Cysteine dependence of *Lactobacillus iners* is a potential therapeutic target for vaginal microbiota modulation

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Abstract

Vaginal microbiota composition affects many facets of reproductive health. *Lactobacillus iners*-dominant microbial communities are associated with poorer outcomes, including higher risk of bacterial vaginosis (BV), compared with vaginal microbiota rich in *Lactobacillus crispatus*. Unfortunately, standard-of-care metronidazole therapy for BV typically results in dominance of *L. iners*, likely contributing to post-treatment relapse. Here we generate an *L. iners* isolate collection comprising 34 previously unreported isolates from 14 South African with and without BV and 4 previously unreported isolates from 3 US women and we report an associated genome catalog comprising 1,218 vaginal *Lactobacillus* isolate genomes and metagenome-assembled genomes (MAGs) from >300 women across four continents. We show that, unlike *L. crispatus*, *L. iners* growth is dependent on L-cysteine *in vitro* and we trace this phenotype to the absence of canonical cysteine biosynthesis pathways and a restricted repertoire of cysteine-related transport mechanisms. We further show cysteine concentrations in cervicovaginal lavage samples correlate with *Lactobacillus* abundance *in vivo* and that cystine uptake inhibitors selectively inhibit *L. iners* growth *in vitro*. Combining an inhibitor with metronidazole promotes *L. crispatus* dominance of defined BV-like communities *in vitro* by suppressing *L. iners* growth. Our findings enable a better understanding of *L. iners* biology and suggest candidate treatments to modulate the vaginal microbiota to improve reproductive health for women globally.

Main text

Female genital tract (FGT) microbiota composition is linked to important women's health and reproductive outcomes including HIV risk[1,2], preterm birth[3], mucosal inflammation[4–6], human papilloma virus (HPV) infection[7], and cervical dysplasia[7]. Diverse, anaerobe-dominant bacterial communities are associated with negative sequelae and are a hallmark of bacterial vaginosis (BV)[8,9], a syndrome involving vaginal discharge and mucosal inflammation that affects up to 58% of women worldwide, with disproportionately high impact in sub-Saharan Africa[10]. By contrast, FGT bacterial communities dominated by *Lactobacillus crispatus* and most other *Lactobacillus* species are generally considered optimal for health[9]. One notable exception involves communities dominated by *Lactobacillus iners*, the most prevalent and abundant FGT species worldwide[11], which are associated with many of the same unfavorable outcomes as BV[1,7,12] and have higher risk of transitioning to BV and BV-like communities[3,13–17]. However factors influencing the balance between *L. iners* and more health-associated lactobacilli are incompletely understood[16,18].

The close associations between vaginal microbiota and disease make microbiota-targeted therapies a key strategy to improve women's health and reproductive outcomes[8]. Microbiota modulation has primarily been studied for BV, where current treatments exhibit low efficacy and high rates of recurrence. Over 50% of women with symptomatic BV who receive standard antibiotic therapy with metronidazole (MTZ) experience recurrence by 12 months, while up to 80% of women with prior history of recurrent BV relapse within 16 weeks[19,20]. MTZ typically shifts BV-associated microbiota towards *L. iners*-dominant communities[17,21–24], which frequently transition back to BV-like communities[13,15,16]. Novel strategies to promote *L. crispatus*-dominance over *L. iners*-dominance during BV treatment are thus a key priority[16,25].

Despite its ubiquity in human populations, *L. iners*' biology remains incompletely characterized[18]. Several features distinguish it from other lactobacilli including a putative virulence factor (inerolysin)[26], lack of D-lactic acid and hydrogen peroxide production[27–29], and a smaller genome size with reduced metabolic potential, suggesting niche adaptation and dependence on exogenous nutrients[28]. However, *L. iners* research has been significantly hampered by its species-defining failure to grow in standard *Lactobacillus* MRS (de Man, Rogosa, Sharpe) media[30], complicating attempts at isolation and *in vitro* characterization[11,18,31]. Consequently, few experimental strains and genomes exist, with many studies reporting little or no success isolating *L. iners* even when culturing from hundreds of clinical samples[32–34].

Here we show that *L. iners*' unique *in vitro* growth limitation is due to a restricted capacity to exploit exogenous cysteine (Cys) sources. Through experiments and analysis of a collection of >1200 diverse *Lactobacillus* genomes, we demonstrate that major vaginal *Lactobacillus* species lack canonical Cys biosynthesis pathways. We find that vaginal Cys concentrations are higher in women without BV and that *in vivo* abundances of both *L. iners* and *L. crispatus* correlate with Cys availability. However, *L. iners* lacks transport mechanisms for Cys and Cys-containing molecules possessed by other *Lactobacillus* species, rendering its growth uniquely susceptible to inhibition by cystine uptake inhibitors. Combining an inhibitor with MTZ promotes *L. crispatus* dominance of defined BV-like bacterial communities by suppressing *L. iners*, identifying a previously undescribed target for therapeutically modulating vaginal microbiota to promote women's health.

RESULTS

L-cysteine supports growth of diverse *L. iners* strains

We investigated the nutritional dependencies underlying *L. iners*' failure to grow in *Lactobacillus* MRS (De Man, Rogosa, and Sharpe) media[31] by testing various nutrient additives. MRS broth supplemented with IsoVitaleX™ (a defined micronutrient mixture) supported robust *L. iners* growth while retaining selectivity against *G. vaginalis* (Fig. 1a). Testing IsoVitaleX™ components individually revealed that L-cysteine (L-Cys) was necessary and sufficient for this growth phenotype (Extended Data Fig. 1a-f; reagent details in Supplementary Table 1 and 2). L-glutamine (L-Gln) was neither necessary nor sufficient, but augmented growth in presence of L-Cys, so subsequent experiments employed a base of L-Gln-supplemented MRS broth ("MRSQ").

We next assessed species-level generalizability of Cys-dependent growth by establishing a large, geographically diverse *L. iners* isolate collection and genome catalog of strains from women both with and without BV. The isolate collection included 4 previously reported US strains from culture repositories, plus 34 previously unreported isolates from 14 South African women and 4 previously unreported isolates from 3 US women, isolated using L-Cys-supplemented MRS or blood agar. The associated genome catalog comprises 327 genomes derived from studies of disparate human populations in the US, South Africa, Sweden, China, and Italy, including 21 "reference" genomes of previously reported isolates retrieved from RefSeq[35], 49 previously unreported isolate genomes, and 257 previously unreported culture-independent metagenome-assembled genomes (MAGs) generated from a combination of both previously published and previously unreported FGT shotgun metagenomic sequencing studies (Hayward et al, manuscript in preparation; Supplementary Table 3 lists isolates tested experimentally in this study; see also additional isolate and genome source information in the Methods, Extended Data Figure 2, and Supplementary Tables 4-11). Species-level phylogenetic reconstruction revealed that *L. iners* genomic diversity was largely independent of geographic source, genome type, or clinical context

(Fig. 1b). Strains broadly representative of species diversity isolated from South African and US women both with and without BV were selected for further experimental characterization (Fig. 1b and Supplementary Table 3). All selected strains grew in MRSQ broth with L-Cys (representative examples in Fig. 1c), confirming Cys-dependent growth as a species-level phenotype.

FGT *Lactobacilli* lack canonical Cys biosynthesis pathways

We investigated whether *L. iners' in vitro* Cys-dependent growth phenotype reflected differences in Cys biosynthetic capacity relative to other common FGT lactobacilli. Bacteria canonically synthesize Cys *de novo* from serine (Ser) via either the Cys synthase pathway (*cysE*, followed by *cysK*, *cysM*, or *cysO*) or reverse transsulfuration pathway (*cbs* or *mccA*, followed by *mccB*; Fig. 2a)[36]. We assessed presence of these pathways in a previously unreported catalog of 1,218 FGT *Lactobacillus* genomes and MAGs, including 327 *L. iners* genomes, along with similarly constructed, geographically diverse catalogs for *L. crispatus* (n = 310 genomes and 198 MAGs), *L. gasseri* (n = 174 genomes and 42 MAGs), *L. jensenii* (n = 80 genomes and 57 MAGs), and *L. vaginalis* (n = 28 genomes and 2 MAGs; see Extended Data Fig. 2 and Supplementary Tables 4-11; Hayward et al, manuscript in preparation). These catalogs represent substantial expansion of known genomic diversity for all species, most notably for *L. iners* with a >10-fold increase in the number of genomes and a 73% increase in pan-genome size compared to RefSeq-derived genomes alone (Supplementary Table 4). No genomes from any species were predicted to encode intact Cys synthase or reverse transsulfuration pathways, although interestingly all non-*iners* species had predicted *mccB* orthologs (Fig. 2b and Supplementary Table 12). Thus, all common FGT *Lactobacillus* species, including *L. iners*, are predicted to lack canonical mechanisms for *de novo* Cys biosynthesis.

To phenotypically test this result, we used isotopic tracing to measure the ability of *L. iners* and *L. crispatus* to convert Ser into Cys *in vitro*. Representative strains were grown in MRSQ broth supplemented with labeled Ser (^{13}C-L-Ser) plus homocysteine or with labeled L-Cys (^{13}C-L-Cys; labeling scheme in Fig. 2a), then cellular amino acid isotopic labeling was analyzed via liquid chromatography-mass spectrometry (LC-MS). Both *L. iners* and *L. crispatus* took up labeled Ser but failed to convert it to Cys (Fig. 2c,d and Supplementary Table 13 and 14), consistent with mechanistic predictions from genomic analysis of the species genome collections (Fig. 2a,b and Supplementary Table 12). By contrast, both strains readily incorporated labeled Cys, supporting the genomic prediction that, despite their different *in vitro* growth phenotypes, both utilize exogenous Cys.

Cys levels correlate with *Lactobacillus* abundance *in vivo*

Since vaginal lactobacilli lack canonical Cys biosynthesis pathways and utilize exogenous Cys, we investigated *in vivo* relationships between vaginal Cys concentrations, BV, and *Lactobacillus* colonization levels. We measured Cys concentrations in cervicovaginal lavage (CVL) fluid from 142 participants in a cohort of South African women aged 18-23 years[1,4,37], 53 of whom were also evaluated for BV by Nugent scoring[38]. Cys concentrations differed strikingly by BV status ($P = 6.3 \times 10^{-9}$), with significantly higher concentrations among women without BV compared to those with BV (Fig. 3a). We explored FGT microbiota composition in this cohort by bacterial 16S rRNA gene sequencing, classifying, individual bacterial communities into four "cervicotypes" (CTs) based on previously established criteria[1]: CT1 (*L. crispatus*-dominant), CT2 (*L. iners*-dominant), CT3 (*Gardnerella*-dominant), and CT4 (non-*Lactobacillus*-dominant, diverse communities typically featuring high *Prevotella* abundance) (Fig. 3b; taxonomy assignments in Supplementary

Table 15). As expected, CT assignment was strongly related to BV status ($P = 1.902 \times 10^{-11}$, two-sided Fisher's Exact Test; Extended Data Fig. 3a). Cys concentration differed markedly between CTs ($P = 2.7 \times 10^{-14}$ overall), with significantly higher concentrations in *Lactobacillus*-dominant CTs compared to CT3 and CT4 (Fig. 3c).

We next examined correlations between Cys concentration and abundance of individual taxa within the microbiota. Analysis of CT-associated genera revealed that *Lactobacillus* relative abundance strongly positively correlated with Cys concentration ($\rho = 0.6$, $P = 5.2 \times 10^{-15}$ by Spearman correlation), while *Prevotella* abundance had equally strong negative correlation ($\rho = -0.57$, $P = 1.9 \times 10^{-13}$) and *Gardnerella* abundance was weakly negatively correlated (Fig. 3d). *L. crispatus* and *L. iners* each positively correlated with Cys when analyzed individually (Extended Data Fig. 3b). To more comprehensively identify taxa associated with Cys, we calculated correlations for each taxon detected in ≥50% of samples at the genus or species level (Extended Data Fig. 3c,d), adjusting for multiple comparisons. *Lactobacillus* (genus-level) and *L. crispatus* and *L. iners* (species-level) were the only taxa positively correlated with Cys (Fig. 3e,f; full statistical results in Supplementary Tables 16 & 17). Other taxa, including various BV-associated bacteria, showed no correlation or significant negative correlation with Cys. Results were similar for the Cys-containing peptides reduced glutathione (GSH) and cysteinylglycine (Extended Data Fig. 4 and Supplementary Tables 16 & 17). Thus, vaginal Cys concentration is higher in women without BV and correlates with abundance of both *L. iners* and *L. crispatus*, consistent with the hypothesis that Cys availability is important for *Lactobacillus* colonization success *in vivo*.

L. iners has limited capacity to use complex L-Cys sources

The species-specific nature of *L. iners*' Cys-dependence *in vitro* appeared discordant with the finding that all FGT *Lactobacillus* species lacked canonical Cys biosynthesis pathways and that *in vivo* abundance of both *L. iners* and *L. crispatus* correlated with vaginal Cys concentrations. We therefore hypothesized that *L. iners'* unique *in vitro* growth phenotype was due to a restricted capacity to utilize exogenous Cys sources in media. Alternatively, Cys might support *L. iners* growth by acting as a chemical reducing agent rather than a direct nutritional supplement. We found that concentrations of free molecular Cys and cystine (its oxidized counterpart) in MRSQ broth were ≤4.6 μM and 0.7 μM respectively, below levels required by Cys-auxotrophic *E. coli* strains[39]. L-cystine supplementation produced similar *L. iners* growth as L-Cys supplementation (Fig. 4a), including in presence of the oxidizing agent hydrogen peroxide (H_2O_2; Extended Data Fig. 5a), demonstrating that L-cystine supports growth by acting as a direct nutritional supplement rather than by chemically reducing the media. Thus *L. iners* does not require a reduced environment to grow when it has access to a bioavailable source of L-Cys (e.g., L-cystine), but L-Cys bioavailability in un-supplemented MRSQ broth is inadequate for *L. iners*.

The ability of non-*iners* lactobacilli to grow in un-supplemented MRS broth implied presence of Cys sources that *L. iners* failed to utilize (MRS contains yeast and beef extracts, which contribute peptides in addition to monomeric amino acids[30,40]). Adding chemical reducing agents to MRSQ broth substantially raised Cys and GSH concentrations (Extended Data Fig. 5b), demonstrating that most Cys and Cys-containing molecules in un-supplemented media exist as mixed disulfide compounds. Addition of chemical reducing agents also supported *L. iners* growth (Extended Data Fig. 5a,c), but in contrast to L-cystine, the oxidized counterparts of these reducing agents did not. Thus, MRS broth contains complex sources of L-Cys, including mixed disulfides, that *L. iners* has restricted capacity to utilize.

L. iners lacks transporters found in other FGT lactobacilli

We hypothesized that *L. iners*'s restricted ability to use exogenous Cys sources reflected a limited repertoire of transport mechanisms for Cys and Cys-containing molecules in comparison to other lactobacilli. Uptake of Cys and Cys-containing molecules in bacteria occurs through multiple known or putative mechanisms including the well-characterized cystine transport systems TcyABC, TcyJKLMN, and TcyP[41], a putative Cys ABC transporter associated with Cys-binding protein CjaA[42,43], and the heterodimeric ABC transporter CydDC encoded by the redox-regulating locus *cydABCD*[44], a system that exports both Cys and GSH to the periplasm in *E. coli*[44] and is proposed to perform glutathione uptake in lactobacilli[45]. Analysis of our *Lactobacillus* genome catalogs revealed that all *Lactobacillus* species except for *L. iners* encoded *cydABCD*, while *L. crispatus*, *L. vaginalis*, and some *L. gasseri* each encoded one or more predicted *cjaA* orthologs and *L. jensenii*, *L. gasseri*, and *L. vaginalis* each encoded one or more cystine transport systems (Fig. 4b and Supplementary Table 12). Low-affinity Cys uptake also occurs via the branched-chain amino acid importer LivJ/LivKHMGF[43], but no genomes possessed *livKHMGF* orthologs except 2 *L. iners* MAGs, nor did any species possess an intact GsiABCD glutathione transport system[46] (Extended Data Fig. 6a and Supplementary Table 12). Thus, each common FGT *Lactobacillus* species except *L. iners* encodes multiple, mechanistically distinct known or putative systems to transport Cys or Cys-containing molecules, while *L. iners*' Cys-related uptake occurs via currently unrecognized mechanisms.

L. iners growth is inhibited by cystine uptake inhibitors

Experimental tools to perform genetic screens in *L. iners* do not currently exist[47], so we used phenotypic approaches to characterize its Cys-related transport mechanisms. Isotopic tracing (Fig. 4c) revealed that *L. iners* incorporated labeled L-cystine ($^{13}C_2$-L-cystine) at levels similar to *L. crispatus* but failed to take up labeled GSH (^{13}C-GSH; synthesis detailed in Supplementary Fig. 1), consistent with predicted absence of GSH transport activity (Fig. 4b and Extended Data Fig. 6a). We therefore assessed whether *L. iners*' limited repertoire of transport mechanisms would cause its growth in L-Cys-supplemented MRSQ broth to be uniquely impacted by the known cystine uptake inhibitors *S*-methyl-L-cysteine (SMC) and seleno-DL-cystine (SDLC)[41]. SMC caused species-specific, dose-dependent growth inhibition of a diverse collection of *L. iners* strains at concentrations of 32-64 mM, while SDLC caused selective inhibition at 0.25 mM (Fig. 4d,e and Extended Data Fig. 6b,c). Inhibition occurred for strains from both US and South African women, and from women both with and without BV (Supplementary Table 3). Inhibitor potency varies for different transporters, but these concentrations appear higher for SMC and slightly higher for SDLC than reported inhibitory concentrations for the TcyJKLMN and TcyP transporters in *Bacillus subtilis*[41]. To confirm that growth inhibition was not an artefact specific to MRS media, we assessed inhibition in NYCIII broth, a serum-containing, nutrient-rich medium that supports growth of diverse fastidious FGT bacteria[48]. Both SMC and SDLC inhibited *L. iners* growth in NYCIII broth, but not growth of *L. crispatus* (Fig. 4f). They also selectively inhibited *L. iners* growth in MRSQ broth treated with chemical reducing agents, supporting the conclusion that reducing agents promote *L. iners* growth by enhancing Cys bioavailability (Extended Data Fig. 6d). Collectively these results from isotopic tracing and growth inhibition assays confirm our genomic prediction that *L. iners* possesses a uniquely restricted repertoire of Cys-related transport mechanisms.

SMC inhibits *L. iners* in competition with *L. crispatus*

To assess the functional significance of *L. iners* growth inhibition by cystine uptake inhibitors, we assessed whether an inhibitor could shift the balance between *L. iners* and *L. crispatus* in culture competition assays. We tested pairwise strain combinations of *L. crispatus* and *L. iners* in L-Cys-supplemented MRSQ broth at varying SMC concentrations, then measured ratios of *L. iners* to *L. crispatus* in the mixed cultures by 16S rRNA gene sequencing. Experimental controls were included to confirm expected growth patterns and absence of significant contamination during sample processing (Extended Data Fig. 7). SMC significantly suppressed *L. iners* relative to *L. crispatus* in a dose-dependent fashion (representative experiments in Fig. 5a, Supplementary Table 18), confirming that it offers selective advantage to *L. crispatus* in direct competition.

