12 APRIL 2020

Growth differences of Lactobacillus crispatus and Gardnerella vaginalis on glucose and glycogen provide novel treatment options against bacterial vaginosis

ALEXANDER WOUDSTRA
SYSTEMS BIOLOGY LAB
STUDENT NR: 2571708 (2571708@STUDENT.VU.NL
BIOMOLECULAR SCIENCES (AM_471127)
BIOINFORMATICS SPECIALIZATION
VU SUPERVISOR: ROSANNE HERTZBERGER
ROSANNE.HERTZBERGER@GMAIL.COM
DAILY SUPERVISOR: JURGEN HAANSTRA
J.R.HAANSTRA@VU.NL
30 EC

Table of contents

Abstract

Background: Bacterial vaginosis is one of the most common vaginal dysbiosis that can occur in women of reproductive age and is caused by a shift in bacterial composition of the vaginal microbiome. In a healthy state, the vaginal microbiome is dominated by lactobacilli including *Lactobacillus crispatus*, while *Gardnerella vaginalis* is one of the main characteristics of bacterial vaginosis. Currently there is a lack of knowledge about the growth of these bacteria, hence a lack of efficient treatments against BV.
Objective: This study investigated the growth of *L. crispatus* and *G. vaginalis* on glucose and glycogen and how these bacteria use these carbon sources. Furthermore, it was explored whether acarbose, an α-glucosidase inhibitor, could be a potential novel treatment against BV.
Methods: Two strains of lactobacilli, 9 and *10,* and *G. vaginalis* were grown on glucose and/or glycogen to identify the growth on these carbon sources. A Glucose Oxidase Peroxidase assay was performed to investigate the carbon source usage of these bacteria. Acarbose was studied as potential novel treatment against bacterial vaginosis by studying its growth inhibitory capacity on glucose and/or glycogen.
Results: *L. crispatus 9* was unable to grow on glycogen, while *L. crispatus 10* was able to grow on both glucose and glycogen. *L. crispatus 10* grows better on glucose than on glycogen. Furthermore, it was shown that *L. crispatus 10* starts to use glucose first for growth before using glycogen. Conversely, *G. vaginalis* grows to a higher optical density on glycogen than on glucose. It was not determined which carbon source it starts to use first for growth. Lastly, it is shown that acarbose is able to inhibit growth of *G. vaginalis* on double carbon sources, while *L. crispatus 10* growth does not seem affected by addition of acarbose.
Conclusion: *L. crispatus* shows a preference for glucose, while the preference of *G. vaginalis* remains unknown. It is suggested that it has a preference for glycogen, due to a higher optical density on glycogen. Furthermore, acarbose shows potential as a novel treatment against bacterial vaginosis due to his ability to inhibit growth of *G. vaginalis* on double carbon sources. Future research is needed to investigate which carbon source *G. vaginalis* prefers growth and how both *G. vaginalis* and *L. crispatus* behave when they are grown together with and or without the addition of acarbose. With these experiments it can be tested whether acarbose is truly a novel treatment against BV.

Introduction

Bacterial vaginosis (BV) is currently one of the most common vaginal dysbiosis that can occur in women of reproductive age[1]. It is estimated that the prevalence amongst the general population worldwide ranges between 23% (in Europe) and 29% (India). Prevalence of BV also shows ethnicity differences which ranged from 23% in non-Hispanic whites to 32% in Mexican Americans in the US [2,3]. This was also found in the Netherlands where a microbiome associated with BV was more prevalent in people of Sub-Saharan African descent then in people of Dutch descent[4]. Although BV is mostly asymptomatic, it can lead to vaginal odor and pain but also to preterm birth when BV is present during pregnancy[5,6].

BV can be defined as the shift in bacterial composition of the vaginal microbiome from lactobacilli to various anaerobic species including *Gardnerella vaginalis*[7]. It has been shown that in reproductive women the most abundant bacteria are the lactobacilli, although the composition of subspecies varies from person to person[8,9]. The vaginal microbiome of individuals is dominated by either *Lactobacillus crispatus, L.iners, L.gasseri or L.jensenii*[10]. These bacteria produce lactic acid from fermenting carbohydrates in order to maintain a low pH (<4.5)[5,9]. However, when the vaginal microbiome consists of various anaerobic species, including *G. vaginalis,* the pH increases, which results in a higher chance of acquiring BV[10]. Furthermore, it has been demonstrated that a high diversity of the vaginal microbiome increases the chance of sexually transmitted infections (STIs) including HIV[11]. In contrast to the microbiome of the skin and mouth, the microbiome of the vagina of healthy women lacks the diversity of the former two[12].

