *Lactobacillus acidophilus* and *Lactobacillus gasseri*. Our genetic and biochemical characterization of lactobacilli BSHs lend to a model of *Lactobacillus* adaptation to the gut. These findings deviate from previous notions that BSHs generally promote colonization and detoxify bile. Rather, we show that BSH enzymatic preferences and the intrinsic chemical features of various BAs determine the toxicity of these molecules during *Lactobacillus* growth. BSHs were able to alter the *Lactobacillus* transcriptome in a BA-dependent manner. Finally, BSHs were able to dictate differences in bacterial competition *in vitro* and *in vivo*, defining their impact on BSH-encoding bacteria within the greater gastrointestinal tract ecosystem. This work emphasizes the importance of considering the enzymatic preferences of BSHs alongside the conjugated/deconjugated BA–bacterial interaction. These results deepen our understanding of the BA–microbiome axis and provide a framework to engineer lactobacilli with improved bile resistance and use probiotics as BA-altering therapeutics.**

*Lactobacillus* | *Acidophilus* | *gasseri* | bile salt hydrolase | bile acid

The human gastrointestinal (GI) tract microbiota plays a critical role in the establishment and maintenance of human health. There is a diversity of microbes that encode millions of genes absent from the human host (1, 2), which are responsible for unique biochemical transformations that occur in the GI tract (3, 4). Perturbations to the gut microbiota and metabolome are associated with the onset and progression of many diseases, such as inflammatory disorders (5–7), metabolic syndromes (8–10), cancers (11–13), and *Clostridioides difficile* infection (14, 15). Bile acids (BAs), a major component of bile, represent an important class of metabolites that shape host physiology, metabolism, and the gut microbiota (16, 17). BAs are produced by host hepatocytes from cholesterol, stored in the gall bladder, and excreted into the proximal small intestine to aid in the solubilization and absorption of lipophilic nutrients and vitamins after the ingestion of food (18, 19). As they transit through the small intestine, the majority of BAs (>95%) are reabsorbed in the terminal ileum and undergo enterohepatic recirculation, where a portion of BAs escape and reach peripheral organs (19). Remaining BAs that travel to the large intestine are either passively absorbed by the epithelium or excreted in the feces. BA receptors, such as the nuclear receptor farnesoid X receptor or the G protein-coupled bile acid receptor 1 (GPBAR1, also known as TGR5) are expressed

throughout the body and are especially abundant in the liver and the GI tract (19–21). BA receptors recognize BAs as agonists or antagonists for receptor signaling and monitor local BA levels to control many aspects of energy metabolism (22), immunity (23, 24), homeostasis (25, 26), and negatively regulate BA synthesis (20, 21).

BAs are weak acids with detergent-like properties that inherently restrict the growth of select bacteria while enriching for others (27–30). Given the dynamic flux of bile through the GI tract, members of the microbiota have evolved strategies to modify BAs, thereby detoxifying BAs and promoting survival (17, 29, 31). Host-derived BAs, referred to as “primary BAs,” are conjugated via an amide bond to either a glycine or taurine (Fig. 1A and *SI Appendix*, Fig. S1). Conjugated BAs are excreted into the proximal small intestine where they are recognized and cleaved by bacterial bile salt hydrolases (BSHs), which liberate the conjugated amino acid and yield deconjugated BAs (31). This step is considered to be a gatekeeper of downstream BA metabolism because deconjugated BAs serve as precursors for microbial-encoded transformations that generate “secondary BAs” (32, 33), which greatly expand the chemical diversity of the BA pool (34). Secondary BAs that undergo enterohepatic recirculation are recycled and reconstituted in the liver before excretion into the GI tract (19). These conjugated secondary BAs also serve as substrates for BSHs, and once deconjugated can undergo further biotransformation. BSH activity is therefore required for the formation and

## Significance

The transformation of bile acids (BAs) by the gut microbiota is increasingly recognized as an important factor shaping host health. The prerequisite step of BA metabolism is carried out by bile salt hydrolases (BSHs), which are encoded by select gut and probiotic bacteria. Despite their prevalence, the utility of harboring a *bsh* is unclear. Here, we investigate the role of BSHs encoded by *Lactobacillus acidophilus* and *Lactobacillus gasseri*. We show that BA type and BSH substrate preferences affect *in vitro* and *in vivo* growth of both species. These findings contribute to a mechanistic understanding of bacterial survival in various BA-rich niches and inform future efforts to leverage BSHs as a therapeutic tool for manipulating the gut microbiota.

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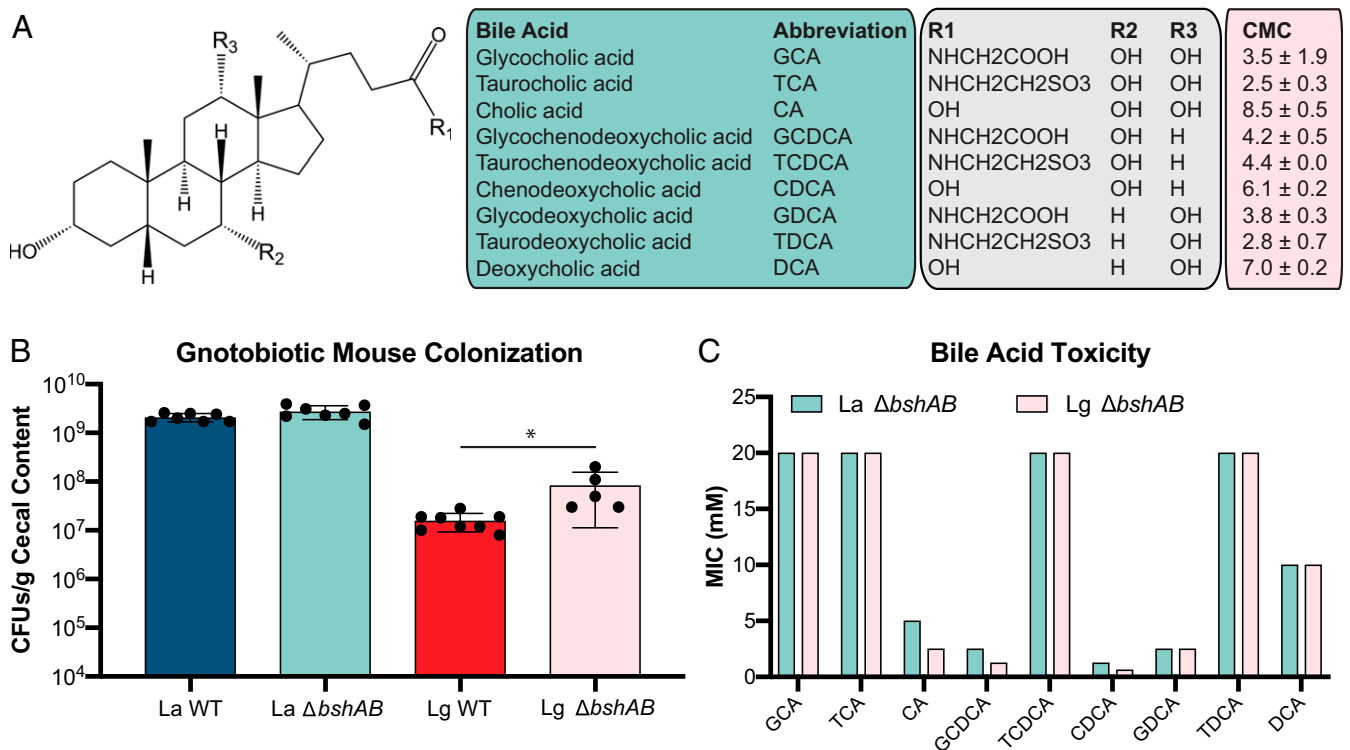
The authors declare no competing interest.

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**Fig. 1.** BA structures differ in inhibition of *Lactobacillus* species. (A) BA structures, abbreviations used in this study. All structures shown in *SI Appendix, Fig. S1*. CMC values calculated in *SI Appendix, Fig. S2* and represent the mean CMC from  $n = 2$  experiments  $\pm$  SEM. (B) WT *L. acidophilus* (La WT),  $\Delta bshAB$  *L. acidophilus* (La  $\Delta bshAB$ ), WT *L. gasseri* (Lg WT), and  $\Delta bshAB$  *L. gasseri* (Lg  $\Delta bshAB$ ) monocolonization of  $n = 5$  to 8 germ-free C57BL/6 mice. CFUs were counted from cecal contents 7 d after colonization. Asterisks represent significant ( $*P < 0.05$ ) differences from WT by Mann–Whitney *U* test. (C) BSH-null *L. acidophilus* and *L. gasseri* ( $\Delta bshAB$ ) strains were used to determine BA MICs. Bars represent mean MICs from  $n = 3$  independent experiments. MICs did not vary between experiments.

upkeep of the BA pool, its many functions, and it is likely a critical mechanism by which many intestinal bacteria shape the GI tract niche they inhabit (26, 35).