SMC plus MTZ enriches *L. crispatus* in mock BV communities

To assess whether cystine uptake inhibitors have potential to augment BV therapy by preferentially promoting *L. crispatus* expansion relative to *L. iners*, we investigated SMC's effects in combination with MTZ on mock BV-like bacterial communities cultured in a rich nutritional milieu. Multiple South African *L. crispatus* isolates unexpectedly failed to grow in NYCIII broth, so we developed a previously unreported nutrient-rich, serum-containing formulation named "S-broth" that supported South African *L. crispatus* strains as well as diverse BV-associated bacteria (Extended Data Fig. 8a). Testing effects of SMC and MTZ on pure cultures of *L. crispatus*, *L. iners*, *G. vaginalis*, *Prevotella bivia*, and *Atopobium* (*Fannyhessea*) *vaginae* grown in S-broth revealed that neither MTZ nor SMC blocked *L. crispatus* growth, SMC inhibited *L. iners*, and

MTZ suppressed both *G. vaginalis* and *P. bivia* (Fig. 5b). The *A. vaginae* strain was relatively MTZ-resistant, but susceptibility was enhanced by addition of SMC.

We next cultured defined communities of these species in S-broth and assessed whether combining SMC with MTZ enhanced *L. crispatus* dominance. Each community contained a different pairwise combination of *L. crispatus* and *L. iners* strains (input ratios as in Supplementary Table 19) and we quantified mock community composition by 16S rRNA gene sequencing (controls shown in Extended Data Fig. 8b,c). Cultivation without inhibitors produced diverse mixtures of all 5 species (representative examples in Fig. 5c). As hypothesized, SMC alone diminished *L. iners* relative abundance, MTZ alone suppressed BV-associated species, and MTZ combined with SMC preferentially favored *L. crispatus*. To quantify *L. crispatus*' competitive advantage, we calculated the ratio of *L. crispatus* relative abundance to the summed abundances of all other taxa (Fig. 5d, Supplementary Table 20). Combining MTZ with SMC increased this ratio significantly more than MTZ alone, suggesting cystine uptake inhibitors could interact with MTZ to promote *L. crispatus* dominance during BV treatment.

DISCUSSION

L. iners has long been recognized as possessing diminished metabolic potential compared to other lactobacilli – indeed, the name "*iners*" refers to relative inertness in biochemical assays[31]. But despite being the most abundant FGT species worldwide and having adverse health associations compared to *L. crispatus*[1,3,52–54,7,11,12,16,18,49–51], *L. iners*' biology remains poorly characterized because it fails to grow in *Lactobacillus* MRS media[31], resulting in a paucity of isolates and genomes[11,18]. Here we resolve this growth defect by identifying a species-specific requirement for Cys supplementation. Employing an isolate collection encompassing both reference and

previously unreported strains and >1200 *Lactobacillus* genomes from diverse geographical and clinical sources, we extend and experimentally confirm predictions from smaller-scale genomic studies suggesting all common FGT lactobacilli lack canonical Cys biosynthesis pathways[55]. We further show that vaginal Cys concentrations strongly correlate with *Lactobacillus* relative abundance *in vivo* in a South African cohort. However, *L. iners* lacks transport mechanisms for Cys and Cys-containing molecules present in other lactobacilli, rendering its growth selectively susceptible to cystine uptake inhibitors. Combining a cystine uptake inhibitor with MTZ (a standard antibiotic used for BV treatment) enhances *L. crispatus* dominance in mock, BV-like bacterial communities by suppressing *L. iners* growth. These advances establish a foundation for future study of *L. iners*' biology, diversity, and effects on human health while identifying a previously undescribed target for therapeutic vaginal microbiota modulation.

The *in vivo* correlation between Cys concentrations and *Lactobacillus* abundance suggests Cys availability may influence FGT microbiota composition. A US-based case-control study comparing women with and without BV found that vaginal Cys concentrations positively correlated with non-*iners* lactobacilli and negatively correlated with various BV-associated species[56]. That study appeared to show a weaker correlation between *L. iners* and Cys than the correlation we identify here[56], which may reflect differences in study design and/or species abundances between the two populations. Since FGT lactobacilli appear to lack *de novo* Cys biosynthetic capacity, we hypothesize that the high Cys concentrations in *Lactobacillus*-dominant states are likely primarily host-derived. Possible mechanisms explaining the correlation could thus include preferential *Lactobacillus* colonization of hosts with high levels of mucosal Cys secretion, induction of host Cys secretion by lactobacilli, Cys degradation by host cells in setting of BV-associated inflammation[57], and/or degradation of Cys by BV-associated bacteria[56] as a means to

outcompete lactobacilli. Further investigation of FGT microbiome-metabolome relationships will be required to fully assess these possibilities.

A striking aspect of our findings is the paucity of transport mechanisms for Cys and Cys-containing molecules in *L. iners* compared to other FGT lactobacilli. In contrast to cystine transporters[41], bacterial Cys transporters are not well characterized due to technical challenges inherent to redox chemistry[43]. Our genome analysis predicted that non-*iners* FGT *Lactobacillus* species each encode multiple known or putative transporters for Cys, cystine, and/or Cys-containing molecules, whereas we identified no predicted transporters for these molecules in *L. iners*. However, both L-Cys and L-cystine supported *L. iners* growth, and isotopic tracing demonstrated uptake when *L. iners* was grown in the presence of labeled L-Cys or L-cystine, pointing to one or more currently unrecognized uptake mechanisms. The finding that cystine uptake inhibitors selectively inhibit *L. iners* growth provides further evidence that it lacks the multiple, mechanistically distinct Cys-related transporters present in other species.

In addition to providing mechanistic insights, our results have important implications for BV treatment. Standard BV therapy with MTZ often results in disease recurrence[19,20,25]. This may be partially due to MTZ's tendency to promote *L. iners*-dominant FGT bacterial communities[17,21–24], which to transition back to BV-like states more frequently than *L. crispatus*-dominant communities[13–17]. We recently showed in a South African population that *L. iners*-dominant communities are a "gateway" for transitions from *Lactobacillus* dominance to more diverse, BV-like communities and our modeling of microbiota dynamics suggested that interventions shifting community transition probabilities towards *L. crispatus* over *L. iners* would most effectively increase the prevalence of *L. crispatus*-dominance within the population[16]. Therapies to promote *L. crispatus* dominance during BV treatment are under active investigation. In a trial of a live *L.*

crispatus biotherapeutic administered after MTZ treatment, BV recurred less frequently among biotherapeutic recipients than placebo recipients[25]. However recurrence in the treatment arm remained high (30% within 3 months)[25]. A small Israeli cases series reported achieving *L. crispatus*-dominance and long-term BV remission in four of five women treated for refractory BV with MTZ plus vaginal microbiome transplants from healthy donors[58], but most patients required multiple rounds of therapy. Interestingly, that study did not report detecting *L. iners* in any donors or recipients – a notable difference from typical FGT microbiota composition in North and South America, Europe, Asia, Africa, and Australia[1,2,23,24,48–54,56,3,59,60,4,5,12,13,17,21,22]. Thus, live biotherapeutic and microbiome transplant approaches for BV show promise but are unlikely to be a panacea. Our study shows that combining MTZ with a previously undescribed *L. iners* growth inhibitor was superior to MTZ alone in promoting *L. crispatus* community dominance of defined, BV-like bacterial communities. The specific inhibitors identified here have potential limitations for use in humans including low potency (SMC) and possible host toxicity (SDLC), and their effects (if any) on the FGT mucosa remain to be characterized. However, our results demonstrate proof of concept for a previously unrecognized therapeutic approach and provide a rationale to identify additional inhibitors targeting *L. iners* through this mechanism. Further development of this approach as an adjunct to antibiotics has potential to improve therapies for microbiota-linked reproductive health conditions worldwide, including for women in low- and middle-income countries where the burden of adverse reproductive health outcomes is greatest.

Methods

Overview of cohorts and sources of metagenomic data

This study includes previously unreported FGT microbiota sequencing data, FGT metabolite concentration data, bacterial isolates, isolate genomes, and/or MAGs assembled from whole-genome shotgun metagenomic data (WGS) from the Females Rising through Education, Support, and Health (FRESH) study, a South African cohort (details below). It also includes previously unreported bacterial isolates, isolate genomes, and/or MAGs assembled from WGS data from participants in 6 US-based cohorts, as well as previously unreported MAGs assembled *de novo* from published WGS data from 5 additional cohorts from the US, Italy, and China (details below).

FRESH cohort characteristics

The FRESH cohort is an ongoing prospective observational study based in Umlazi, South Africa, that enrolls 18–23-year-old, HIV-uninfected, non-pregnant, otherwise healthy women willing to have frequent HIV testing and 3-monthly collection of blood and mucosal specimens during study enrollment. Exclusion criteria included hemoglobin level <10 g/dL, any chronic medical condition or other conflict likely to prevent adherence to the study protocol, and/or enrollment in any other study that involves frequent blood sampling or that might otherwise interfere with the FRESH study protocol. Study characteristics and inclusion and exclusion criteria have also been described in detail elsewhere[1,4,37]. The study protocol was approved by the Massachusetts General Hospital Institutional Review Board (2012P001812/MGH) and the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (UKZN; Ethics Reference Number BF131/11). Participants were recruited at local sites frequented by young people including shopping malls, cafes, and nightclubs. All participants provided written informed consent. The study protocol includes non-

monetary benefits encompassing job-skills, health, and empowerment training, a light meal during study site visits, and cumulative monetary compensation over a 36-week period of 3,700 rand (~$280 US dollars), which helps defray transportation expenses to twice-weekly study site visits. Participant self-selection bias may potentially occur based on participants' perception of their own HIV risk, but it is unknown whether or how such a bias would affect FGT microbiota composition or metabolite concentrations.

FGT sample collection within the FRESH study included multiple cervical swab samples (Puritan 6" Sterile Standard Foam Swab w/Polystyrene Handle), which were collected by swabbing the ectocervix in two full revolutions under direct visualization during speculum exam. The swabs were then used to make a slide preparation for Gram stain analysis, were cryopreserved in thioglycolate broth with 20% glycerol for bacterial isolation or were frozen without cryopreservatives for bacterial isolation or nucleic acid extraction and microbiota profiling. Cervicovaginal lavage (CVL) samples were collected using a flexible plastic bulb pipette to dispense 5 mL of sterile normal saline into the vaginal vault, wash the cervix four times, then re-aspirate the fluid into a 15 mL conical tube. CVL and swab samples were stored on ice for 1-4 hours during transport to the processing laboratory at the Nelson R. Mandela School of Medicine at UKZN, after which swabs were stored at -80°C and CVL samples were centrifuged at 700xg for 10 minutes at 4°C, then supernatants were transferred to cryovials and stored at -80°C.

FRESH cohort microbiota and metabolite profiling data

Paired bacterial microbiota composition and cervicovaginal metabolite concentrations (Figure 3 and Extended Data Figures 3 and 4) were determined for 143 HIV-uninfected FRESH study participants consecutively sampled in May through October of 2017 (methodological details

below). The sample size was initially selected for microbiota profiling based on a separate, unpublished investigation of microbiome-immune parameters; paired CVL samples from these participants were later used for FGT metabolite concentration measurements due to availability of paired microbiota sequencing data. No *a priori* sample size calculation was performed in relation to analysis of FGT microbiota-cysteine correlations because it was (to our knowledge) the first investigation of this association in a sub-Saharan African cohort and most studies in non-African populations have used case-control designs that are not adapted to evaluate general population-level correlations – we therefore lacked information upon which to make informed estimates about metabolite distributions for power calculations. However the cohort was of similar or larger size as other reported studies of the FGT metabolome[56,61,62]. One of the 143 participants was excluded from analysis of vaginal metabolite concentrations based on the pre-specified criterion of low bacterial 16S rRNA gene sequence read count (<10,000 reads). Assessment of BV status among this group of FRESH study participants using the Nugent scoring method[38] was performed sporadically prior to September 2017, when a protocol change instituted universal BV testing for all participants. We restricted analysis of BV-metabolite associations (Figure 3a and Extended Data Figures 4a,b) to the subset of 53 out of 143 participants assessed under the universal testing protocol to minimize risk of bias. Nugent scoring was performed at Global Laboratories (now Neuberg Global Laboratories, Durban, South Africa), an accredited commercial laboratory diagnostics company, by trained laboratory technologists blinded to all participant information other than study ID.

Sources of bacterial isolates and MAGs

This study reports previously undescribed bacterial isolates used in experiments (experimentally studied strains are listed in Supplementary Table 3, isolation details are described below) and analyzes a genome catalog including previously unreported *Lactobacillus* bacterial isolate genomes and MAGs (Figures 1b, 2b, 4b, and Extended Data Figures 2 and 6a; see also Supplementary Tables 4-11. The genome catalog was abstracted from a more extensive, unpublished, global characterization of FGT microbiome phylogenetic structure and function analyzing a combination of published bacterial isolate genome and WGS data, as well as previously unreported US and South African genomic and metagenomic data (manuscript in preparation by M.R. Hayward, S.M. Bloom, F.X. Ceasar, U. Das Adhikari, S.M.R. Demby, K.L. Dong, M. Dong, J.A. Elsherbini, M.T. France, M.S. Ghebremichael, T. Gumbi, F.A. Hussain, C. Huttenhower, N. Ismail, N.A. Mafunda, J.M. Marrazzo, C.M. Mitchell, T. Ndung'u, J. Ravel, D.A. Relman, J.K. Rice, J.M. Urbach, J. Xu, N. Xulu, and D.S. Kwon; additional methodological details are provided below). The previously undescribed datasets contributing to the analysis by Hayward et al include isolates and MAGs from 138 total FRESH study participants (6 of whom contributed both isolates and MAGs, 11 of whom contributed isolates only, and 121 of whom contributed MAGs only). This number included 35 participants from among the 143 participants listed above, as well as 103 additional FRESH study participants for whom microbiota composition based on bacterial 16S rRNA gene-based profiling has previously been described[1,4].

Additional previously unreported US-based primary isolates tested experimentally (Supplementary Table 3), as well as bacterial isolate genomes and WGS sequence data that served as the source of MAGs (Supplementary Tables 5, 6, 8, 9, and 11) were obtained via the Vaginal Microbiome Research Consortium (VMRC; https://vmrc4health.org/). The VMRC data and isolates were derived from two different IRB-approved protocols. The first of these IRB-approved

protocols (Stanford University IRB protocol #21956; principal investigator D.A.R.) encompasses two previously described[63] cohorts that enrolled 135 pregnant women aged at least 18 years presenting for obstetrical care at either Lucille Packard Children's Hospital at Stanford University or at the University of Alabama, Birmingham, who provided written informed consent. Exclusion criteria included antibiotic or probiotic use within twelve weeks prior to enrollment, significant immunosuppression, or (retrospectively) delivery of a baby who had congenital defects. Vaginal swab specimens were collected during pregnancy and (in the Stanford cohort) in the post-partum period as well. Cumulative monetary compensation totaled as much as $350 per participant. Isolates and MAGs obtained from participants enrolled via this protocol were derived from 18 total study participants, 11 of which contributed both isolates and MAGs, 5 of which contributed isolates only, and 2 of which contributed MAGs only. The second VMRC-associated cohort (University of Maryland Baltimore IRB protocol #HP-00041351; principal investigator J. Ravel), which has also been previously described[23], enrolled 135 non-pregnant, HIV-uninfected women at University of Alabama, Birmingham, who were recruited via newspaper advertisements, fliers, general gynecology clinics, and the Jefferson County STD clinic. Participants were aged 18-45 with regular menses who provided written informed consent. Exclusion criteria included antibiotic or antimycotic use within 30 days prior to enrollment, pregnancy or current lactation, active infection with *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, or syphilis, symptomatic BV or vulvovaginal candidiasis for which treatment was requested at time of enrollment, immunocompromised status (including HIV), significant affective or psychotic disorders or intellectual limitations precluding informed consent, history of prior or pending hysterectomy or pelvic radiotherapy, non-fluency in English, current use of NuvaRing® contraception, recent receipt of a bacterial vaccine, or concurrent participation in other trials

involving blinded administration of antibiotics or topical microbicides. Participants self-collected daily vaginal swabs longitudinally over 10 weeks. Cumulative monetary compensation totaled $440 per participant. Isolates and MAGs obtained from participants enrolled via this protocol were derived from 43 total study participants, 11 of which contributed both isolates and MAGs, 12 of which contributed isolates only, and 20 of which contributed MAGs only.

Additional previously unreported US-derived genomes of 111 non-iners Lactobacillus isolates (Supplementary Tables 5, 6, 8, 9, and 11) were generated from samples collected as part of the Vaginal Health Project (VHP) study and the isolates were provided as a kind gift from VHP principal investigator Dr. Jeanne M. Marrazzo[64]. The VHP was an IRB-approved longitudinal study of BV dynamics based in Seattle, Washington, USA, that enrolled women aged 16-30 years who reported having sex with ≥ 1 female partner in the prior year. Recruitment occurred through media, advertisements, and community referrals. Participants provided written informed consent and attended at least quarterly study visits over a year at which mid-vaginal swabs were collected, with more frequent study visits if BV was diagnosed via a combination of Amsel criteria[65] and Nugent scoring[38,64]. Cumulative monetary compensation totaled $100 per participant. Bacteria were subsequently isolated from cryopreserved swabs via cultivation on Lactobacillus MRS agar (thus L. iners was not isolated). For isolate genome sequencing, a subset of 6 participants were retrospectively selected from the overall VHP cohort based on presence of both BV and cultivable lactobacilli at the baseline study visits and genome sequencing was performed on 15 to 32 isolates per participant, isolated from samples collected at between 4 and 6 study visits per participant.

Other previously unreported US-derived genomes of 4 non-*iners Lactobacillus* isolates (Supplementary Tables 5, 6, 8, 9, and 11) were derived from 4 participants in the V2 cohort[66], an IRB-approved cohort at Massachusetts General Hospital (MGH) in Boston, MA, (MGH IRB

Protocol #2014P001066) that enrolled women presenting for gynecological care at MGH who provided written informed consent, excluding those with HIV or who were otherwise immunocompromised. Participants received no monetary compensation and ages ranged from 24-38 years. Samples were collected via swabs.

MAGs were assembled from previously unreported US-based WGS data from a single participant (Supplementary Tables 5, 6, 8, 9, and 11) in an IRB-approved Female Reproductive Tract biopsy protocol based at the Ragon Institute of MGH, MIT and Harvard in Cambridge, MA, (MGH IRB Protocol #2012P001812). The protocol enrolls non-pregnant, premenopausal women aged 18 to 50 years with normal Pap smears, normal results on routine screening laboratory tests, and willingness to provide informed consent. Exclusion criteria included history of severe gynecologic disease, symptomatic vaginal infection, treatment for vaginal infection within the prior 30 days, being <8 weeks postpartum, sexual intercourse, tampon use or application of vaginal medicines within the prior 2 days, use of immunomodulatory therapy, prior receipt of an HIV vaccine, history of significant medical illness, or history of requiring antibiotics prophylaxis for invasive procedures. Samples collected included cervical and mid-vaginal swabs, with optional consent for cervical and vaginal biopsy. Participants were compensated $150 if no biopsy was performed and $250 if biopsy was performed. The participant for whom MAGs were assembled from WGS data was 26 years old and HIV uninfected.

Additional previously unreported MAGs (Supplementary Tables 5, 6, 8, and 9-11) were assembled from previously reported FGT WGS data from three additional US cohorts (95 total participants; NCBI BioProject accession numbers PRJNA48479, and PRJNA288562)[67–70], one Italian cohort (10 participants; NCBI BioProject accession number PRJNA352475)[71], and one Chinese cohort (4 participants; NCBI BioProject accession number PRJEB24147)[72].

