Characterization of vaginal microbial enzymes identifies amylopullulanases that support growth of *Lactobacillus crispatus* on glycogen

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The healthy human vaginal microbiota is generally dominated by *Lactobacilli*, and the transition to a more diverse community of anaerobic microbes is associated with a number of health risks. While the mechanisms underlying the stability of *Lactobacillus*-dominated vaginal communities are not fully understood, competition for nutrients is a likely contributing factor. Glycogen secreted by epithelial cells is widely believed to support the growth of vaginal microbes. However, the mechanism by which bacteria access sugars from this complex polymer is unclear, with evidence to support a role for both microbial and human enzymes. To shed light on the potential contribution from microbial enzymes, here we biochemically characterize six glycogen-degrading enzymes predicted to be secreted by vaginal bacteria and confirm their ability to support the growth of an amylase-deficient strain of *L. crispatus* on glycogen. We reveal significant differences in the pH tolerance between enzymes from different organisms, suggesting the adaptation of *Lactobacilli* to an acidic vaginal environment. Using a simple assay specific for the

microbial enzymes, we confirm their presence in cervicovaginal lavage samples. Finally, we demonstrate the selective inhibition of glycogen-degrading enzymes from two vaginal microbes associated with dysbiosis. This work provides biochemical evidence to support the role of vaginal bacterial amylase enzymes in the breakdown of glycogen, providing insight into factors that shape the vaginal microbiota and highlighting the possibility of manipulating community structure via non-antibiotic small molecules.

Dysbiosis within the human vaginal microbiota is associated with adverse health outcomes¹. The bacterial composition of this community can be classified taxonomically based on 16S rRNA sequencing into one of five Community State Types (CSTs).² CST I-III and V are dominated by a single species of *Lactobacillus*: *L. crispatus*, *L. gasseri*, *L. iners*, or *L. jensenii*, respectively. CST-IV, by contrast, consists of a diverse group of anaerobic microbes, including species of *Gardnerella*, *Prevotella*, and *Mobiluncus*. The *Lactobacillus*-dominated CSTs are associated with a vaginal pH below 4.5, low Nugent scores, and low levels of inflammation³, whereas CST-IV is often associated with a higher pH and a number of health sequelae, including HIV acquisition⁴, bacterial vaginosis⁵, and preterm birth⁶. Thus, the prevailing view of the “healthy” vaginal bacterial community is one containing a high proportion of *Lactobacillus* species. However, it is important to note that CST IV is overrepresented in healthy Hispanic and Black women, and is not necessarily indicative of dysbiosis.⁷ It has also recently been revealed that even CSTs dominated by a single species have significant intra-species genetic variation⁸, with multiple strains of the same species co-occurring. The factors contributing to the stability of different communities, and what causes transitions between the different CSTs, remain poorly understood⁹. Overall, it has become clear that community composition alone is insufficient to predict health outcomes, and resolving these questions requires understanding specific functions encoded by vaginal bacteria.

One function known to influence the composition and stability of host-associated microbial communities is the liberation of carbohydrates from specific dietary or host-derived sources by

glycoside hydrolases. This has been well established within the human gut microbiota,¹⁰ where the presence of an extracellular glycoside hydrolase in *B. ovatus*, was beneficial for the success of the organism *in vivo*.¹¹ In addition, co-occurring bacteria have been shown to rely on a glycoside hydrolase ‘producer’ species for community access to carbon sources.^{11,12,13} Compared to the gut, microbial carbohydrate metabolism in the vaginal environment is poorly understood. It is widely believed that glycogen secreted by vaginal epithelial cells supports the colonization of vaginal *Lactobacilli*¹⁴, since free glycogen levels in vaginal samples have been associated with *Lactobacillus* dominance and a low vaginal pH¹⁵. However, until recently, attempts to obtain vaginal *Lactobacillus* isolates that were capable of growth on glycogen were largely unsuccessful.^{16,17} This difficulty raised the important question of how vaginal bacteria access this carbon source.

Glycogen consists of linear chains of $\alpha(1-4)$ linked glucose moieties, with periodic $\alpha(1-6)$ branches. Metabolism of glycogen requires an extracellular glycoside hydrolase to release shorter glucose polymers (maltodextrins) for import into the cell. Several vaginal *Lactobacilli* have been shown to utilize maltodextrins for growth, leading to the initial hypothesis that a non-*Lactobacillus* glycoside hydrolase in the vaginal environment releases these oligosaccharides.¹⁸ The detection of human α -amylase via ELISA in cervicovaginal lavages (CVLs) by Spear et al. lent support to this proposal, and in the same study those authors went on to demonstrate that amylase-containing human saliva could support *L. crispatus* growth on glycogen.¹⁸ How human amylase, which is produced predominantly in the pancreas and salivary glands,¹⁸ comes to be found in genital fluid, has not yet been established. Further analysis of the CVLs in that study revealed that most samples had reduced amylase activity at low pH, consistent with the pH profile of human amylase.¹⁹ In a small fraction of samples, though, activity increased as the pH became more acidic, suggesting the presence of other glycogen-degrading enzymes in the vaginal environment.¹⁹