Currently, there still is a lack of understanding about the growth of various lactobacilli and *G. vaginalis*. It has been shown that there are high levels of glycogen present in the vaginal lumen[13]. It was thought that *L. crispatus* is not able to breakdown glycogen by itself, but that it relies on the host or other bacterial species to degrade glycogen into glucose, maltose and other oligosaccharides[14,15]. Recently however, one study succeeded in growing *L. crispatus* with extracellular glycogen. This study also identified a putative pullulanase type I gene that is responsible for the breakdown of glycogen. However, this gene is not present in every variant of *L. crispatus*[10]. The pullulanase type I enzyme is involved in catalyzing the hydrolysis of α-(1,6) linked branches that are present in the structure of glycogen[16]. In a study that reviewed the growth of *G. vaginalis,* it was indicated that this bacteria is the dominant component of the BV biofilm, hereby potentially allowing other anaerobic bacteria to colonize the vagina as well[17]. One study suggested that *G. vaginalis* uses an α -1,4- glucan phosphorylase enzyme to cut glycogen into smaller mono- and oligosaccharides. Furthermore, a recent study identified in the genome of *G. vaginalis* different domains that express pullulanases (fig 1)[18,19].

Due to the lack of understanding of the vaginal microbiome there still is no efficient treatment found for people with BV[1,20]. Currently metronidazole and clindamycin are used, but some *G. vaginalis* have become resistant against these antibiotics. Furthermore, these antibiotics target a broad spectrum of anaerobic bacteria, hereby also targeting bacteria that are good for health[21].