Bacterial culture conditions and additives for bacterial growth media

Bacterial cultivation was performed at 35-37°C under anaerobic conditions using an AS-580 anaerobic chamber (Anaerobe Systems) in an atmosphere of 5% carbon dioxide, 5% hydrogen, and 90% nitrogen (Airgas®). All media and other culture reagents were pre-reduced (deoxygenated) by placement overnight in the anaerobic chamber prior to use. Nutritional additives or inhibitors (reagents listed in Supplementary Table 1; additives prepared as in Supplementary Table 2) were added to autoclave-sterilized broth media after cooling to room temperature (broth media), then broth was re-sterilized by passage through a 0.22 μm vacuum filter before being transferred to the anaerobic chamber to pre-reduce (deoxygenate) for use in experiments. For testing of nutrient pools (Extended Data Figure 1), a single concentrated stock comprising all pool constituents was added to media as per Supplementary Table 2 rather than stocks of each additive being added separately. In contrast to other additives, stocks of hydrogen peroxide (H_2O_2) and metronidazole (MTZ) were added to pre-reduced broth media as freshly prepared, filter-sterilized stock solutions immediately prior to inoculation of bacterial cultures due to potential volatility or chemical instability. For preparation of solid media, filter-sterilized additives were added to autoclave-sterilized agar media after cooling to 50°C, mixed using a magnetic stir/hot plate, then used to pour agar plates.

Bacterial broth culture media

Growth characteristics and nutritional requirements distinguishing *L. iners* from other *Lactobacillus* species were investigated using *Lactobacillus* MRS broth. We tested two different commercial MRS formulations (BD Difco™ and Hardy Criterion). The BD Difco™-formulated

media is referred to as “MRS” while the Hardy Criterion-formulated media is referred to as “HMRS” throughout this paper. The broth was prepared by autoclaving according to manufacturer instructions and allowed to cool to room temperature. We confirmed that *L. crispatus*, *L. jensenii*, and *L. gasseri* grew rapidly and robustly in both formulations whereas *L. iners* failed to grow even after >10 days in HMRS and grew only after a prolonged delay of ~48-72 hours in BD Difco™-formulated MRS. Supplementation of MRS broth (BD Difco™) with IsoVitaleX™ (2% v/v), L-Cys or L-Cys-containing mixtures (augmented by L-Gln) enabled robust growth of *L. iners* by 20-36 hours, depending on the experimental strain (Fig. 1a,c, and Extended Data Fig. 1a-e), while supplementation of HMRS broth had similar pro-growth effects after a more prolonged lag phase (Extended Data Fig. 1f). Unless otherwise indicated, figures depict growth in MRS (BD Difco™) broth; key growth results were reproduced where shown using HMRS broth. MRS broth supplemented with L-Gln (1.1 mM) is abbreviated “MRSQ” (or “HMRSQ” for L-Gln-supplemented HMRS broth) in figures and text.

NYCIII broth (ATCC medium 1685) was prepared using a slightly modified version of the standard ATCC protocol. Pre-media consisted of 4 g/L HEPES, 15 g/L Proteose Peptone No.3 and 5 g/L Sodium Chloride in 875 mL distilled water, was pH-adjusted to 7.3 and autoclaved. Prior to use, complete NYCIII broth was prepared from autoclaved, cooled pre-media by adding dextrose (3g/45mL) at 7.5% v/v, yeast extract solution at 2.5% v/v, and heat inactivated horse serum at 10% v/v, then sterilized by passage through a 0.22 μm vacuum filter. Where indicated, NYCIII broth was supplemented with IsoVitaleX™ (2% v/v), Vitamin K1-Hemin Solution (5% v/v), and/or Tween-80 (1 g/L); volumes of distilled water in the pre-media were decreased accordingly to ensure all other components were present at standard concentrations in the final solution.

"S-broth" pre-media consisting of 37 g/L BHI Broth, yeast extract (powder) 10 g/L, and dextrose 1 g/L was brought to a boil in 880 mL distilled water, then autoclaved (121°C for 15 min) and cooled, followed by addition of fetal bovine serum 5% v/v, Vitamin K1-Hemin Solution 5% v/v, and IsoVitaleX™ Enrichment 2% v/v to a final volume of 1L. The complete broth was then sterilized by passage through a 0.22-micron vacuum filter. Unless otherwise indicated, S-broth was supplemented with 1g/L Tween-80.

Except where otherwise indicated, lactobacilli were propagated in MRSQ broth with L-Cys (4 mM), *Prevotella* species were propagated in Wilkins-Chalgren Anaerobe Broth (Thermo Scientific™), and other species including *Gardnerella vaginalis* were propagated in NYCIII broth.

Bacterial solid culture media

Non-*iners Lactobacillus* isolates were maintained and quantitatively titered on *Lactobacillus* MRS Agar plates (purchased as prepared media from Hardy Diagnostics). Most other species, including *L. iners*, were maintained and titered on Columbia Blood Agar ("CBA"; purchased as prepared media from Hardy Diagnostics). *Prevotella* species were maintained on CBA agar or CDC Anaerobe Laked Sheep Blood Agar with Kanamycin and Vancomycin ("LKV"; purchased as prepared media from BD BBL™). For bacterial isolations from primary clinical samples, we used commercial CBA and LKV agar plates and prepared in-house MRS agar plates by autoclaving *Lactobacillus* MRS Broth (BD DIFCO™) in Noble Agar (BD DIFCO™, 2% w/v), cooling to 50°C with agitation, then supplementing with IsoVitaleX™ Enrichment (BD BBL™; 2% v/v) with or without Vitamin K1-Hemin Solution (BD BBL™; 5% v/v), or with freshly filter-sterilized stocks of L-Cys and L-Gln (at final concentrations of 4 mM and 1.1 mM respectively). All solid media

was stored at 4°C in the dark after purchase or preparation until being introduced to the anaerobic chamber for deoxygenation and use.

Bacterial isolate sources and isolation protocols

Experiments were performed using a combination of previously reported bacterial strains (n = 9 strains), previously unreported South African primary isolates from cervicovaginal samples from the FRESH cohort (n = 20 strains), and previously unreported US strains from cervicovaginal samples (n = 4 strains; See Supplementary Table 3 and additional details below). Isolation of South African strains from FRESH cohort samples was performed at the Ragon Institute. Cervicovaginal swabs were thawed in the anaerobic chamber. Swabs that had been frozen dry (without cryopreservatives) were immersed in 500uL of room temperature pre-reduced PBS. Swabs that had been frozen in cryopreservative solution were rapidly thawed directly without additional dilution. Thawed swabs were agitated vigorously within the solution for 30 seconds using sterile forceps to dislodge bacteria, then removed from the cryovial using a sterile micropipette tip to strain excess fluid from the swab. The solution was then serially diluted in 10-fold dilutions in sterile, pre-reduced PBS and dilutions were plated on *Lactobacillus* MRS agar plates (with or without nutritional additives), CBA, and LKV agar. After 2-5 days incubation (depending on growth rate), colonies were selected based on distinct morphology, and individual representative colonies were sub-cultured onto reduced agar plates, then further clonally sub-cultured into broth media. Aliquots from the purified broth cultures were frozen at -80ºC in 20% glycerol, with additional aliquots reserved for nucleic acid extraction and sequence-based taxonomic identification as described below. *L. iners* colonies were preferentially identified for isolation from primary swab cultures on supplemented MRS or CBA plates based on characteristic morphology

after 3-4 days incubation (circular, white-translucent colonies between 0.5 and 2 mm in diameter with entire vs slightly irregular edges, smooth surface, and convex/umbonate contour that were non-hemolytic on CBA after ~4 days). In experienced hands (S.M.B. and N.A.M.), morphology-based identification of L. iners colonies for isolation from CBA plates resulted in equally high or higher rates of L. iners recovery from clinical samples compared to isolation using plates with supplemented MRS media (which had lower CFU yield than CBA), enabling us to perform targeted isolations of *L. iners* from >80% of attempted clinical samples, including BV-associated, anaerobe-dominated samples known to have <5% *L. iners* relative abundance based on 16S rRNA gene sequencing. Isolated bacteria were identified based on Illumina-based sequencing of genomic DNA and/or Sanger sequencing of the full-length bacterial 16S rRNA gene as described below. BV status of the samples from which isolates were derived was determined based on Nugent scoring when available (Supplementary Table 3).

The following 4 experimental strains were obtained through BEI Resources, NIAID, NIH as part of the Human Microbiome Project: *L. iners*, Strain LEAF 2053A-b, HM-705; *L. iners*, Strain LEAF 3008A-a, HM-708; *L. iners*, Strain UPII 143-D, HM-126; *L. crispatus*, Strain JV-V01, HM-103. Other strains (4 in total) were obtained from the American Type Culture Collection or the Culture Collection University of Gothenburg culture repositories as indicated (Supplementary Table 3). Genomes corresponding to these strains were obtained from RefSeq. Information about the BV status of the samples from which these isolates and other isolate genomes were derived was obtained (when available) from metadata accompanying the isolate genome entries in RefSeq or the Genomes OnLine Database (GOLD; https://gold.jgi.doe.gov/) or from associated information from the reference strain repositories from which they were obtained. One previously reported US-based experimental strain of *L. crispatus*[1] was genome-sequenced for

this project using the methods described below. Additional previously unreported US-based strains used experimentally in this study were provided by Jacques Ravel and the VMRC (4 strains; strain details are reported in Supplementary Table 3). Unless otherwise indicated, all growth, growth inhibition, isotopic tracing, and mock community experiments employed *L. crispatus* strain 233, *L. iners* strain F1, and *G. vaginalis* strain ATCC 14018. Reference bacterial strains used experimentally in this study are available from the indicated culture repositories (see Supplementary table 3) and previously unreported strains used in these experiments are available upon reasonable request to the corresponding author.

Nucleic acid extraction

Total nucleic acids from cervicovaginal swab samples were extracted with a phenol-chloroform method, which includes a bead beating process to disrupt bacteria as previously described[4,60]. Swabs were thawed on ice, transferred into a solution consisting of phenol:chloroform:isoamyl alcohol ("PCI", 25:24:1, pH 7.9, Ambion) and 20% sodium dodecyl sulfate (Fisher) in Tris-EDTA buffer with sterile 0.1mm glass beads (BioSpec), vigorously rubbed against the walls of the tube to dislodge microbial material, then incubated on ice for 5-10 minutes. Swabs were then removed, squeezing out excess fluid by pressing the swab against the side of the tube using a sterile pipette tip as it was withdrawn. A bead beater was used to homogenize samples for 2 minutes at 4°C, then centrifuged at 6,800xg for 3 minutes at 4°C. The aqueous phase was transferred to a clean tube with equal volume of PCI solution, mixed by vortexing, centrifuged again at 16,000xg for 5 minutes at 4°C, and the aqueous phase was transferred to a clean tube, precipitated using 0.8 volume of -20°C isopropanol (molecular biology grade, Sigma) with 0.08 volume (relative to initial sample) 3M sodium acetate at pH 5.5 (Life Technologies), inverted to mix, and incubated

overnight at -20°C. Samples were then centrifuged for 30 minutes at 21,100xg 4°C, then washed in 0.5 mL 100% ethanol (Decon), and centrifuged for 15 minutes at 21,100xg at 4°C. The ethanol supernatant was discarded, the pellet allowed to dry, and the sample was resuspended in 20 μL molecular-grade Tris-EDTA buffer. Genomic DNA (gDNA) from bacterial isolates or mock communities cultured *in vitro* was extracted using a plate-based adaptation of the above protocol that included a bead beating process and combined phenol-chloroform isolation with Qiagen QIAamp 96 DNA QIAcube HT kit (Qiagen) procedures.

Bacterial full-length 16S rRNA gene PCR and Sanger sequencing

Bacterial isolate identity was confirmed using near-full-length bacterial 16S rRNA gene was PCR-amplified from gDNA of individual bacterial isolates using the broad-range primers Bact-8F (5'-AGAGTTTGATCCTGGCTCAG-3') and Bact-1510R (5'-CGGTTACCTTGTTACGACTT-3'; Integrated DNA Technologies, Inc.). PCR amplicons were confirmed via agarose gel electrophoresis, then purified and directly Sanger-sequenced on an ABI3730XL DNA Analyzer at the MGH Center for Computational and Integrative Biology DNA Core using the Bact-8F and Bact-1510R PCR primers as sequencing primers in separate forward and reverse sequencing reactions. Isolates identities were determined by BLAST searches against the NCBI nucleotide collection (nt) database and Bacterial 16S Ribosomal RNA RefSeq Targeted Loci Project database (NCBI accession PRJNA33175). Isolate identities were further confirmed in most cases by bacterial whole genome sequencing as detailed below.

Shotgun library preparation

Shotgun sequencing libraries for cultured bacterial isolate genomes or culture-independent swab samples were prepared following a modified protocol of Baym et. al[73] using the Nextera DNA Library Preparation Kit (Illumina) and KAPA HiFi Library Amplification Kit (Kapa Biosystems). Briefly, DNA concentration of each sample was standardized to 0.6 ng/µL after quantification with SYBR Green I. Simultaneous fragmentation and sequencing adaptor incorporation was performed by mixing 0.6 ng DNA with 1.25 µL TD buffer and 0.25 µL TDE1 (Tagment DNA Enzyme, Nextera) and incubating for 10 min at 55°C. Tagmented DNA fragments were PCR-amplified using KAPA high fidelity library amplification reagents and primers incorporating Illumina adaptor sequences and sample barcodes. Products were pooled, purified with magnetic beads, and paired-end sequenced on an Illumina NextSeq with a 300-cycle kit.

Culture-independent bacterial 16S rRNA gene amplification and Illumina MiSeq sequencing

Bacterial microbiota composition in study participants and results of bacterial growth competition experiments were determined via Illumina-based amplicon sequencing of the V4 region of the bacterial 16S rRNA gene. The V4 region of the bacterial 16S rRNA gene was PCR-amplified following standard protocols[4,60,74]. Samples were amplified using 0.5 units of Q5 High-Fidelity DNA Polymerase (NEB) in 25 µl reaction with 1X Q5 Reaction Buffer, 0.2 mM dNTPs (Sigma), 200 pM 515F primer (5'- AATGATACGGCGACCACCGAGACGTACGTACGGTGTGCCAGCMGCCGCGGTAA-3', where the underlined sequence represents the region of complementarity to the bacterial 16S rRNA gene; IDT) and 200 pM barcoded 806R primer (5'-CAAGCAGAAGACGGCATACGAGATXXXXXXXXXXXXAGTCAGTCAGCCGGACTAC

HVGGGTWTCTAAT-3', where the underlined sequence represents the region of complementarity to the bacterial 16S rRNA gene and the X characters represent the barcode position; IDT) in PCR-clean water (Invitrogen Ultra Pure DNase/RNase-Free Distilled water). A water-template negative control reaction was performed in parallel for each barcode master mix. Additional blank extraction and amplification controls performed using unique barcoded primers were also included in sequencing libraries. DNA from clinical swab samples for FGT microbiota profiling was amplified in triplicate reactions that were then combined prior to library pooling to minimize stochastic amplification biases. DNA from *in vitro* mock community experiments (for which each experimental condition had been cultured in multiple replicates) was amplified in a single reaction per replicate culture. Amplification was performed at 98°C for 30 seconds, followed by 30 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 20 s, with a final 2 min extension at 72°C. PCR products were checked via agarose gel electrophoresis in parallel with the matching water-template control reactions to confirm successful target amplification and absence of background amplification. Gel band strength was used to semi-quantitatively estimate relative amplicon concentrations for library pooling. To prepare the sequencing libraries, 3-20 μl of individual PCR products (adjusted based on estimated relative amplicon concentration) were combined into 100 μl sub-pools and purified using an UltraClean 96 PCR Cleanup Kit (Qiagen). Blank extractions, water-template, and (for *in vitro* experiments) blank media controls were included in the sequencing libraries although they did not produce visible PCR bands. Concentration of the sub-pools were quantified using a Nanodrop (Thermo Scientific), then pooled at equal molar concentrations to assemble the final library. The pooled library was diluted and supplemented with 10% PhiX according to standard Illumina protocols, then single-end sequenced on an Illumina MiSeq using a v2 300-cycle sequencing kit with addition of custom Earth

Microbiome Project sequencing primers (Read 1 sequencing primer: 5'-ACGTACGTACGGTGTGCCAGCMGCCGCGGTAA-3'; read 2 sequencing primer: 5'-ACGTACGTACCCGGACTACHVGGGTWTCTAAT-3'; index sequencing primer: 5'- ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3'; IDT)[74].

Genome and meta-genome sequence processing and assembly

We constructed *Lactobacillus* genome catalogs from 127 reference isolate genomes, as well as from genome sequences of previously unreported bacterial strains and MAGs assembled from culture-independent WGS sequences of genital tract samples derived from the cohorts detailed above (Hayward et al, manuscript in preparation; see cohort descriptions above and details in Supplementary Tables 4-11). Briefly, we retrieved all reference genomes annotated as *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus jensenii*, *Lactobacillus gasseri*, or *Lactobacillus vaginalis* that were deposited in the NCBI RefSeq database[35] as of February 2020 (Supplementary Table 7). Genomes of previously unreported FGT strains from the South African FRESH cohort (isolated as described above) and of non-*iners Lactobacillus* strains isolated from the US-based VHP and V2 cohorts were sequenced on an Illumina NextSeq at the Ragon Institute as described above. Additional US FGT *Lactobacillus* isolate genomes were provided by the VMRC from the cohorts detailed above. FGT WGS metagenomic samples from cohorts in South Africa, the US, Italy, and China (see above) were used to generate MAGS. Sequence reads from isolate genomes and WGS samples were trimmed and filtered to high quality reads. Human reads from WGS samples were removed by mapping to GRCh38 (Genbank accession GCA_000001405.15). We assembled genomes and MAGs from high quality reads, then binned contigs and removed contamination. Bin completeness, contamination, and strain heterogeneity

metrics were determined, then genomes and MAGs were assigned quality scores based on criteria from the Genome Standards Consortium[75]. Species genome bins (SGBs) were determined based on 95% pairwise absolute nucleotide identity (ANI).

Lactobacillus genome taxonomy assignment

We assigned taxonomy to SGBs with FGT *Lactobacillus* genomes based on presence of reference genomes with defined NCBI taxonomy, confirming that each contained reference genomes only from a single species. Of note, for both *L. jensenii* and *L. gasseri*, publicly available reference genomes classified in RefSeq as belonging to these species actually segregate into two separate SGBs per species, indicating that *L. jensenii* and *L. gasseri* (as traditionally defined) each comprise two distinct genomic species (Hayward et al, manuscript in preparation)[76]. However we analyzed *L. jensenii* and *L. gasseri* as single species units in this study to correspond with prevailing paradigms in literature on 16S rRNA gene-based FGT microbiota profiling[77].

Cervicovaginal *Lactobacillus* pan-genome construction and analysis

We constructed a cervicovaginal *Lactobacillus* pan-genome using genes from catalogs of *L. iners*, *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. vaginalis* genomes and MAGs. To maximize comprehensiveness of the pan-genome, we included genomes and MAGs classified as high-quality (>90% completeness and <5% contamination) or medium-quality (≥50% completeness and <10% contamination) assemblies based on Genome Standards Consortium criteria[75]. Some of the medium-quality assemblies are classified as "partial" by NCBI standards based on size criteria. Genes were identified within individual genomes by Prokka v1.14.5[78], which predicts genes using Prodigal v2.6.3[79]. The resulting genes were clustered into a comprehensive pan-genome at 95%

nucleotide identity using Roary v3.13.0[80] to generate a multi-fasta file containing gene sequences and a per-genome gene presence-absence table[81]. The pan-genome was annotated with eggNOG 5.0 using eggNOG-mapper v2[82,83]. We used custom R scripts to parse the eggNOG output for genes predicted to encode enzymatic or transporter activities of interest based on gene names, KEGG numbers, KEGG reaction numbers, Clusters of Orthologous Groups (COGs), EC numbers, Transporter Classification (TC) numbers, and GO (Gene Ontology) terms, followed by manual curation of the initial search results and BLAST-based sequence review for genes with unclear annotations[81]. We then determined the number of genomes from each species predicted to encode each gene function of interest. Since the Genome Standards Consortium criteria for high- and medium-quality genomes and MAGs allow for low-level sequence contamination within assemblies[75], we restricted gene presence-absence analysis to gene sequences of interest that were detected in at least two genomes or MAGs from a species in order to exclude singleton contaminant sequences from our analysis. Importantly, including partial genomes and MAGs with completeness as low as 50% maximizes genome catalog diversity, thus increasing pan-genome size and sensitivity for detecting genes of interest within each species, but also results in a fraction of genomes and MAGs appearing to lack universally present genes due to incompleteness of assemblies. The absence of intact Cys synthase and reverse transsulfuration pathways in these species determined by EggNOG-based analysis (Figure 2b) was further supported by results of BLAST[84] searches against the genome collections using gene sequences of interest from related species (see custom code posted on Zenodo[81]).