In addition to human amylase, recent evidence suggests a potential role for bacterial enzymes in the breakdown of vaginal glycogen. The characterization of a small number of CVLs via proteomics and in-gel amylase assay after native polyacrylamide gel electrophoresis (PAGE) revealed the presence of several putative bacterial glycoside hydrolases, as well as the human enzyme.²⁰ In addition, Van der Veer and co-workers recently isolated several *L. crispatus* strains that could grow on glycogen. These authors suggested a putative secreted Type 1 pullulanase (PulA, WP_003549917.1) as the source of amylase activity, based on strain-to-strain variation in its predicted signal peptide which correlates with growth on glycogen²¹. Type 1 pullulanases hydrolyze the $\alpha(1-6)$ linkages in pullulan and other branched oligosaccharides, and this activity enables the release of maltodextrins from highly branched glycogen.²² Homologs of PulA are present in a variety of vaginal genomes,⁹ suggesting this enzyme might not be limited to *Lactobacillus* isolates, and highlighting the potential for competition for glycogen and/or cross-feeding. Notably, the proteomics study identified putative pullulanases from *Lactobacillus iners* and *Gardnerella vaginalis* in CVLs.²⁰ However, the predicted $\alpha(1-6)$ specificity of Type I pullulanases raises questions regarding the fate of the remaining glycogen backbone and how longer branches are hydrolyzed. The identification of these bacterial enzymes also raises questions about the relative role of the human amylase in the vaginal ecosystem. However, interpretation of all of these results is confounded by the fact that none of the reported bacterial enzymes have been verified biochemically. A better understanding of glycogen metabolism within the vaginal microbiota would benefit from a detailed characterization of these enzymatic activities.

Here, we report the biochemical characterization of six signal peptide-containing PulA homologs from vaginal microbes representing *Lactobacillus*-dominated CSTs (I and III) and the diverse CST-IV community. We show that all of these proteins are glycogen-degrading enzymes (GDEs) that support growth of amylase-deficient *L. crispatus* on glycogen. We further characterize the substrate scope of each enzyme, finding that several should be reannotated as amylopullulanases. Using an enzymatic assay selective for pullulanases, an activity not exhibited

by human amylase, we provide evidence that these enzymes are present in CVLs. We also determine the pH optimum of each enzyme and the spectrum of oligosaccharide produced. Finally, we demonstrate selective inhibition of these enzymes, suggesting the possibility of therapeutic interventions for targeted vaginal microbial community modulation. Overall, this work provides mechanistic insight into the bacterial metabolism of an abundant carbon source in the vaginal microbiota.

Results

Identification and purification of bacterial extracellular GDEs

To identify candidate vaginal microbial GDEs, we conducted a BlastP search of genomes from 151 vaginal isolates in the IMG database using the putative Type I pullulanase identified by Van der Veer et al in *L. crispatus* (PulA) as our query sequence²¹ (WP_003549917.1), with a cut-off of 35% amino acid identity. Hits were further narrowed to those containing a putative signal peptide, since glycogen degradation occurs extracellularly²³. 69 potential homologs were identified across a range of bacteria (Supplementary File 1), including among others *Lactobacillus crispatus* (8/9 strains, average 99% amino acid identity), *Lactobacillus iners* (12/13 strains, average 46% identity), *Mobiluncus mulieris* (2/4 strains, average 43% identity), *Prevotella bivia* (2/2 strains, 40% identity), and *Gardnerella vaginalis* (15/18 strains, average 38% identity). Interestingly, gene neighborhood analysis revealed another signal peptide-containing glycoside hydrolase (GH 13) immediately next to the *P. bivia* *pulA* (25% identity to PulA), so this sequence was added to our analysis. Each of these bacteria has been previously associated with health or disease, thus subsequent efforts focused on this set of proteins. It should be noted that we also detected potential homologs with lower amino acid identity (Supplementary File 1), including one from *Streptococcus agalacticae* (SAG0089_06185, 33% identity), and one significantly smaller protein in *Gardnerella vaginalis* (HMPREF0424_1317) homologous to a recently reported α -glucosidase enzyme from *Gardnerella* spp. that is active on maltose and other oligosaccharides, but lacks the ability to degrade glycogen.²⁴ PFAM domain analysis revealed that all six candidates

contain either an S-layer protein A domain (SlpA), a cell wall binding domain (CWB), or transmembrane helices (TM), suggesting they are located on the cell surface^{25,26,27}. Additionally, each protein contains at least one α -amylase catalytic domain (PF00128), which is a member of the glycoside hydrolase 13 enzyme family known to catalyze the cleavage of various glycosidic bonds²⁸. Interestingly, the *G. vaginalis* enzyme contains two unique amylase domains. In addition, several of the proteins possess putative carbohydrate-binding domains, including the pullulanase domain (PUD; PF03714), which is common among bacterial pullulanase enzymes.²⁹ Additional carbohydrate binding modules from the CaZy database found in these enzymes include CBM25 and CBM48, which are responsible for binding different linear and cyclic α -glucans related to starch and glycogen^{28,30} and for multivalent binding to soluble amylopectin and pullulan³¹ (**Fig. 1a**).

Fig. 1: Purified glycogen-degrading enzymes support *L. crispatus* growth on glycogen. **a.** Predicted domains in putative vaginal microbial extracellular GDEs. Abbreviations: **SP**, Signal Peptide; **PUD**, Bacterial Pullulanase-Associated Domain; **CBM**, Carbohydrate Binding Module 48(*L. crispatus*, *L. iners*, *G. vaginalis*, *M. mulieris*) Carbohydrate Binding Module 21(*G. vaginalis*); **SlpA**, Surface Layer Protein A; **α-Amylase**, α-Amylase Catalytic Domain; **C-Malt**, Cyclomaltodextrinase Domain; **CWB**, Cell Wall Binding Repeat 2; **TM**, Transmembrane Helices. **b.** Growth of *L. crispatus* C0176A1 (*pulA*⁻, JAEDCG000000000) and MV-1A-US (*pulA*⁺) on different carbon sources. **c.** *L. crispatus* C0176A1 (*pulA*⁻) grown on Oyster glycogen supplemented with 200 nM purified *L. crispatus* PulA. **d.** Heat map of OD₆₀₀ values of *L. crispatus* C0176A1 (*pulA*⁻) supplemented with 200 to 400 nM purified protein after 24 h of growth. All growth curves are representative of three biological replicates with 3 or 4 technical replicates each day. Error bars represent one standard deviation above and below the mean of all data collected.

