Bacterial vaginosis is associated with increased amylolytic activity

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Abstract

Glycogen is the most abundant vaginal carbohydrate in reproductive aged women. Reduced vaginal glycogen is associated with lower levels of *Lactobacillus crispatus*, overgrowth of fastidious anaerobes such as *Gardnerella vaginalis* and increased risk of adverse reproductive and sexual health outcomes. Here we show that *Gardnerella vaginalis*, *Lactobacillus iners* and *Lactobacillus crispatus* can autonomously utilize glycogen as a source for growth. Using an ungelatinized and labeled form of raw amylose, a more degradation-resistant α-1,4-glucan, we were able to discriminate between the alpha-glucosidase activities of common vaginal bacterial species. The bacteria *Lactobacillus crispatus*, *Lactobacillus iners, Gardnerella piotii* as well as several other common vaginal species were not capable of raw amylose degradation, while *Gardnerella vaginalis*, *Gardnerella swidsinskii* and *Gardnerella leopoldii* were, with the latter two having the highest degradation rates. In contrast to the glycogen-degrading activity we previously identified in *Lactobacillus crispatus*, this *Gardnerella* alpha-glucosidase activity was not cell-bound and not repressed in the presence of glucose. Raw amylose degradation activity in vaginal swabs was strongly associated with bacterial vaginosis as assessed by Nugent scoring. Overall, our results show that the dysbiotic microbiota of bacterial vaginosis is associated with increased amylolytic activity, which is also found in pure cultures of *Gardnerella* species, but not in other common vaginal bacteria.

Introduction

The vaginal mucosa of reproductive aged women is rich in glycogen (1, 2). Through shedding and lysis of the superficial vaginal epithelial cells, glycogen is released in the vaginal lumen where it is the primary carbon and energy source for bacteria colonizing the vagina. Glycogen levels were found to strongly depend on the vaginal microbiota composition. The more prevalent *Lactobacillus*-dominated vaginal microbiota is associated with higher levels of glycogen compared to the more diverse *Lactobacillus*-deprived microbiota with higher levels of fastidious anaerobes such as *Gardnerella*, *Fannyhessea* and *Prevotella*. This microbial state is also referred to as bacterial vaginosis (BV) and is a general risk factor for adverse reproductive and sexual health outcomes, such as HIV infection (3), HPV infection (progression) (4), subfertility (5), pelvic inflammatory disease (6), pregnancy loss (7) and preterm birth (8). Therefore, it is important to develop simple, low-cost screening options for bacterial vaginosis.

Gardnerella has been identified as a hallmark genus in bacterial vaginosis. Although this microbial state has a much higher bacterial diversity compared to the *Lactobacillus* dominated microbiota, *Gardnerella* alone can cause many of the (clinical) manifestations of bacterial vaginosis, including exfoliation of the epithelial wall lining the vagina and thinning of the mucus leading to abnormal secretions (9–11).

Alpha-glucosidases are enzymes that catalyze the hydrolysis of α1-4 or α1-6 linkages between glucose units of alpha-glucans. Alpha-glucosidases of vaginal bacteria are hypothesized to take a central role in carbon and energy metabolism by extracellular degradation of host-associated glycogen followed by transport and metabolism of the resulting smaller dextrins. Previously, we have elucidated the genetic basis, regulation and prevalence of one of these alpha-glucosidases: the *L. crispatus* amylopullulanase (12–14). Strikingly, we found a relatively high number of *L. crispatus* strains to be deficient for this amylopullulanase and identified frequently occurring mutations in its gene in the genomes of these strains as well as in

databases of the vaginal metagenome (14, 15). Insight in the ability of other common vaginal microbes to digest alpha-glucans such as vaginal glycogen remains forthcoming.

Most studies characterizing the alpha-glucosidases of vaginal bacteria have used soluble starch or glycogen (16–18). Yet, for the gut microbiome it was shown that the structure of the alpha-glucan has an important role in distinguishing between enzymes of different bacterial taxa. Animals fed with alpha-glucans, that are more resistant to degradation due to a denser structure and a relatively lower degree of branching, show an increase of amylolytic species such as *Bifidobacteria* (19, 20). These bacteria dispose of a type II amylase-pullulanase fusion protein capable of hydrolyzing resistant starch (21, 22). A heterologously expressed homologue of this enzyme in *Gardnerella* was found to degrade amylose, pullulan, glycogen and starch (23). Recent research showed that 14 out of 15 tested *Gardnerella* strains carried a full copy of this gene (17) and that isolates of four different *Gardnerella* species can transport and metabolize the small maltodextrins derived from glycogen breakdown (24).

Here we characterized the ability of common vaginal bacteria *L. crispatus*, *L. iners* and *Gardnerella* species to degrade a more resistant alpha-glucan: raw amylose. We show that *Gardnerella* species can be distinguished from common vaginal lactobacilli by their ability to break down raw labeled amylose. We further show that the degradation is significantly associated with vaginal dysbiosis.

Results

***Gardnerella* and *Lactobacillus iners* can use glycogen as the sole carbon source for growth.**

The optical density of *L. crispatus*, *L. iners* and *G. vaginalis* after anaerobic growth in NYCIII medium with glucose or glycogen is shown in Figure 1. As expected, and previously shown, the amylopullulanase-deficient strain of *L. crispatus* did not grow on glycogen, whereas the amylopullulanase-sufficient strain grew to a comparable optical density on glycogen as on glucose. The *L. iners* strain showed an equal optical density on glycogen compared to glucose, while the two *G. vaginalis* strains showed a higher optical density on glycogen compared to glucose. No increase in optical density was observed compared to the cell-free control when growing isolates of either species in NYCIII medium without glucose or glycogen (results not shown).