Maximum likelihood phylogenetic distances and phylogenetic reconstruction

Phylogenetic reconstruction of *L. iners* genomes and MAGs (Fig. 1b) was performed using assemblies with >60% completeness and <5% contamination to maximize robustness of the analysis. fetchMG v1.0[85] was used to extract DNA sequences for each of forty single-copy universal bacterial marker genes from each genome (called using Prodigal[79]). Genome assemblies containing fewer than 10 of the 40 universal marker genes were omitted from the subsequent alignment. A phylogenetic reconstruction was then produced using ETE3 v3.1.1 (parameters: "*ete3 build -w clustalo_default-trimal-gappyout-none-none -m cog_85-alg_concat_default-fasttree_default*")[86], and the tree was visualized using iTOL v4[87]. An additional phylogenetic reconstruction encompassing other major FGT *Lactobacillus* species (Extended Data Fig. 2a) was performed using *L. iners* genomes and MAGs as well as isolate genomes from *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. vaginalis*, filtered based on the same quality and completeness criteria.

Preparation of bacterial inocula for growth experiments

For initial experiments identifying cysteine and cystine as key nutrient requirements for *L. iners* growth (Fig. 1a,c, Fig 4a, and Extended Data Fig. 1), bacteria from frozen stocks were plated on solid media, incubated for 3 days, then suspended in sterile, pre-reduced Dulbecco's phosphate-buffered saline (PBS), adjusted to an OD600 of 0.3+/-0.05, and then inoculated into the indicated broth media formulations for measurement of growth kinetics. In subsequent growth experiments for *Lactobacillus* species, experimental bacterial inocula were prepared from liquid starter cultures in MRSQ broth with L-Cys (4 mM) that were incubated for 18-20 hours. The starter cultures were then pelleted by centrifugation for 10 min at 3716 x g, spent media was decanted, and bacteria were washed 2 times by re-resuspending in sterile, pre-reduced PBS followed by centrifugation to avoid carryover of nutrients from the original starter culture media. Washed bacteria were

resuspended in PBS, adjusted to OD600 0.3 (± 0.05), then inoculated into experimental media at 3.5% (v/v), equating to bacterial titers ranging from ~$1x10^5$ to $1x10^6$ colony-forming units (C.F.U.) per mL, depending on the experimental species and strain.

Growth kinetics quantification

We found that *Lactobacillus* growth kinetics in broth media were adversely affected by either continuous or intermittent agitation, therefore we grew broth cultures without agitation. Mono-culture growth kinetics were assessed using separate cultures prepared in parallel for each experimental timepoint to avoid repeatedly agitating a single culture by performing serial measurements. For timepoint series lasting ≤48 hours, broth cultures were grown in a volume of 250 μL in technical triplicate in clear 96-well flat bottom plates (Falcon), with a blank for each media condition per plate. Measurements were taken at ~16, 20, 24, 28, ~42, and 48 hours by removing plates from the anaerobic chamber, pipetting to re-suspend bacteria, and measuring OD600 on a Tecan Infinite® M1000 PRO plate reader. Due to slower growth kinetics in the HMRS broth formulation, *L. iners* growth measurements in HMRS were taken daily for up to 10 days, precluding use of 96-well plates due to evaporation at later timepoints. Bacteria were therefore incubated in low-evaporation 1.2 mL 96-well cluster tubes (Corning™) in triplicate, with separate parallel culture plates used for each experimental timepoint, then 250 μL from each culture was transferred to a clear 96-well flat-bottom Falcon plate for optical density measurement. Data from mono-culture growth experiments are depicted as median ± range for the three replicates. For each bacterial strain and media condition, figures depict representative results from 1 of ≥2 independent experiments with distinct batches of freshly prepared media and freshly prepared bacterial input

inocula unless otherwise indicated. Growth data collection and analysis were not performed blind to the conditions of the experiments.

Inhibitor experiments and analysis

To test mono-culture growth inhibition, bacteria were cultured in media containing inhibitors at varying concentrations as indicated, including a reference (no-inhibitor) control. Since growth kinetics differed between species and strains, inhibition was determined for each strain at the first timepoint fulfilling the European Commission on Antimicrobial Susceptibility Testing (EUCAST) criterion of "definite turbidity" in the reference control (which we defined experimentally as OD600 >0.2), unless otherwise specified. At the selected timepoint for each strain, the median OD600 value for the reference control was set to a reference value of 100%, which was used to calculate percentage growth in the other conditions. Growth experiments employing metronidazole (MTZ) used a concentration of 50 μg/mL, approximating the concentration in 0.75% intravaginal MTZ gel (a first-line treatment for BV)[25]. Data from inhibition experiments are depicted as median ± range for three technical replicates. For each bacterial strain and media condition, figures depict representative results from 1 of ≥2 independent experiments with distinct batches of freshly prepared media and freshly prepared bacterial input inocula, unless otherwise indicated. Inhibition data collection and analysis were not performed blind to the conditions of the experiments.

Competition and mock community culture experiments

For pairwise competition between *L. iners* and *L. crispatus* strains in MRS broth ± SMC and for mock BV-like community experiments with *L. iners* (multiple strains), *L. crispatus* (multiple

strains), *G. vaginalis* (ATCC 14018), *P. bivia* (ATCC 29303), and *A. vaginae* (0795_578_1_1_BHK4; see Supplementary Table 3) in S-broth ± SMC and/or MTZ, bacteria were initially prepared in individual suspensions in broth as described above for bacterial monoculture growth and inhibition experiments. Aliquots of the mono-bacterial suspensions were mixed in defined ratios, then mixtures were divided into replicate cultures for incubation (5 replicates per conditions for MRSQ experiments; 6 replicates per condition for S-broth experiments) by adding 150 µL of mixture per well into V-bottom 96-well plates (Falcon). C.F.U. titers were determined for each input mono-bacterial suspension as described above and used to calculate starting ratios within the mixed cultures. At 28 hours, cultures were harvested by centrifuging at 4700xg for 25 minutes at 4C, spent media was decanted, and pellets were frozen for later DNA extraction and analysis. Relative growth within mixed cultures was assessed by bacterial 16S rRNA gene sequencing as described below. Aliquots of mono-bacterial suspensions in the corresponding media type were cultured separately to confirm expected growth patterns (e.g., Fig. 5b and Extended Data Fig. 7a).

Processing of Illumina MiSeq 16S rRNA gene sequences

Demultiplexing of Illumina MiSeq bacterial 16S rRNA gene sequence data was performed using QIIME 1 version 1.9.1[88]. Mapping files in standard QIIME 1 format were created and validated using validate_mapping_file.py, sequences were demultiplexed using split_libraries_fastq.py with parameter store_demultiplexed_fastq and no quality filtering or trimming, and demultiplexed sequences were organized into individual fastq files using split_sequence_file_on_sample_ids.py[60]. We then used dada2 version 1.6.0[89] in R used to filter and trim reads at positions 10 (left) and 230 (right) using the filterAndTrim function with

parameters truncQ = 11, MaxEE = 2, and MaxN = 0. Sequences were then inferred and initial taxonomy assigned using the dada2 assignTaxonomy function employing the RDP training database rdp_train_set_16.fa.gz (https://www.mothur.org/wiki/RDP_reference_files). Taxonomic assignments were refined and extended via extensive manual review; see Supplementary Table 15 for amplicon sequence variant (ASV) taxonomy. The denoised dada2 results with final taxonomic assignment were analyzed in R using phyloseq version 1.30.0[90] and custom R scripts. Initial sequencing-based analysis of FGT microbiota composition of FRESH cohort participants, taxonomy assignment, and cervicotype assignments were performed blinded to participants' corresponding Nugent scores and metabolite concentrations in CVL fluid.

16S rRNA gene-based microbiome analysis

For 16S rRNA gene-based microbiome profiling of clinical specimens, microbial communities were classified into four cervicotypes (CTs) as previously defined in a non-overlapping subset of participants from the FRESH cohort: CT1 includes communities with >50% relative abundance of non-*iners Lactobacillus* species (which consists almost entirely of *L. crispatus* in this population[4]); CT2 consists of communities in which *L. iners* is the most dominant taxon; CT3 consists of communities in which the genus *Gardnerella* is the most dominant taxon; and CT4 consists of communities dominated by other species, typically featuring high abundance of one or more *Prevotella* species[1]. Although *L. jensenii* and *L. gasseri* were detected in microbial communities of some FRESH study participants by both sequencing and cultivation, they were not dominant in any individuals, a common finding among studies of sub-Saharan African populations[1,4,6,50] that contrasts with observations in most North American and European cohorts, where a consistent minority of women tend to have *L. gasseri*-dominant or *L. jensenii*-dominant communities[12,49,59,77].

For further sequence processing and analysis, amplicon sequence variants (ASVs) that could not be defined at least to the level of taxonomic class were pruned from the dataset and one sample was excluded due to sequence read count <10,000. The remaining 142 samples were rarefied without replacement to a uniform depth of 16,603 reads (the minimum read count among remaining samples), yielding a relative abundance limit of detection (L.D.) of 6.02×10^{-5}. ASVs were collapsed at the species or genus level as indicated for further visualization and statistical analysis. In plots displaying taxon relative abundance using a logarithmic scale, a pseudocount equal to 0.5 x L.D. was added to assist visualization of any taxon with read count 0. Correlation analysis between bacterial relative abundances and cervicovaginal metabolite concentrations was restricted to taxa detected (≥1 sequence read after rarefaction) in ≥50% of samples (Extended Data Fig. 3c,d).

Sequence analysis for *in vitro* competition experiments and mock communities

For pairwise competitions between *L. crispatus* and *L. iners*, 16S rRNA gene sequences were generated, processed, and annotated as described above. Ratios of *L. iners* reads to *L. crispatus* reads in each sample were calculated. Significance of between-group differences for each mixture was determined by 1-way ANOVA. Significance of pairwise differences between the positive control condition (MRSQ + L-Cys without inhibitor) and each experimental condition was calculated using Dunnett's test and all significant pairwise comparisons were plotted (see Supplementary Table 18 for numeric p-values). Results of representative competition experiments are shown in Fig. 5a.

For mock BV-like community experiments, 16S rRNA gene sequences were generated, processed, and annotated as described above. Relative abundances of each experimental strain

were determined for individual replicates and displayed in Fig. 5d. To assess *L. crispatus* enrichment, read counts of all experimental strains except *L. crispatus* were summed for each replicate sample, then ratios of *L. crispatus* to the summed taxa were calculated. Significance of between-group differences for each mixture was determined by 1-way ANOVA, and significance of pairwise comparisons were calculated using Tukey's test, with significance of selected pairwise comparisons plotted in Fig. 5e and shown in detail in Supplementary Table 20. Competition and mock community assay data collection and analysis were not performed blind to the conditions of the experiments.

Measurement and analysis of metabolites in cervicovaginal lavage fluid

Concentrations of Cys, reduced glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine), and cysteinylglycine (Cys-Gly) were measured in CVL supernatants from the 143 FRESH cohort participants whose microbiome composition was concurrently profiled by 16S rRNA gene sequencing in this study. CVL supernatant samples underwent a single freeze-thaw cycle during preparation of aliquots for metabolite analysis. Metabolites of interest were quantified using ultraperformance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) by Metabolon, Inc., as part of an untargeted metabolomics analysis. To avoid bias in assay performance, measurements were performed after randomized reordering of samples by laboratory staff at Metabolon, Inc., who were blinded to all participant information except sample ID. Metabolon's method generates relative instead of absolute metabolite concentrations. For analysis, concentrations were volume-normalized and median values were adjusted to 1, then percent missingness (reflecting samples with analyte concentrations below the limit of detection, or L.D.) was calculated for each analyte. For analytes with missingness >0%, analyte limit of detection was

inferred as equaling the lowest measured relative concentration in the cohort, then missing values were imputed at half the limit of detection. The Shapiro-Wilk test of normality was then performed on relative concentration values after log-transformation. In subsequent analysis of concentration differences between cervicotypes, differences were analyzed by 1-way ANOVA with post-hoc Tukey test for Cys (which had 0% missingness and was normally distributed, Fig. **3a,c**) and by Kruskal-Wallis test with post-hoc Dunn's test for GSH and Cys-Gly (which had 21.7% and 7.0% missingness respectively and were therefore not normally distributed after imputation, Extended Data Fig. 4**a-d**). We calculated Spearman rank-order correlations between individual metabolites and bacterial taxa at both the genus and species levels, adjusting p-values for multiple comparisons where indicated using the Bonferroni method via the R stats package function p.adjust(). The R package DescTools was used to calculate confidence intervals for the Spearman correlation coefficients (ρ), including Bonferroni-corrected confidence levels at (1 - 0.05/n), where n represents the number of taxa (Fig. 3e,f, Extended Data Fig. 4e,f, and Supplementary Tables 16 & 17).

Quantification of oxidized and reduced cysteine and glutathione in MRS

Oxidized and reduced glutathione and cysteine were quantified by UPLC-MS/MS (Waters Acquity/TQ-S), according to a modification of the protocol by Sutton et al[91]. Briefly, 100 µL anaerobic broth was mixed with 90 µL buffer (100 mM ammonium bicarbonate, pH 7.4), followed by 10 µL fresh *N*-ethylmaleimide solution (25 mg/mL in ethanol), and allowed to react at room temperature for 10 minutes. Samples were then diluted 100-fold in water before analysis by UPLC-MS/MS, with a 1 µL injection volume. Separation was carried out with an Acquity BEH/C18 UPLC column (Waters 186002350), with the following gradient: 0 minutes, 2% B; 6 minutes, 98%

B; 7 minutes, 98% B; 7.1 minutes, 2% B; 9 minutes, 2% B. Mobile phase A was water with 0.1% formic acid, and B was acetonitrile with 0.1% formic acid. Compounds were quantified by comparison of peak area to authentic standards processed via the same method.

Synthesis and analysis of isotopically labeled cystine, GSSG and GSH

^{13}C-labeled cystine (1,1'-$^{13}C_2$-L-cystine) was prepared from 1-^{13}C-L-cysteine (Cambridge Isotope Labs, CLM-3852-0.5) via a modification of the procedure of Hill, Coy, and Lewandowski[92]. Briefly, 100 mg of labeled cysteine was mixed with 200 µL 10% NaOH, and 800 µL 3% hydrogen peroxide. The resulting precipitate was filtered and washed extensively with ice cold water, then finally redissolved in 1 M aqueous HCl. Complete oxidation was confirmed by UPLC-MS/MS analysis of the resulting solution, as described above.

Labeled glutathione-(1-^{13}C-cysteine) was prepared enzymatically using *E. coli* GshA and GshB overexpressed and purified as N-terminal 6xHis fusions. *gshA* and *gshB* were amplified from genomic DNA of *E. coli* BL21(DE3) using Q5 polymerase (New England Biolabs) and the following primers (purchased from Millipore-Sigma): HIS-gshA-F, gcctggtgccgcgcggcagcATCCCGGACGTATCACAG; HIS-gshA-R, cagcttcctttcgggctttgTCAGGCGTGTTTTTCCAGCC; HIS-gshB-F gcctggtgccgcgcggcagcATCAAGCTCGGCATCGTGAT; and HIS-gshB-R, cagcttcctttcgggctttgTTACTGCTGCTGTAAACGTGC, according to the manufacturer's instructions. The expression vector backbone pET28 was amplified from purified stock using primers: pET28-F, GCTGCCGCGCGGCACCAG; and pET28-R, CAAAGCCCGAAAGGAAGCTG. After DpnI digest and purification, expression plasmids pETgshA and pETgshB were assembled using HiFi Assembly MasterMix (NEB) and transformed

into chemically competent *E. coli* DH5a. Positive colonies were verified via Sanger sequencing, and correctly assembled plasmids were transformed into *E. coli* BL21(DE3). Cells for protein expression were inoculated from overnight culture with 100-fold dilution into 4 L flasks containing 1 L LB broth, and grown at 37°C and 200 RPM until reaching OD600 ~ 0.5, after which they were induced with 0.5 mM IPTG, and grown for a further 3 hours at 37 ºC. Pellets were harvested by centrifugation at 6,000 g for 10 minutes, then resuspended in ice cold 98% Buffer A (50 mM HEPES, 300 mM KCl, 10% glycerol, pH 7.5) and 2% Buffer B (Buffer A with 500 mM imidazole). Cells were lysed by three passages through an Emulsiflex C5 (Avestin) at 15,000 psi, and lysates were clarified by centrifugation at 20,000 g, 4°C for 30 minutes. Target proteins were bound to 1 mL Ni-NTA His-Bind resin (Millipore Sigma) in gravity columns, then washed with 5 mL wash buffer (90% Buffer A, 10% Buffer B) and eluted with 3 mL elution buffer (100% Buffer B). Fractions containing purified proteins were identified via SDS-PAGE, then combined and dialyzed overnight against Buffer A with 3.5k MWCO Slide-A-Lyzer Cassettes (ThermoFisher), then concentrated by centrifugation through 3k MWCO Microsep spin filters (Pall).

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine) synthesis was carried out in 100 mM sodium phosphate buffer (pH 7.2), with 80 mM 1-^{13}C-L-cysteine, 120 mM glycine, 120 mM glutamic acid, 40 mM $MgCl_2$, and 100 mM ATP, using 50 mg 1-^{13}C-L-cysteine, 1 mg each of GshA and GshB, incubating overnight at 37°C. The resulting glutathione-(1-^{13}C-cysteine) was treated with 3% hydrogen peroxide to produce the oxidized form of glutathione (GSSG) for purification via preparative HPLC on a Thermo Scientific Dionex UltiMate 3000 HPLC system with a Thermo Scientific Hypersil GOLD aQ C18 preparative column (20 x 20 mm, 5 µm) using a gradient from 100% A (water + 0.1% formic acid) to 70% B (acetonitrile + 0.1% formic acid). Fractions containing labeled GSSG ($^{13}C_2$-GSSG) were pooled and lyophilized. Analysis of the

purified product was conducted by ^{1}H NMR (400 MHz) in the Magnetic Resonance Laboratory in the Harvard University Department of Chemistry and Chemical Biology on a Jeol J-400, as well as by LC-MS/MS as described above, and by high-resolution LC-MS using an Agilent 6530 Q-TOF (confirming expected product characteristics as detailed in HMDB: https://doi.org/10.13018/BMSE000906). This analysis confirmed the authenticity of the desired product and complete conversion of cysteine, and also revealed contamination with ADP, which was not removed by preparative HPLC (Supplementary Fig. 1). This material was used without further purification. Reduced labeled glutathione (^{13}C-GSH, 4 mM) for use in experiment measuring GSH uptake was produced from the oxidized form ($^{13}C_2$-GSSG) by treating a stock solution of 81.6 mM labeled GSSG with TCEP at a 0.9:1 molar ratio to reduce the disulfide bonds prior to addition to MRSQ broth.