Purified GDEs support the growth of *L. crispatus* on glycogen

The domain analysis of these enzymes suggested they act on extracellular carbohydrate polymers. To test this hypothesis, we heterologously expressed and purified each protein,

removing the signal peptides for better expression in *E. coli* (**SI Fig. 1, SI Fig. 2**). We then investigated each enzyme's ability to support growth of a strain of *L. crispatus* that lacks *pulA* in its genome and is unable grow on glycogen (**Fig. 1b; SI Fig. 3**). When purified *L. crispatus* PulA was added to the medium at the time of inoculation, growth on glycogen was recovered (**Fig. 1c; SI Fig. 3**). This provides direct evidence that PulA is necessary for *L. crispatus* growth on glycogen, as suggested in previous work.²¹ We next expanded this analysis to include the other five purified extracellular glycoside hydrolases. Each enzyme supported the growth of *pulA*-deficient *L. crispatus* on glycogen (**Fig. 1d; SI Fig. 3**), although titers were slightly lower for both *P. bivia* enzymes, suggesting they are not as efficient at glycogen degradation. Combining the two *P. bivia* enzymes did not affect the overall growth rate (data not shown).

Kinetic characterization reveals different glycosidic linkage and substrate preferences

Having demonstrated the glycogen degrading activity of these enzymes indirectly through growth complementation, we next performed kinetic characterization using a variety of glucose polymers in order to determine the specificity of each enzyme for the different glycosidic linkages in glycogen, and their substrate preference (**Table 1, SI Fig. 4**). In addition to glycogen, we tested amylose, which consists solely of $\alpha(1-4)$ linkages, and pullulan, a polymer that consists of maltotriose units connected by $\alpha(1-6)$ linkages. Consistent with the results of the growth supplementation experiment, all enzymes were active on glycogen. In addition, all were active on pullulan, suggesting the ability to cleave $\alpha(1,6)$ linkages, which was later confirmed by liquid chromatography-mass spectrometry (LC-MS) analysis of the products (discussed below). However, the enzymes differed in their activity toward the $\alpha(1-4)$ linkages in amylose, with *L. crispatus*, *L. iners*, *G. vaginalis* and *P. bivia* GH13 enzymes showing activity while the *M. mulieris* PulA and *P. bivia* PulA were inactive (**Table 1, SI Fig. 4**).

In general, the measured kinetic parameters were consistent with prior values observed for bacterial enzymes that process these substrates (glycogen^{32,33,34}, amylose^{35,36}, pullulan^{37,35}).

Comparing the specificity constants for each substrate revealed that glycogen is the preferred substrate for the *G. vaginalis* and *L. iners* PulA. The *L. crispatus* PulA had similar specificity constants for both pullulan and glycogen, with a lower specificity for amylose. Other enzymes, including *P. bivia* PulA and *M. mulieris* PulA, had higher specificity constants for pullulan in comparison to glycogen and amylose, while the *P. bivia* GH 13 enzyme preferred amylose (**Table 1; SI Fig. 4**). Taken together, these data demonstrate that both *Lactobacillus* PulA enzymes, the *G. vaginalis* PulA enzyme, and the glycoside hydrolase from *P. bivia* can cleave α -1,4 linkages and likely also α -1,6 linkages needed for the complete utilization of glycogen, and support their reassignment as amylopullulanases (EC. 3.2. 1.1/41, reviewed in³⁸). In contrast, the lack of activity of the *M. mulieris* and *P. bivia* PulA enzymes on amylose identifies them as strictly pullulanases and may explain their reduced ability to complement *L. crispatus* growth on glycogen (**Fig. 1d**).

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (mg mL^{-1})	Specificity Constant ($mL\ mg^{-1}\ s^{-1}$)	Classification
<i>L. crispatus</i> PulA	Glycogen	65 ± 4	0.091 ± 0.026	710 ± 210	Amylopullulanase
	Amylose	33 ± 7	0.25 ± 0.14	130 ± 80	
	Pullulan	100 ± 10	0.15 ± 0.05	700 ± 250	
<i>L. iners</i> PulA	Glycogen	51 ± 5	0.10 ± 0.05	500 ± 220	Amylopullulanase
	Amylose	30 ± 4	0.20 ± 0.07	150 ± 60	
	Pullulan	27 ± 9	0.93 ± 0.56	29 ± 20	
<i>G. vaginalis</i> PulA	Glycogen	450 ± 40	0.098 ± 0.044	4600 ± 2100	Amylopullulanase
	Amylose	110 ± 10	0.27 ± 0.06	390 ± 90	
	Pullulan	220 ± 40	0.42 ± 0.170	520 ± 230	
<i>M. mulieris</i> PulA	Glycogen	57 ± 9	6.1 ± 1.7	9.5 ± 3.0	Pullulanase
	Amylose	NA	NA	NA	
	Pullulan	150 ± 20	0.33 ± 0.14	440 ± 200	
<i>P. bivia</i> PulA	Glycogen	0.81 ± 0.31	11 ± 7	0.077 ± 0.058	Pullulanase
	Amylose	NA	NA	NA	
	Pullulan	60 ± 6	0.24 ± 0.07	250 ± 70	
<i>P. bivia</i> GH13	Glycogen	5.1 ± 0.60	3.8 ± 1.0	1.4 ± 0.3	Amylopullulanase
	Amylose	210 ± 50	0.68 ± 0.33	310 ± 160	
	Pullulan	120 ± 30	1.5 ± 0.6	79 ± 38	

Table 1: Kinetic analysis of vaginal microbial glycogen-degrading enzymes on various carbohydrate polymers at pH 5.5. Values are representative of three experimental replicates over two days. Error range represents one standard deviation above and below the mean.

Site-directed mutagenesis of G. vaginalis PulA confirms the contribution of both active sites to enzyme activity

G. vaginalis PulA contains two α -amylase catalytic domains, suggesting there may be two functional active sites. To probe the activity of each domain individually, active site mutants of each catalytic domain as well as a double active site mutant were constructed by mutating the critical catalytic aspartate residue to alanine (**SI Fig. 5**). The specific activities of the single active site mutants were significantly reduced, retaining approximately 5% of wild-type activity, and the

double mutant was completely inactive (**SI Fig. 5**). This result confirms the individual activity of each active site and suggests they may act synergistically to process glycogen.