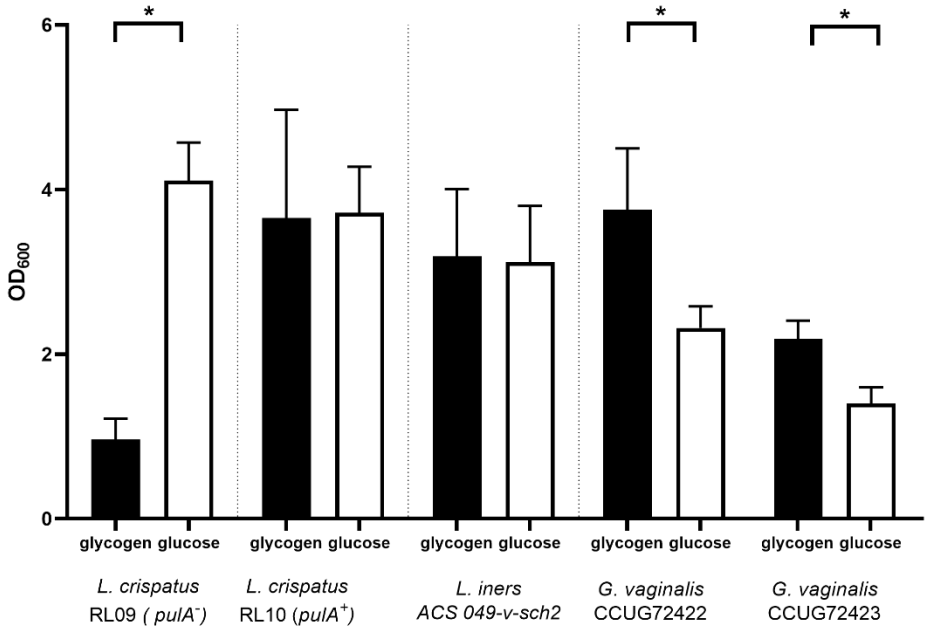

Figure 1: Optical density after 48 hours of growth in liquid NYCIII medium with 5 g/L glucose (white bars) or 5 g/L glycogen (black bars). All measurements were performed in 3 biological replicates in separate experiments on separate days except for *G. vaginalis* CCUG72423 where data from two biological replicates is used. Data represent average and error bars are the standard deviation. Paired t-test was used to compare averages between glycogen and glucose. * = $p < 0,05$.

Multi-well assay with undissolved labeled amylose to measure enzymatic rate of alpha-glucosidases.

Next, we further characterized alpha-glucosidase activity of *L. crispatus*, *L. iners* and *Gardnerella vaginalis* using a quantitative amylase assay with raw amylose labelled with a covalently linked azure cross-linked (AZCL) chromogenic substrate (25) in a microtiter plate. This assay is conventionally used to study amylase activity using agarose plates or in dissolved form but is applied here using undissolved granules in a xanthan gum buffer to keep the substrate dispersed.

The release rate of blue dye is dependent on the enzymatic digestion rate of the labelled glycan (see a video of the dye diffusion in Supplementary Video S2 and calibration curves in Supplementary Figure S3). No pretreatment, purification or dialysis of the sample is required, and the assay is suitable for use in multi-well format in a plate reader as well as for visual detection.

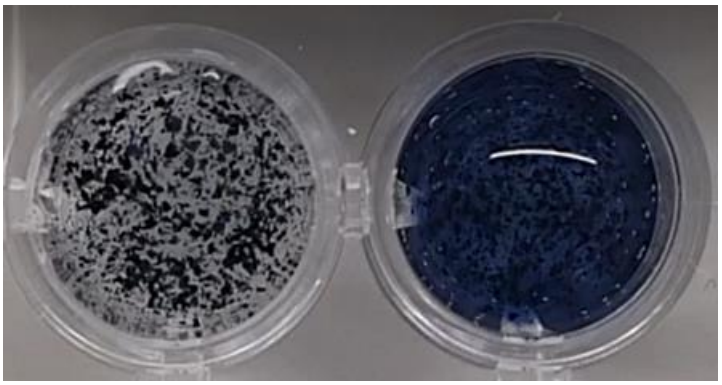

Figure 2: The AZCL-amylose substrate in xanthan gum buffer with buffer (negative control, left) or after mixing with saliva (positive control, right). Still image from the video of the enzymatic assay Supplementary Video S2.

***Gardnerella vaginalis* shows extracellular amylose-degrading activity**

In Figure 3, the enzymatic activity in pellets and supernatants as determined by the amylose degradation assay is shown. All cultures had a minimal OD600 of 1.0. The amylose degradation assay showed that the *G. vaginalis* strains were capable of degrading the raw amylose substrate, while in *L. crispatus* and *L. iners* cultures, no increase of optical density

due to labeled amylose digestion was observed. The activity was mostly found in supernatants of *G. vaginalis* and to a limited extent associated with the cell pellet. The activity was also present in *G. vaginalis* cultures grown on medium with glucose, indicating that *G. vaginalis* alpha-glucosidase activity is not subject to carbon-catabolite repression, as observed for the glycogen-degrading activity of *L. crispatus*.

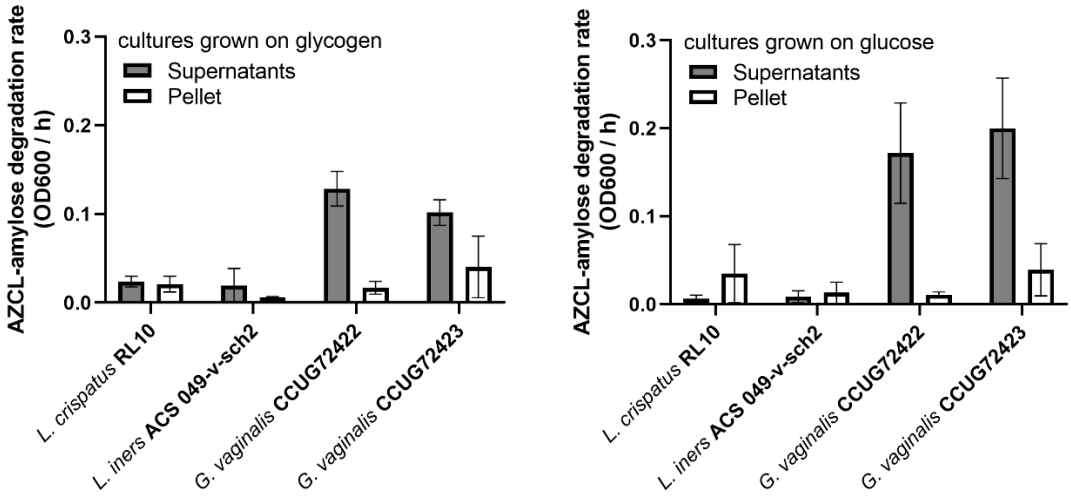

Figure 3: AZCL-amylose degradation rate expressed as the increase of optical density at 600 nm per hour in resuspended cell pellets (white bars) and supernatants (grey bars) from *Lactobacillus crispatus* RL10 (*pulA*$^{+}$), *Lactobacillus iners* ACS 049-v-Sch2 and *Gardnerella vaginalis* strains CCUG 72422 and CCUG 72423. Cultures were grown anaerobically in NYCIII medium with glycogen (left panel) or glucose (right panel) for 48 hours. Bars represent the mean and error bars represent standard deviation of three independent biological replicates, except for *G. vaginalis* CCUG72423 on glucose (two biological replicates) and *G. vaginalis* CCUG72422 on glycogen (four biological replicates).