Quantification and isotopic analysis of amino acids

For isotopic analysis of proteinogenic amino acids, *L. crispatus* (strain 233) and *L. iners* (strain F1) grown in MRS/Q broth supplemented with isotopically labeled or unlabeled substrates were harvested via centrifugation. The pellets washed 3 times with water and resuspended in 0.6 mL of 6 N aqueous HCl and heated at 100°C overnight to facilitate cell lysis, protein hydrolysis, and Cys oxidation. The hydrolysate was dried under air, then resuspended in 100 µL of 90% acetonitrile, 10% water. For determination of amino acid concentration in media, samples were prepared by diluting media 10-100-fold in 90:10 acetonitrile:water, followed by centrifugation to remove precipitated material. All amino acid samples were analyzed via UPLC-MS/MS using instrumentation described above. Separation was carried out with an Acquity BEH/Amide UPLC column (Waters 186004800) in HILIC mode, with the following gradient: 0 minutes, 90% B; 0.3

minutes, 73% B; 1 minute, 73% B; 1.5 minutes, 30% B; 2 minutes, 30% B; 2.2 minutes, 90% B; 4 minutes, 90% B. Mobile phase A was water with 0.1% formic acid, and B was acetonitrile with 0.1% formic acid. Quantification of amino acids was confirmed by comparison of peak area to authentic standards (Sigma # AAS18-5ML) processed via the same method. Isotopic distribution data were corrected for the natural abundance of ^{13}C using IsoCor v2.1.3[93] (Supplementary Tables 13 & 14). Isotopic assay data collection and analysis were not performed blind to the conditions of the experiments.

Statistics, software, and visualization

Data analysis, statistics, and visualization were performed in R v3.6.3, except where otherwise indicated, using packages including seqinr v4.2.5, tidyverse, v1.3.1, knitr v1.33, ggpubr v0.4.0, DescTools v0.99.41, gtools v3.8.2, gridExtra v2.3, cowplot v1.1.1, scales v1.1.1, grid v3.6.3, broom v0.7.6, e1071 v1.7.6, and table1 v1.4. All p-values are two-sided with statistical significance defined at $\alpha = 0.05$ unless otherwise indicated.

Data availability

Compressed directories containing data files sufficient to reproduce (1) analysis of pan-genome composition and gene content, (2) analysis for human FGT microbiota-metabolite analysis, and (3) analysis for competition cultures of *L. iners* and *L. crispatus* and mixed community cultures are posted at zenodo.org under DOI https://zenodo.org/record/5900469[81]. The dataset containing the raw Illumina MiSeq read data for genital tract bacterial 16S rRNA gene profiling analyzed in this study (Figure 3 and Extended Data Figures 3 & 4) is available in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA729907. The dataset containing the raw Illumina MiSeq

read data for the bacterial 16S rRNA gene sequences from competition cultures of *L. iners* and *L. crispatus* (Fig. 5a) and from mixed community cultures (Figs 5c&d) is available in the NCBI SRA under BioProject PRJNA777644. The taxonomic assignments used for amplicon sequence variants (ASVs) from bacterial 16S rRNA gene sequencing are supplied in Supplementary Table 15. The *Lactobacillus* genomic catalogs included a total of 1,091 previously unreported isolate genomes, partial genomes, and MAGs from multiple human cohorts, as detailed above. The assemblies are available in the NCBI SRA under BioProjects PRJNA799384, PRJNA799634, PRJNA799626, PRJNA799445, PRJNA799630, PRJNA799633, PRJNA799642, PRJNA799746, PRJNA799744, PRJNA799737, PRJNA799762, and PRJNA797778; additional details on the individual studies associated with these BioProjects are contained in Supplementary Tables 5, 6, and 10, and individual NCBI BioSample accession numbers for each of the 1,091 assemblies are listed in Supplementary Table 8. In addition, the genome catalogs included 127 previously reported isolate genomes that were retrieved from RefSeq; the individual accession numbers for these genomes are listed in Supplementary Table 7. The raw and corrected cystine and serine isotopologue measurements associated with Fig 2c,d and 4c are available in Supplementary Tables 13 and 14. Some metadata related to previously reported isolate genomes was obtained from corresponding entries in RefSeq (https://www.ncbi.nlm.nih.gov/refseq/) or the Genomes OnLine Database (GOLD; https://gold.jgi.doe.gov/). Source data are supplied for plots and phylogenetic trees, including for Figures 1-5 and Extended Data Figures 1-8.

Code availability

Compressed directories containing R analysis code sufficient to reproduce (1) analysis of pan-genome composition and gene content, (2) analysis for human FGT microbiota-metabolite

analysis, and (3) analysis for competition cultures of *L. iners* and *L. crispatus* and mixed community cultures is available at zenodo.org under DOI https://zenodo.org/record/5900469[81]. Each compressed directory contains a README file describing dependencies and other details, an R Project file, an R Markdown file containing the analysis code with additional information, and associated sub-directories used in the analysis.

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Author Contributions

S.M.B. and D.S.K. conceived the overall study and guided it throughout with input from B.M.W., E.P.B., and C.M.M.; S.M.B., N.A.M., and J.K.R. performed primary bacterial isolations; S.M.B., N.A.M., J.F.F., B.M.W., A.J.M., X.W., N.C., and C.M.M. contributed to media design and production and/or bacterial growth and inhibition experiments; B.M.W. and E.P.B. synthesized

labeled glutathione; B.M.W., S.M.B., N.A.M., and E.P.B. designed, performed, and/or analyzed measurements of media composition and isotopic tracing experiments; S.M.B., N.A.M., and J.X. performed nucleic acid extractions and sequencing; S.M.B. performed bacterial 16S rRNA gene sequencing analysis; M.R.H. and D.A.R. performed bacterial isolate genomic and metagenomic sequence analysis and assembly, genome catalog development, and phylogenetic reconstructions; S.M.B., M.R.H., F.A.H., and B.M.W. conceived and/or performed genomic pathway analysis; S.M.B. and A.B.A. performed analysis of *in vivo* metabolite data; K.L.D., M.D., T.G., F.X.C., T.N., N.I., S.M.B., N.X., M.S.G., and D.S.K. contributed to clinical cohort design, cohort performance, and/or sample acquisition and processing efforts; S.M.B., B.M.W., M.R.H., N.A.M., and D.S.K. wrote the paper, and all authors reviewed, offered input to the writing, and approved the manuscript.

Ethics declaration

Competing interests

All authors declare no competing interests.

Fig. 1. *Lactobacillus iners* requires L-cysteine supplementation to grow in conditions that support other vaginal lactobacilli. a, Growth of *Lactobacillus crispatus*, *L. iners* (strain F1), and *Gardnerella vaginalis* in *Lactobacillus* MRS broth ± supplementation with 2% IsoVitaleX™. **b**, Unrooted phylogenetic tree of 198 *L. iners* genomes from previously unreported and reference bacterial isolates and previously undescribed culture-independent metagenome-assembled genomes (MAGs) derived from geographically diverse populations. Red dots indicate isolates experimentally studied in this work. Genome type, geographic origin, and BV status of the source samples are shown. The tree depicts only genome assemblies exceeding certain quality thresholds to ensure robustness of the phylogenetic reconstruction (see **Methods**); additional strains and genomes were included in other analyses (**Supplementary Tables 3-11** and **Extended Data Fig. 2**). **c**, Growth of 8 representative US (prefix "A") or South African (prefix "F") *L. iners* strains cultured in MRS broth supplemented as indicated with IsoVitaleX™ (2%), L-cysteine (L-Cys, 4 mM), and/or L-glutamine (L-Gln, 1.1 mM). In **1a,c** growth was assessed by optical density at 600 nm (OD600) and plotted as median (± range) for 3 replicates from 1 of ≥2 independent experiments per strain and media condition.

Fig. 2. Vaginal lactobacilli lack canonical Cys biosynthesis pathways. a, Canonical bacterial Cys biosynthesis pathways. Cysteine synthase pathway: serine *O*-acetyltransferase (encoded by *cysE*), then cysteine synthase (*cysK*, *cysM*, or *cysO*). Reverse transsulfuration pathway: cystathionine β-synthase (*cbs*) or *O*-acetylserine-dependent cystathionine β-synthase (*mccA*), then cystathionine γ-lyase (*mccB*). Asterisks indicate position of ^{13}C label in isotopic tracing experiments. **b,** Predicted presence of genes encoding cysteine biosynthesis enzymes within the cysteine synthase or reverse transsulfuration pathways in isolate genomes and MAGs of the

indicated FGT *Lactobacillus* species (n = number of genomes; detailed results in Supplementary Table 12). Assessment of gene presence was based on annotations from eggNOG 5.0 performed using eggNOG-mapper v2[82,83]. Results were supported by BLAST[84] searches of the genomes for sequences of interest[81]. **c,** Serine isotopic enrichment in *L. crispatus* and *L. iners* grown for 24 hours in L-Gln-supplemented MRS broth ("MRSQ") containing no isotopic tracer (supplemented with unlabeled L-Cys 4 mM) or supplemented with 1-^{13}C-L-serine (^{13}C-L-Ser) + homocysteine (both 4 mM). Plot depicts fractional abundance of unlabeled (m+0, Ser) and labeled (m+1, ^{13}C-Ser) isotopologues in cellular hydrolysates. **d,** Cystine isotopic enrichment in lysates of *L. crispatus* or *L. iners* cultured as shown in **(c)** or in broth supplemented with 1-^{13}C-L-cysteine (^{13}C-L-Cys; 4 mM). An oxidation step during sample processing converts Cys to cystine for quantification. Plot depicts fractional abundance of cystine isotopologues: unlabeled (m+0; cystine), partially labeled (m+1; $^{13}C_1$-L-cystine), and fully labeled (m+2; $^{13}C_2$-L-cystine) isotopologues. Data in **2c**,**d** depict median values of 3 replicates per group and were un-replicated (full data in Supplementary Tables 13 and 14).

Fig. 3. Vaginal Cys concentrations are higher in women without BV and correlate with *Lactobacillus*-dominant microbiota. a, Relative Cys concentration by BV status in cervicovaginal lavage (CVL) fluid from 53 South African women (21 without BV, 24 with BV, and 8 intermediate by Nugent method[38]). Differences in log-transformed concentrations determined by one-way analysis of variance (ANOVA) with post-hoc Tukey's test; all significant pairwise differences at confidence level 0.95 are shown (No BV-BV: $P < 1 \times 10^{-7}$; Intermediate-BV: P = 0.0060). **b,** FGT bacterial microbiota composition among 142 HIV-uninfected South African women (including the 53 from **a**), determined by bacterial 16S rRNA gene sequencing

(taxonomy assignments in Supplementary Table 15). Bacterial communities were classified into "cervicotypes" (CTs) using previously defined criteria[1]. **c,** Relative Cys concentrations per CT in CVL fluid from women in **(b)**. Significance determined by one-way ANOVA with post-hoc Tukey's test; all significant pairwise differences are shown. CT-CT3: P = 0.000120; CT1-CT4: P < 1 x 10^{-7}; CT2-CT3: P = 0,00898; CT2-CT4: P < 1 x 10^{-7}; CT-CT4: P = 0.0279. In **a,c**, box center lines, edges, and whiskers signify median, interquartile range (IQR), minima and maxima, respectively. **d,** Two-tailed Spearman rank correlation between Cys concentrations and bacterial relative abundances of the genera *Lactobacillus*, *Gardnerella*, and *Prevotella*, showing correlation coefficients (ρ) with unadjusted p-values. Linear regression lines (solid blue) with 95% confidence intervals calculated from log-transformed abundances and concentrations are shown to assist visualization (*Lactobacillus*: y = 0.37 + 0.32x; *Gardnerella*: y = -0.25 – 0.15x; *Prevotella*: y = -0.47 – 0.27x). Red dotted line represents limit of detection (L.D.). **e,f** Two-tailed Spearman correlation coefficients (ρ) with adjusted confidence intervals between Cys concentrations and relative abundances of each genus **(e)** or species **(f)** detected at >50% prevalence in cohort (n = 142). P-values and confidence intervals in **e,**f were adjusted for multiple comparisons using the Bonferroni method at significance level 0.05/n (full statistical results in Supplementary Tables 16 & 17). Significance of adjusted P-values depicted as * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

Fig. 4. *L. iners* lacks Cys-related transport mechanisms present in other lactobacilli and is selectively inhibited by cystine uptake inhibitors. a, Growth of *L. crispatus*, *G. vaginalis*, and representative *L. iners* strains in MRSQ broth ± L-Cys (4 mM) or L-cystine (2 mM). **b,** Predicted presence of putative Cys transport-related gene *cjaA*, cystine transport loci *tcyABC*, *tcyJKLMN*,

and *tcyP*, and the Cys/GSH transport/redox homeostasis locus *cydABCD* locus in FGT *Lactobacillus* species genomes and MAGs (n = number of genomes; detailed results in Supplementary Table 12). TcyBC takes up glutathione in some species. **c,** Cystine isotopic enrichment in *L. crispatus* or *L. iners* grown in MRSQ broth containing labeled L-cystine ($^{13}C_2$-L-cystine; 2 mM) or GSH (^{13}C-GSH; 4 mM; synthesis described in Supplementary Fig. 1). Plot depicts isotopologue median fractional abundance and was un-replicated. (Full data in Supplementary Tables 13 & 14). **d,** Growth of representative *Lactobacillus* strains in L-Cys-supplemented MRSQ broth containing the cystine uptake inhibitor *S*-methyl-L-cysteine (SMC). Percentages calculated relative to median OD600 measurement in L-Cys-containing no-inhibitor control. **e,** Median growth inhibition of non-*iners Lactobacillus* strains (*L. crispatus*, n = 7; *L. jensenii*, n = 2; *L. gasseri*, n = 1) or *L. iners* (n = 16) by SMC in L-Cys-supplemented MRSQ broth. Significance of differences between *L. iners* and non-*iners* strains was determined by two-sided t-test. Box center lines, edges, and whiskers signify the median, IQR, minima and maxima respectively. **f,** Growth inhibition of *L. crispatus* and *L. iners* by SMC (128 mM) or SDLC (2 mM) in NYCIII broth. Plots in **a,d,f** depict median (± range) for 3 replicates per condition and are representative of ≥2 independent experiments per condition.

Fig. 5. SMC inhibits *L. iners* in competition with *L. crispatus* and combining SMC with metronidazole enhances *L. crispatus* dominance of mock BV-like communities. a, Ratios of *L. iners* to *L. crispatus* in representative mixed culture competition assays after 28 hours incubation in L-Cys-supplemented MRSQ broth with varying concentrations of SMC. Ratios were determined via sequencing the bacterial 16S rRNA gene in DNA isolated from the mixed cultures; plots depict results for 5 replicates per condition. Between-group differences were determined by

one-way ANOVA. Pairwise comparisons to the reference condition ("Ref": no-inhibitor, L-Cys-supplemented control) were calculated using Dunnett's test. All significant comparisons are shown (full statistical results in Supplementary Table 18). Input ratios of colony-forming units (C.F.U.) of *L. iners* to *L. crispatus* were 8.01:1, 1.08:1, and 5.96:1 for the mixes containing *L. iners* strains A4, F1, and F3 respectively. **b,** Growth inhibition of *L. crispatus*, *L. iners*, and three BV-associated species at 48 hours incubation in S-broth with or without SMC and/or metronidazole (MTZ). Plots depict median (± range) for 3 replicates per condition and are representative of 2 independent experiments per condition. **c,** Relative abundance of bacterial species in three representative mock BV-like communities (each with a different *L. iners* strain) grown in S-broth with or without SMC and/or MTZ at 28 hours incubation. Composition was determined by bacterial 16S rRNA gene sequencing. Input ratios of strains for each community are shown in Supplementary Table 19. **d,** Ratios of *L. crispatus* to the sum of all other taxa in the mock communities shown in **(c)**. **(c,d)** depict 6 replicates per condition. Between-group differences in **(d)** were determined via one-way ANOVA with post-hoc Tukey test; selected pairwise differences are shown (full statistical results in Supplementary Table 20). In 5a,e significance is depicted as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. **(b,d)** Box center lines, edges, and whiskers signify the median, IQR, minima and maxima respectively.