The Lactobacillus GDEs maintain activity at low pH

Lactobacillus-dominant communities are typically associated with a lower vaginal pH than mixed anaerobic communities due to their high production of lactic acid, which excludes other microbes¹⁵. We therefore hypothesized that GDEs from *Lactobacilli* may have evolved to maintain activity at a lower pH than those from other vaginal bacteria. Measuring specific activity on glycogen, we screened a pH range from 2.5 to 8.0 (**Fig. 2**). Five of the GDEs have a pH optimum between 5.5 and 6.0, which is consistent with values observed for other characterized bacterial pullulanases and amylopullulanases.³⁹ *P. bivia* PulA had a slightly lower pH optimum of between 4.5 and 5 (**Fig. 2**). We observed that most of the enzymes from vaginal anaerobes – *G. vaginalis* PulA, *M. mulieris* PulA, and *P. bivia* GH 13 – have almost no activity at pH 4.0, which is within the range of a healthy vaginal community.⁴⁰ Critically, however, the *Lactobacillus crispatus*, *Lactobacillus iners* and *Prevotella bivia* PulAs retain 45%, 62% and 71% of their maximal activity at pH 4.0, suggesting these enzymes are better suited for a low pH environment. The ability to compete effectively for host-derived glycogen at low pH may potentially contribute to the stability of *Lactobacillus*-dominant communities.

Fig. 2: *Lactobacillus* amylopullulanases are adapted to a low pH environment pH profiles of six extracellular glycogen-degrading enzymes. Buffer systems consisted of glycine (pH = 2.5 to 3.5), sodium acetate (pH = 4.0 to 5.0), MES (pH = 5.5 to 6.5), HEPES (pH = 7.0 to 8.0). Data is representative of three experimental replicates over two days. Error bars represent one standard deviation above and below the mean.

Breakdown product analysis reveals distinct oligosaccharide product production by GDEs

Next, we sought to characterize the oligosaccharides produced by each enzyme. Following overnight incubations with glycogen, amylose, and pullulan, oligosaccharide production was quantified by LC-MS (**Fig. 3**). Both human amylase and the *P. bivia* GH 13 produced predominantly maltose and a relatively small amount of glucose from glycogen and amylose. In contrast, the enzymes annotated as Type I Pullulanases (PulA homologs) produced longer oligosaccharides in addition to maltose, including maltotriose and in some cases maltotetraose. These results are similar to those observed for previously characterized bacterial amylopullulanases^{41,38}. Amongst the pullulanases, maltotetraose was not detected in the *G. vaginalis* PulA reaction, and was only detected at a low level in *M. mulieris* and *P. bivia*. However, the *Lactobacillus*-derived PulA enzymes produced a higher relative amount of maltotetraose when acting on amylose or glycogen. During incubation with pullulan, all of the bacterial enzymes

produced predominantly maltotriose, however the human salivary amylase was not active on this substrate. The sole production of maltotriose is common among enzymes that degrade pullulan^{37,39,41} and provides direct confirmation that vaginal bacterial GDEs can cleave α -1,6 linkages. This observation further corroborates the functional assignments of these enzymes as either amylopullulanases or pullulanases (**Table 1**).

Fig. 3: *Lactobacillus crispatus* amylopullulanase produces unique breakdown products a. Polymer breakdown products generated following overnight incubation with purified enzyme. LC-MS analysis is representative of three experimental replicates over three days. Error bars represent one standard deviation above and below the mean. Protein abbreviations are as follows, **MM**, *M. mulieris* PulA; **LC**, *L. crispatus* PulA; **LI**, *L. iners* PulA; **PBG**, *P. bivia* GH13, **PBP**, *P. bivia* PulA; **GV**, *G. vaginalis* PulA; **HA**, Human Amylase

Acarbose selectively inhibits GDEs from G. vaginalis and P. bivia

Given the clear biochemical differences revealed in the pH optimum and breakdown product analysis of the bacterial GDEs, and their role in enabling growth on glycogen, we hypothesized that these enzymes may be good targets for possible therapeutic intervention aimed at

establishing a *Lactobacillus*-dominant community. We therefore turned our focus to identifying inhibitors for the GDEs from microbes associated with dysbiosis and screened each purified enzyme against a panel of four clinically relevant human amylase inhibitors. Of the compounds tested, only acarbose and acarviosin showed any inhibition (**Fig. 4a**). We then determined IC₅₀ values for the inhibition of each enzyme by acarbose, the most promising inhibitor. Acarbose inhibited *G. vaginalis* PulA, *P. bivia* PulA, and *P. bivia* GH 13 enzymes with IC₅₀ values of 120 ± 30 µM, 420 ± 90 µM, and 0.84 ± 0.05 µM, respectively, while the *L. crispatus*, *L. iners*, and *M. mulieris* enzymes were largely unaffected (**Fig. 4b**). For comparison, the IC₅₀ of acarbose towards human amylase is approximately 11 µM.⁴²

Since acarbose selectively inhibited GDEs from CST-IV-associated microbes, as a first step toward community modulation, we characterized its effect on the growth of several vaginal microbes. While acarbose inhibited *G. vaginalis* growth on glycogen (IC₅₀ = 0.2 µM), it also inhibited *L. crispatus* growth on maltose (IC₅₀ = 22 µM) and glycogen (IC₅₀ = 0.1 µM), despite the fact that the *L. crispatus* PulA was not affected *in vitro*. Interestingly, growth was not affected when glucose was the primary carbon source (**Fig. 4c**). These data suggest there are additional *L. crispatus* enzymes involved in maltodextrin metabolism that are inhibited by acarbose. Despite potently inhibiting the *P. bivia* GH13 *in vitro*, acarbose had no impact on *P. bivia* growth on any substrate. (**Fig 4c**). This suggests that PulA, which was less inhibited *in vitro*, is the predominant source of extracellular amylase activity, and that, unlike in *L. crispatus*, intracellular maltodextrin catabolism in *P. bivia* is not affected by acarbose. Overall, although acarbose is not a suitable candidate for community modulation due to its broad target spectrum, these results highlight differences between the glycogen-degrading enzymes that may be targeted for selective inhibition.