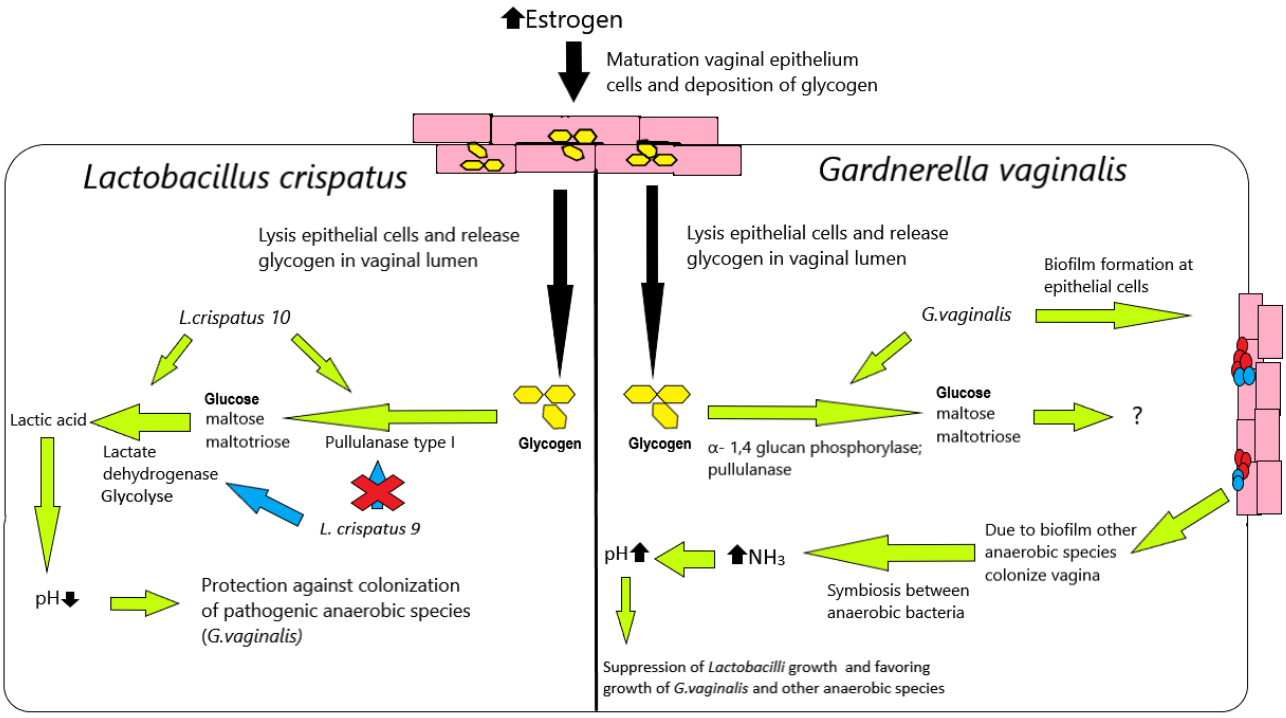


Figure 1: Schematic overview of the proposed glycogen usage by *L. crispatus* and *G. vaginalis.* In bold (glycogen and glucose) are the carbohydrates that are measured in this study. During puberty the amount of estrogen increases, hereby causing the maturation of epithelial cells in the vagina and deposition of glycogen. Lysis of epithelial cells causes the release of glycogen in the vaginal lumen. Glycogen is processed by *L. crispatus 10* due to activation of a putative pullulanase type I gene. *L. crispatus 9* cannot process glycogen due to a deletion in this gene. Pullulanase type I cuts glycogen outside the cell into glucose, maltose and maltotriose hereby increasing the levels of these carbohydrates in the vaginal lumen[16]. These carbohydrates are taken up by *L. crispatus* and further processed by lactate dehydrogenase in the glycolysis into lactic acid that lowers the pH which provides an optimal environment for growth of *Lactobacilli* and protection against other (pathogenic) anaerobic species[5]. *G. vaginalis* uses the enzymes alpha 1,4- glucan phosphorylase and pullulanase to cut glycogen into glucose, maltose and maltotriose[18,19]. These are probably used for growth, but their actual fate remains unknown. *G. vaginalis* is also considered as one of the main characteristics of BV by forming biofilm at vaginal epithelial cells. Forming biofilm protects *G. vaginalis* against lactobacilli, but also provides a doorway for other anaerobic species to colononize the vagina. The symbiotic nature between the anaerobic bacteria result in various products including ammonia (NH_3) that is utilized by *G. vaginalis* for growth. Furthermore, ammonia results in an increase in pH, hereby suppressing growth of lactobacilli and favoring growth of *G. vaginalis* and other anaerobic bacteria[17].

The aim of this study was to acquire a better understanding of the growth of *L. crispatus 9*, *L.crispatus 10* and *G. vaginalis* on glucose and glycogen. Because multiple *L. crispatus* strains can be isolated from the same patient, it was explored whether there is a difference in growth between *L. crispatus 9* and *L. crispatus 10*. Moreover, we investigated whether acarbose is a suitable drug that can be used as replacement for antibiotics. Acarbose is an α- glucosidase inhibitor that is primarily used in China against diabetis mellitus type II[22]. Acarbose shows structural similarity with glycogen, whereas the difference is that in acarbose two glucose molecules can be bound together by a nitrogen-carbon bond instead of an oxygen –carbon bond (fig 2A and B). Due to this difference acarbose has a higher affinity for binding to amyloglucosidases hereby competitively inhibiting the degradation of glycogen into glucose molecules (fig 2C)[23]. It was hypothesized that *L. crispatus* and *G. vaginalis* have a preference for either glucose or glycogen as primary supply source for growth. Furthermore, due to this preference for

a particular carbon source, it was also hypothesized that acarbose has a different inhibitory effect on the growth of these bacteria. To answer these questions concerning the growth of these bacteria and the usage of glucose and glycogen, the first part of this study will discuss the growth of different *L. crispatus* strains and *G. vaginalis*. These bacteria are grown on either glucose and glycogen to investigate whether there is a difference in growth. The second part will investigate the usage of glucose and glycogen over time of *L. crispatus* and *G. vaginalis*. The bacteria are grown on double carbon sources (glucose and glycogen) and the usage of these carbon sources is investigated by a Glucose Oxidase Peroxidase assay . The last part will explore the influence of acarbose on growth of these bacteria. The bacteria are grown on glucose and/or glycogen with or without addition of acarbose to investigate whether acarbose could be a new potential treatment against BV.