Gardnerella species show variation in amylolytic activity

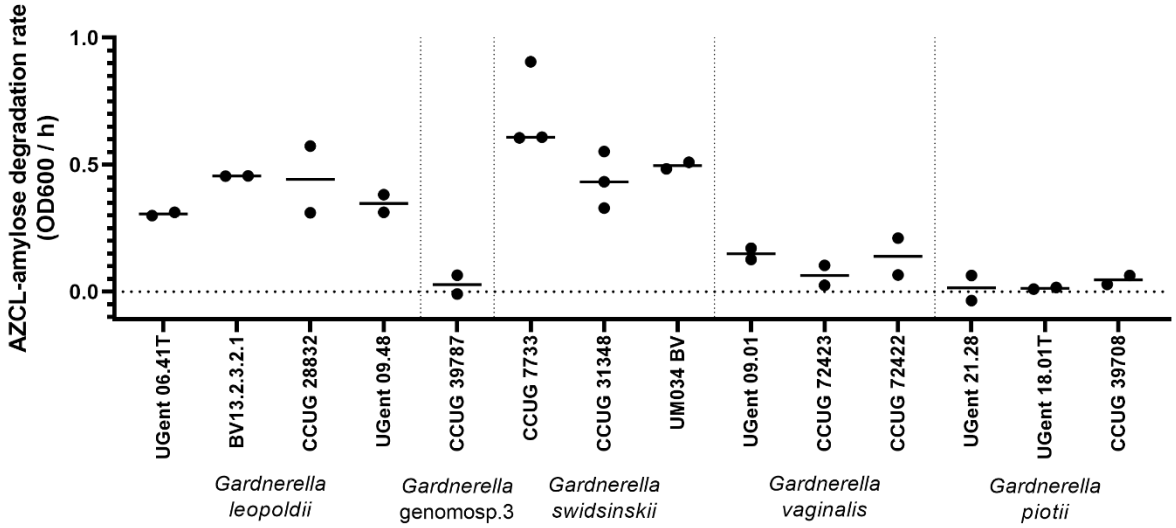

Figure 4: AZCL-amylose degradation rate by optical density increase at 600 nanometer of cultures (cells in growth medium) of various human vaginal isolates grown for 48 hours on NYC III medium with glucose. Data points represent biological replicates that were corrected for a medium control.

AZCL-amylose degradation activity of different *Gardnerella* species is shown in Figure 4. Activity varied across *Gardnerella* species: none of the three *G. piotii* isolates showed degradation activity in the assay whereas *G. swidsinskii* and *G. leopoldii* showed increased activity compared to *G. vaginalis*. When all biological replicates of the three *G. vaginalis* strains (6 measurements) were compared to all biological replicates of the *G. piotii* strains taken together (6 measurements) a significant difference was observed between the degradation of strains belonging to these species (Mann Whitney $p<0.05$). No amylase activity was observed in any of the lactobacilli or other vaginal genera such as *Fannyhessea*, *Prevotella*, *Bifidobacterium* and *Candida* (Supplementary Figure 4).

AZCL-amylose degradation rate is associated with Nugent score but not with abundance of specific *Gardnerella* species.

The substantial differences in raw amylose degradation activity between three of the most common vaginal species prompted us to study the presence of this enzymatic activity in vaginal

samples. We analyzed amylose degradation in 96 vaginal samples from a collection of swabs obtained from women visiting an outpatient Sexually Transmitted Disease clinic in Amsterdam, as described previously (12). The degradation rate in clinical samples across Nugent categorization is shown in Figure 5.

Degradation of amylose was strongly associated with Nugent group. Namely, samples from women with Nugent score between 7-10 (bacterial vaginosis) showed a mean amylose degradation rate of 0,41 OD600/h +/- 0,27 compared to 0,22 OD600/h +/- 0,18 (mean +/- standard deviation) in women with Nugent score 0-3 (indicating a *Lactobacillus* dominated microbiome) (Mann-Whitney, p=0,0001). Women with an intermediate Nugent score (4-6) showed a mean degradation rate of 0,33 +/- 0,33 OD600/h, which was also significantly lower compared to women with bacterial vaginosis (*p*-value 0,039).