While BSHs are broadly distributed in members of the gut microbiota as well as some pathogens (36–38), many studies have focused on characterizing BSH activity from *Lactobacillus* species (39–47). Harboring a *bsh* gene is considered a desirable trait of probiotic lactobacilli due to the dogma that BSH activity contributes to bile resistance and has a hypocholesterolemic effect (39, 48). Recent work has demonstrated that lactobacilli often harbor multiple distinct BSHs as a strategy to adapt to their host niche in the gut, making them a rich source of *bsh* diversity (44). Additionally, taxonomic profiling of BSHs demonstrated that lactobacilli encode for several prominent and highly active BSH phylotypes (49). Furthermore, lactobacilli are associated with increased BSH function (50). Despite the apparent importance of lactobacilli in the GI tract, the relationship between BSH activity and *Lactobacillus* host colonization is still not well understood, and the assertion that BSH activity reduces BA toxicity is poorly supported in the literature (38, 51, 52). Additionally, the use of qualitative plate precipitation assays and heterogeneous bile preparations, such as ox-gal, have impeded a mechanistic understanding of *Lactobacillus*–BSH–BA interactions in vitro and in vivo (53).

Here, we investigate, characterize, and redefine the role of dual BSHs encoded by *Lactobacillus acidophilus* NCFM and *Lactobacillus gasseri* ATCC 33323 with respect to bacterial fitness and BA tolerance in vitro and in vivo. Leveraging bacterial genetics and biochemical approaches, we describe how key differences in BSH substrate preferences impact *Lactobacillus* growth, physiology, and tune the global transcriptional response

to BAs. In marked contrast to the previously ascribed functions of BSHs, we report conditions in which BSHs increase the toxicity of BAs. Finally, we show that BSHs can be used to alter microbial dynamics between *bsh*<sup>+</sup> and *bsh*<sup>−</sup> lactobacilli. This work reexamines the relationship between BSH-encoding lactobacilli and the BA determinants that mediate colonization of the GI tract and provides a rational basis for the manipulation of BA metabolism that impacts the gut microbiota and host health.

## Results

### BA Conjugation and Structure Determine Toxicity against *Lactobacillus*.

*L. acidophilus* NCK1909 and *L. gasseri* NCK2253 each encode two BSHs, *bshA* and *bshB*. To investigate the contribution of these enzymes to *Lactobacillus* fitness in vivo, BSH-null strains ( $\Delta bshAB$ ) were constructed by deleting both *bsh* genes by allelic exchange (54). WT and  $\Delta bshAB$  strains of *L. acidophilus* and *L. gasseri* were monoassociated in germ-free mice for 7 d when ceca were harvested. Given that BSH expression is reported to promote survival in the presence of conjugated BAs (38, 51, 52), we hypothesized that WT strains would better colonize the germ-free GI tract, which lacks microbial BSHs, relative to  $\Delta bshAB$  strains. Cecal content was enumerated for lactobacilli and the BA pool was quantified using targeted liquid chromatography/mass spectrometry (LC/MS). Evidence of WT *bsh* expression was exhibited by the increased abundance of deconjugated BAs and the coordinated decrease in conjugated BAs compared to the  $\Delta bshAB$  mutant (*SI Appendix, Fig. S2*). While no difference in colonization was detected between *L. acidophilus* strains, *L. gasseri*  $\Delta bshAB$  colonized significantly better than WT (Fig. 1B). This suggests that BSH activity in *L. gasseri* may be detrimental to the colonization of the

GI tract, a finding that contradicts the assumed role of these enzymes (38, 51, 52).

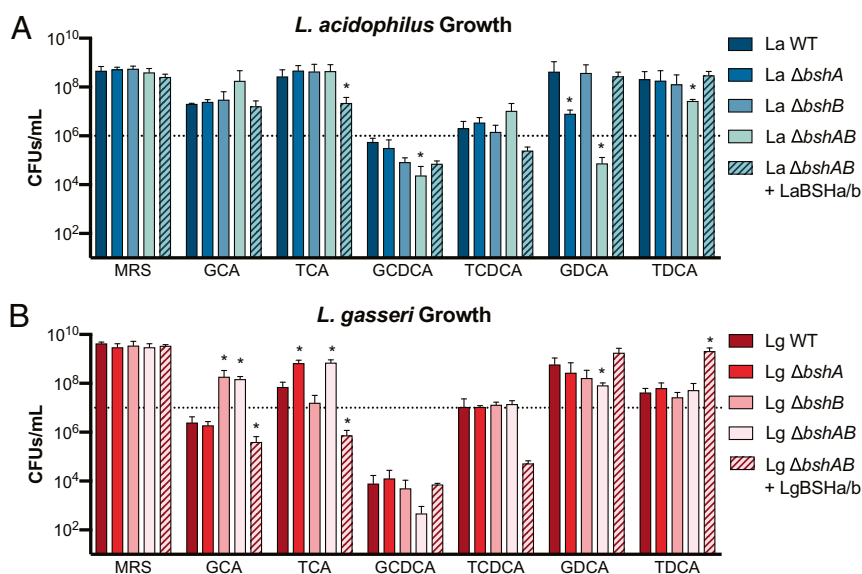
Given this observation, we sought to better define the interaction between *Lactobacillus bsh* and the glycine or taurine conjugation of three of the most prominent BAs found in the human GI tract: Cholic acid (CA), chenodeoxycholic acid (CDCA), and deoxycholic acid (DCA), illustrated in Fig. 1A. While the BA pool is comprised of many chemically distinct BAs, their structures vary by the mere presence or absence of a hydroxyl or conjugated amino acid. The detergent properties of BAs similarly vary and can induce membrane damage through lipid solubilization, surface protein disruption, or lysis (28). In order to understand the relative detergent properties of BAs, we quantified the critical micelle concentration (CMC) of the BAs used in this study (Fig. 1A and *SI Appendix*, Fig. S2). In general, conjugation lowered the CMC of a given BA, indicating an increased potential for membrane solubilization and damage (28, 55, 56).

To determine whether BA CMCs correlated with toxicity, the minimum inhibitory concentration (MIC) of each BA was determined for the *L. acidophilus*  $\Delta bshAB$  and *L. gasseri*  $\Delta bshAB$  mutants to measure the baseline toxicity of individual BAs (Fig. 1C). However, the presence or absence of BSHs did not alter lactobacilli inhibition from deconjugated BAs (*SI Appendix*, Fig. S4). BSHs are reported to detoxify BAs through deconjugation and our CMC data suggested that the conjugated BAs displayed a greater potential to induce membrane damage, therefore we hypothesized that lactobacilli would display a decreased tolerance for conjugated BAs. Both lactobacilli were resistant to the highest concentrations of glycocholic acid and taurocholic acid (GCA and TCA) tested, yet CA was considerably more toxic relative to its conjugated forms (Fig. 1C). *Lactobacillus* inhibition from taurochenodeoxycholic acid (TCDCA) and taurodeoxycholic acid (TDCA) was only achieved at high concentrations, whereas glycochenodeoxycholic acid (GCDCA) and glycodeoxycholic acid (GDCA) were  $\sim 10\times$  more inhibitory. To our knowledge this difference in BA toxicity based on the conjugation and sterol core has never been tested and illustrates that conjugation is an overlooked and potentially important selective pressure exerted by the BA pool on the GI tract microbiota.

CDCA was the most inhibitory BA tested, whereas DCA did not potently inhibit either strain. Notably the deconjugation of GDCA to DCA represents the only conversion that outright supports the role of BSHs in detoxifying bile. Additionally, *L. acidophilus* was twice as resistant to the deconjugated primary BAs CA and CDCA compared to *L. gasseri*, potentially explaining the lack of a difference between WT and  $\Delta bshAB$  *L. acidophilus* in Fig. 1C. Given these MIC results, we concluded that neither the conjugated amino acid nor the CMC of a BA predicted its toxicity, so we hypothesized that BA inhibition of lactobacilli occurs in a manner not limited to the detergent properties of the molecule.