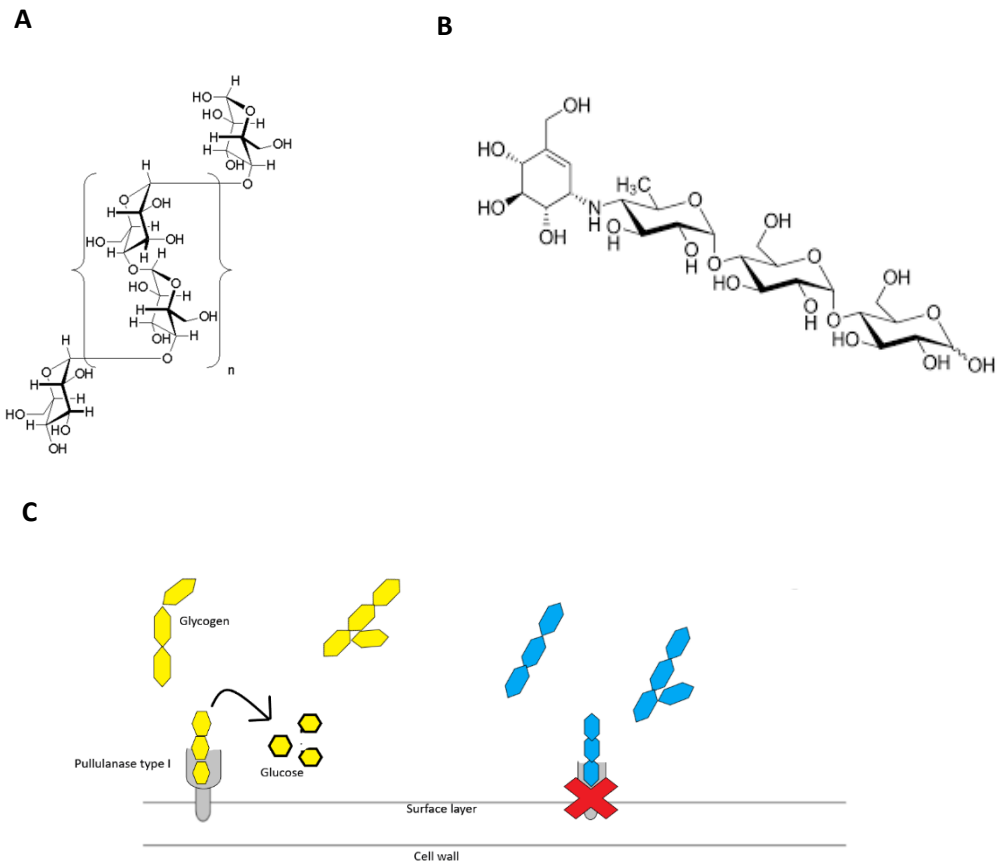


Figure 2: Structure of **A** glycogen , **B** acarbose and **C** the expected method of action of acarbose against an α- glucosidase. Normally glycogen binds to extracellular pullulanase type I that cuts it into smaller glucose, maltose and maltotriose molecules. Acarbose has almost the same structure as glycogen. However, acarbose has a higher affinity for binding to amyloglucosidases. It binds reversibly to the pullulanase receptor hereby blocking the entry for glycogen. The figure is adapted from Moller *et al. (2017)*[16].

Methods

Samples

L. crispatus 9 (RL-09), L. crispatus 10 (RL-10) and *G. vaginalis (DSM 4944)* were isolated from vaginal swabs of women with or without BV. *L. crispatus* strains were obtained from the Sexually Transmitted Infections Clinic in Amsterdam and the Netherlands Organisation for Applied Scientific Research (TNO)[24]. *G. vaginalis* strains were obtained from the DSM collection[25]. The isolates were stored in 20% glycerol at -80°C.

Growth media

L. crispatus and *G. vaginalis* strains were grown on NYCIII agar plates. NYCIII agar plates contained HEPES (2.7 g/L), Proteose Peptone No.3 (16.7 g/L), Yeast extract (4,2 g/L), NaCl (5,6 g/L), glucose monohydrate (6,1 g/L), 1% agar and after autoclaving 50 ml heat inactivated horse serum. The compounds were solved in 450 ml demiwater. The pH before autoclaving was between 7.1 and 7.3.
For 1.1x carbo-deprived liquid NYCIII medium the compounds were the same as described above except that there was no glucose and agar added. Furthermore, 400 ml demiwater was used instead of 450 ml.

Growth experiment

Standard Inoculation conditions and Optical Density measurements *L. crispatus* and *G. vaginalis growth*

L. crispatus 10 and *G. vaginalis* were inoculated according to this protocol. When there was a deviation from this method, it is stated in the respective growth experiment section. Both bacteria were acquired from the -80 stocks. The bacteria were inoculated in the anaerobic chamber on NYCIII plates. These plates were incubated anaerobically at 37°C for three days. After a minimal of three days of growth colonies were scraped from the plate and precultures for growth experiments were prepared that consisted of NYCIII liquid medium and 5 g/L glucose. *G. vaginalis* precultures were grown for two days at 37°C, while *L. crispatus 10* precultures were grown for 1 day at 37°C. A cell free control preculture was included with 5 g/L glucose.

During the growth experiments the Eppendorf tubes were stored in an incubator set on 37°C. This incubator was situated in the anaerobic chamber. Every 2 hours 100 µL of each sample was transported to a F-bottom 96 wells plate. The OD590 was measured with either the multiskan GO or OMEGA fluostar plate reader. When exponential growth was noticed, the OD590 was measured every hour. When OD590 reached 0.8, the sample was diluted 5x in PBS.

CO_2 conditions anaerobic chamber growth check

L. crispatus 10 strain was directly inoculated from -80°C in 1 ml NYCIII liquid medium and 5 g/L glucose. Precultures were stored in an anaerobic jar with a cell free control consisting of 5 g/L glucose. The jar was made anaerobic by 3x filling the environment with 5% CO_2/N_2 and vacuum pulling. The jar was stored at 37°C for two days. After two days 50 µL from the preculture was transported to 1 ml NYCIII liquid medium with 5 g/L glucose and different concentrations of bicarbonate (0 mM, 0.25 mM, 2.5 mM and 25 mM). OD was measured according to standard protocol. During this experiment the sample was not diluted 5x in PBS.

L. crispatus 9 and 10 growth comparison

L. crispatus 9 and *10* precultures were prepared with colonies directly inoculated from the -80 stock in the laminar flow cabinet. These precultures were stored in an anaerobic jar together with a cell free control consisting of 5 g/L glucose. The anaerobic jar was made anaerobic by 3x vacuum pulling and filling the environment with 5% CO_2/N_2. The anaerobic jar was stored for 2 days at 37°C. After two days 50 µL of preculture was transported to 1 ml NYCIII liquid medium with either 5 g/L glucose or 5 g/L glycogen in an Eppendorf tube. OD was measured according to the standard protocol.