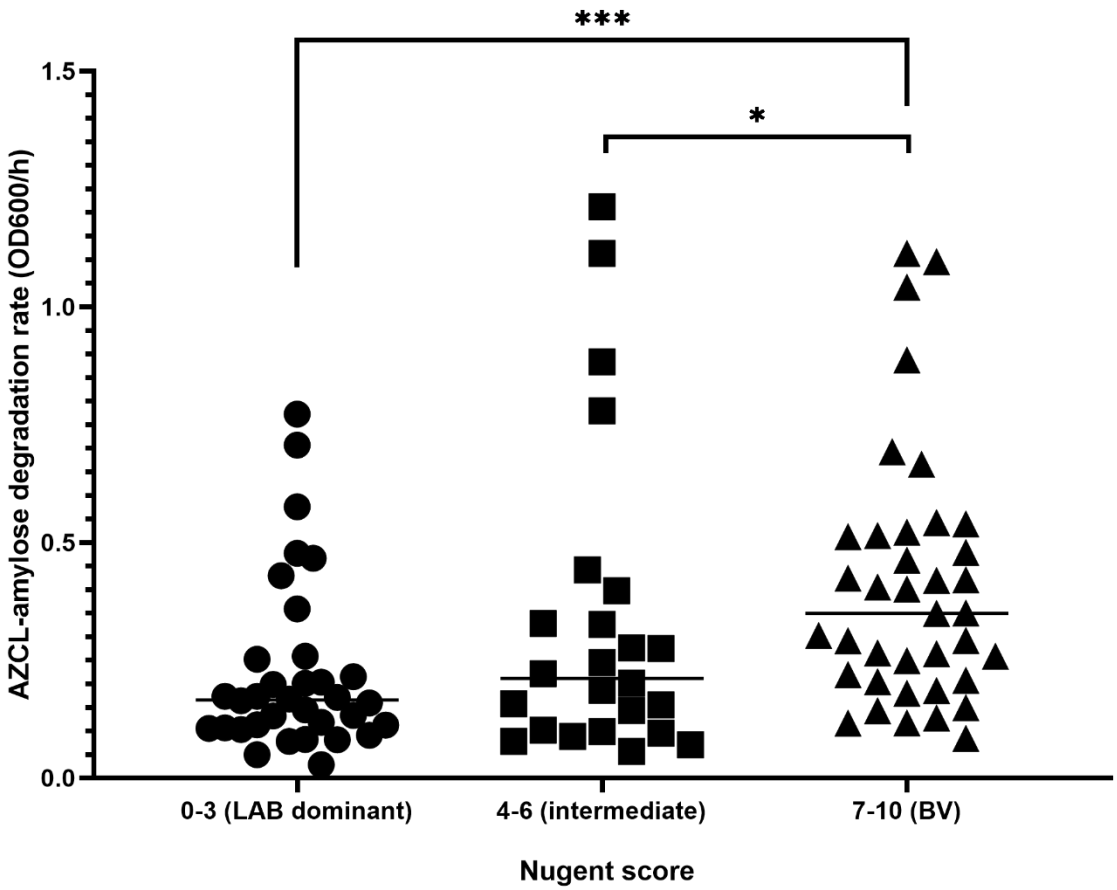

Figure 5: AZCL-amylose degradation rate of 96 vaginal samples with *Lactobacillus* (LAB)-dominant microbiome (Nugent score 0-3), intermediate microbiome (Nugent-score 4-6) or bacterial vaginosis

(Nugent score 7-10). *p*-values were calculated by Mann-Whitney test and displayed as * $p < 0.05$ and *** $p < 0.0005$. Horizontal bars indicate the mean.

In growth experiments, a higher amylose degradation rate was observed in cultures of *Gardnerella* species compared to *Lactobacillus* species. Therefore, we hypothesized that the observed difference in samples from women with high Nugent scores (bacterial vaginosis) compared to women with low Nugent scores may be due to higher *Gardnerella* abundance in the high Nugent score group. We therefore assessed whether the measured amylose degradation rate was associated with *Gardnerella* abundance and found a positive correlation between AZCL-amylose degradation rate and levels of *Gardnerella* and *Fannyhessea* (r of 0,24 and 0,23, *p*-value of 0,016 and 0,028, respectively) that was nevertheless non-significant after Bonferroni correction for multiple testing (4 tests). Since *Gardnerella* showed strong interspecies differences in amylolytic activity *in vitro*, we continued to quantify the various *Gardnerella* species using species-specific qPCR. Given the high amylose degradation rate of *G. leopoldii* and *G. swidsinskii* strains and the low rate in strains of *G. piotii* (Figure 4), we expected to find a high abundance of *G. leopoldii* and *G. swidsinskii* in vaginal samples with high Nugent scores and high AZCL-amylose degradation rate, and samples with high Nugent score but low degradation rates to have less *G. leopoldii* and *swidsinskii*. However, none of the *Gardnerella* spp. concentrations showed a significant correlation with AZCL-amylose degradation rate of the vaginal samples, using a *p*-value threshold that was corrected using Bonferroni for multiple testing (Table 1).

Table 1: correlation between raw AZCL-amylose degradation rate and bacterial abundance in vaginal samples measured by two different qPCR protocols. Spearman's rank correlation coefficient (R_s) for log10 transformed genome equivalents and AZCL-amylose degradation rate with approximate *p*-value (*p*-value of < 0,013 are considered significant after Bonferroni correction for multiple testing).

Species	Assay	R_s (*p*-value)
Gardnerella	BV diagnostic PCR	0.245 (0.016)
Fannyhessea	BV diagnostic PCR	0.224 (0.028)
Lactobacillus	BV diagnostic PCR	-0.035 (0.734)
Bacteria	BV diagnostic PCR	0.211 (0.084)
Gardnerella leopoldii	*Gardnerella* species-specific qPCR	0.084 (0.417)
Gardnerella piotii	*Gardnerella* species-specific qPCR	0.096 (0.352)
Gardnerella swidsinskii	*Gardnerella* species-specific qPCR	0.192 (0.060)
Gardnerella vaginalis	*Gardnerella* species-specific qPCR	0.238 (0.020)

Discussion

In this study we set out to characterize extracellular alpha-glucosidases of key member species of the vaginal microbiota. These enzymes catalyze the degradation of α1-4 and/or α1-6 linkages between glucose units of alpha-glucans such as amylopectin, glycogen and amylose. The female reproductive tract between puberty and menopause is rich in glycogen (26). Vaginal bacteria that express and secrete glycogen-degrading enzymes gain access to these carbon and energy stores in a general nutrient-poor environment.

Most *L. crispatus* strains express an amylopullulanase that is capable of degrading starch and allows growth on glycogen as a single carbon source (13–15). Despite the ability to degrade starch, here we showed that *L. crispatus* is unable to break down a more degradation resistant alpha-glucan: raw amylose. Amylose consists of α1-4 linked glucose units and has a denser structure compared to α-glucans such as amylopectin, pullulan and glycogen due to the absence of branches (that are introduced by α1-6 linkages). Vaginal lactobacilli as well as other common vaginal species, such as *Fannyhessea vaginae*, *Prevotella bivia* and *Candida albicans* were all unable to degrade raw labeled amylose. Furthermore, we saw profound differences in the raw amylolytic activity of various *Gardnerella* species, which went previously undetected using glycogen as a substrate (17).

These findings are contrasting previously published results on vaginal bacterial alpha-glucosidases. A characterization of a variety of amylopullulanases from vaginal species showed that alpha-glucosidases of *L. crispatus, L. iners, G. vaginalis* and *Prevotella bivia* were capable of amylose degradation in addition to glycogen and pullulan, when heterologously expressed in *Escherichia coli* (23). The difference may stem from the type of amylose used. Woolston *et al*. used amylose dissolved in DMSO which may alter the crystal structure and therefore the physical accessibility, whereas in our study we have used ungelatinized labeled amylose kernels. A second difference may be caused by the cleavage of the signal peptide in their study and the heterologous expression in *E. coli* which may lead to posttranscriptional modification of these enzymes.