**BSH Activity Impacts *Lactobacillus* Growth in a Substrate-Dependent Manner.** To assess the relative contribution of each *bsh* to *Lactobacillus* fitness in vitro, single and double *bsh* deletion mutants were grown anaerobically in the presence of conjugated BAs and CFUs were enumerated after 24 h (Fig. 2). All conjugated BAs were used within approximate ranges of concentrations found in the GI tract (57). *L. acidophilus* and *L. gasseri* growth varied by several orders of magnitude depending on the BA to which they were exposed. WT lactobacilli growth was improved compared to their respective  $\Delta bshAB$  strains in several conditions tested (*L. acidophilus* with GDCA and TDCA in Fig. 2A; *L. gasseri* with GDCA in Fig. 2B). Notably, WT and  $\Delta bshB$  *L. acidophilus* growth in the presence of GDCA was robust and comparable to de Man, Rogosa, and Sharpe (MRS) medium alone, while the  $\Delta bshA$  and  $\Delta bshAB$  strains were substantially inhibited or killed. Whereas, TDCA elicited a slightly different result by requiring the loss of both *L. acidophilus* BSHs in order to observe a growth defect. For both *L. acidophilus* and *L. gasseri*, WT CFUs were less than the starting CFUs, indicating that there was death over the 24-h experiment. Comparatively, the *bsh* mutants displayed a larger drop in CFUs demonstrating that BSH activity limited bacterial death when exposed to GCDCA as opposed to limiting growth and highlighting a different strategy by which BSHs can promote lactobacilli survival and presumably host colonization.

Given that the MICs in Fig. 1C demonstrated that deconjugation could increase BA toxicity, it was plausible that BSH activity impaired lactobacilli growth as well (*L. acidophilus* with GCA and TCDCA in Fig. 2A; *L. gasseri* with GCA and TCA in



**Fig. 2.** BSHs impact *Lactobacillus* fitness in a genotypic and bile acid specific-manner. (A) *L. acidophilus* and (B) *L. gasseri* BSH mutants grown for 24 h in MRS, 5 mM GCA, 5 mM TCA, 2.5 mM GCDCA, 2.5 mM TCDCA, 2.5 (A)/1.25 (B) mM GDCA, or 5 mM TDCA. Error bars represent SD from  $n = 4$  independent experiments. Dashed lined denotes the approximate starting CFUs/mL at 0 h. Asterisks represent significant ( $*P < 0.05$ ) differences from WT by Mann-Whitney *U* test.



Fig. 2B). Interestingly, the *L. gasseri*  $\Delta bshA$  and  $\Delta bshB$  single mutants exhibited enhanced growth when exposed to TCA and GCA, respectively, hinting that LgBSHa and LgBSHb (*bshA* and *bshB* encoded by *L. gasseri*) have complementary preferences for each type of conjugation. Despite the fact that our findings were limited to a single time point and starting BA per culture, this screen for lactobacilli growth allowed us to broadly examine and identify conditions where BSHs may be beneficial or detrimental. That such a widely penetrant enzyme in the microbiota could be mal-adapting bacteria to GI tract stress is a surprising finding that has yet to be reported. We also investigated whether BSHs could impact *Lactobacillus* growth in the presence of deconjugated BAs, but no differences were observed (SI Appendix, Fig. S5). Overall, we concluded that the impact of BSH-catalyzed deconjugation on *Lactobacillus* fitness was context-specific and depended on the conjugated amino acid, the BA core, and the *Lactobacillus bsh*, which is much more of a dynamic relationship than previously thought.

**BSH Activity and Specificity Determines Tolerance to Various BAs.** We next hypothesized that differences in substrate specificity could explain why the *bshA* and *bshB* of *L. acidophilus* and *L. gasseri* varied in their contributions to bacterial fitness observed in Fig. 2. Hereafter, *bshA* and *bshB* encoded by *L. acidophilus* and *L. gasseri* are referred to as LaBSHa and LaBSHb (57% amino acid identity), and LgBSHa, and LgBSHb (31% amino acid identity), accordingly. LaBSHa, LaBSHb, LgBSHa, and LgBSHb were recombinantly expressed, purified, and assayed for activity on a panel of conjugated BAs using the Ninhydrin reaction to quantify amino acid release (SI Appendix, Fig. S6). All BSHs displayed acidic pH optima, a common feature of these enzymes, and a practical attribute when in acidic environments relevant for lactic acid bacteria (SI Appendix, Fig. S7) (58). Both *L. acidophilus* BSHs displayed a distinct preference for glycine-conjugated BAs; however, the activity of LaBSHa was  $\sim 10\times$  greater than that of LaBSHb (Fig. 3 A and B). Both BSHs showed preference for GDCA, potentially explaining the large gap in GDCA-supplemented growth between WT *L. acidophilus* and the  $\Delta bshA$  or  $\Delta bshAB$ . The comparatively weaker activity on taurine-conjugated BAs like TDCA supports the need for collective and concerted activity of both LaBSHa and LaBSHb. In contrast, the *L. gasseri* BSHs LgBSHa and LgBSHb displayed orthogonal functions; LgBSHa preferred taurine-conjugated BAs, whereas LgBSHb was selective for glycine-conjugates (Fig. 3 C and D). These enzymes' preferences biochemically elucidate the phenotypes of  $\Delta bshA$  with TCA and  $\Delta bshB$  with GCA (Fig. 2B), and similarly highlight the benefit of encoding multiple *bsh* homologs. Despite the patterns between BSH substrate preferences and *bsh* mutant phenotypes, it is still unknown whether the in vitro BSH activity and substrate preferences are mirrored in bacterial cultures and in vivo.

BSHs are cytoplasmic enzymes that limit the availability of conjugated BAs on which they can act (48). The deconjugated BAs generated inside the cell are either internally sequestered or are disposed of via efflux; however, the localization of their toxic effects has not been defined (48, 59, 60). To investigate whether intracellular BSH compartmentalization is critical for their function or throttles their access to conjugated substrates, equimolar amount of purified BSHs were spiked into the  $\Delta bshAB$  mutant cultures of *L. acidophilus* and *L. gasseri*. Supplementing the lactobacilli  $\Delta bshAB$  cultures with BSHs functionally complemented or overcomplemented growth of WT in several conditions (see *L. acidophilus* with GCA, GCDCA, GDCA, TDCA in Fig. 2A; *L. gasseri* with GCA, TCA, GCDCA, GDCA in Fig. 2B). In other cases, exogenous BSHs distorted  $\Delta bshAB$  growth or inhibited growth beyond that of WT (TCA in Fig. 2A; TCDCA, TDCA in Fig. 2B). These instances represent conditions in which exogenous BSHs have more access to BAs and are

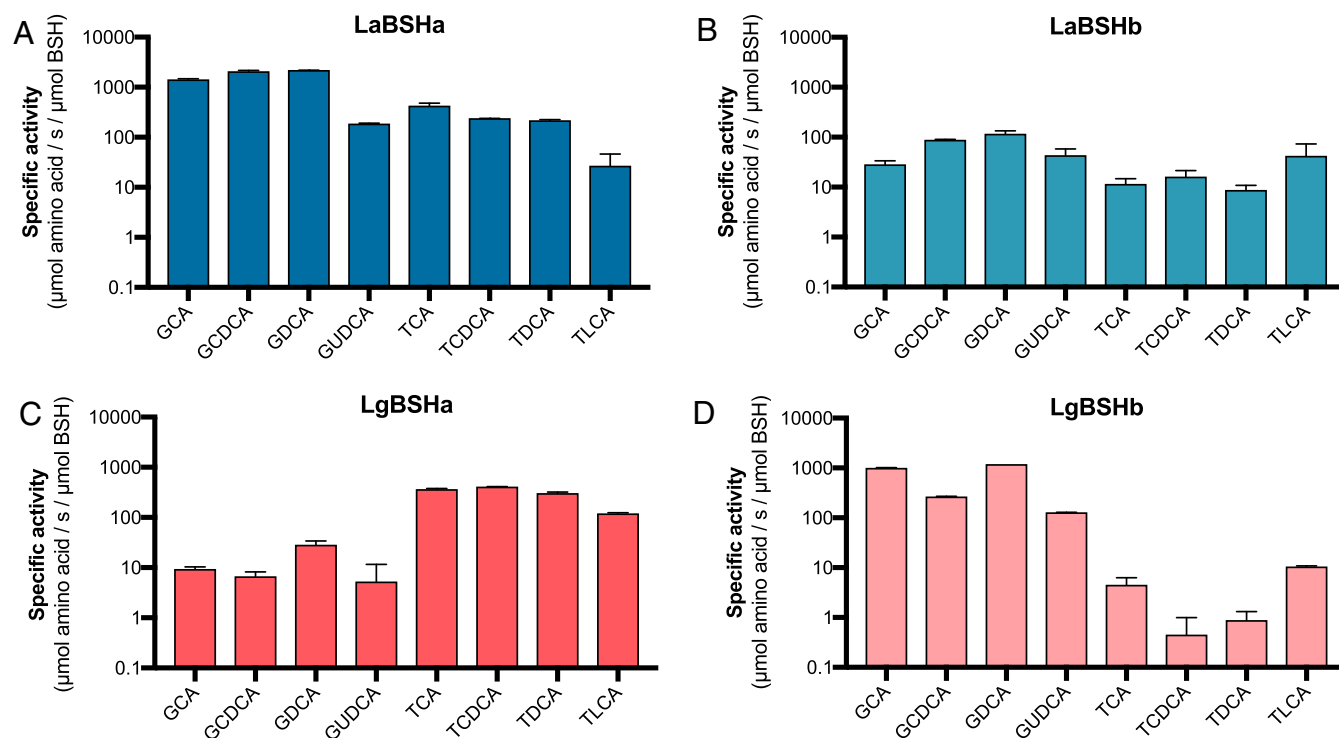
in greater abundance than in WT conditions where BSHs are restricted to the cytoplasm and are produced as a function of *Lactobacillus* growth. Thus, their activity is artificially inflated. Overall, while we have found that BSHs can display differences in their enzymatic preferences, their cytoplasmic expression can mask the full extent of their selectivity and catalysis.