Temperature growth check

Two NYCIII plates were inoculated by taking a scoop of *L. crispatus* 10 -80 stock and stored for three days at 37°C. After three days one plate was taken out and cycled in the anaerobic chamber, while the other stayed at 37°C. From the plate in the anaerobic chamber a preculture was prepared and stored for 1 day at 37°C. The plate in the anaerobic chamber stayed there for two days. After one day OD590 of the preculture was measured with OMEGA fluostar plate reader. After two days the plate at 37°C was taken out and cycled in anaerobic chamber. From both plates precultures were prepared and stored for 1 day at 37°C. After 1 day OD590 was measured for both precultures (See supplement 3 for Method Scheme).

G. vaginalis and GOPOD growth

Pre- growth of *G. vaginalis* was carried out according to the standard protocol. After 2 days 100 µL preculture sample was transported to 1 ml NYCIII liquid medium with 5 g/L glucose or 5 g/L glycogen. Two cell free controls with either 5 g/L glucose or 5 g/L glycogen was included. OD was measured according to the standard protocol.

For GOPOD pre- growth of *G. vaginalis* and *L. crispatus 10* were carried out according to the standard protocol. Before the growth experiments 100 µL of *G. vaginalis* or *L. crispatus 10* preculture was transported to 1 ml NYCIII liquid medium with 2 g/L glucose and 2 g/L glycogen. A cell free control with 2 g/L glucose and 2 g/L glycogen was also included. OD was measured according to the standard

protocol. Additionally, after each GOPOD growth OD measurement 100 µL of the sample was pippeted back from the F-bottom 96 wells plate into a new Eppendorf tube. These tubes were centrifuged at 15000 rpm and supernatant was collected and stored at -20°C for the GOPOD assay.

Acarbose growth inhibition

Pre- growth of *G. vaginalis* and *L. crispatus 10* was carried out according to the standard protocol. For growth experiments 100 µL *L. crispatus 10* or *G. vaginalis* preculture was transported to 1 ml NYCIII liquid medium with 2 g/L glucose and/or 2 g/L glycogen and either no or 100 µM acarbose. A cell free control was added that included 2 g/L glucose, 2 g/L glycogen and 100 µM acarbose. OD was measured according to the standard protocol.
Furthermore*, L. crispatus 10* was grown in 1 ml NYCIII liquid medium with different concentrations acarbose (0 µM, 25 µM, 50 µM and 100 µM) and 2 g/L glycogen. OD was measured according to standard protocol.

Glucose Peroxidase assay

For the Glucose Peroxidase (GOPOD) assay a GOPOD reagent was prepared with 0.5 ml glucose oxidase (5 mg/ml), 0.5 ml horse radish peroxidase (5 mg/ml), 5 ml 4-hydroxybenzoic acid (7,3 mM) and 2.5 ml 4-aminoantipyrine (20 mM). Ice cold PBS was added to an end volume of 50 ml. The reagent was aliquoted in 2 ml and stored at -20°C.
10 µL of 100x diluted amyloglucosidase was added to 40 µL sample in a V-bottom 96 wells. Furthermore, a glycogen standard curve was used between 0 and 2 mg/ml. For every sample and standard curve an amylase buffer control without amyloglucosidase was included. In the samples without amyloglucosidase no conversion of glycogen to glucose takes place. The plate was sealed off with parafilm and sterile adhesive film. After sealing, the plate was incubated for 1 hour in a 60°C incubator. During the incubation step, the GOPOD reagent was taken from the -20°C and stored in ice water for thawing. After 1 hour the plate was checked for condense forming. When condense was formed, the plate was centrifuged for 1 minute at 3000 rpm. After the condense check 5 µL of the sample in the V-bottom 96 wells plate was transported to a F-bottom 96 wells plate. 95 µL of GOPOD reagent was added to each well and a glucose calibration curve was included. The OD was measured at an absorbance of 510 nm in a Multiskan GO plate reader for 200 runs of 1 minute. To calculate the glycogen concentration the OD510 of the control wells (with amylase buffer) was substracted from the OD510 of the sample wells (with amyloglucosidase). The signal was divided by the slope of the calibration curve to calculate the concentration of glucose and glycogen.

Statistical analysis

Values are presented by mean ± standard deviation (SD). Data were tested for normality with the Shapiro- Wilkinson test. For comparison between two different groups an independent student t-test was performed with Welsh correction for assuming unequal standard deviation. A p-value < 0.05 was considered as statistically significant. Statistical analysis and plots were carried out by Graphpad Prism 7.04 for Windows, Graphpad Software, La Jolla California USA, www.graphpad.com.

Results

Growth experiments

The aim of this study was to investigate the growth of *L. crispatus* and *G. vaginalis* on glucose and glycogen and how they are using these carbon sources. Furthermore, it was explored whether acarbose could be a potential novel treatment against BV.

CO_2 Concentration anaerobic chamber was sufficient for growth anaerobic bacteria

Before we could start with the actual growth experiments to answer our aim we first wanted to know whether the bacteria can grow in the anaerobic chamber with 5% CO2/N2. This is important because then we know that the standard conditions in the chamber are sufficient. To answer this question *L. crispatus 10* was grown on 5 g/L glucose and was further subjected to various concentrations bicarbonate. Adding extra bicarbonate supplies bacteria with an additional single carbon source (CO_2). Figure S1 shows that extra bicarbonate did not result in greater growth for *L. crispatus.* Furthermore, *L. crispatus* without extra bicarbonate was able to grow to almost the same OD as *L. crispatus* with 25 µM bicarbonate after 24h. This indicated that the anaerobic chamber had a sufficient concentration of CO_2 for the growth of anaerobic bacteria.