Previously, resistant starches (such as high amylose corn starch) were found to modulate the gut microbiome. High amylose corn starch is degraded by specific gut genera, one of which is *Bifidobacterium* that is closely related to the genus *Gardnerella*. The amylopullulanase of bifidobacteria has the same carbohydrate-binding, amylase and pullulanase domains, N-terminal signal peptide and C-terminal transmembrane domain as the *Gardnerella* gene that is likely responsible for the observed raw amylose degradation activity (17, 23).

Using raw amylose we found strong differences in amylolytic activity between *Gardnerella* species and other vaginal genera. Besides substrate degradation we also found differences between the regulation. Alpha-glucosidase activity of *G. vaginalis* was in this study also observed in cultures grown on glucose whereas we previously found *L. crispatus* amylopullulanase to be repressed in the presence of the products of glycogen degradation such as glucose, maltose or maltotriose (14).

Apart from the absence of catabolite repression, and the ability to degrade raw amylose, we identified a third characteristic that was found to be distinctive: unlike to what we previously found for *L. crispatus* (14), the *Gardnerella* amylolytic activity was mostly found in the extracellular environment and only to a minor extent cell-bound. This is not consistent with the presence of a C-terminal transmembrane domain in the candidate amylopullulanase gene. We do consider that this could be the result of the linkages in our chemically inaccessible form of the labeled substrate and that there may be in fact some cell-associated activity that is incapable of accessing the linkages embedded in the granules. However, this phenomenon has been described previously for the *Gardnerella* sialidase gene *nanH*. The *nanH* gene also has a C-terminal transmembrane domain, yet the activity of the gene product is exclusively found in spent medium (27). Further experiments are required to understand the exact location of these enzymes and potential cleavage of the transmembrane domain.

Although the association between AZCL-amylose degradation rate and Nugent categorization was high, we did not find evidence that the association was related to the abundance of any specific *Gardnerella* species. For example, samples that had high abundance of *G. swidsinskii*

were equally likely to show high or low amylose degradation activity compared to samples that were high in *G. piotii* even though the former *Gardnerella* species had much higher activity in culture compared to the latter. Some samples with no detectable *G. swidsinskii* or *G. leopoldii* were amongst the top 10% of samples in terms of amylase degradation.

Many have speculated that there is a significant role of the host in vaginal glycogen hydrolysis and demonstrated the presence of human amylase in vaginal samples with different microbial community states (16, 18, 28). Apart from microbial amylases, human amylase may contribute to the observed amylose degradation activity in the vagina. Likely, human amylase activity, if any, is not present at biologically relevant levels in *Lactobacillus*-dominated vaginal environments, given the very low levels of AZCL-amylose breakdown in most of the samples with low Nugent scores (0-3). We cannot exclude the presence of human amylase activity in samples with higher Nugent scores. Since *in vitro* we did not observe any raw amylose degradation in several other vaginal bacterial strains commonly associated with bacterial vaginosis, such as *Fannyhessae, Prevotella* or vaginal *Bifidobacteria* the origin of this activity remains elusive.

To further identify the contribution of *Gardnerella* in glycogen degradation in the vaginal environment it is now crucial to analyze the genetic basis for this activity. We especially focused on the amylopullulanase gene that was confirmed to encode a 200 kDa pullulanase-amylase enzyme by heterologous expression in two previous studies (Woolston and Bhandari). The amylopullulanase gene sequence of the strains used in this study show extensive genetic variation but no apparent genetic disruptions coinciding with the (in)ability of a *Gardnerella* strain to degrade raw amylose (not shown). One of the *G. piotii* strains (UGent 18.01) had a full copy of the amylopullulanase gene whereas another strain did not (UGent 21.28). Both strains were not capable of AZCL-amylose degradation. *G. leopoldii* and *G. swidsinskii* strains had a smaller gene, confirmed to be an amylase (17), upstream of the amylopullulanase gene which may explain the higher amylase activity observed in these strains. However, we also detected one *G. leopoldii* and one *G. swidsinskii* strain had

inconsistent high amylose activity. These strains were thus not described here due to high variability in degradation rates between experiments. Future experiments should focus on the identification, expression and regulation of the genetic basis of the *Gardnerella* amylase activity observed in this study, as well as the factors that impact its activity in different strains to elucidate the underlying mechanism.

Taken together, the characteristics of the *Gardnerella* amyloglucosidase could explain the low glycogen levels in samples with bacterial vaginosis (26). Lactobacilli may in contrast only 'graze' the accessible linkages of the glycogen molecules and cease amylopullulanase expression as soon as the smaller maltodextrins start to accumulate (14). Overgrowth of *Gardnerella* typically seen in BV in combination with the metabolic differences may add to the reduced glycogen concentrations in BV.

Lastly, we consider that the secreted nature of the glycogen-degrading activity may mean that these enzymes do not remain confined to the vaginal space but may travel to the tissues of the higher reproductive tract. The enzymes could hypothetically migrate through the cervix to access the endometrium lining of the uterus. Glycogen stores of the endometrium and fallopian tubes function as an early carbohydrate source for the implanted blastocyst in many animals (29). *Gardnerella* enzymes degrading endometrial glycogen may impact the chances of successful implantation.

Previously, *Gardnerella* sialidase and cytolysin were found to be linked to clinical characteristics of bacterial vaginosis, namely mucus thinning and epithelial exfoliation (9, 11). Here we consider *Gardnerella*'s glycogen metabolism in the vagina as a third factor that links colonization and outgrowth of this specific bacterium to a BV-characteristic: low luminal glycogen levels. Furthermore, glycogen hydrolysis in addition with epithelial exfoliation and mucus degradation may explain the extensive mucosal barrier degradation that is associated with *Gardnerella* colonization.

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Author contributions

Conceptualization, R.H., R.K. and W.L.; Methodology and data analysis, R.H., L. H., L. van S., W.L., R.K., P.C., S.B.; Experiments: R.H., L. H., L. van S.; Data curation: R.H., L.H., L. van S.; Writing—original draft preparation, R.H.; Writing review and editing, R.H., L.H., S.B., P.C., R.K.; Supervision: S.B., P.C., R.K. All authors have read and agreed to the published version of the manuscript.