**BSHs Alter *Lactobacillus* Membrane Physiology.** BAs are known to impair bacterial membrane integrity through several means (28). While there was no obvious relationship between the tested BA CMC and MIC for *L. acidophilus* and *L. gasseri* (Fig. 1A and C), this did not rule out that BAs could be damaging membranes and we hypothesized that BSH activity could be curbing or augmenting this damage to the respective benefit or detriment of the bacteria. Using propidium iodide (PI), a membrane impermeant nucleic acid stain commonly used to assess viability, we attempted to describe how BSH activity could impact the integrity and permeabilization of *Lactobacillus* membranes in the presence of BAs by acutely exposing midlog-grown lactobacilli (61–63).

In several different conditions, *L. acidophilus* membrane structure was affected by BSH expression (Fig. 4A). GDCA exposure resulted in increased  $\Delta bshAB$  fluorescence, which is bolstered by the increased killing of the strain compared to WT, but it is unclear how much of the fluorescence is due to permeabilized versus nonviable cells, since the  $\Delta bshAB$  culture displayed a reduction in CFUs compared to the inoculum after a 24-h culture (Fig. 2A). Exposure to GCA and TCDCA significantly increased the PI fluorescence of WT compared to  $\Delta bshAB$ . However, this trend opposed the patterns of growth seen in Fig. 2A, revealing that for *L. acidophilus*, BA-induced membrane damage does not necessarily reflect the molecule's toxic effects on growth. *L. gasseri* WT PI fluorescence was greater than  $\Delta bshAB$  when exposed to GCA (Fig. 4B), which underpins WT inhibition of growth with GCA and possibly death (Fig. 2B). Despite no robust BSH-dependent changes in growth with TCDCA and TDCA, both substrates induced greater membrane damage in WT *L. gasseri*. Altogether, these results indicate that quantifying membrane damage, rather than growth, may relay more sensitive functional information about BA toxicity in certain cases. These data also demonstrate how BSH expression can regulate lactobacilli membrane integrity and exposure to membrane-damaging BAs, although the exact mechanisms of membrane disruption are unknown and likely vary in a substrate-dependent manner.

#### **BSH Expression Alters *Lactobacillus* Transcriptome during BA Exposure.**

Given that both lactobacilli varied in their physiological responses to BA exposure, we wanted to better understand how these bacteria differentially responded to the toxic effects of BAs when equipped with or without a BSH. Previous studies have demonstrated that BA-exposure can lead to transcriptional responses in pathways responsible for membrane organization (64), and we hypothesized that these would be similarly active due to the changes in membrane integrity seen in Fig. 3. We compared whole-transcriptome expression levels for *L. acidophilus* and *L. gasseri* during logarithmic growth containing no BAs, CA, GCA, or TCA using RNA sequencing (RNA-seq). While the  $\log_2$  ratio changes were relatively low, indicating there were not drastic transcriptional changes, there were some significant ( $P < 0.05$ ) differences depending on the gene or condition. Our data determined that the gene-expression levels for *bshA* from *L. acidophilus* was increased similarly ( $\log_2$  ratio  $\sim 1.10$ ) after growth in all three BAs compared to MRS alone, but there were minimal changes in the expression of *bshB* (SI Appendix, Fig. S8A). *L. gasseri bshA* expression was significantly increased in media containing TCA and GCA ( $\log_2$  ratio 1.11 and 0.80, respectively) and lower expression in CA ( $\log_2$  ratio  $-0.66$ ) (SI Appendix, Fig. S8B), indicating positive transcriptional regulation in the presence



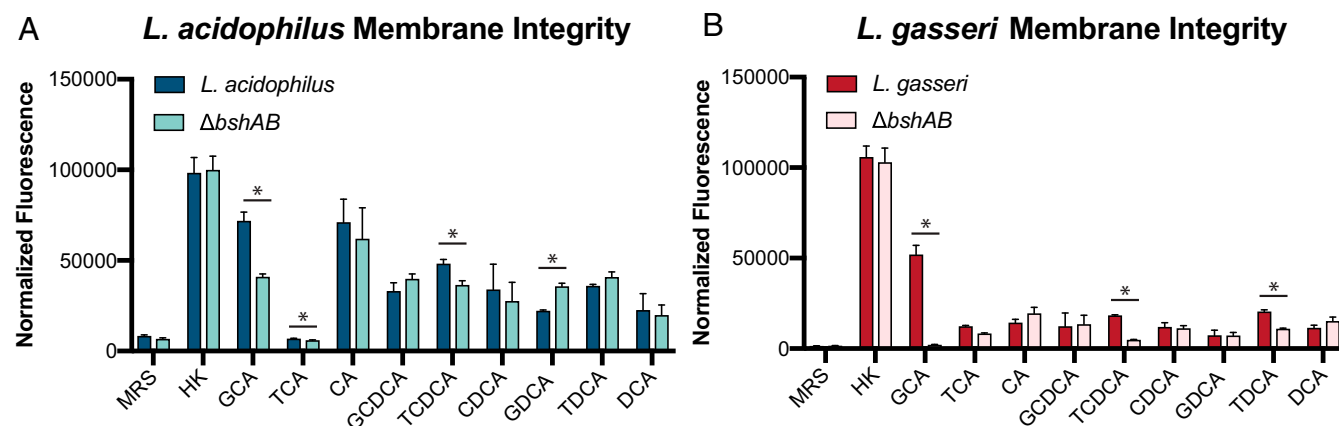
**Fig. 3.** *Lactobacillus* BSHs display variable preferences for bile acid conjugation. Average specific activities from (A) LaBSHa, (B) LaBSHb, (C) LgBSHa, and (D) LgBSHb were determined by the Ninhydrin assay on the same panel of conjugated BAs used in Fig. 2 supplemented with glycooursodeoxycholic acid (GUDCA) and tauroolithocholic acid (TLCA). Error bars represent SD from  $n = 3$  independent experiments.

of conjugated BAs. Interestingly, *bshB* expression from *L. gasseri* was significantly decreased after growth in MRS containing CA and TCA ( $\log_2$  ratio  $-0.41$  and  $-0.40$ , respectively) but not GCA, a preferred substrate of this enzyme.

We next determined the differential gene expression for both  $\Delta bshAB$  mutant and WT after growth with CA, GCA, or TCA compared to MRS alone. Relatively few genes were differentially expressed (SI Appendix, Figs. S8 and S9). *L. acidophilus* exposure to GCA had the greatest effect on gene expression with 20 genes differentially expressed (SI Appendix, Figs. S8A and S9). Notably,

this included genes encoding a transporter, an ABC transporter operon, surface proteins and *mmnE* and *mmnG* involved in tRNA regulation, and other cell-surface proteins (SI Appendix, Fig. S8A and Table S1). These cell surface and transporter proteins may contribute to the efflux of the BAs from the cell and are responding to the detergent effect of the BAs.