L. crispatus 9 is not able to grow on glycogen and L. crispatus 10 grows better on glucose than on glycogen.

Because we now know that the standard conditions in the anaerobic chamber were sufficient enough for the growth of our bacteria we could investigate whether there is a difference in growth between two different lactobacilli strains. To answer this question, *L. crispatus 9* and *L. crispatus 10* were inoculated directly from -80 were grown on either 5 g/L glucose or 5 g/L glycogen. Figure 3 shows the comparison between final growth OD for 24 hours of *L. crispatus 9 and L. crispatus 10* on glucose and glycogen. It was shown that there was a significant difference in growth of *L. crispatus 9* between glucose and glycogen (mean glucose = 0.58 ± 0.026 versus mean glycogen = 0.12 ± 0.01, p = 0.003). There was no significant difference between growth on glucose or glycogen for *L. crispatus 10 (*mean glucose= 0.51 ± 0.11 versus mean glycogen = 0.48 ± 0.11, p = 0.72*).* This indicates that both strains can grow on glucose, but that only *L. crispatus 10* can grow on glycogen.

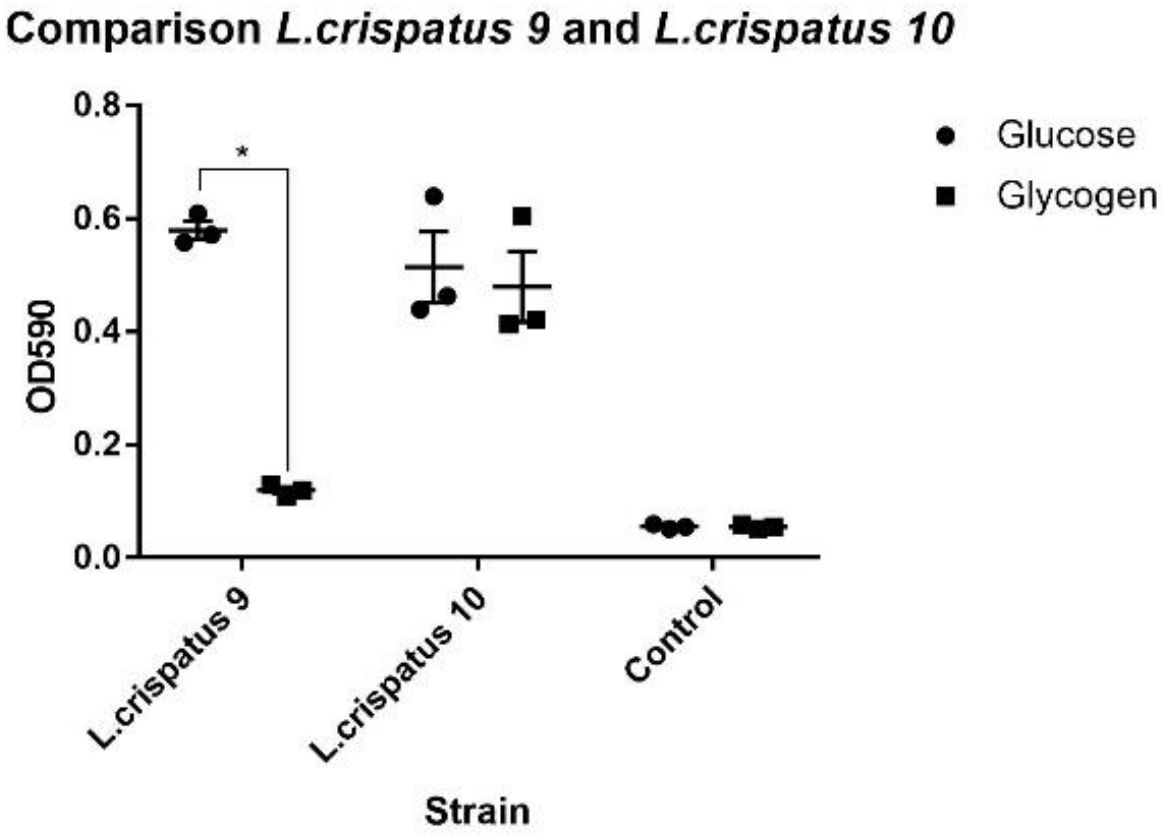


Figure 3: Final OD growth for 24h of *L. crispatus 9* on 5 g/L glucose or 5 g/L glycogen (mean glucose = 0.58 ± 0.03 versus mean glycogen = 0.12 ± 0.01, p = 0.003) and *L. crispatus 10* on 5 g/L glucose or 5 g/L glycogen (mean glucose = 0.51 ± 0.11 versus mean glycogen = 0.48 ± 0.11, p = 0.72). Every data point represents an experiment. The horizontal line represents the mean, while the vertical lines represent the standard deviation. Two cell free controls were used with either 5 g/L glucose or 5 g/L glycogen.