Methods

Bacterial cultivation

For this study we selected various vaginal strains isolated from vaginal samples. The isolation and characterization of the *Lactobacillus crispatus* amylopullulanase-sufficient strain RL10 and amylopullulanase-deficient strain RL09 were previously described (12–14). *Lactobacillus iners* ACS 049-v-Sch2 was isolated from a vaginal sample and identified using MALDI-TOF. Various *Gardnerella* strains whose identification and genome sequences were previously published (30) were included as well as several other members of the vaginal ecology who were also identified using MALDI-TOF. An overview of all strains can be found in Supplementary Table S1.

Cultivation of bacteria was carried out in two laboratories (Ghent University and Vrije Universiteit (VU) Amsterdam) with different equipment. The results from the VU Amsterdam laboratory are depicted in Figure 1, 2, 3, 5, S1, S2 and S3. The results from experiments by Ghent University are depicted in Figure 4 and S4. All strains were anaerobically cultivated from -80 °C glycerol stocks (VU Amsterdam: NYCIII medium + 20% glycerol, Ghent: TSB + 15% glycerol) on agar plates under conditions indicated in Table S1. From these plates colonies were picked to inoculate liquid New York City (NYC)-III broth with 10% horse serum as described previously (14) or YEPG broth for *Candida albicans*. All cultures were handled under anaerobic conditions (VU Amsterdam: N_2 + 10% CO_2, Ghent University: 10% H_2, 10% CO_2, 80% N_2) in an anaerobic chamber (VU Amsterdam: PLAS Labs 855 NB; Ghent university: Bugbox Plus). For the three most common vaginal bacterial species -*Gardnerella vaginalis, Lactobacillus iners* and *Lactobacillus crispatus*- we studied growth on glycogen by comparing optical density after anaerobic incubation in NYCIII medium with glucose or glycogen. To verify whether any other medium components resulted in growth we inoculated these species also in NYCIII medium without glucose or glycogen supplemented.

In Amsterdam the optical density of cultures after 48 hours of growth was determined by diluting in saline and measuring the turbidity at 600 nm with a 1 mL cuvette using a UV-VIS spectrophotometer (Cary 50 Bio, Varian), subtracting the cell-free medium control. In Ghent the optical density was measured by diluting the culture in phosphate-buffered saline in a 96-well microtiter plate and measuring the turbidity in a plate reader at 600 nm (GloMax® Explorer Multimode Microplate Reader, Promega).

AZCL-amylose assay

AZCL-amylose was used in a raw ungelatinized form and suspended in an amylase buffer with xanthan gum to disperse the granules. After mixing with the sample (either vaginal sample of bacterial culture, spent medium or resuspended cell pellet) the increase in optical density in a microtiter plate was measured at 600 nm. The lack of heating or other pretreatment results in the substrate retaining a coarse, granular, undissolved structure. Pelleting is prevented in this assay by suspension in a viscous buffer containing xanthan gum (see Supplementary Figure S2). Xanthan gum (Sigma Life Sciences) was dissolved at 5 mg/mL in amylase buffer (100 mM sodium acetate buffer containing 5 mM $CaCl_2$, pH adjusted with HCl to 5,3). This solution was autoclaved at 121 °C for 20 minutes and kept at room temperature. AZCL-amylose (O-AZAMYF) is commercially available in two forms: regular and fine. The granules in the ‘fine’ product are small enough to pipet with regular pipet tips to dose and mix with the sample. This solution was stable without sedimentation of the raw amylose granules for up to 48 hours. AZCL-amylose was dispersed in the xanthan gum/amylase buffer prior to each experiment by shortly vortexing.

The AZCL-amylose assay was also carried out at both Ghent University and VU Amsterdam, with different equipment. In both protocols the assay was carried out in a flat bottom 96-well plate covered with adhesive film. A total of 190 µL reagent solution was added to 10 µL sample and mixed by carefully pipetting up and down. The plate was incubated in a plate reader (Amsterdam: Multiskan microplate photometer, Thermo Fisher; Ghent University: GloMax® Explorer Multimode Microplate Reader, Promega) at 37 °C and absorption was measured at

600 nm. Maximum rate was taken as the maximum slope of a three-hour time frame (including 19 datapoints). The enzyme assay was validated using *Aspergillus oryzae* amylase (Sigma-Aldrich) solution in amylase buffer with 150 units/µL where one unit corresponds to the amount of enzyme needed to cleave 1 µmol of maltose per minute at pH 6.0 and 25 °C.

AZCL-amylose assay video

The video of the assay shown in Supplementary materials S2 was made by prewarming the substrate suspension and microtiter plate to 40 °C after which 10 µL of saliva or amylase buffer (negative control) was added and filmed at room temperature on the bench using a Samsung smartphone. The footage was clipped to include only the two relevant wells and accelerated 50 times.

AZCL-amylose assay on bacterial cultures

To study the prevalence of raw amylose degradation activity amongst *Gardnerella* species, we screened a set of 14 human vaginal *Gardnerella* strains, including *Gardnerella leopoldii, G. swidsinskii, G. vaginalis* and *G. piotii* and *Gardnerella* genomospecies 3. We compared these to isolates of other common species colonizing the human vagina including *Candida albicans*, *Fannyhessea vaginae* (previously known as *Atopobium vaginae*), *Prevotella bivia, Bifidobacterium* and several *Lactobacillus* species.

For this enzymatic assay with bacterial cultures we only included cultures with sufficient growth with a minimal OD600 of 1,0 (VU Amsterdam) or 0,2 (Ghent University). Cultures were either used without centrifugation or centrifuged for 1 minute at 20,000 rcf at room temperature to separate cells. Spent medium was aspirated and mixed with the substrate and cell pellets were resuspended in sterile phosphate-buffered saline and mixed with the substrate. In experiments with full cultures (shown in figure 4 and S4) a correction with a NYCIII medium control was included. For one biological replicate this medium control was not available so instead the *L. iners* measurement was taken as the baseline since this species was shown in all experiments to have no activity compared to the medium control.

In one experiment the optical density and AZCL-amylose degradation rates of *Gardnerella* cultures on NYCIII with glucose were omitted because of lack of sufficient growth. One other biological replicate was omitted (optical densities as well as AZCL-degradation rate) since the optical densities were more than twice the usual optical densities. We ruled out contamination but the reason of the unusually high optical density remained unexplained and was not observed afterwards. Therefore, we decided to leave the data out.

One *G. leopoldii* (4042406) and one *G. swidsinskii* strain (GS10234) showed highly discrepant results between different experiments and were omitted for this reason.