The exposure of *L. gasseri* to CA had the largest effect on gene expression, with 8 and 25 genes differentially expressed in the WT and  $\Delta bshAB$  strains, respectively, indicating a deconjugated BA-specific transcriptomic response (SI Appendix, Figs. S8B and



**Fig. 4.** BSH activity alters membrane integrity and global transcriptome in a BA-specific manner. (A and B) PI staining to assess membrane integrity of midlog ( $OD_{600\text{ nm}} = 0.7$ ) grown *Lactobacillus* exposed to various BAs or heat killed (HK). BA concentrations were the same as those used in Fig. 2. Normalized fluorescence was calculated by subtracting background PI fluorescence and normalizing to the starting  $OD_{600\text{ nm}}$  at BA exposure. Bars represent average fluorescence from  $n = 3$  independent experiments and error bars represent SD. Asterisks represent significant ( $*P < 0.05$ ) differences between groups by Welch's  $t$  test.

S9 and Table S1). Loss of *bshAB* resulted in a stronger differential expression for 21 genes after growth in CA (SI Appendix, Figs. S8B and S9), indicating a lack of the *bsh* genes resulted in an altered transcriptional response to compensate for loss of potential BSH function. Eleven of the 21 genes were up-regulated and 7 of these genes are part of a putative pyrimidine biosynthetic pathway (SI Appendix, Fig. S8B and Table S1). As we have shown CA to be more inhibitory compared to TCA and GCA, it may be expected that the largest stress and transcriptional response was in response to CA. Altogether, our analyses of BA exposure illustrate that the *Lactobacillus* transcriptional response is weak but pleiotropic, nonetheless, with cell surface proteins being one of the most common to be differentially regulated, although many of their exact functions remain unclear.

**BSH-Driven Competition Is Niche- and BA-Specific.** In the GI tract, bacterial competition is an underlying force that shapes the microbiota composition. Competitive strategies for antagonizing or restricting nutrients from competitors have been studied considerably more than those that involve the remediation of environmental stressors, such as BAs. Given that BA biotransformations are a communal activity in the microbiota, we wanted to understand how BSH activity could impact bacterial dynamics and relative fitness between WT and  $\Delta bshAB$  lactobacilli in coculture. In order to differentiate and enumerate the competing strains, spontaneous rifampicin- and streptomycin-resistant WT and  $\Delta bshAB$  clones, respectively, were isolated with no detected growth defects on MRS or on select BAs (SI Appendix, Fig. S10). We reasoned that these in vitro competitions could model bacterial interactions in vivo. To further probe these dynamics, exogenous BSHs were also included in cocultures to model the role of extraneous BSHs encoded by other members of the microbiota.

Despite there being favorable conditions for the  $\Delta bshAB$  mutant over WT *L. acidophilus* and *L. gasseri* in monoculture, there was no instance where  $\Delta bshAB$  displayed greater relative fitness in coculture (Fig. 5A and B). However, there were several BAs that enriched for WT growth. WT *L. acidophilus* BSH activity led to  $\Delta bshAB$  being outcompeted when in the presence of GCDCA and GDCA. These two results mirror the trends from monoculture (Fig. 2A), and show that WT *L. acidophilus* can use its BSHs to benefit itself, but when toxic deconjugated BAs are generated, competitors are equally damaged. WT *L. gasseri* dominated cocultures in the presence of GCA and TCA (Fig. 5B), a puzzling result given that in monoculture, WT growth is significantly inhibited by these BAs compared to  $\Delta bshAB$  (Fig. 2B). Supplementing the competition experiments with exogenous BSHs altered competitive dynamics by neutralizing the fitness advantages of WT lactobacilli, except in the case of WT *L. acidophilus* with TCA, which unpredictably benefited from the added deconjugation (Fig. 5A). These results indicate that *bsh* expression can benefit competing lactobacilli, but this advantage is dependent on the type of BA present as well as the existing level of BSH activity.

Our previous observations that BSH activity could be detrimental to lactobacilli fitness did not align with the results of our in vitro competition experiments, so to further understand *Lactobacillus* BSH-driven competition in a more complex and natural environment, we repeated the germ-free mouse colonization experiment by coassociating mice with WT and  $\Delta bshAB$  strains of *L. acidophilus* and *L. gasseri* (Fig. 5C). We predicted that competing these strains in vivo would more closely approximate bacterial competition in the wild and reveal BSH function in the presence of a dynamic flux of BAs. The bacterial load in the ceca after 7 d of colonization demonstrated that  $\Delta bshAB$  could compete in the murine GI tract better than WT for both species. While *L. gasseri* mirrored its trend seen in monoassociated mice, *L. acidophilus* coassociation better supported the growth of the *bsh*-null strain (Figs. 1B and 5C). We also tested if the presence of a more complex microbiota and BA pool would change the

role of BSH activity during bacterial competition by competing WT and  $\Delta bshAB$  strains in ex vivo conventional mouse cecal content (Fig. 5D).  $\Delta bshAB$  strains were more competitive than WT after 48 h of growth. Together, these data demonstrate that BSH activity can drive bacterial competition between strains in an environment-specific fashion, an important consideration for gut colonization and fitness. Additionally, the outcomes from both in vivo gnotobiotic and ex vivo conventional mouse studies support a departure from the dogma dictating that deconjugation is used for BA detoxification.

## Discussion

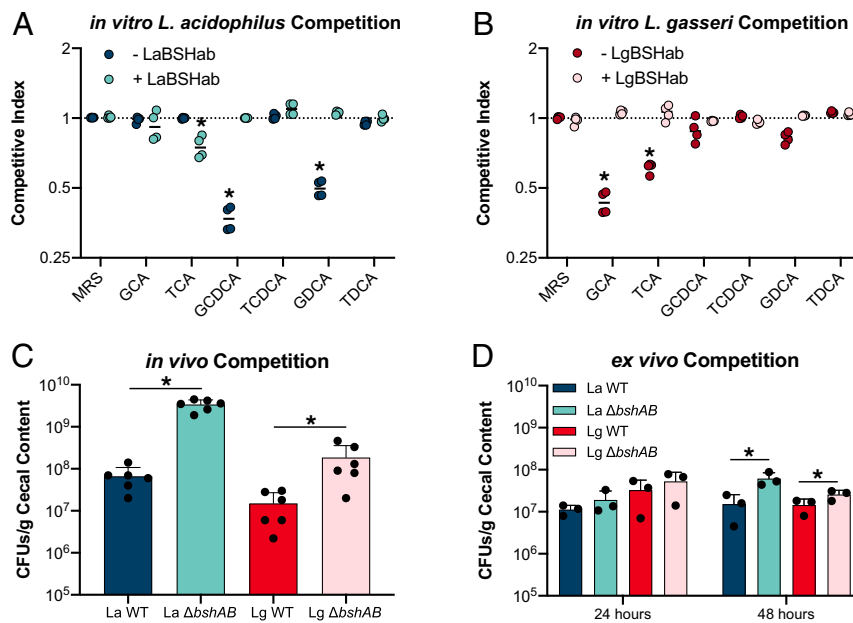
BAs are a diverse group of metabolites that dynamically flux through the GI tract. Their metabolism by resident microbes is a means of cross-talk between the host and its microbiota that has substantial consequences for human health (6, 13, 65–67). Despite the importance of BSHs in initiating the collective metabolism of BAs in the gut, their exact purpose for and impact on the bacteria that encode them is less clear. A large body of work has surveyed the in vitro BSH specificities from many genera of bacteria, especially those encoded by *Lactobacillus* (58, 68–70), while far fewer studies have genetically assessed the contribution of BSH activity to bacterial fitness (36, 38, 71, 72). Our work has aimed to provide a link between the two by combining genetic and biochemical methods in tandem, whereas early approaches characterizing *L. acidophilus* BSH activity had lower sensitivity and lacked the same level of resolution (53). Consequently, our findings contradict the purported role of BSHs as enzymes that detoxify BAs and demonstrate that this may be an overgeneralization of their activity in vivo (SI Appendix, Fig. S11A).

Given the complex and pleiotropic effects of BA deconjugation (i.e., shifts in local microbiota composition and function, modulation of intestinal physiology) it is possible that the bile-detoxifying effects of BSHs may be overstated for *bsh*<sup>+</sup> bacteria. *Listeria monocytogenes*, *Listeria innocua*, and *Brucella abortus* all display increased colonization of the murine GI tract and resistance to BA mixtures due to BSH expression (36, 38, 71, 72). It is unclear how or if these bacteria differ from commensal lactobacilli in their response to individual BAs, but perhaps BSH-promoted survival is a trait enriched in pathogenic genera or that works alongside other protective mechanisms to deal with BA stress (60, 64, 73, 74). Nevertheless, our findings show that BSH expression in vivo may come at a fitness cost due to the increase in toxic deconjugated BAs.