Fig. 4: Selective inhibition of vaginal microbial GDEs **a.** Effects of known amylase inhibitors (1 mM) on the activities of purified GDEs toward a BODIPY fluorescent starch substrate. **b.** Inhibitory activity of acarbose toward purified extracellular amylases. A BODIPY fluorescent starch substrate was used and activity was normalized to a no inhibitor control. Data are representative of three experimental replicates over two days. Error bars represent one standard deviation above and below the mean of the three trials. **c.** Bacteria were grown in the presence of the indicated concentrations of acarbose in media containing either glucose, maltose or glycogen as the primary carbohydrate source. Growth in the presence of inhibitor was normalized to the untreated control. IC_{50} values were calculated using a least-squares regression of the normalized values. ND (Not determined) is indicated when the resulting curve fit was poor and an IC_{50} value could not be confidently determined. Data are representative of at least two biological replicates over two days and error bars are one standard deviation above and below the mean.

Analysis of CVLs shows activity of human and microbial enzymes

Having identified *bona fide* amylopullulanases in vaginal microbial genomes, we next sought to understand i) if they are expressed in the vaginal environment and ii) the relative contribution of these enzymes compared to the human amylase found in previous work. To do this, we analyzed 21 human cervicovaginal lavage (CVL) sample supernatants and pellets spanning a range of Nugent scores. First, we compared total amylase activity in the samples to

the concentration of human amylase, as determined via ELISA. Across a range of pHs, there was a strong correlation between these measurements (R^2 values; pH 4.4 = 0.832; pH 5.5 = 0.956; pH 6.8 = 0.985) (**Fig. 5a**), suggesting that the majority of the amylase activity in these samples is of human origin. To rule out the possibility that the ELISA signal arose from cross-reactivity of the antibodies with conserved structural features of one or more of the microbial enzymes identified here, we assayed our purified enzymes using the same kit, and found no cross-reactivity at enzyme concentrations as high as 1 μ M (**SI Fig. 6**). Though we cannot rule out the possibility that the antibodies react with additional microbial amylases not identified here, these data strongly corroborate previous findings of human amylase in vaginal samples and suggest that the contribution of this host enzyme in shaping the vaginal microbiome should not be overlooked, despite the existence of microbial enzymes with related activities.

We next attempted to determine if any of the microbial GDEs were present by assaying for activity using pullulan as a substrate, taking advantage of the fact that all the enzymes we examined were active on pullulan, whereas the human amylase was not (**Fig. 3**). Due to limited availability of sample material, our analysis was constrained to a small number of CVLs with high amylase activity (**Fig. 5a**). In one out of the four samples assayed (sample 2171), there was significant enzymatic production of maltotriose from pullulan (**Fig. 5b, SI Fig. 7**). Notably, in this sample significant amylase activity was retained even at low pH, which is inconsistent with the pH profile of the human enzyme. The majority of the pullulanase activity was present in the pellet, consistent with the predicted cell wall localization of the bacterial GDEs. Additionally, in these samples the activity of the supernatant on glycogen was similar to the activity of the pellet on pullulan, suggesting that microbial enzyme(s) constitutes a significant fraction of the total activity. These results demonstrate that vaginal microbial amylopullulanase enzymes contribute to carbohydrate breakdown activity in patient samples, and highlight a need to include both supernatants and microbial pellets in future assays with clinical samples so as not to disregard bacterial cell wall-associated activity.

Fig. 5: Human CVLs samples contain human and microbial amylase activity **a.** Activity of human CVL supernatants correlated with human amylase detection by ELISA at all pH levels. Red circled point denotes sample 2171. Activity was determined using three experimental replicates over two days for each sample. Error bars are representative of one standard deviation above and below the mean. **c.** Four most active human CVL samples degrading carbohydrate polymers into maltotriose detected by LC-MS analysis. Data are representative of two experimental replicates over two days and the error bars are one standard deviation above and below the mean.

Discussion

In this study, we characterized six glycogen-degrading enzymes from vaginal bacterial isolates. Our results demonstrate that, in addition to relying on human amylase, some vaginal bacteria possess alternative enzymes for accessing glycogen. A critical finding of this work is that, despite sharing a common annotation, the substrate preferences of the different PulAs are quite distinct. While the *L. crispatus*, *L. iners* and *G. vaginalis* amylopullulanases had the highest specificity for glycogen, enzymes from other organisms were most active on amylose or pullulan

and had comparatively high K_m values for glycogen. This is consistent with the unique carbohydrate binding modules in each protein and may suggest adaptation to structurally distinct glucose polymers in the vaginal environment. Further, since the oligosaccharides produced from glycogen breakdown are released extracellularly and may act as ‘public goods’⁴³, the differences in the product distribution of these enzymes may suggest differential availability of oligosaccharides between community state types, supporting the growth of distinct non-glycogen-degrading bacteria via cross-feeding, as has been shown within the gut microbiota¹³. A better understanding of the structure of glycogen within the vaginal environment, and whether it is different among CSTs, is needed to further evaluate this possibility.

Our work also suggests a potential mechanism for *L. crispatus* dominance in a low pH environment. The higher relative activity of the *L. crispatus* amylopullulanase at the healthy vaginal pH (~3.5-4) may provide an advantage in accessing glycogen, facilitating exclusion of bacteria with GDEs which are less active under these conditions, and/or bacteria relying on glycogen breakdown products released by human amylase, which is also minimally active in this pH range. Critically, the pH optimum of amylases cannot be predicted from primary sequence analysis⁴⁴, further highlighting the need for biochemical characterization to support bioinformatic interrogations of microbial function within communities.