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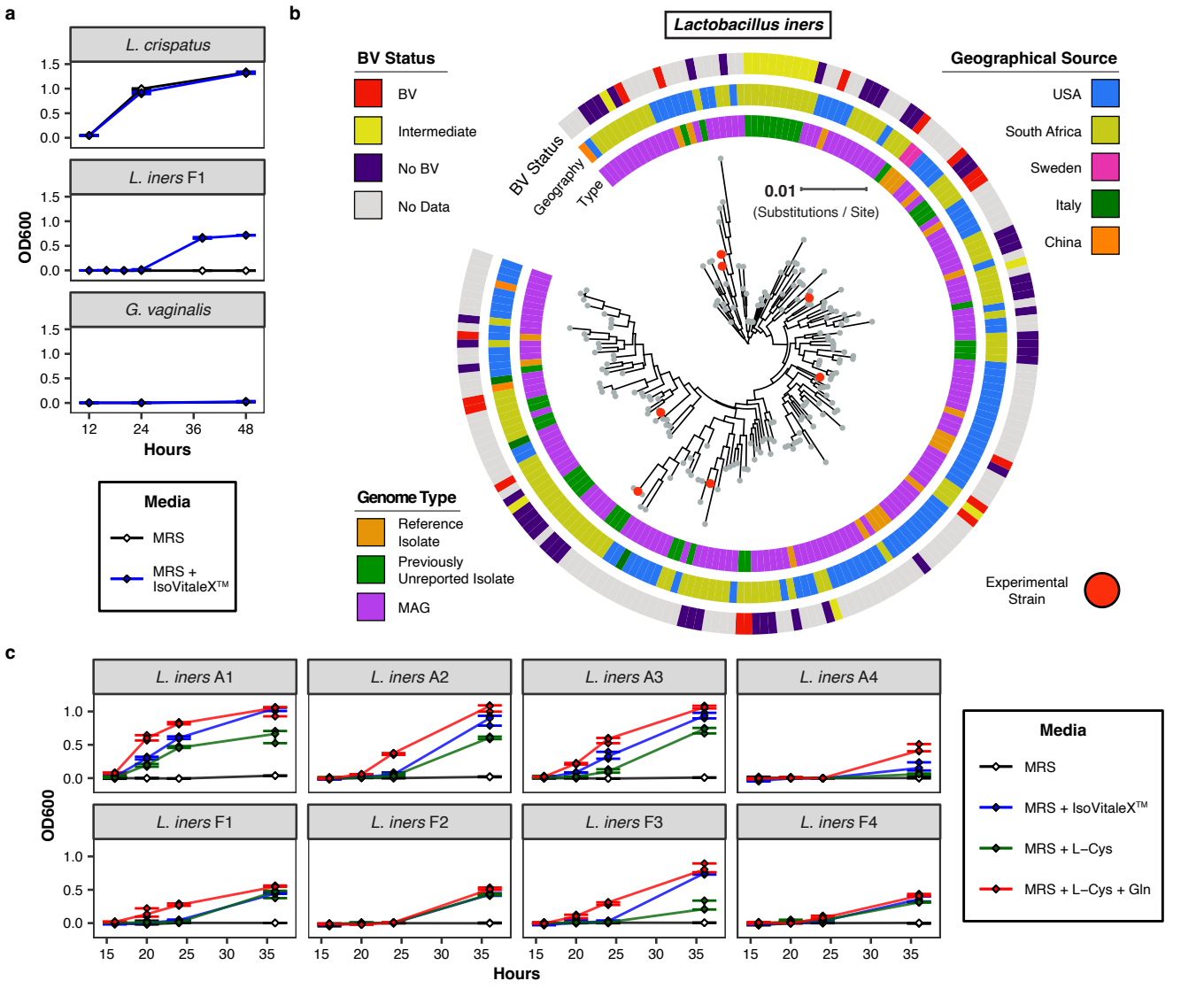
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No BV
No Data
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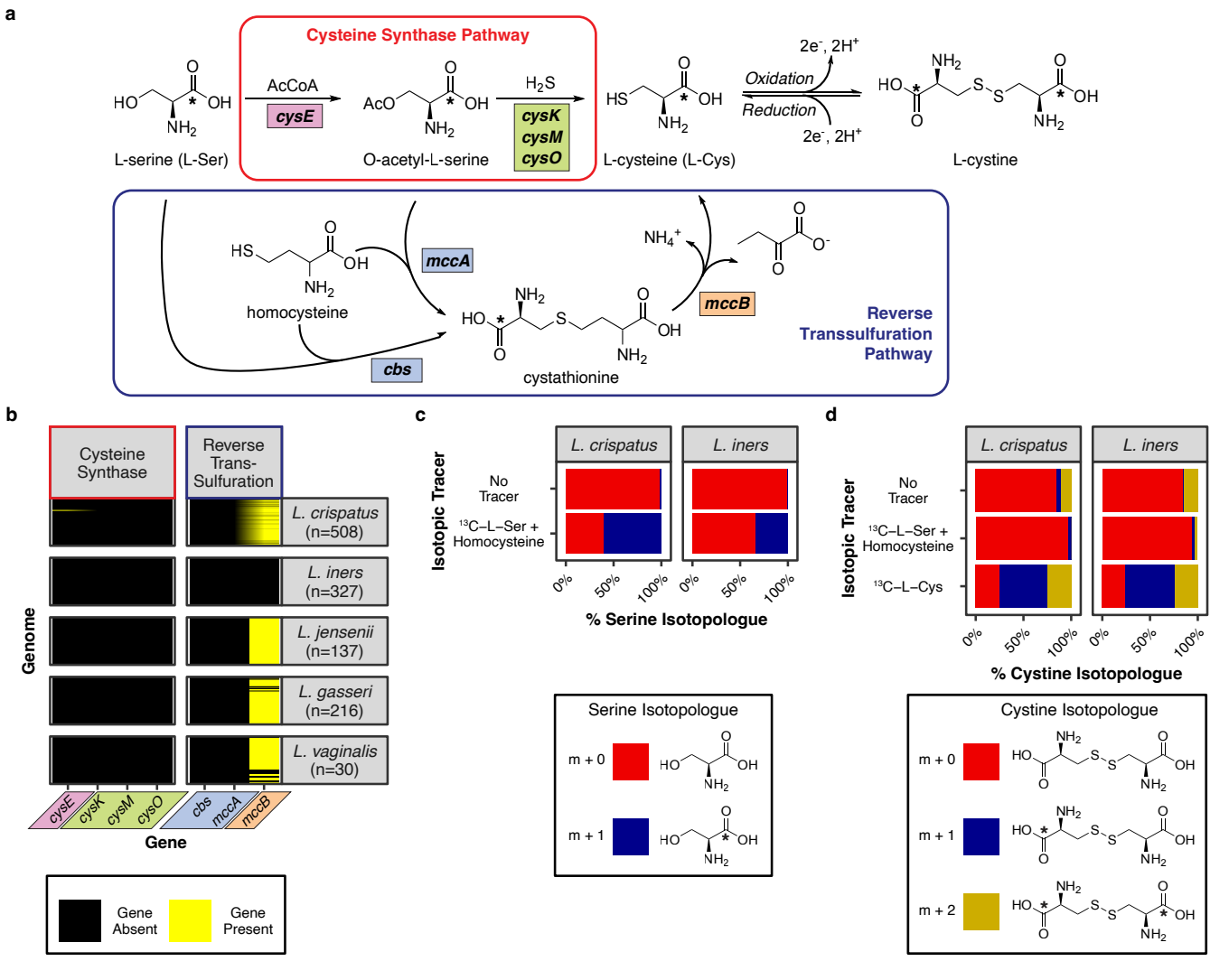
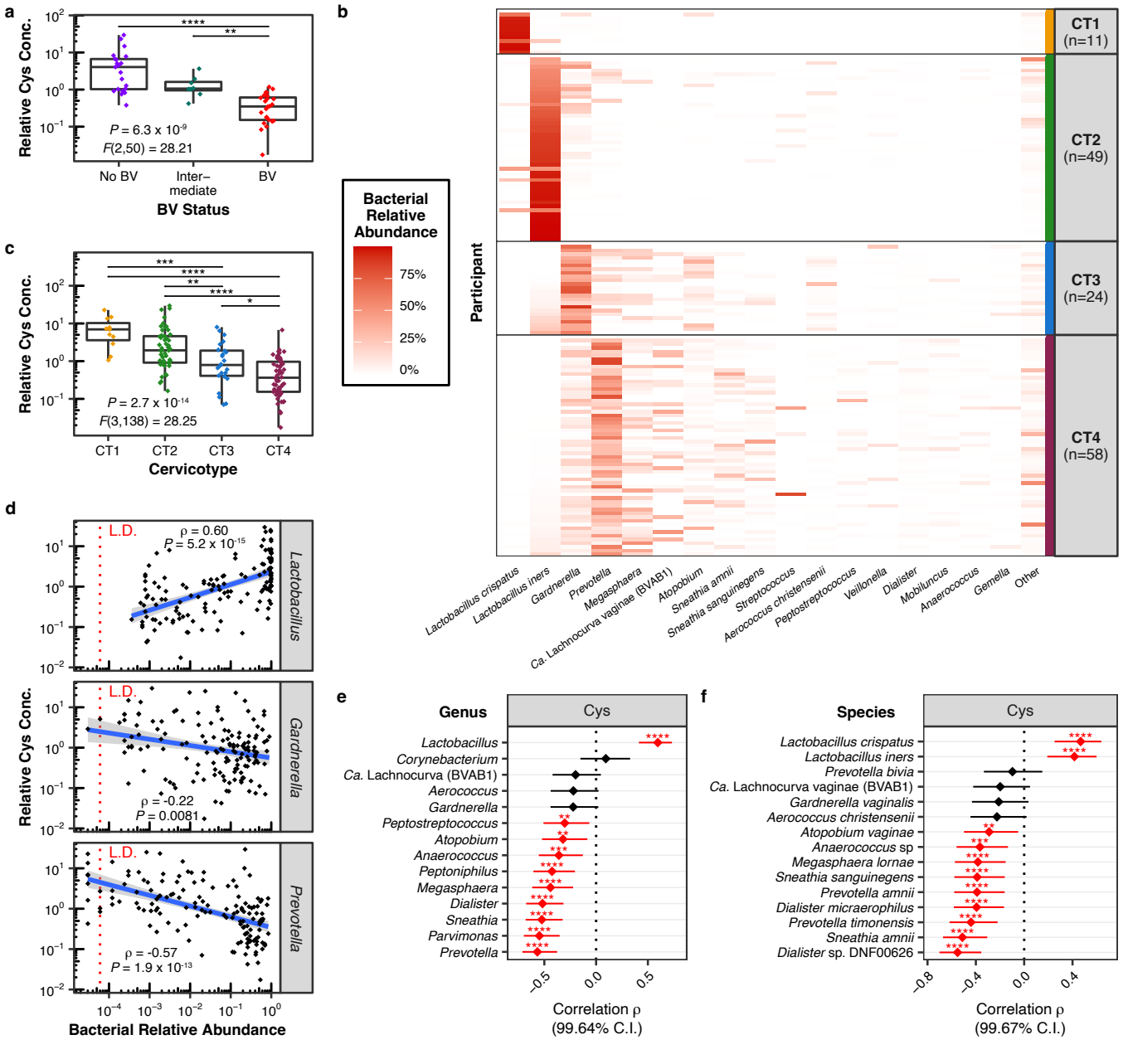
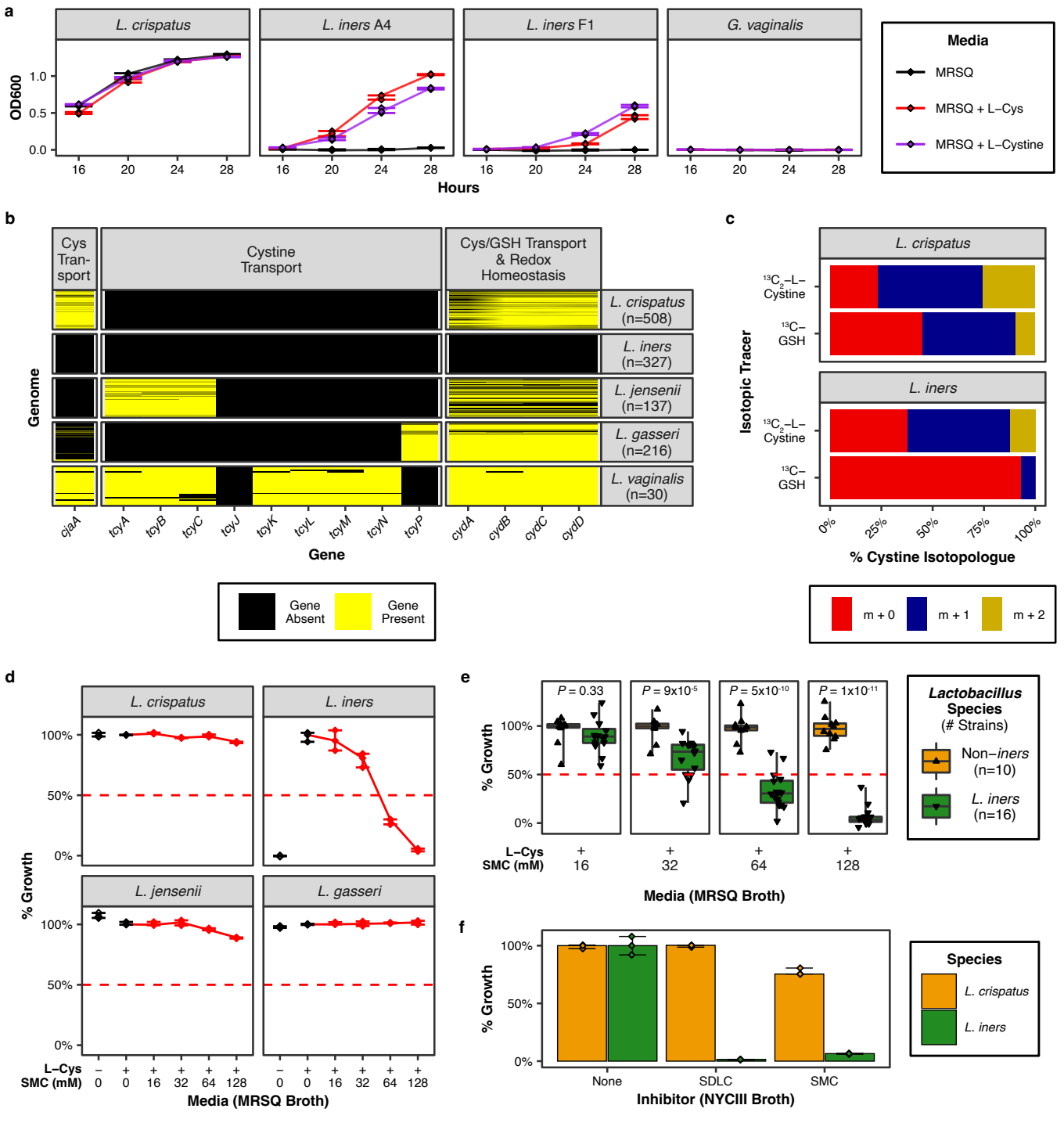
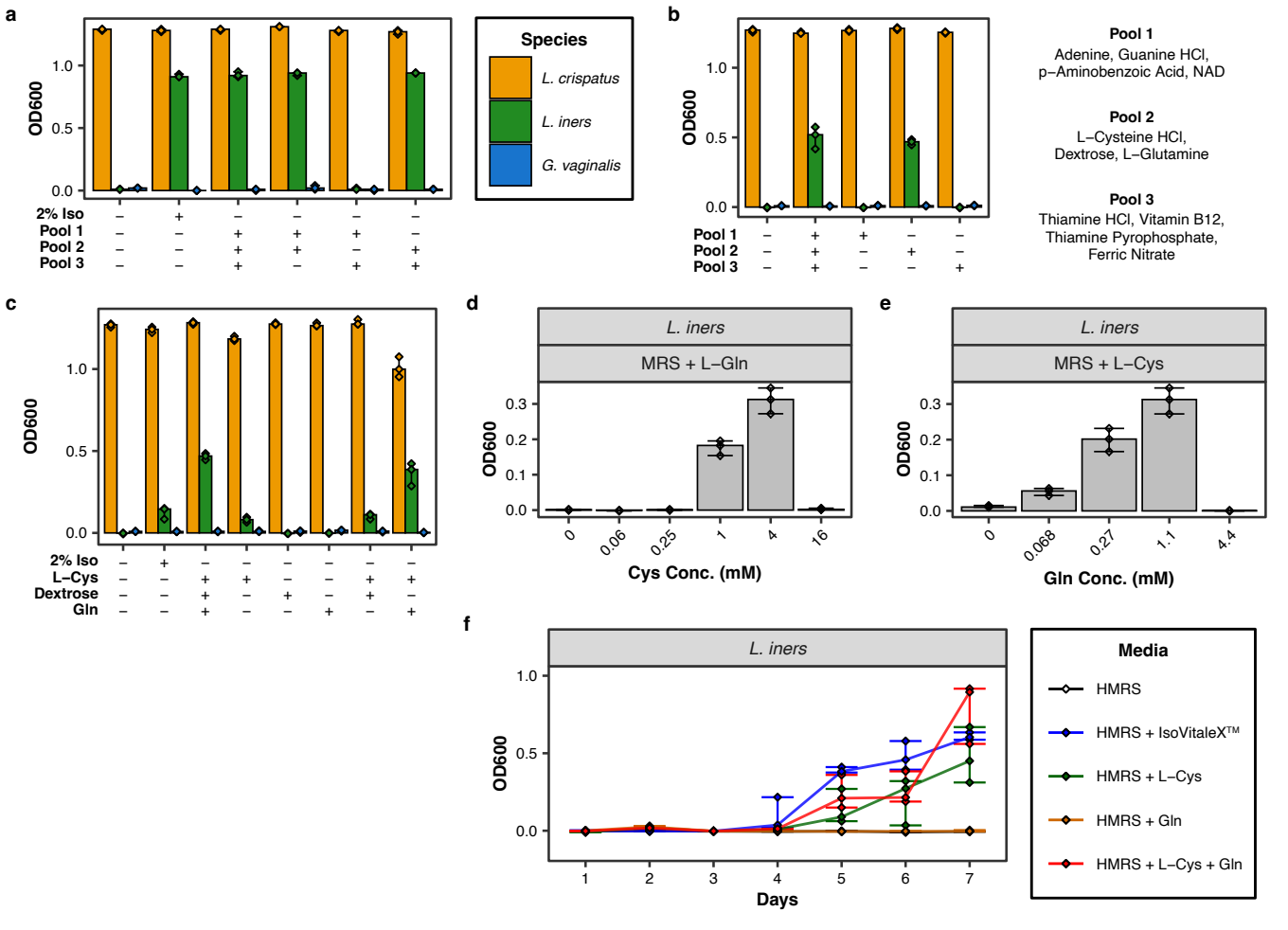
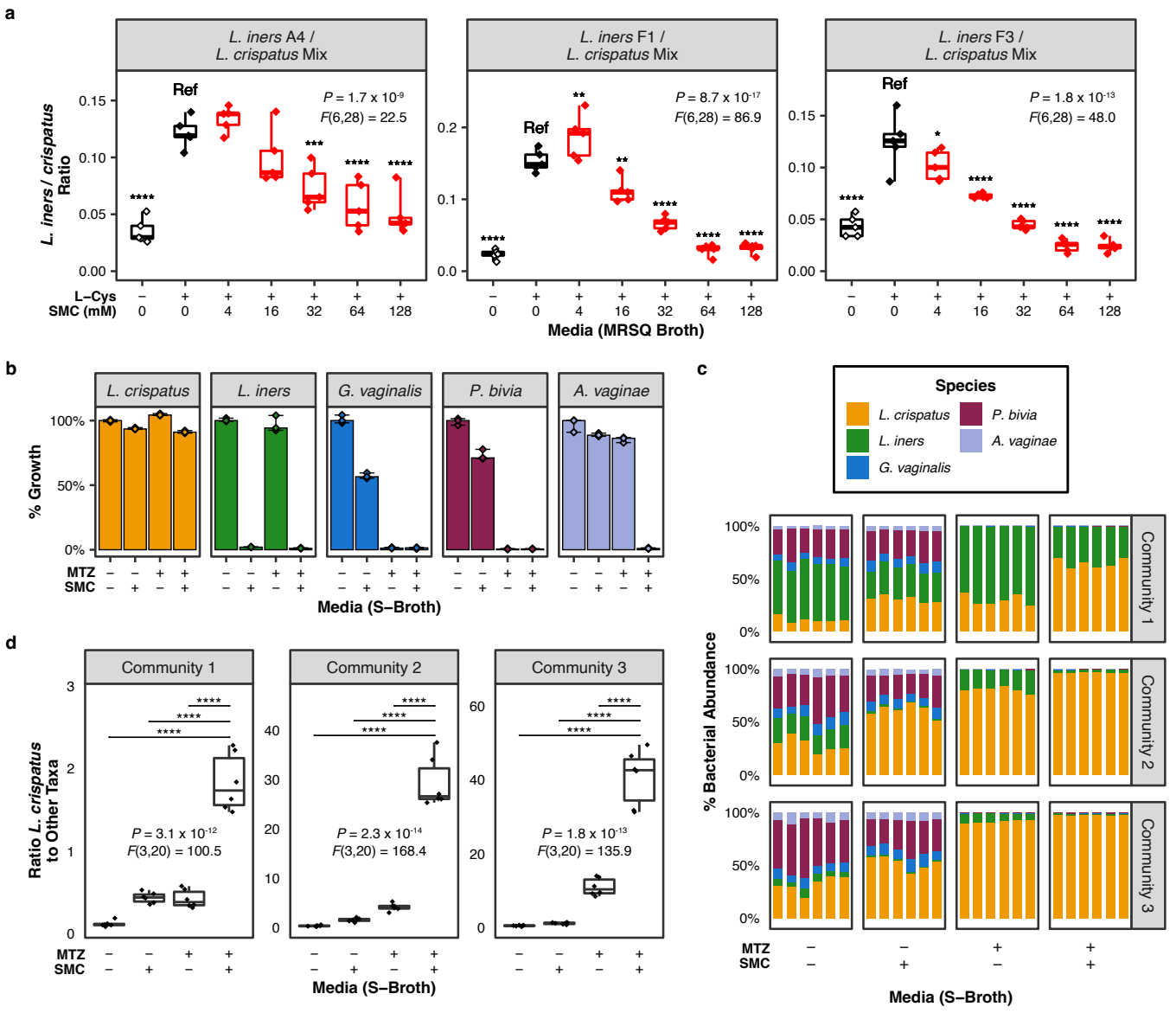
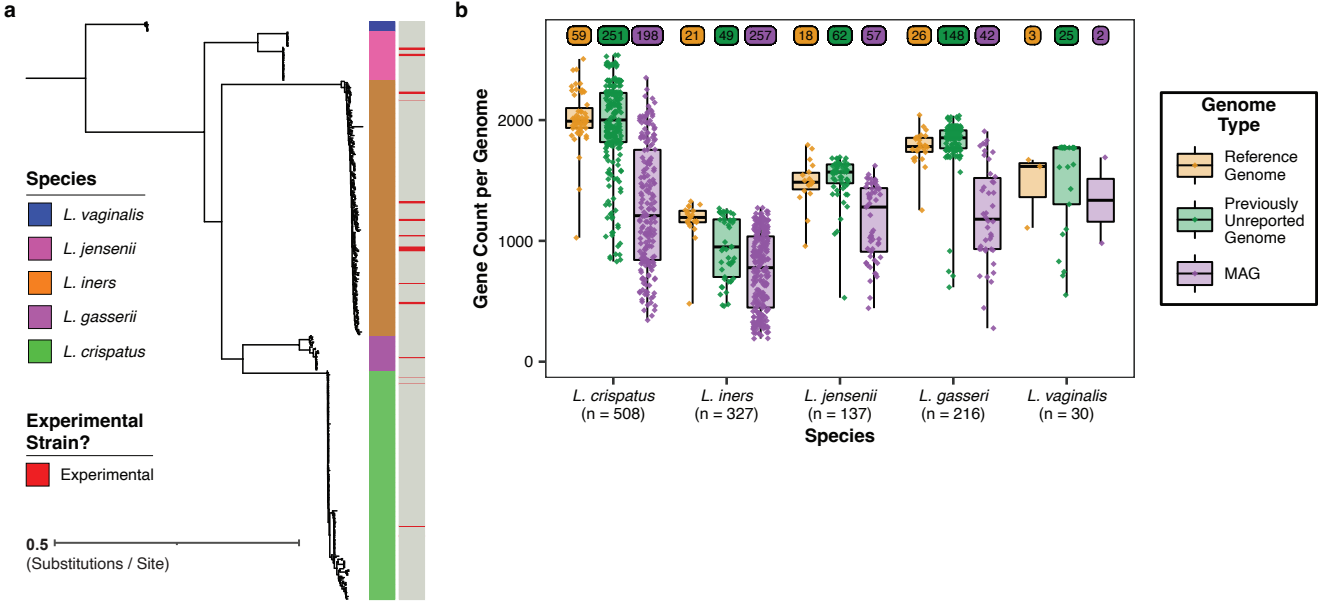

a
Cysteine Synthase Pathway
AcCoA
cysE
H2S
cysK
cysM
cysO
L-serine (L-Ser)
O-acetyl-L-serine
L-cysteine (L-Cys)
Oxidation
Reduction
2e-, 2H+
2e-, 2H+
L-cystine
mccA
cbs
homocysteine
cystathionine
mccB
NH4+
Reverse
Transsulfuration
Pathway
b
Cysteine
Synthase
Reverse
Trans-
Sulfuration
L. crispatus
(n=508)
L. iners
(n=327)
L. jensenii
(n=137)
L. gasseri
(n=216)
L. vaginalis
(n=30)
Genome
cysE
cysK
cysM
cysO
cbs
mccA
mccB
Gene
Gene
Absent
Gene
Present
c
L. crispatus
L. iners
Isotopic Tracer
No
Tracer
13C–L–Ser +
Homocysteine
0%
50%
100%
% Serine Isotopologue
Serine Isotopologue
m + 0
m + 1
d
L. crispatus
L. iners
Isotopic Tracer
No
Tracer
13C–L–Ser +
Homocysteine
13C–L–Cys
0%
50%
100%
% Cystine Isotopologue
Cystine Isotopologue
m + 0
m + 1
m + 2