While it is known that BAs can restrict the growth of bacteria, we have found that the type of conjugated amino acid dictates much of this toxicity, whereas measuring detergent-like properties (via the CMC) was not predictive of its ability to disrupt bacterial membranes or inhibit *Lactobacillus* growth (Fig. 1A and C). These data suggest that the variable levels of glycine:taurine conjugation in bile, for example in different hosts or as a result of diet (75, 76), may play an underappreciated role in shaping the microbiota. Additionally, the glycine/taurine bias that BSHs exhibit may be an evolved trait to alter the levels of glycine/taurine conjugated BA exposure, thereby adjusting the overall toxicity of pooled conjugated and deconjugated BAs. While we chose to focus on prominent BAs found in humans, recent work identifying phenylalanine, tyrosine, and leucine-conjugated BAs in mouse and human GI tracts raise many new questions about the effects of these molecules on members of the microbiota (77).

To our knowledge, our finding that BSH substrate specificity can affect *Lactobacillus* growth in a conjugation-dependent manner provides evidence explaining why many lactobacilli encode multiple BSHs (Figs. 1C and 2). *L. gasseri* exemplifies this by correspondingly encoding a BSH specific for glycine and taurine, thereby broadening the substrate range of the strain, whereas the *L. acidophilus* BSHs seemingly display an additive effect (Fig. 3B). Perhaps an expanded and complementary repertoire of





**Fig. 5.** BSH activity drives competitive dynamics in vitro and in vivo. (A and B) CIs for *Lactobacillus* cocultures anaerobically for 24 h in the presence of various BAs. BA concentrations were the same as those used as in Fig. 2. CIs were calculated as follows:  $CI = \text{Final}[\text{Log}_{10}(\Delta bshAB \text{ CFUs})/\text{Log}_{10}(\text{WT CFUs})]/\text{Initial}[\text{Log}_{10}(\Delta bshAB \text{ CFUs})/\text{Log}_{10}(\text{WT CFUs})]$ . Dashed lines denote a CI = 0. Asterisks represent significant ( $P < 0.05$ ) differences between groups by Mann–Whitney *U* test. (C) *L. acidophilus* and *L. gasseri* WT/ $\Delta bshAB$  cocolonization of  $n = 6$  germ-free C57BL/6 mice. CFUs were counted from cecal contents 7 d after colonization. Asterisks represent significant ( $P < 0.05$ ) differences from WT by Mann–Whitney *U* test. (D) Cocolonization of  $n = 3$  cecal contents from conventional C57BL/6 mice. Asterisks represent significant ( $*P < 0.05$ ) differences from WT by unpaired *t* test.

*bsh* is a competitive feature for intestinal bacteria and a noteworthy signature of gut adaptation that may differ in terms of substrate preferences across hosts with distinct BA pools. This difference in specificity may also have consequences for the host, and previous work has suggested that BSH substrate selectivity can directly modulate host physiology (65). Two recent studies positively or negatively associated specific BSH phylotypes with metabolic and inflammatory diseases that are known to be regulated by BAs (49, 70). Differences in substrate specificity may underlie the protective vs. pathogenic effects of BSHs in the human GI tract or at least serve as a marker for health. Furthermore, whether the *bsh*-expressing bacteria benefit from establishing these disease niches remains to be seen.

Bacteria have many strategies to cooperate or compete for nutrients and space in the harsh gut environment (78). While BAs are a source of cellular stress that can shape the microbiota, the roles of BA-altering enzymes, such as BSHs, had not been previously tested for their roles in competition. Our in vivo and ex vivo competition studies surprisingly show that both the *L. acidophilus* and *L. gasseri*  $\Delta bshAB$  mutants can outcompete their WT opponents in the gnotobiotic and conventional mouse ceca (Fig. 5 C and D and *SI Appendix, Fig. S11B*). These findings agree with our observation that WT BSH activity can increase the toxicity of BAs during growth (Fig. 2); however, coculture competitions on individual BAs suggested that BSH could provide a competitive growth advantage under specific conditions (Fig. 5 A and B). We speculate that the murine pool of taurine-conjugated and deconjugated primary BAs may explain the disagreement in these data. Additionally, while we chose not to focus on the murine-exclusive muricholic acids, they may underlie some of the unexpected findings in this study and are an important molecule to consider in future studies. That mice somewhat mimic the human BA pool argues for a combined approach using in vivo models as well as in vitro assays supplemented with purified BAs alone or in combination in future studies.

The utility of BSHs as promoters of probiotic colonization and as treatments for disease by reducing serum cholesterol (79), or

intervening during obesity (41, 80), is disputed and uncertain. A limited knowledge of the *Lactobacillus*–BSH–BA relationship, and the enzymatic specificity of a given BSH may explain why the therapeutic success of BSHs and probiotics have had mixed results (81). As next-generation probiotics are engineered using genome-editing tools such as CRISPR-Cas (82), BSHs are promising targets for modification with the potential to improve human health using engineered biotherapeutics. Still, knowledge gaps remain about these enzymes that limit their ability to be used medically. Our findings have provided a foundational understanding of how BSHs equip probiotic lactobacilli to deal with BA stress. Future studies are needed to elucidate the role of BSHs in adapting *Lactobacillus* and other gut commensals to the competitive environment within the microbiota, and similarly how BSH activity alters the structure and function of that ecosystem.

## Materials and Methods

**Bacterial Strains and Growth Conditions.** For the construction of *bsh* mutants, *L. acidophilus* and *L. gasseri* strains were grown statically under ambient atmospheric conditions in MRS (Difco Laboratories) broth or glucose semi-defined medium (83) at 37 °C or 42 °C. Brain–heart infusion (Difco) broth was used for growth of *Escherichia coli* strains at 37 °C with aeration. Solid media contained 1.5% (wt/vol) bacteriological agar (Difco) and relevant antibiotics were included in the media where required. Bacterial strain and plasmid details are provided in *SI Appendix, Table S2*. During experiments, strains were grown statically in a 37 °C Coy anaerobic chamber (5% H<sub>2</sub>/10% CO<sub>2</sub>/85% N<sub>2</sub>). *Lactobacillus* growth experiments were carried out anaerobically in static MRS media at pH 6.4 for 24 h. Briefly, 2× MRS was diluted with water and aqueous suspensions of BAs (Sigma-Aldrich) to achieve a range of BA concentrations. Endpoint CFUs were determined by diluting cultures in PBS and enumerating on MRS plates. Growth curves were performed in a Tecan plate reader within the anaerobic chamber. Clear flat-bottom plates contained 200  $\mu$ L of media per well were incubated at 37 °C for 24 h.

**DNA Manipulations and Sequence Analysis.** All oligonucleotides used in this study were synthesized by Integrated DNA Technologies (IDT) and are listed in *SI Appendix, Table S3*. Molecular-grade reagents were purchased from Roche and New England Biolabs. Genomic DNA was isolated using the ZR Fungal/Bacterial DNA miniprep kit (Zymo Research). Plasmid DNA from

*E. coli* was isolated using the QIAprep Spin Miniprep Kit (Qiagen). Chemically competent *E. coli* EC101 and EC1000 (SI Appendix, Table S2) cells were prepared as described previously (84), and electrocompetent *Lactobacillus* cells were prepared as described by Walker et al. (85). PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen), QIAquick PCR Purification Kit (Qiagen), Monarch PCR & DNA Cleanup Kit (New England Biolabs), or Monarch DNA Gel Extraction Kit (New England Biolabs). Genewiz performed DNA Sanger sequencing.