Though our work shows that *Lactobacillus crispatus* encodes an enzyme that allows growth on glycogen, interesting questions remain about how other common vaginal *Lactobacilli* lacking a *pulA* homolog can dominate a community, for example *Lactobacillus jensenii* (CST V) and *Lactobacillus gasseri* (CST II). Possibly these microbes rely on cross-feeding of oligosaccharides produced by human amylase or GDEs from other vaginal microbes, since they are capable of growth on a range of maltooligosaccharides¹⁸.

Our efforts to identify inhibitors for bacterial GDEs demonstrate that these enzymes have sufficient biochemical diversity to be selectively targeted. Unfortunately, growth assays in bacterial cultures suggest these existing amylase inhibitors have additional effects on

maltodextrin metabolism that limit our ability to target specific bacteria. Future work could examine other differences in maltooligosaccharide catabolism between vaginal strains, and phenotypic screening approaches may help identify inhibitors that favor the growth of *Lactobacillus* strains through the collective inhibition of amylases from both bacteria associated with dysbiosis, and the host.

Finally, our characterization of patient CVLs demonstrated that microbial GDEs are not only found in vaginal microbial genomes, but are likely also expressed and active in their native environment. Understanding the relative contribution of human and microbial enzymes to glycogen catabolism may provide insight into microbial community dynamics. Our results, and those from other efforts (Forney, Spear)^{20,18} show that the presence of bacterial glycoside hydrolases in patient samples is highly variable, thus this line of investigation would benefit from testing larger numbers of clinical samples. Yet current methods for analyzing the specific contribution from microbial enzymes rely either on metaproteomics or zymography,²⁰ which are not widely accessible in clinical labs. Here we showed that a simple biochemical pullulanase assay can be used to identify microbial-specific activity in clinical samples, and we anticipate this methodology will find broad utility in the analysis of patient samples to gain further insight into potential roles of bacterial GDEs in community stability. Overall, the ecologically relevant insight gained from this investigation highlights the need for complementing bioinformatic analysis with detailed biochemical enzyme characterization.

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Author Contributions

EPB and BMW conceived the study. BMW and DJJ designed and conducted enzyme purification and biochemical characterization experiments. BMW, DJJ, and EPB wrote the manuscript. DJJ and MIH designed and conducted bacterial growth experiments. All authors contributed to the interpretation of data, were involved in the revision of the manuscript, and approved the final manuscript. BMW and DJJ contributed equally to the study.

Competing Interests

The authors declare no competing interests

Methods

Reagents

Unless otherwise noted, commercial chemicals were of the highest purity available and purchased from Sigma-Aldrich. Acetonitrile for LC-MS was purchased from Honeywell-Burdick & Jackson.

Identification and cloning of glycogen degrading enzymes

Homologs of Pula in *L. crispatus*²¹ (EEU28204.2) were identified by BlastP search of genomes from vaginal isolates in the IMG database⁴⁵ using an E-value cut-off of 1×10^{-5} . Hits were further curated by removing proteins with no predicted signal peptide (SignalP v5.0⁴⁶). A panel of six candidates with >35% amino acid identity from microbes associated with health or disease were

selected for further study. The strains were obtained and genomic DNA was extracted with a DNeasy UltraClean Microbial Kit (Qiagen). Genes were amplified via PCR with primers designed to remove the signal peptide (**SI Fig. 2**) and cloned into the *E. coli* expression vector pET28a (Novagen) via Gibson assembly (New England Biolabs, NEB) to generate an N-terminal His₆-tagged gene. All plasmids were verified via Sanger sequencing (Eton Biosciences) and transformed into the expression host BL21 (DE3) (*P. bivia* enzymes) or ArcticExpress (DE3) (all other enzymes) for expression and purification. Complete lists of plasmids and primers are provided in Supplementary Tables 1 and 2, respectively.

Purification of glycogen degrading enzymes

Cultures containing expression plasmids were grown in 2-6 L of LB (Research Products International, RPI) containing 50 µg/mL kanamycin. Once cultures reached an optical density at 600 nm (OD₆₀₀) of 0.6-0.8, they were cooled to 15 °C and induced with 250 µM IPTG (Teknova). After 16 h at 15 °C, cells were harvested (6720 x g for 10 min at 4 °C) and the pellets were stored at -20 °C until use. Pellets were resuspended in 98% Buffer A (50 mM HEPES, 300 mM KCl, 10% glycerol, pH 7.8), 2% Buffer B (50 mM HEPES, 300 mM KCl, 10% glycerol, 500 mM imidazole, pH 7.8) supplemented with EDTA-free protease inhibitor cocktail (Sigma). Cells were lysed via homogenization (3 x 15,000 psi, Emulsiflex-C3, Avestin) and lysates were clarified (16,000 x g for 45 min at 4 °C) before being loaded onto a 5 mL HisTrap column (GE Healthcare). This was followed by one column volume (c.v.) of 2% Buffer B, and 2 c.v. of 10% Buffer B. Protein was eluted using a linear gradient from 10 to 100% Buffer B over 20 c.v., and protein-containing fractions and purity was determined by SDS-PAGE (Biorad) The following day, protein-containing fractions were pooled, concentrated to a volume of approximately 1 mL in a spin concentrator (Millipore), and purified by size exclusion chromatography (GE Healthcare, Superdex 200) in 100% buffer A. Fractions were again analyzed by SDS-PAGE and protein-containing fractions were pooled, concentrated (Millipore, Amicon 30 kDa), flash frozen in liquid nitrogen, and stored

at -80 °C until use. Protein concentration was determined using a bradford assay (biorad).
Molecular weights used for concentration determination was done using the native protein
sequences predicted in EXPASY.