Vaginal samples

Vaginal swabs were collected from patients visiting the outpatient clinic for Sexually Transmitted Disease of the Public Health Service in Amsterdam (The Netherlands) as described previously (12). Ethics approval was obtained by the AMC ethics review board (document reference number W12_086 # 12.17.0104). The procedure for consent to participate for use of vaginal swabs for scientific research has been approved (reference number W15_159 # 15.0193). The swabs were eluted in Amies buffer and 15% glycerol and cysteine were added for cryoprotection to preserve live bacteria and enzymes for subsequent isolation and analysis. The samples were stored at -80 °C.

Samples for this study were selected based on Nugent scores choosing one third of samples with scores between 0-3 (*Lactobacillus* dominated), one third with scores between 4-6 (intermediate) and one third with a Nugent score between 7-10 (bacterial vaginosis).

AZCL-amylose assay on vaginal samples

For the amylose assay samples were thawed, vortexed and pipetted but not homogenized or centrifuged, which means that mucus and other solid material was still present in the sample when pipetted. 10 µL of the liquid fraction was transferred to a microtiter plate to which 190 µL of the AZCL-amylose in the amylase buffer with xanthan gum was added. The assay and rate calculation was carried out as described above.

DNA extraction and bacterial quantification in vaginal samples

The commercially available multiplex PCR for BV diagnosis (ATRiDa, The Netherlands) was performed according to instructions of the manufacturer (31, 32) on isolated DNA from vaginal samples targeting total *Lactobacillus*, total *Gardnerella*, total *Fannyhessea* and total bacterial load. Both the DNA extraction as well as the qPCR reaction were performed as described previously (33). This same extracted DNA was utilized for *G. vaginalis*, *G. piotii*, *G. leopoldii* and *G. swidsinskii* specific qPCR assays (34). To account for the multiple testing, we adjusted the *p*-value threshold by Bonferroni-correction leading to a required *p*-value of 0,013 (p=,05 divided by 4 outcomes) for the BV diagnostic PCR and the *Gardnerella* species-specific qPCR (also 4 outcomes). As expected, *Gardnerella, Fannyhessea* and total bacterial abundance were strongly associated with Nugent score, and these species had a higher abundance in samples with Nugent score 7-10 compared to samples with a Nugent score of 0-3, while lactobacilli were more abundant in women with low Nugent scores compared to women with high Nugent scores. All correlations between the genera and Nugent score group are shown in Table S3.

We used the log transformed data to find correlations between AZCL-amylose degradation rate and abundance of any of the targets. All data processing and statistical analysis were performed in Microsoft Excel and GraphPad Prism 9.5.0.

References

1. Cruickshank, R.1934. The Conversion of the Glycogen of the Vagina Into Lactic Acid. J. Pathol. Bacteriol. 39: 213–219.

2. Mirmonsef P, Hotton AL, Gilbert D, Gioia CJ, Maric D, Hope TJ, Landay AL, Spear GT. 2016. Glycogen Levels in Undiluted Genital Fluid and Their Relationship to Vaginal pH, Estrogen, and Progesterone. PloS One 11:e0153553.

3. Gosmann C, Anahtar MN, Handley SA, Farcasanu M, Abu-Ali G, Bowman BA, Padavattan N, Desai C, Droit L, Moodley A, Dong M, Chen Y, Ismail N, Ndung'u T, Ghebremichael MS, Wesemann DR, Mitchell C, Dong KL, Huttenhower C, Walker BD, Virgin HW, Kwon DS. 2017. Lactobacillus-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased HIV Acquisition in Young South African Women. Immunity 46:29–37.

4. Lin D, Kouzy R, Abi Jaoude J, Noticewala SS, Delgado Medrano AY, Klopp AH, Taniguchi CM, Colbert LE. 2020. Microbiome factors in HPV-driven carcinogenesis and cancers. PLoS Pathog 16:e1008524.

5. Vergaro P, Tiscornia G, Barragán M, García D, Rodriguez A, Santaló J, Vassena R. 2019. Vaginal microbiota profile at the time of embryo transfer does not affect live birth rate in IVF cycles with donated oocytes. Reprod Biomed Online 38:883–891.

6. Haggerty CL, Hillier SL, Bass DC, Ness RB, PID Evaluation and Clinical Health study investigators. 2004. Bacterial vaginosis and anaerobic bacteria are associated with endometritis. Clin Infect Dis Off Publ Infect Dis Soc Am 39:990–995.

7. Brown RG, Al-Memar M, Marchesi JR, Lee YS, Smith A, Chan D, Lewis H, Kindinger L, Terzidou V, Bourne T, Bennett PR, MacIntyre DA. 2019. Establishment of vaginal microbiota composition in early pregnancy and its association with subsequent preterm prelabor rupture of the fetal membranes. Transl Res. 207:30-43.

8. Gudnadottir U, Debelius JW, Du J, Hugerth LW, Danielsson H, Schuppe-Koistinen I, Fransson E, Brusselaers N. 2022. The vaginal microbiome and the risk of preterm birth: a systematic review and network meta-analysis. Sci Rep 12:7926.

9. Gilbert NM, Lewis WG, Lewis AL. 2013. Clinical features of bacterial vaginosis in a murine model of vaginal infection with *Gardnerella vaginalis*. PloS One 8:e59539.

10. Gelber SE, Aguilar JL, Lewis KLT, Ratner AJ. 2008. Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. J Bacteriol 190:3896–3903.

11. Lewis WG, Robinson LS, Gilbert NM, Perry JC, Lewis AL. 2013. Degradation, foraging, and depletion of mucus sialoglycans by the vagina-adapted Actinobacterium *Gardnerella vaginalis*. J Biol Chem 288:12067–12079.

12. Dols JAM, Molenaar D, van der Helm JJ, Caspers MPM, de Kat Angelino-Bart A, Schuren FHJ, Speksnijder AGCL, Westerhoff HV, Richardus JH, Boon ME, Reid G, de Vries HJC, Kort R. 2016. Molecular assessment of bacterial vaginosis by *Lactobacillus* abundance and species diversity. BMC Infect Dis. 16:180.

13. van der Veer C, Hertzberger RY, Bruisten SM, Tytgat HLP, Swanenburg J, de Kat Angelino-Bart A, Schuren F, Molenaar D, Reid G, de Vries H, Kort R. 2019. Comparative

genomics of human *Lactobacillus crispatus* isolates reveals genes for glycosylation and glycogen degradation: implications for in vivo dominance of the vaginal microbiota. Microbiome 7:49.