**Deletion of *bsh* Genes from *L. acidophilus* and *L. gasseri*.** Strains of *L. acidophilus* and *L. gasseri* with deletions in a single *bsh* gene ( $\Delta bshA$  or  $\Delta bshB$ ) and deletions in both *bsh* genes ( $\Delta bshAB$ ) were constructed using the *upp*-based counterselective gene replacement system (54). For each targeted *bsh* gene the flanking regions (FRs) were amplified by PCR. The FRs for *L. acidophilus bshA* (LBA0892) were amplified with PCR primer pairs La\_BshA\_SOEA\_F/R (678 bp) and La\_BshA\_SOEB\_F/R (624 bp) (SI Appendix, Table S2). PCR primer pairs La\_BshB\_SOEA\_F/R (665 bp) and La\_BshB\_SOEB\_F/R (743 bp) were used to amplify the FRs for *bshB* (LBA1078) (SI Appendix, Table S2). For *L. gasseri* the FRs for *bshA* (LGAS\_00260) were amplified with PCR primer pairs Lg\_BshA\_SOEA\_F/R (749 bp) and Lg\_BshA\_SOEB\_F/R (663 bp) and Lg\_BshB\_SOEA\_F/R (755 bp) and Lg\_BshB\_SOEB\_F/R (669 bp) for *bshB* (SI Appendix, Table S2). Each FR for each *bsh* gene was purified and PCR products joined by splicing using overlap extension (SOE) PCR. Each SOE product was digested with SacI and BamHI, purified and ligated into the SacI and BamHI sites of pTRK935. Ligation mixtures were transformed into *E. coli* EC101 or EC1000. Potential transformants were screened with primer pair *upp\_ScF* and *upp\_ScR* (SI Appendix, Table S3). Sequence integrity of the plasmid insert was confirmed by Sanger sequencing. Plasmids were designated pTRK1123 (FRs of *bshA* in *L. acidophilus*), pTRK1124 (FRs of *bshB* in *L. acidophilus*), pTRK1206 (FRs of *bshA* in *L. gasseri*), and pTRK1207 (FRs of *bshB* in *L. gasseri*) (SI Appendix, Table S2). To construct strains with a single *bsh* deletion in *L. acidophilus*, plasmids pTRK1123 or pTRK1124 were electroporated into NCK1910, which harbors the helper plasmid pTRK669 that provides *repA* in *trans* for the replication of pTRK1123 and pTRK1124. Procedures to isolate and confirm plasmid-free recombinants after targeted plasmid integration into the chromosome and double-cross-over recombination were performed as described previously (54). The confirmed mutant strains with a deletion in *bshA* or *bshB* were designated *L. acidophilus* NCK2521 and NCK2522, respectively. To create a double-deletion mutant in *bshA* and *bshB*, the helper plasmid pTRK669 was electroporated into NCK2521. Subsequently, pTRK1124 was electroporated into NCK2521 harboring pTRK669 to facilitate deletion in *bshB* using the same protocols described in Goh et al. (54), resulting in strain *L. acidophilus* NCK2523 with a deletion in both *bshA* and *bshB*. Single deletions in *bshA* or *bshB* and deletions in both *bsh* genes ( $\Delta bshAB$ ) were similarly made for *L. gasseri* using NCK2254 as the base strain with plasmids pTRK1206 and pTRK1207 resulting in *L. gasseri* NCK2678 ( $\Delta bshA$ ), *L. gasseri* NCK2679 ( $\Delta bshB$ ), and *L. gasseri* NCK2680 ( $\Delta bshAB$ ).

**RNA Isolation.** Total RNA was isolated from *L. acidophilus* NCK1909 (WT), NCK2523 ( $\Delta bshAB$ ), and *L. gasseri* NCK2253 (WT) and NCK2680 ( $\Delta bshAB$ ) grown to midlog phase (OD 600 nm ~0.7) in MRS containing no BA, CA (0.3125 mM), TCA (1.25 mM), or GCA (1.25 mM). Cells were harvested from two biological replicates for each condition by centrifugation (10 mL, 1,717  $\times$  g for 10 min at room temperature), pellets flash frozen and stored at  $-80^\circ\text{C}$ . Total RNA was extracted using Tri-Reagent (Life Technologies) and purified with the Zymo Direct-Zol RNA Miniprep Kit (Zymo Research). Briefly, the cell pellets were resuspended in 1 mL of Tri-Reagent, added to a screw cap tube containing beads (0.1-mm glass beads, Bio-Spec) and bead-beated for 5 min (five times each for 1-min intervals with 1 min on ice after each interval). Samples were then left at room temperature for 5 min and subsequently centrifuged at 16,873  $\times$  g for 20 min at 4  $^\circ\text{C}$ . Total RNA was purified using the Zymo Direct-Zol RNA Miniprep Kit (Zymo Research) according to the manufacturer's instructions. DNA was removed by incubating samples with Turbo DNase as described by the manufacturer (Ambion), purified using the RNA Clean and Concentrator kit (Zymo Research), and checked for integrity by capillary electrophoresis on the Agilent Bioanalyzer (Agilent Technologies). Samples were confirmed to be DNA-free by PCR with *L. acidophilus* NCFM and *L. gasseri* ATCC33323 gene-specific primers.

**RNA-Seq and Transcriptional Analysis.** Library preparation and sequencing was performed at The High-Throughput Sequencing and Genotyping Unit of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. The Ribozero Bacteria kit (Illumina) was used to remove rRNA followed by library preparation with the TruSeq Stranded RNA Sample Prep kit (Illumina). Libraries were then quantitated via qPCR and sequenced on one lane for 101

cycles from each end of the fragments on a NovaSeq. 6000 using a using a NovaSeq SP reagent kit; reads were 100 nt in length. Fastq files were generated and demultiplexed with the bcl2fastq v2.20 Conversion Software (Illumina). Adapter sequences were removed and raw sequences assessed for quality using FastQC v0.11.9. Subsequent processes were performed with Geneious Prime 2020.0.5 using default settings. RNA sequence reads for *L. acidophilus* NCK1909 and NCK2523 were mapped to the *L. acidophilus* NCFM genome (NC\_006814). *L. gasseri* NCK2253 and NCK2680 sequence reads were mapped to the *L. gasseri* ATCC 33323 genome (NC\_008530). Expression levels were calculated based on the normalized transcripts per million and differential expression was performed with DESeq2 in Geneious. Genes were considered differentially expressed when they had a  $\log_2$  ratio  $\leq -2$  or  $\geq 2$  and a  $P < 0.05$ , unless otherwise stated.

**BA Critical Micelle Concentrations.** CMCs were determined for the BAs used in this study using Optimizer blueBALLS (G-Biosciences) according to the manufacturer's instructions and Devlin et al. (63). Eight 1.5-mL Epi tubes containing 250  $\mu\text{L}$  of BA ranging from 1 to 14 mM and two Optimizer blueBALLS were set up in duplicate and were statically incubated for 16 h at room temperature with intermittent vortexing. To read the reactions, tubes were centrifuged for 5 min at 6,000  $\times$  g and supernatants were removed and centrifuged at the same speed once more. Of the supernatants, 200  $\mu\text{L}$  were removed, added to clear flat-bottom 96-well plates, and were read at 630 nm in a Tecan Infinite F200 Pro plate reader. The amount of solubilized dye in the supernatant is related to the quantity of BA micelles present. Absorbance values were plotted using GraphPad Prism and were fitted to a five-parameter logistic curve, where the computed inflection point represented by the  $\text{Log}_{10}\text{EC}_{50}$  corresponds to the CMC (86).

**Minimum Inhibitory Concentrations.** BA tolerance measured by MIC was adapted from the bile tolerance assay by Jacobsen et al. (87). Overnight *lactobacilli* cultures ( $10^7$  to  $10^8$  CFUs/mL) were inoculated 1% into MRS containing a range of BA concentrations. Cultures were anaerobically incubated for 24 h at 37  $^\circ\text{C}$ . Following incubation, cultures were serially diluted in PBS and plated on MRS agar to determine if the concentration of BA tested inhibited growth relative to the starting inoculum.

**Animals and Housing.** Animal experiments were conducted in the Laboratory Animal Facilities located on the North Carolina State University (NCSU) College of Veterinary Medicine (CVM) campus. The animal facilities are equipped with a full-time animal care staff coordinated by the Laboratory Animal Resources division at NCSU. The NCSU CVM is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. The Institutional Animal Care and Use Committee at the NCSU CVM approved this study. Trained animal handlers in the facility fed and assessed the status of animals several times per day.

**Gnotobiotic Mouse Colonization.** Five- to 8-wk-old germ-free C57BL/6J mice (male and female) were challenged once with  $10^9$  CFUs of *Lactobacillus* via oral gavage. Seven days postgavage, animals were humanely killed via  $\text{CO}_2$  asphyxiation. Cecal contents were collected and a portion was immediately frozen in liquid  $\text{N}_2$  for downstream metabolomics and in parallel a sample was freshly diluted 1:10 (wt/vol) in sterile PBS. The diluted contents were serially diluted and plated onto MRS to enumerate lactobacilli.