L. crispatus purified protein growth recovery assay

MRS broth containing glucose (BD Difco) was prepared according to the manufacturer's protocol.
For growth assays on different carbon sources, MRS broth without glucose (Food Check
Systems) was prepared according to the manufacturer's recipe and supplemented with either 2%
D-glucose (Sigma), 2% maltose monohydrate (VWR), or 5% glycogen from oyster (Sigma). Each
media type was filter sterilized (.2 µm) and left inside an anaerobic chamber with an atmosphere
of 2.5 % H₂, 5 % CO₂, 92.5 % N₂ (Coy Labs) overnight for equilibration. Starter cultures of *L.*
crispatus C0176A1 (*pulA*⁻) and *L. crispatus* MV-1A-US (*pulA*⁺) were inoculated into MRS media
(BD Difco) in hungate tubes (VWR) and incubated overnight at 37 °C. The next day, purified
protein was thawed and added to 5% glycogen MRS media to a concentration ranging between
200-400 nM. After protein addition, the media was again filter sterilized before use. As a negative
control, protein boiled at 100 °C for 15 min was also used in the assay. Immediately after protein
addition, 50 µL of each media type was aliquoted into a 384-well TC-treated, clear microplate
(Corning). 1 µL of overnight culture was used to inoculate each well. The plate was sealed (VWR),
and growth was monitored in a plate reader (Biotek) inside of an anaerobic chamber (Coy Labs)
for 24 h by measuring OD₆₀₀ every 15 min while incubating at 37 °C. Growth conditions contained
2.5% H₂, 97.5 % N₂ with oxygen levels maintained below 20 ppm. Three experimental replicates
over three days were performed with three to four technical replicates on each day.

Kinetic analysis of glycogen-degrading enzymes

Kinetic analysis of glycogen-degrading enzymes was preformed using a previously described
reducing sugar assay,³⁷ modified for a 96-well format. 300 µL reactions were set up containing

substrate (0.0048-10 mg mL⁻¹ glycogen; 0.0012-1.25 mg mL⁻¹ Pullulan (Megazyme); or 0.0048-1.25 mg mL⁻¹ amylose in a final concentration of 2% DMSO), 0.8-700 nM enzyme, and reaction buffer (20 mM sodium acetate, pH 5.5, 0.5 mM CaCl₂). Reactions were incubated at 37 °C for 15 min and 50 µl aliquots were removed (2, 5, 7.5, 10, 15 min) into 125 µL of the BCA stop solution (0.4 M sodium carbonate, pH 10.7, 2.5 mM CuSO₄, 2.5 mM 4,4'-dicarboxy-1,2'-biquinoline, 6 mM L-serine). After 30 min incubation at 80 °C, 125 µL was transferred to a TC-treated flat bottom plate (Greiner Bio) and absorbances were read at 540 nm. A maltose standard curve (0.000610-0.625 mg mL⁻¹) was used to quantify hydrolysis activity. Initial velocities were calculated via linear regression selecting the data points that produced the highest initial rate utilizing at least three data points. K_M and k_{cat} parameters were determined by fitting the Michaelis-Menten equation to the initial velocity data using nonlinear regression (Graphpad Prism 8). Replicates consisted of three experimental trials over two days.

Enzyme pH profile and G. vaginalis Pula active site mutant activity on Glycogen

The reducing sugar assay described above was used to determine the dependence of activity on pH. Reactions contained 1.25 mg mL⁻¹ glycogen, 0.9 to 1050 nM enzyme, and assay buffer ranging in pH from 2.5 to 8.0 (20 mM glycine, 0.5 mM CaCl₂, pH 2.5-3.5; 20 mM sodium acetate, 0.5 mM CaCl₂, pH 4.0-5.0; 20 mM MES, 0.5 mM CaCl₂, pH 5.5-6.5; 20 mM HEPES, 0.5 mM CaCl₂, pH 7.0-8.0). Initial velocities were determined for each condition and normalized to enzyme concentration. A maltose standard curve (0.000610-0.625 mg mL⁻¹) was used to quantify hydrolysis. Replicates consisted of three experimental replicates over two days. Specific activity *G. vaginalis* Pula active site mutants were performed as described above at pH 5.5.

Polysaccharide breakdown product analysis and growth studies

To measure polysaccharide breakdown products, reactions were set up containing 10 mg mL⁻¹ substrate and 500 nM enzyme, all dissolved in assay buffer (20 mM sodium acetate, 0.5 mM

CaCl₂, pH 5.5) and incubated at 37 °C overnight. The next day, samples were quenched with a 10-fold dilution into 90% LC-MS grade acetonitrile. The plates were centrifuged (3220 x g for 10 min, 4 °C) and the samples were diluted 1000-fold into LC-MS grade acetonitrile before analysis by LC-MS. An ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) system model Xevo TQ-S (Waters) was used in this study. The mass spectrometer system consists of a triple quadrupole equipped with an electrospray ionization probe (ESI). 5 µL of sample was injected onto an Acquity BEH/Amide UPLC Column heated to 40 °C (Waters, 1.7 µm, 130Å, 2.1 mm x 50 mm). A flow rate of 0.5 ml min⁻¹ was used. The following gradient was applied: 0-1.0 min at 97 % B (acetonitrile with 0.1% formic acid) and 3% A (H₂O with 0.1% formic acid) isocratic, 1.0-4.0 min 97-30% B, 4.0-5.0 min at 30% B isocratic, 5.0-5.1 min at 30-97% B, 5.1-7.0 min at 97% B isocratic. Carbohydrate products were detected by ESI in positive mode (capillary voltage 3.10 kV; cone voltage 42 V; source offset voltage 50 V; desolvation temperature 500 °C; desolvation gas flow 1000 L/hr; cone gas flow 150 L/hr; nebuliser 7.0 bar). See supplementary information for compound specific detection parameters (**SI table 3**). For quantification, standards of glucose, maltose (VWR), maltotriose (Carbsynth), maltotetraose (Carbsynth), and maltopentaose (Carbsynth) were prepared ranging from 0.001-10 µg mL⁻¹ in 9:1 acetonitrile:water. Oligosaccharide peak areas were quantified using the standard curve. These data consisted of three experimental replicates run over three different days.