From the previous growth experiment it became clear that there is a difference in growth between different *L. crispatus* strains. However, it could be that the growth temporarily stops after 24h, due to the production of too much lactic acid and not because there is a lack of carbon sources(figure S2). Based on this hypothesis, it is decided to grow *L. crispatus 10* on a lower glucose or glycogen concentration. *L. crispatus 10* was pre-grown on a NYCIII plate at 37°C for three days, inoculated in a preculture consisting of 1 ml NYCIII liquid medium and 5 g/L glucose and then inoculated in the anaerobic chamber with 2 g/L glucose or 2 g/L glycogen. Figure 4 shows that there was no significant difference in final OD between growth on glucose or glycogen (mean glucose = 0.39 ± 0.028 versus mean glycogen = 0.29 ± 0.029, p = 0.07). However, in every experiment it was noticed that *L. crispatus* grew to a higher OD on glucose than on glycogen suggesting that *L. crispatus* has a preference for glucose as a carbon source for growth.
In the experiment with 5 g/L glucose or glycogen it was noticed that it took a long time for *L. crispatus 10* to start growing *(*figure S2*).* Furthermore, it was noticed that the OD of the precultures were different each day (data not shown). It was argued that temperature could play a role in length of the lag phase of these bacteria. It is hypothesized that when bacteria are at room temperature they go into stationary phase. When the temperature rises again it takes the bacteria a certain time to start growing. An

experiment was designed where bacteria were grown on two NYCIII plates at 37°C. After 3 days one plate was kept at room temperature and one at 37°C for two days. Then the precultures from these plates were compared (figure S3). It shows that the preculture from the room temperature plate only had an OD590 of 0.2 while the preculture from the 37°C plate had an OD590 of 0.87 (fig S4) after overnight growth. After this observation we decided to grow our bacteria first on plate before making a preculture instead of making a preculture directly from the -80°C stock.

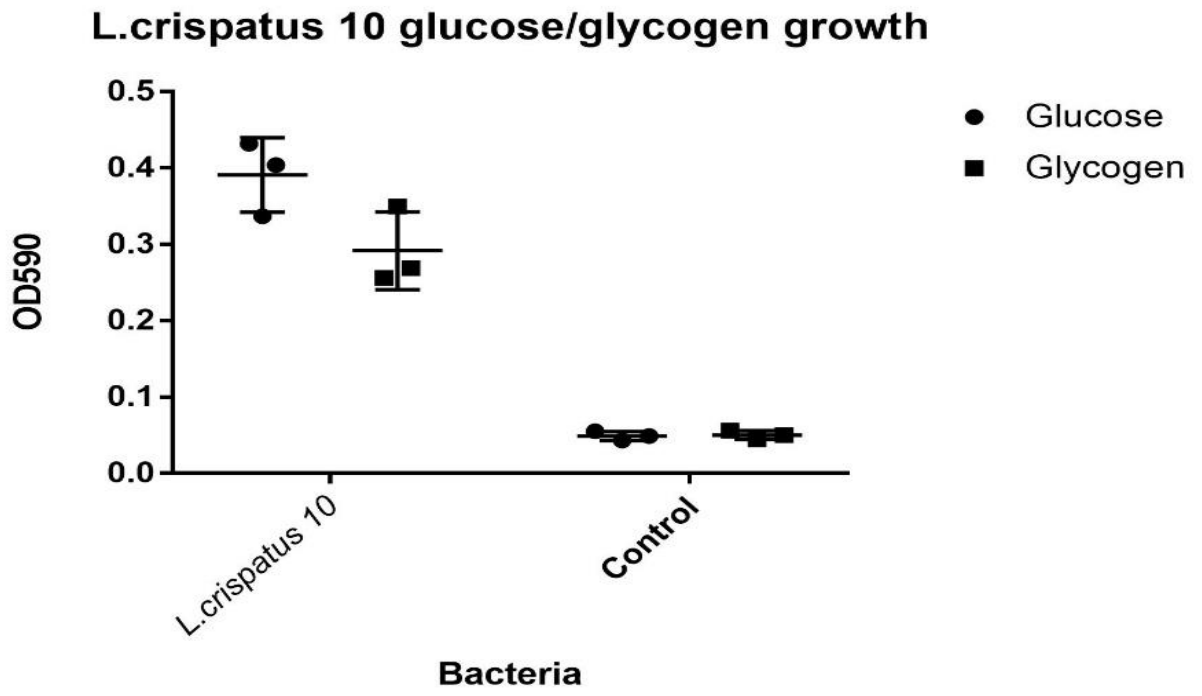


Figure 4: Final OD growth for 26h of *L.crispatus 10* on 2 g/L glucose or 2 g/L glycogen (*mean glucose = 0.39 ± 0.028 versus mean glycogen = 0.29 ± 0.029, p = 0.07)*. Every data point represents an experiment. The horizontal line represents the mean, while the vertical lines represent the standard deviation. Two cell free controls were used with 2 g/L glucose or 2 g/L glycogen.