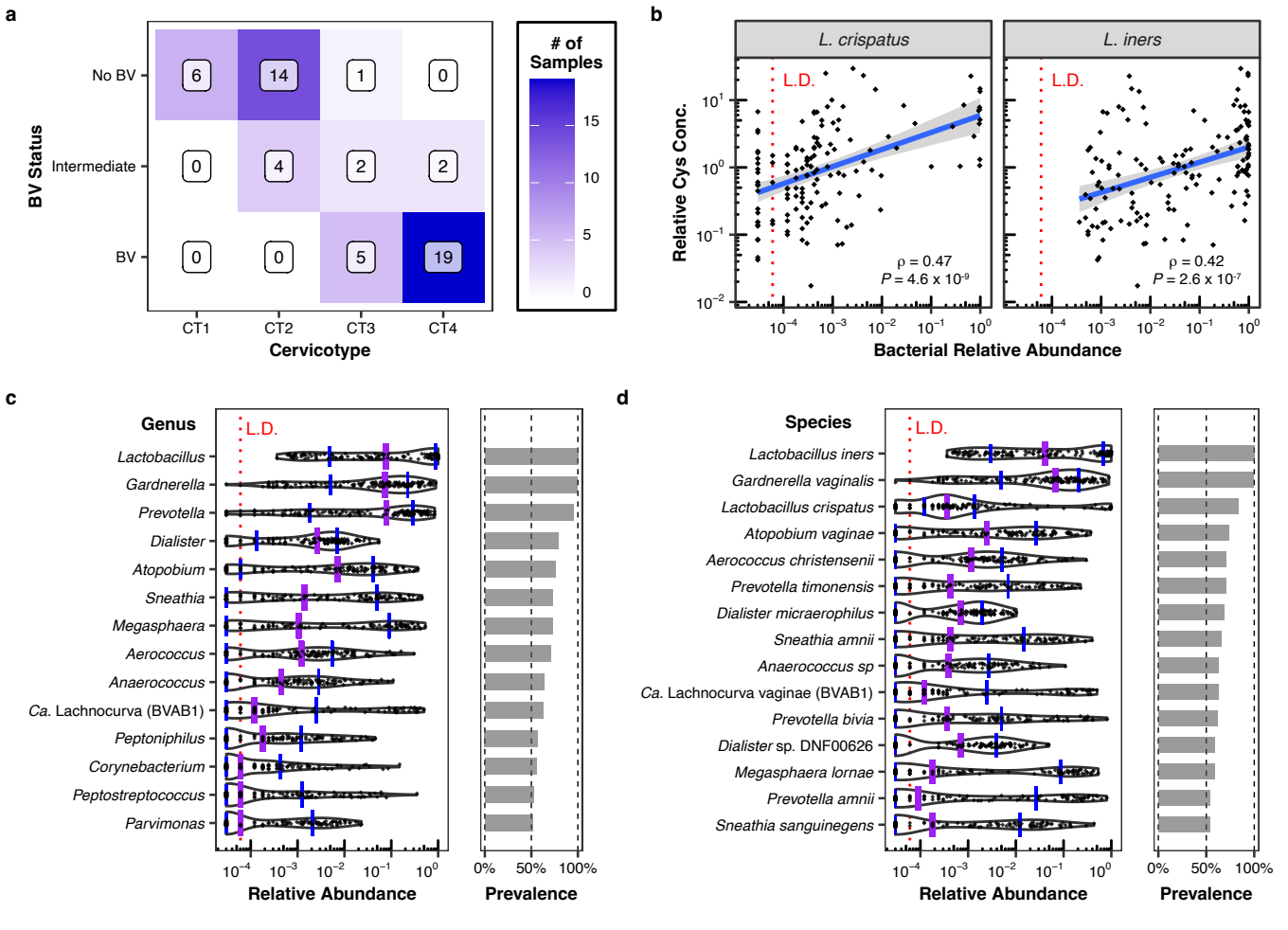

a
Relative Cys Conc.
****
**
P = 6.3 x 10-9
F(2,50) = 28.21
No BV
Inter–mediate
BV
BV Status
c
Relative Cys Conc.
***
****
**
****
*
P = 2.7 x 10-14
F(3,138) = 28.25
CT1
CT2
CT3
CT4
Cervicotype
b
Bacterial Relative Abundance
75%
50%
25%
0%
Participant
CT1 (n=11)
CT2 (n=49)
CT3 (n=24)
CT4 (n=58)
Lactobacillus crispatus
Lactobacillus iners
Gardnerella
Prevotella
Megasphaera
Ca. Lachnocurva vaginae (BVAB1)
Atopobium
Sneathia amnii
Sneathia sanguinegens
Streptococcus
Aerococcus christensenii
Peptostreptococcus
Veillonella
Dialister
Mobiluncus
Anaerococcus
Gemella
Other
d
L.D.
ρ = 0.60
P = 5.2 x 10-15
Lactobacillus
ρ = -0.22
P = 0.0081
Gardnerella
ρ = -0.57
P = 1.9 x 10-13
Prevotella
Relative Cys Conc.
Bacterial Relative Abundance
e
Genus
Cys
Lactobacillus
Corynebacterium
Ca. Lachnocurva (BVAB1)
Aerococcus
Gardnerella
Peptostreptococcus
Atopobium
Anaerococcus
Peptoniphilus
Megasphaera
Dialister
Sneathia
Parvimonas
Prevotella
Correlation ρ
(99.64% C.I.)
f
Species
Cys
Lactobacillus crispatus
Lactobacillus iners
Prevotella bivia
Ca. Lachnocurva vaginae (BVAB1)
Gardnerella vaginalis
Aerococcus christensenii
Atopobium vaginae
Anaerococcus sp
Megasphaera lornae
Sneathia sanguinegens
Prevotella amnii
Dialister micraerophilus
Prevotella timonensis
Sneathia amnii
Dialister sp. DNF00626
Correlation ρ
(99.67% C.I.)

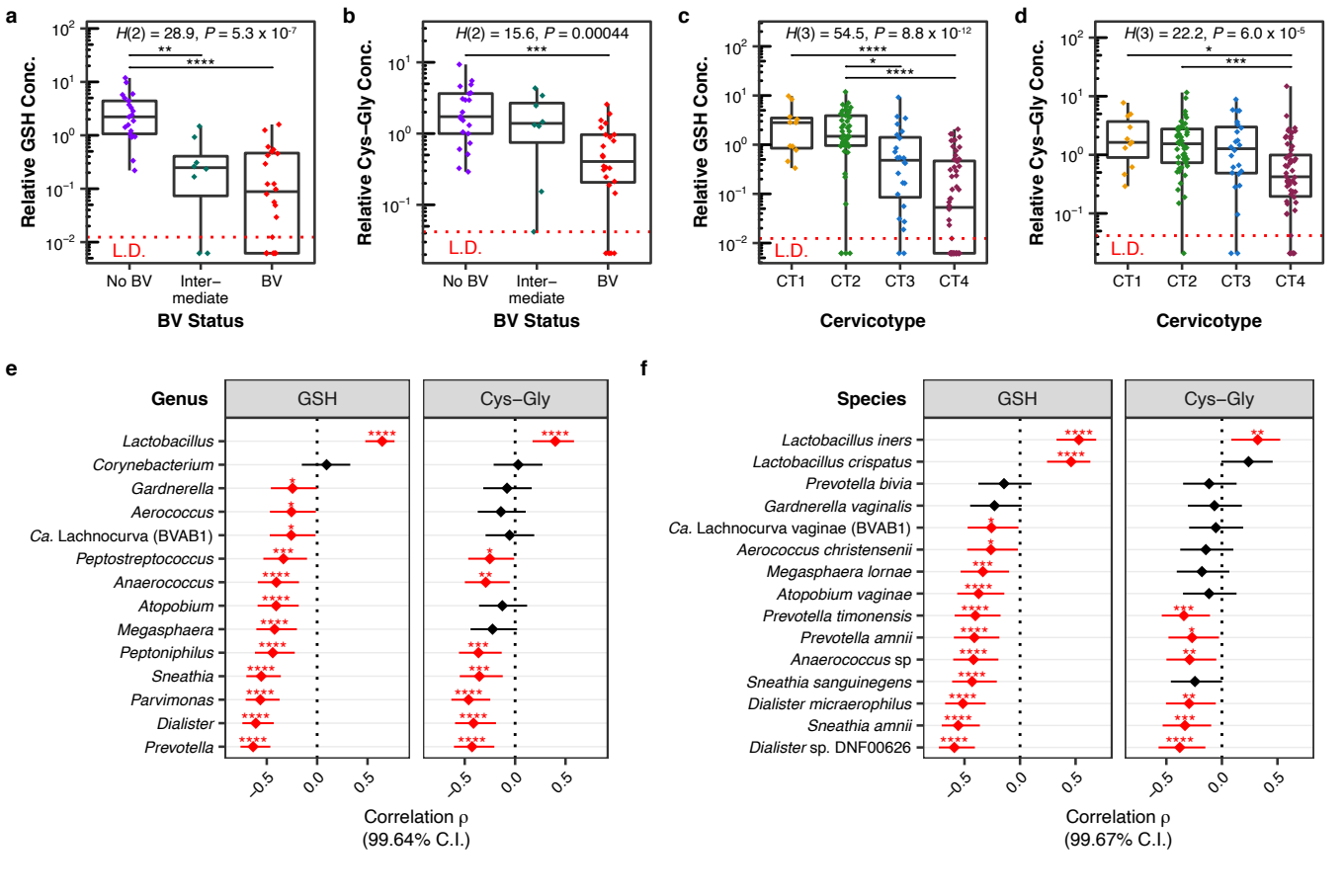

a
L. crispatus
L. iners A4
L. iners F1
G. vaginalis
OD600
1.0
0.5
0.0
16
20
24
28
Hours
Media
MRSQ
MRSQ + L–Cys
MRSQ + L–Cystine
b
Cys Tran-sport
Cystine Transport
Cys/GSH Transport & Redox Homeostasis
L. crispatus (n=508)
L. iners (n=327)
L. jensenii (n=137)
L. gasseri (n=216)
L. vaginalis (n=30)
Genome
cjaA
tcyA
tcyB
tcyC
tcyJ
tcyK
tcyL
tcyM
tcyN
tcyP
cydA
cydB
cydC
cydD
Gene
Gene Absent
Gene Present
c
L. crispatus
L. iners
$^{13}C_2$–L–Cystine
^{13}C–GSH
Isotopic Tracer
0%
25%
50%
75%
100%
% Cystine Isotopologue
m + 0
m + 1
m + 2
d
L. crispatus
L. iners
L. jensenii
L. gasseri
% Growth
100%
50%
0%
L–Cys
SMC (mM)
0
16
32
64
128
Media (MRSQ Broth)
e
P = 0.33
P = 9x10-5
P = 5x10-10
P = 1x10-11
% Growth
L–Cys
SMC (mM)
16
32
64
128
Media (MRSQ Broth)
Lactobacillus Species (# Strains)
Non–iners (n=10)
L. iners (n=16)
f
% Growth
None
SDLC
SMC
Inhibitor (NYCIII Broth)
Species
L. crispatus
L. iners

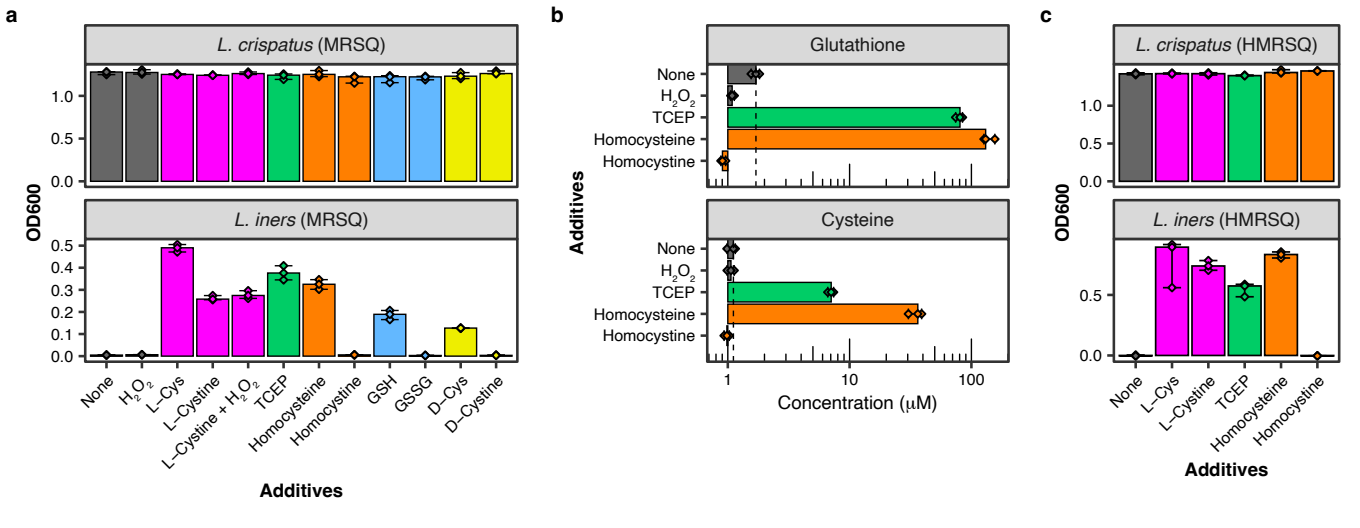

a
OD600
1.0
0.5
0.0
2% Iso
Pool 1
Pool 2
Pool 3
Species
L. crispatus
L. iners
G. vaginalis
b
OD600
1.0
0.5
0.0
Pool 1
Pool 2
Pool 3
Pool 1
Adenine, Guanine HCl,
p–Aminobenzoic Acid, NAD
Pool 2
L–Cysteine HCl,
Dextrose, L–Glutamine
Pool 3
Thiamine HCl, Vitamin B12,
Thiamine Pyrophosphate,
Ferric Nitrate
c
OD600
1.0
0.5
0.0
2% Iso
L–Cys
Dextrose
Gln
d
L. iners
MRS + L–Gln
OD600
0.3
0.2
0.1
0.0
0
0.06
0.25
1
4
16
Cys Conc. (mM)
e
L. iners
MRS + L–Cys
OD600
0.3
0.2
0.1
0.0
0
0.068
0.27
1.1
4.4
Gln Conc. (mM)
f
L. iners
OD600
1.0
0.5
0.0
1
2
3
4
5
6
7
Days
Media
HMRS
HMRS + IsoVitaleX™
HMRS + L–Cys
HMRS + Gln
HMRS + L–Cys + Gln