**BA Metabolomics.** BA analysis was performed with a UPLC-electrospray ionization (ESI)-MS/MS consisting of a Vanquish UPLC system (Thermo Fisher Scientific) coupled with an Altis TSQ triple quadrupole mass spectrometer (Thermo Fisher Scientific) equipped with a heated ESI source, which enabled the measurement of 20 BAs using the Biocrates BAs kit (Biocrates Life Sciences). To ensure accuracy and precision, the method provides seven calibration standards, a mixture of nine isotope-labeled internal standards, and three quality-control samples. For cecal samples, 0.5-mm ceramic beads (Omni International) were added to 2.0-mL centrifuge tubes containing 50 to 250 mg of sample. Three times the sample volume of extraction buffer containing 20 mM phosphate buffer and ethanol was added to each sample. The samples were homogenized using a genie disrupter (Scientific Industries) for 15 min after vortex mixing for 30 min. Samples were then centrifuged at 15,000 rpm and 4  $^\circ\text{C}$  for 10 min and then stored at  $-80^\circ\text{C}$  awaiting further processing. On the day of data acquisition, samples were thawed to room temperature and another three times the sample volume of extraction buffer was added. Samples were subjected to vortex mixing for 10 min followed by centrifugation at 15,000 rpm and 4  $^\circ\text{C}$  for 15 min. The supernatant was removed and used for analysis. Ten microliters of plasma or extracted fecal, cecal, or ileal matter was added together with 10  $\mu\text{L}$  of the



internal standards mixture onto filter spots suspended in the wells of a 96-well filter plate (PALL AcroPrep, PTFE 0.2  $\mu\text{m}$ ) fixed on top of a deep-well plate and extracted with 100  $\mu\text{L}$  methanol by shaking at 600 rpm for 20 min. Elution of the methanol extracts was performed using a positive-pressure manifold (Waters) into the lower receiving deep-well plate, which was then detached from the upper filter plate. After adding 50  $\mu\text{L}$  Milli-Q water to the extracts and shaking briefly (600 rpm, 5 min), the sample plate was analyzed by UPLC-MS/MS. All target BAs were baseline-separated using a Restek Raptor C18 column (1.7 mm, 2.1  $\times$  50 mm). Mobile phase A was 5 mM ammonium acetate and mobile phase B was 1:1 methanol/acetonitrile. The gradient program initially started at 0.5 mL/min 35% B with a stepped gradient to 55%B over 4.6 min. The flow rate then increased to 0.8 mL/min at 85%B for an additional 2 min followed by re-equilibration at initial conditions for a total run time of 8.5 min. Samples, standards, blanks and quality controls were analyzed (20- $\mu\text{L}$  injections) in negative ion mode (spray voltage 2.5 kV, ion transfer tube temperature 325  $^{\circ}\text{C}$ , vaporizer temperature 350  $^{\circ}\text{C}$ , sheath gas 50 a.u., aux gas 10 a.u., sweep gas 1 a.u.). MS/MS acquisition was performed in multiple reaction monitoring mode using two specific *m/z* transitions per analyte. BA concentrations were calculated in QuanBrowser (Thermo Fisher Scientific) and MetIDQ (Biocrates Life Sciences). All calibration curves had  $R^2$  values between 0.9911 and 0.9993 and the relative SD (RSD) of internal standards (ITSD) were below 15%.

**Recombinant BSH Cloning and Protein Expression.** LaBSHa, LgBSHa, and LgBSHb were PCR-amplified from genomic DNA for cloning into pETite C-His vector (Lucigen) according to the manufacturer's instructions. Due to poor expression, LaBSHb was codon-optimized and synthesized (IDT). BSH-expressing pETite plasmids were transformed into Rosetta (DE3) plysS cells (EMD Biosciences) and plated on LB agar containing 30  $\mu\text{g mL}^{-1}$  kanamycin (Kan) and 20  $\mu\text{g mL}^{-1}$  chloramphenicol (Cam). After 16 to 20 h of growth at 37  $^{\circ}\text{C}$ , colonies were scraped up and were used to inoculate 1 L of terrific broth plus Kan and Cam for growth at 37  $^{\circ}\text{C}$ . Cultures were grown to an OD 600 nm of  $\sim$ 0.6 before 0.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was added and cells were grown 30  $^{\circ}\text{C}$  for an additional 16 h. Cells were harvested by centrifugation and cell pellets were flash frozen in liquid  $\text{N}_2$  until purification.

**Recombinant Protein Purification.** Cell pellets were resuspended in 50 mL of lysis buffer (50 mM Na  $\text{PO}_4$  buffer, 300 mM NaCl, 20 mM imidazole, protease inhibitor tablet [Roche], 10 mM 2-mercaptoethanol, pH 8.0) and were lysed by sonication. Lysates were clarified by centrifugation at 25,000  $\times g$  for 30 min at 4  $^{\circ}\text{C}$  to remove intact cells. His-tagged BSHs were purified using gravity columns containing 4 mL of fresh HisPur cobalt resin (Thermo Scientific) equilibrated in wash buffer (50 mM Na  $\text{PO}_4$  buffer, 300 mM NaCl, 20 mM imidazole, pH 8.0). Lysates were allowed to drip through the columns at a rate of  $\sim$ 1 mL per min. Bound protein was washed using 2  $\times$  20 mL of wash buffer. BSHs were eluted using 10 mL of wash buffer containing 150 mM imidazole and 10 mM DTT. Enzymes were immediately flash frozen in liquid  $\text{N}_2$  to prevent further oxidation. Enzyme concentrations were quantified using Qubit Protein Assay Kit and protein purity was assessed using 10% or 4 to 20% SDS/PAGE gels (Thermo Scientific).

**BSH Activity Assays.** Enzymatic assays were set up according to a previously described two-step method (88). Enzymatic reactions were carried out at

37  $^{\circ}\text{C}$  in 50- $\mu\text{L}$  volumes containing 0.1 M sodium phosphate pH 6.0, 10 mM DTT, 9 mM BA, and 1 to 10 nM BSH. Reactions were quenched using an equal volume of 15% (wt/vol) trichloroacetic acid and were centrifuged at 12,000  $\times g$  for 2 min at room temperature to pellet precipitate. To determine the quantity of released amino acid by ninhydrin reaction, 25  $\mu\text{L}$  of the quenched reaction was added to 475  $\mu\text{L}$  of Ninhydrin buffer (0.3 mL glycerol, 0.05 mL of 0.5 M citrate buffer pH 5.5. 0.125 mL of 0.5 M citrate buffer pH 5.5 with 1% [wt/vol] Ninhydrin). The Ninhydrin reaction was developed by boiling for 14 min and cooled on ice for 3 min. A standard curve of glycine or taurine was prepared for each assay. Absorbance at 570 nm was measured in a clear flat-bottom plate in a Tecan Infinite F200 Pro plate reader.

**PI Staining.** WT and  $\Delta bshAB$  strains of *L. acidophilus* and *L. gasseri* were captured at midlog (OD 600 nm = 0.8), then back-diluted to a final OD 600 nm = 0.1 into MRS containing BAs for 3 h. Following exposure, bacteria were stained with PI using slight modifications to a previous method (62, 63). Bacteria were heat-killed at 80  $^{\circ}\text{C}$  for 10 min as positive controls for PI staining. Sub-MIC concentrations of deconjugated BAs were included to control for strain-dependent differences in membrane permeabilization. PI fluorescence was measured from flat-bottom clear plates (excitation: 540 nm; emission: 610 nm) and was normalized to OD 600 nm.

**Coculture Competition Assays.** In vitro competition experiments were performed identical to monoculture growth assays. MRS agar containing 100  $\mu\text{g/mL}$  of rifampicin and 500  $\mu\text{g/mL}$  of streptomycin were used to select for WT (RifR) and  $\Delta bshAB$  (StrR) strains, respectively (89, 90). Competing bacteria were inoculated 1:1 and competitive indexes (CI) were used to present relative fitness across conditions. CI was calculated as:

$$CI = \frac{\text{Final } [\text{Log}_{10} (\Delta bshAB \text{ CFUs}) / \text{Log}_{10} (\text{WT CFUs})]}{\text{Initial } [\text{Log}_{10} (\Delta bshAB \text{ CFUs}) / \text{Log}_{10} (\text{WT CFUs})]}$$

Ex vivo competition experiments were performed in triplicate by inoculating cecal content with  $10^5$  CFUs of WT (RifR) and  $\Delta bshAB$  (StrR) strains/gram of content. *Lactobacillus* Selection Agar (BD) supplemented with rifampicin or streptomycin was used to select for and enumerate the test strains separately. Cecal contents were harvested from 5 to 8 wk-old WT C57BL/6J mice (Jackson Laboratory) as described above in the gnotobiotic mouse colonization methods.

**Data Availability.** The RNA-sequence data has been deposited to the Sequence Read Archive database, <https://www.ncbi.nlm.nih.gov/bioproject> (BioProject ID PRJNA639594 and accession numbers SRR12018454–SRR12018485).

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