Inhibitor growth assays

Inhibition growth assays were performed in an anaerobic chamber (Coy Labs) with an atmosphere of 2.5 % H₂, 5 % CO₂, 92.5 % N₂. Bacteria were inoculated from single colonies into a peptone-yeast extract base broth (PYTs) consisting of proteose peptone (20 g L⁻¹), yeast extract (10 g L⁻¹), MgSO₄ (0.008 g L⁻¹), K₂HPO₄ (0.04 g L⁻¹), KH₂PO₄ (0.04 g L⁻¹), NaHCO₃ (0.4 g L⁻¹), vitamin K (0.0025 g L⁻¹), hemin (0.005 g L⁻¹), L-cysteine • HCl (0.25 g L⁻¹), Tween 80 (0.25 mL L⁻¹), horse serum (50 mL L⁻¹), and glucose (2 g L⁻¹), and incubated at 37 °C for approximately 24 h. Cultures

were adjusted to OD₆₀₀ 0.4-0.5 , sub-cultured at a 1:50 dilution into fresh PYTs (without glucose) with the indicated carbohydrates added to a final concentration of 2 g L⁻¹. Glycogen was from oyster (Sigma, G8751). Assays were performed in duplicate in 384-well plates, sealed with BreathEasy gas permeable membranes (Diversified Biotech) under anaerobic conditions. Bacterial growth was monitored by measuring the OD₆₀₀ at 1 h intervals for 48 h in a BioTek Epoch2 plate reader. Data are representative of at least two independent experiments performed on separate days. Data were normalized to blank (uninoculated) media. For inhibition assays, bacteria were cultivated as above, with the addition of acarbose at the indicated concentrations. Half maximal inhibitory concentrations (IC₅₀) were calculated using growth data from each tested concentration of inhibitor taken from the time the untreated control reached stationary phase. Data were then normalized to the untreated control, and IC₅₀ values were calculated using a least squares regression (Graphpad Prism 8).

Patient CVL analysis

Patient CVLs were obtained from Dr. Caroline Mitchell at Massachusetts General Hospital (IRB: 2014P001066). CVLs were collected using 3 mL of sterile saline, washed over the cervix and vaginal walls with a transfer pipette, and then re-aspirated. Samples were centrifuged (10,000 x g for 10 min at 4 °C) and the supernatants were decanted and used in the assay. Purified proteins were diluted in Buffer A (50 mM HEPES, 300 mM KCl, 10% glycerol, pH 7.8) to 1 µM, then used in the assay. Human salivary amylase was purchased from Sigma Aldrich (A1031-1KU). Human amylase was detected in CVLs using an ELISA for human pancreatic amylase (Abcam ab137969) according to manufacturer's instructions. Two experimental replicates were conducted over two days, and two technical replicates of the standard curve were also measured each day.

Activity of CVL supernatants was determined using the EnzCheck Ultra Amylase Assay Kit (ThermoFisher, E33651). The fluorescent substrate was prepared according to the kit

instructions using three different buffers (20 mM sodium acetate, 0.5 mM CaCl₂, pH 4.4; 20 mM sodium acetate, 0.5 mM CaCl₂, pH 5.5; 20 mM MES, 0.5 mM CaCl₂, pH 6.8). 10 µL of CVL supernatant was added to each well of a clear-bottom black 96-well plate and then diluted with 40 µL of buffer. The reactions were initiated with 50 µL of substrate and incubated for 30 min at 37 °C. Readings were taken every 51 s by monitoring an excitation of 485 nm and an emission of 528 nm. Initial rates were calculated in the plate reader software (Biotek) by determining the highest slope that covered at least 5 data points.

CVL cell pellets were resuspended in approximately 100 µL of filter sterilized (0.2 µM) resuspension buffer (PBS + 10 % DMSO, pH 7.2). 10 µL of sample (CVL supernatants and pellets) was added to 190 µL of 10 mg mL⁻¹ substrate (glycogen or pullulan) that had been resuspended in assay buffer (20 mM sodium acetate, 0.5 mM CaCl₂, pH 5.5). The reactions were incubated at 37 °C and timepoints at 3, 5, 8, and 24 h were taken by diluting 100-fold into 9:1 acetonitrile:water. Samples were further diluted 1,000-fold in LC-MS grade acetonitrile and analyzed by LC-MS as described above for the detection of maltotriose. Samples were normalized to a no enzyme control. Two experimental replicates over two days were tested for each sample.

Inhibitor screening and IC₅₀ determination for acarbose

The inhibitory effect of a panel of four small-molecule inhibitors was determined using a modification of the assay above. For initial screening, enzymes were preincubated with 1 mM each inhibitor for 15 min at room temperature. For IC₅₀ analysis, enzyme (2.5-50 nM) was preincubated for 15 min at room temperature with acarbose (Abcam) ranging from 0.366 µM to 3000 µM in a total volume of 50 µL. The reactions were initiated with 50 µL of substrate and incubated for 30 min at 37 °C monitoring with an excitation of 485 nm and an emission of 528 nm. Initial rates were calculated in the plate reader software (Biotek) by determining the highest slope that covered at least 8 data points. Percent activity was calculated by normalizing the activity to

568 a no inhibitor control. IC₅₀ values were calculated using a nonlinear fitting of the data to the
 569 inhibitor vs normalized response function (GraphPad Prism 8). Error associated with the IC₅₀
 570 values represents 95% confidence intervals.

571

572 *Data Availability*

573 All raw data that support the findings of this study are available from the corresponding author
 574 upon reasonable request.

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