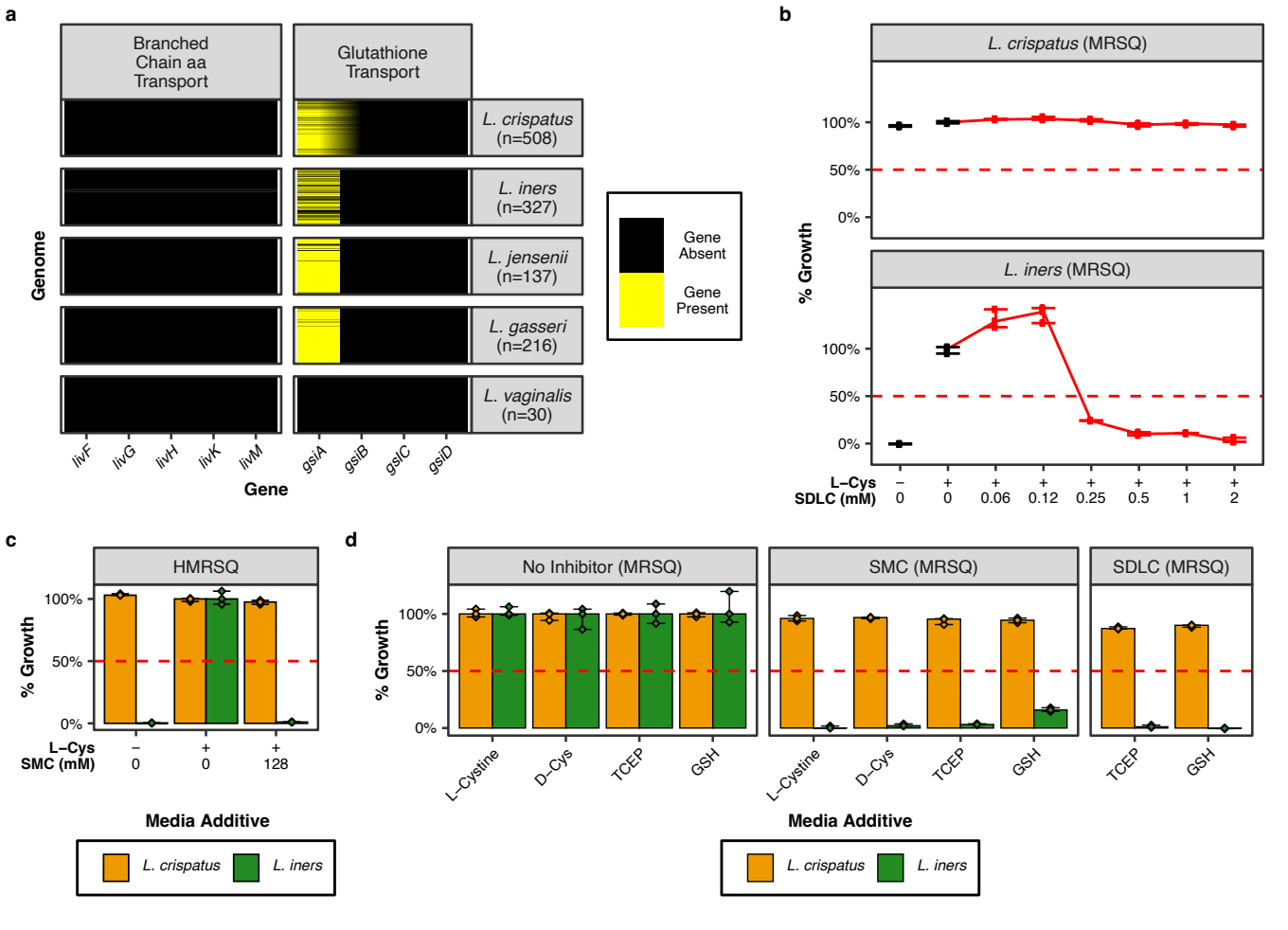

a
L. iners A4 / L. crispatus Mix
L. iners F1 / L. crispatus Mix
L. iners F3 / L. crispatus Mix
L. iners / crispatus Ratio
Ref
P = 1.7 x 10-9
F(6,28) = 22.5
P = 8.7 x 10-17
F(6,28) = 86.9
P = 1.8 x 10-13
F(6,28) = 48.0
L–Cys
SMC (mM)
Media (MRSQ Broth)
b
L. crispatus
L. iners
G. vaginalis
P. bivia
A. vaginae
% Growth
MTZ
SMC
Media (S–Broth)
c
Species
L. crispatus
L. iners
G. vaginalis
P. bivia
A. vaginae
% Bacterial Abundance
Community 1
Community 2
Community 3
MTZ
SMC
Media (S–Broth)
d
Community 1
Community 2
Community 3
Ratio L. crispatus to Other Taxa
P = 3.1 x 10-12
F(3,20) = 100.5
P = 2.3 x 10-14
F(3,20) = 168.4
P = 1.8 x 10-13
F(3,20) = 135.9
MTZ
SMC
Media (S–Broth)

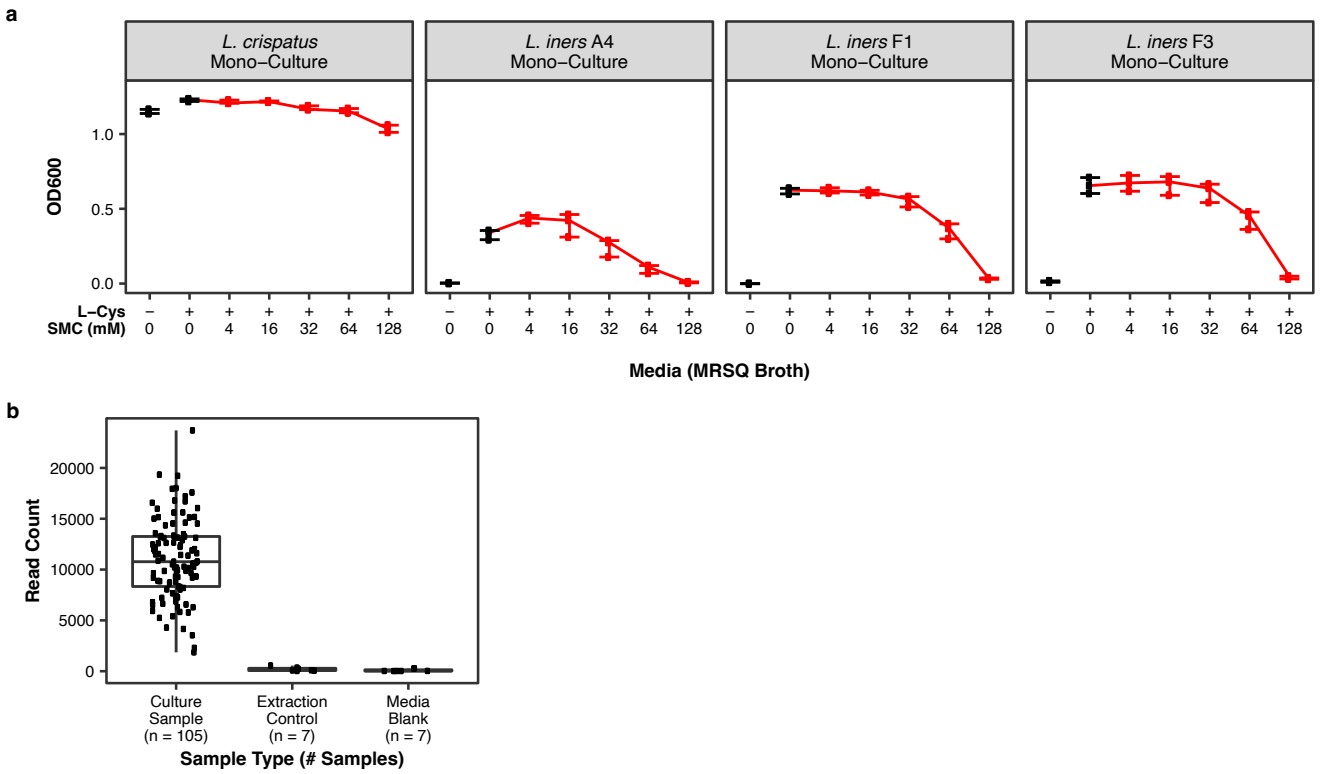

a
Species
L. vaginalis
L. jensenii
L. iners
L. gasserii
L. crispatus
Experimental Strain?
Experimental
0.5
(Substitutions / Site)
b
59
251
198
21
49
257
18
62
57
26
148
42
3
25
2
Gene Count per Genome
2000
1000
0
L. crispatus
(n = 508)
L. iners
(n = 327)
L. jensenii
(n = 137)
L. gasseri
(n = 216)
L. vaginalis
(n = 30)
Species
Genome Type
Reference Genome
Previously Unreported Genome
MAG

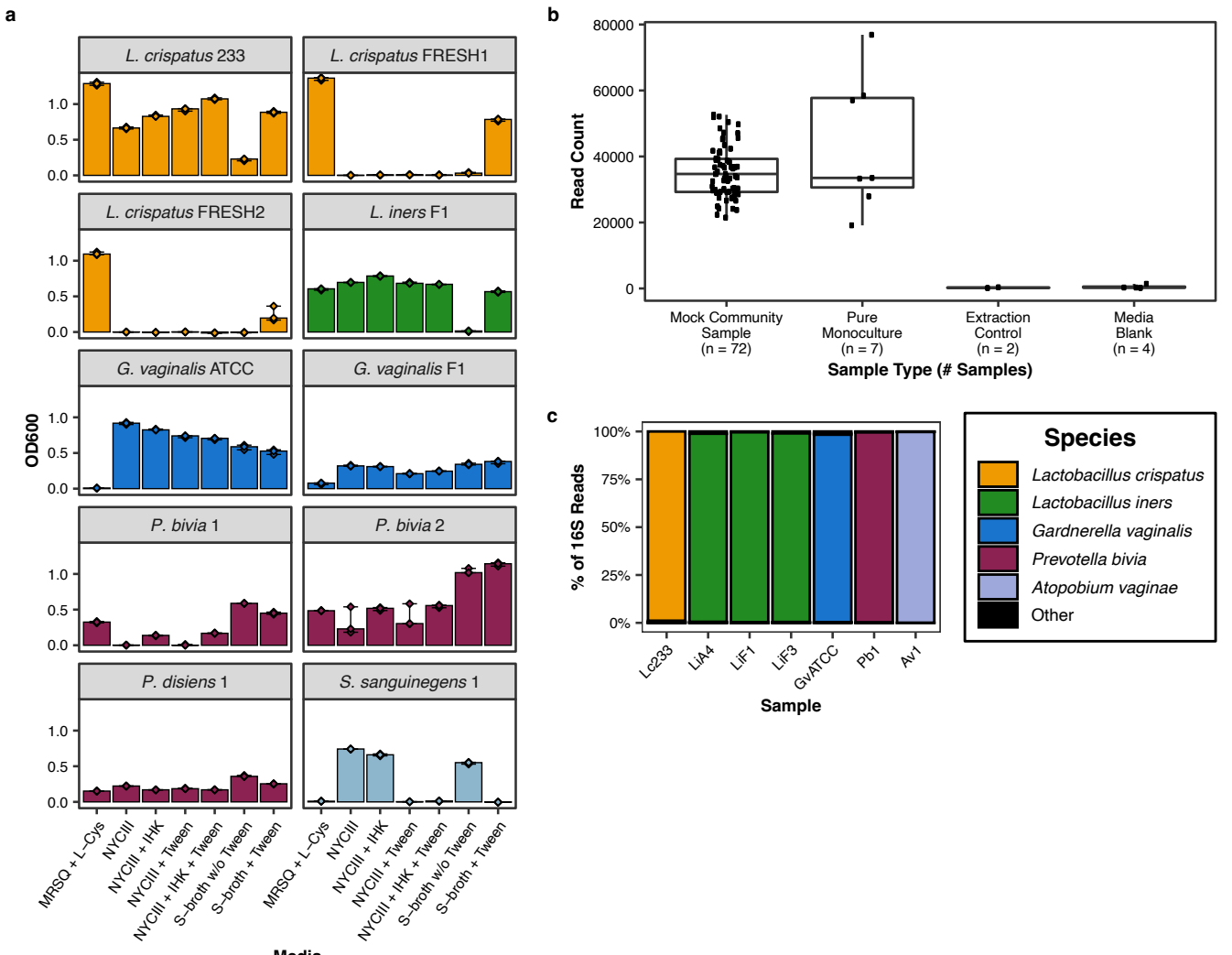

a
BV Status
No BV
Intermediate
BV
6
14
1
0
0
4
2
2
0
0
5
19
CT1
CT2
CT3
CT4
Cervicotype
# of Samples
15
10
5
0
b
L. crispatus
L. iners
L.D.
L.D.
Relative Cys Conc.
ρ = 0.47
P = 4.6 x 10-9
ρ = 0.42
P = 2.6 x 10-7
Bacterial Relative Abundance
c
Genus
L.D.
Lactobacillus
Gardnerella
Prevotella
Dialister
Atopobium
Sneathia
Megasphaera
Aerococcus
Anaerococcus
Ca. Lachnocurva (BVAB1)
Peptoniphilus
Corynebacterium
Peptostreptococcus
Parvimonas
Relative Abundance
Prevalence
0%
50%
100%
d
Species
L.D.
Lactobacillus iners
Gardnerella vaginalis
Lactobacillus crispatus
Atopobium vaginae
Aerococcus christensenii
Prevotella timonensis
Dialister micraerophilus
Sneathia amnii
Anaerococcus sp
Ca. Lachnocurva vaginae (BVAB1)
Prevotella bivia
Dialister sp. DNF00626
Megasphaera lornae
Prevotella amnii
Sneathia sanguinegens
Relative Abundance
Prevalence
0%
50%
100%

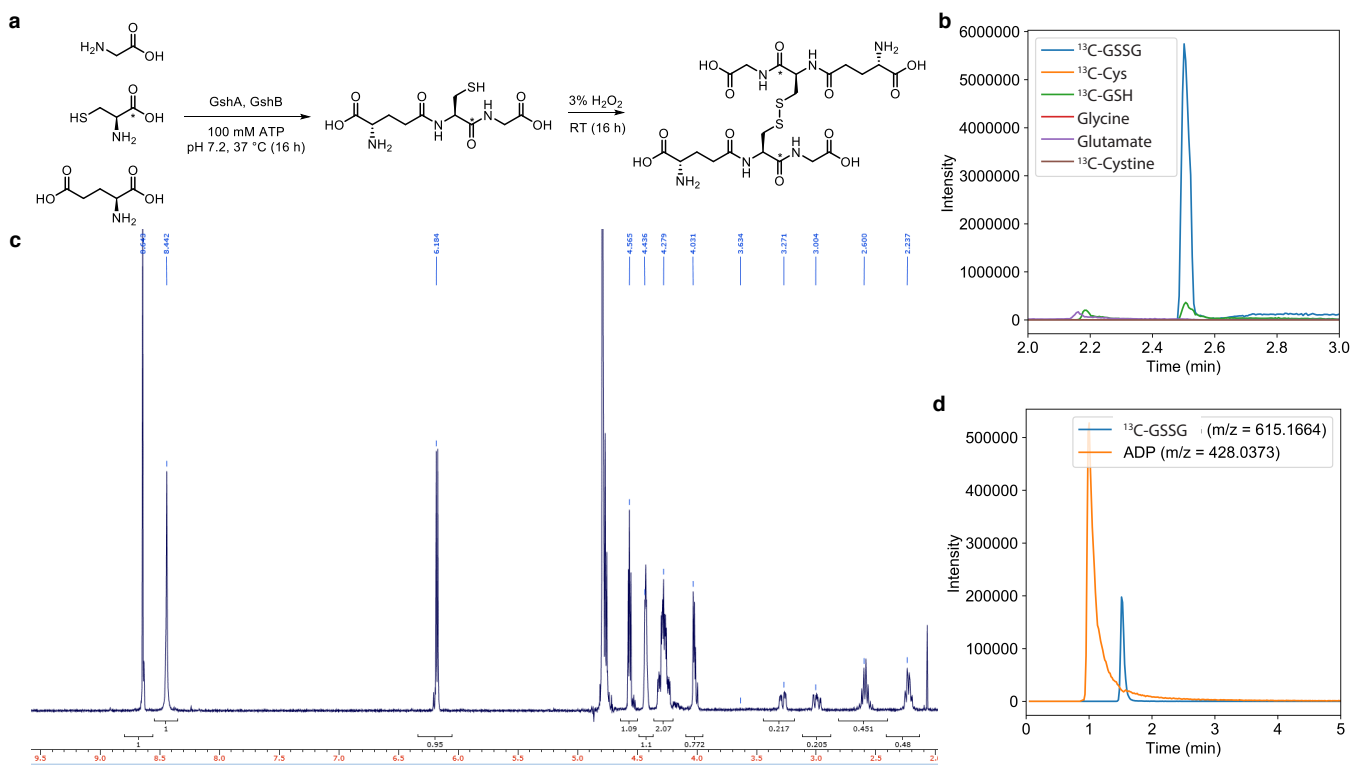

a
Relative GSH Conc.
H(2) = 28.9, P = 5.3 x 10-7
**
****
L.D.
No BV
Inter–mediate
BV
BV Status
b
Relative Cys–Gly Conc.
H(2) = 15.6, P = 0.00044
***
L.D.
No BV
Inter–mediate
BV
BV Status
c
Relative GSH Conc.
H(3) = 54.5, P = 8.8 x 10-12
****
*
****
L.D.
CT1
CT2
CT3
CT4
Cervicotype
d
Relative Cys–Gly Conc.
H(3) = 22.2, P = 6.0 x 10-5
*
***
L.D.
CT1
CT2
CT3
CT4
Cervicotype
e
Genus
GSH
Cys–Gly
Lactobacillus
Corynebacterium
Gardnerella
Aerococcus
Ca. Lachnocurva (BVAB1)
Peptostreptococcus
Anaerococcus
Atopobium
Megasphaera
Peptoniphilus
Sneathia
Parvimonas
Dialister
Prevotella
–0.5
0.0
0.5
Correlation ρ
(99.64% C.I.)
f
Species
GSH
Cys–Gly
Lactobacillus iners
Lactobacillus crispatus
Prevotella bivia
Gardnerella vaginalis
Ca. Lachnocurva vaginae (BVAB1)
Aerococcus christensenii
Megasphaera lornae
Atopobium vaginae
Prevotella timonensis
Prevotella amnii
Anaerococcus sp
Sneathia sanguinegens
Dialister micraerophilus
Sneathia amnii
Dialister sp. DNF00626
–0.5
0.0
0.5
Correlation ρ
(99.67% C.I.)


a
L. crispatus (MRSQ)
L. iners (MRSQ)
OD600
1.0
0.5
0.0
0.5
0.4
0.3
0.2
0.1
0.0
None
H2O2
L–Cys
L–Cystine
L–Cystine + H2O2
TCEP
Homocysteine
Homocystine
GSH
GSSG
D–Cys
D–Cystine
Additives
b
Glutathione
Cysteine
None
H2O2
TCEP
Homocysteine
Homocystine
Additives
1
10
100
Concentration (μM)
c
L. crispatus (HMRSQ)
L. iners (HMRSQ)
OD600
1.0
0.5
0.0
0.5
0.0
None
L–Cys
L–Cystine
TCEP
Homocysteine
Homocystine
Additives


a
Branched Chain aa Transport
Glutathione Transport
L. crispatus (n=508)
L. iners (n=327)
L. jensenii (n=137)
L. gasseri (n=216)
L. vaginalis (n=30)
Genome
livF
livG
livH
livK
livM
gsiA
gsiB
gsiC
gsiD
Gene
Gene Absent
Gene Present
b
L. crispatus (MRSQ)
L. iners (MRSQ)
% Growth
100%
50%
0%
L–Cys
SDLC (mM)
0
0
0.06
0.12
0.25
0.5
1
2
c
HMRSQ
% Growth
L–Cys
SMC (mM)
0
0
128
Media Additive
L. crispatus
L. iners
d
No Inhibitor (MRSQ)
SMC (MRSQ)
SDLC (MRSQ)
% Growth
L–Cystine
D–Cys
TCEP
GSH
Media Additive
L. crispatus
L. iners


a
L. crispatus Mono−Culture
L. iners A4 Mono−Culture
L. iners F1 Mono−Culture
L. iners F3 Mono−Culture
OD600
1.0
0.5
0.0
L−Cys
SMC (mM)
0
4
16
32
64
128
Media (MRSQ Broth)
b
Read Count
20000
15000
10000
5000
0
Culture Sample (n = 105)
Extraction Control (n = 7)
Media Blank (n = 7)
Sample Type (# Samples)


a
L. crispatus 233
L. crispatus FRESH1
L. crispatus FRESH2
L. iners F1
G. vaginalis ATCC
G. vaginalis F1
P. bivia 1
P. bivia 2
P. disiens 1
S. sanguinegens 1
OD600
0.0
0.5
1.0
MRSQ + L–Cys
NYCIII
NYCIII + IHK
NYCIII + Tween
NYCIII + IHK + Tween
S–broth w/o Tween
S–broth + Tween
Media
b
Read Count
0
20000
40000
60000
80000
Mock Community Sample (n = 72)
Pure Monoculture (n = 7)
Extraction Control (n = 2)
Media Blank (n = 4)
Sample Type (# Samples)
c
% of 16S Reads
0%
25%
50%
75%
100%
Lc233
LiA4
LiF1
LiF3
GvATCC
Pb1
Av1
Sample
Species
Lactobacillus crispatus
Lactobacillus iners
Gardnerella vaginalis
Prevotella bivia
Atopobium vaginae
Other


a
GshA, GshB
100 mM ATP
pH 7.2, 37 °C (16 h)
3% H2O2
RT (16 h)
b
13C-GSSG
13C-Cys
13C-GSH
Glycine
Glutamate
13C-Cystine
Intensity
Time (min)
c
d
13C-GSSG (m/z = 615.1664)
ADP (m/z = 428.0373)
Intensity
Time (min)