14. Hertzberger R, May A, Kramer G, van Vondelen I, Molenaar D, Kort R. 2022. Genetic Elements Orchestrating *Lactobacillus crispatus* Glycogen Metabolism in the Vagina. 10. Int J Mol Sci 23:5590.

15. Sycuro L, Lithgow K, Cochinamogulos A, Muirhead K, Konschuh S, Oluoch L, Mugo N, Roxby A. 2023. Glycogen-Degrading pullulanase a is variably present and correlated with lactic acid in vaginal samples from young african women. Am J Obstet Gynecol 228:S788.

16. Nunn Kenetta L., Clair Geremy C., Adkins Joshua N., Engbrecht Kristin, Fillmore Thomas, Forney Larry J., Young Vincent B. 2020. Amylases in the Human Vagina. mSphere 5:e00943-20.

17. Bhandari P, Tingley J, Abbott DW, Hill JE. 2022. Glycogen degrading activities of catalytic domains of α-amylase and α-amylase-pullulanase enzymes conserved in *Gardnerella* spp. from the vaginal microbiome. bioRxiv10.19.512974

18. Spear GT, French AL, Gilbert D, Zariffard MR, Mirmonsef P, Sullivan TH, Spear WW, Landay A, Micci S, Lee BH, Hamaker BR. 2014. Human alpha-amylase present in lower-genital-tract mucosal fluid processes glycogen to support vaginal colonization by *Lactobacillus*. J Infect Dis 210:1019–1028.

19. Brown I, Warhurst M, Arcot J, Playne M, Illman RJ, Topping DL. 1997. Fecal Numbers of *Bifidobacteria* Are Higher in Pigs Fed *Bifidobacterium longum* with a High Amylose Cornstarch Than with a Low Amylose Cornstarch. J Nutr 127:1822–1827.

20. Sybille T, June Z, Michael K, Roy M, Maria L. M. 2013. The intestinal microbiota in aged mice is modulated by dietary resistant starch and correlated with improvements in host responses. FEMS Microbiol Ecol 83:299–309.

21. Jung D-H, Seo D-H, Kim Y-J, Chung W-H, Nam Y-D, Park C-S. 2020. The presence of resistant starch-degrading amylases in *Bifidobacterium adolescentis* of the human gut. Int J Biol Macromol 161:389–397.

22. Maier TV, Lucio M, Lee LH, VerBerkmoes NC, Brislawn CJ, Bernhardt J, Lamendella R, McDermott JE, Bergeron N, Heinzmann SS, Morton JT, González A, Ackermann G, Knight R, Riedel K, Krauss RM, Schmitt-Kopplin P, Jansson JK. 2017. Impact of Dietary Resistant Starch on the Human Gut Microbiome, Metaproteome, and Metabolome. mBio 8:e01343-17.

23. Woolston BM, Jenkins DJ, Hood-Pishchany MI, Nahoum SR, Balskus EP. 2021. Characterization of vaginal microbial enzymes identifies amylopullulanases that support growth of *Lactobacillus crispatus* on glycogen. bioRxiv 07.19.452977

24. Bhandari P, Hill JE. 2022. Transport and utilization of glycogen breakdown products by *Gardnerella* spp. from the human vaginal microbiome. bioRxiv 11.01.514706

25. McCleary BV. 1980. New chromogenic substrates for the assay of alpha-amylase and (1→4)-β-d-glucanase. Carbohydr Res 86:97–104.

26. Mirmonsef P, Hotton AL, Gilbert D, Burgad D, Landay A, Weber KM, Cohen M, Ravel J, Spear GT. 2014. Free glycogen in vaginal fluids is associated with *Lactobacillus* colonization and low vaginal pH. PloS One 9:e102467.

27. Robinson LS, Schwebke J, Lewis WG, Lewis AL. 2019. Identification and characterization of NanH2 and NanH3, enzymes responsible for sialidase activity in the vaginal bacterium *Gardnerella vaginalis*. J Biol Chem 294:5230–5245.

28. Nasioudis D, Beghini J, Bongiovanni AM, Giraldo PC, Linhares IM, Witkin SS. 2015. alpha-Amylase in Vaginal Fluid: Association With Conditions Favorable to Dominance of *Lactobacillus*. Reprod Sci 22:1393-8.

29. Dean M. 2019. Glycogen in the uterus and fallopian tubes is an important source of glucose during early pregnancydagger. Biol Reprod 101:297–305.

30. Vaneechoutte M, Guschin A, Van Simaey L, Gansemans Y, Van Nieuwerburgh F, Cools P 2019. Emended description of *Gardnerella vaginalis* and description of *Gardnerella leopoldii* sp. nov., *Gardnerella piotii* sp. nov. and *Gardnerella swidsinskii* sp. nov., with delineation of 13 genomic species within the genus *Gardnerella*. Int J Syst Evol Microbiol 69:679–687.

31. Shipitsyna E, Khusnutdinova T, Budilovskaya O, Krysanova A, Shalepo K, Savicheva A, Unemo M. 2020. Bacterial vaginosis-associated vaginal microbiota is an age-independent risk factor for *Chlamydia trachomatis*, *Mycoplasma genitalium* and *Trichomonas vaginalis* infections in low-risk women, St. Petersburg, Russia. Eur J Clin Microbiol Infect Dis. 39:1221-1230.

32. Rumyantseva T, Shipitsyna E, Guschin A, Unemo M. 2016. Evaluation and subsequent optimizations of the quantitative AmpliSens Florocenosis/Bacterial vaginosis-FRT multiplex real-time PCR assay for diagnosis of bacterial vaginosis. APMIS Acta Pathol Microbiol Immunol Scand 124:1099–1108.

33. van der Veer C, van Houdt R, van Dam A, de Vries H, Bruisten S. 2018. Accuracy of a commercial multiplex PCR for the diagnosis of bacterial vaginosis. J Med Microbiol 67:1265-1270.

34. Latka A, Van Simaey L, Reynders M, Cools P, Rogier T, Lebbe B, Corsini L, Landlinger C, Vaneechoutte M. 2022. Optimization of propidium monoazide qPCR (viability-qPCR) to quantify the killing by the *Gardnerella*-Specific endolysin PM-477, directly in vaginal samples from women with bacterial vaginosis. Antibiotics 11:111.