L. crispatus 10 uses glucose prior to glycogen

In the previous section we saw that *L. crispatus* 10 can grow to a higher OD on glucose than on glycogen, which could indicate that glucose is the preferred carbon source for growth of *L. crispatus 10*. Next, we wanted to know whether glucose is the first carbon source used for growth when glycogen is also present . To answer this question, *L. crispatus 10* was grown on 2 g/L glucose and 2 g/L glycogen. A Glucose Oxidase Peroxidase (GOPOD) assay was used to investigate the utilization of these carbon sources. This assay required some troubleshooting before it could be used to study glucose and glycogen usage (see supplement 6). Figure 5A shows the growth curve of *L. crispatus 10* on double carbon sources. During growth *L. crispatus* 10 uses glucose immediately, while glycogen starts decreasing after approximately 2 hours (Fig 5B). Thus it is suggested that glucose acts as the preferred carbon source for *L. crispatus 10* growth.

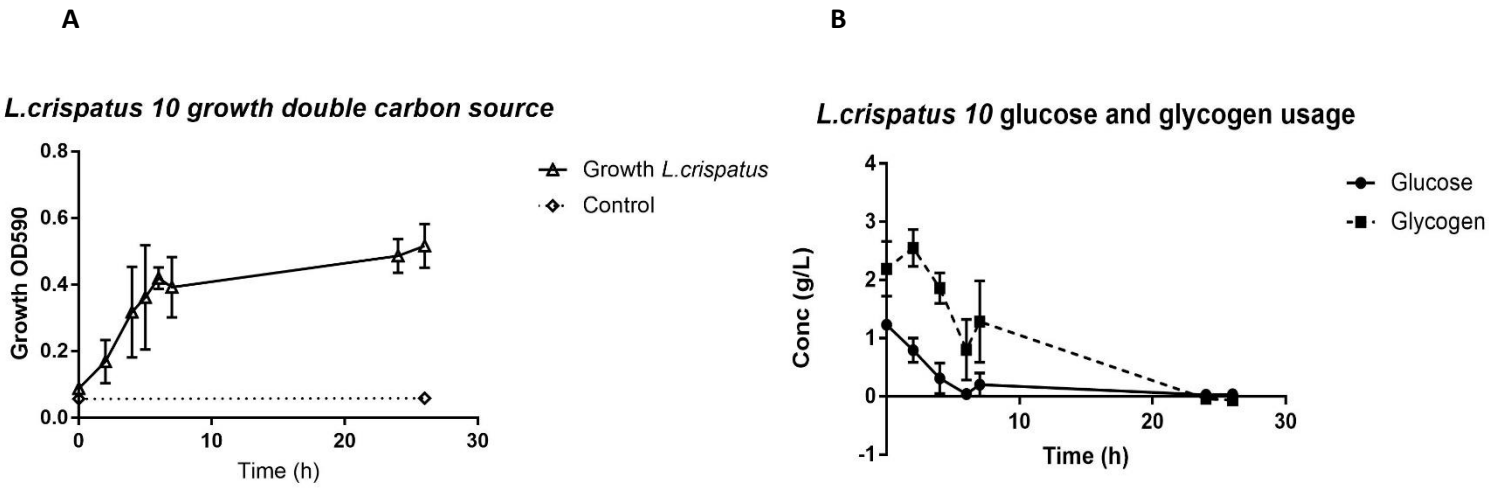


Figure 5: Glucose Oxidase Peroxidase assay of *L. crispatus* 10. **A** *L. crispatus 10* growth on 2 g/L glucose and 2 g/L glycogen. A cell free control was used with 2 g/L glucose and 2 g/L glycogen **B** Glucose and glycogen usage over time by *L. crispatus 10.* This experiment was carried out in triplicate. The vertical lines represent the standard deviation.

G. vaginalis is able to grow to a higher final OD on glycogen than on glucose.

Now that we know that *L. crispatus 10* favors glucose over glycogen as carbon source for growth, we want to investigate which carbon source *G. vaginalis* prefers. *G. vaginalis* is considered as one of the most prominent members of BV. One study suggested that *G. vaginalis* consists of virulence factors that assist in creating a biofilm and allow it to compete with lactobacilli in the vagina[17]. However, it could also be that *G. vaginalis* can compete with *L. crispatus* for the same carbon sources. To answer our question *G. vaginalis* was grown on either 5 g/L glucose or 5 g/L glycogen. *G. vaginalis* has a slightly higher final OD when grown on glycogen compared to growth on glucose, but it was not significant (mean = 0,86± 0.35 versus mean = 0.6± 0.19, p = 0.27) (fig 6). However, in all experiments *G. vaginalis* grew to a higher final OD on glycogen than on glucose which could indicate that *G. vaginalis* shows a preference for glycogen as a carbon source for growth.

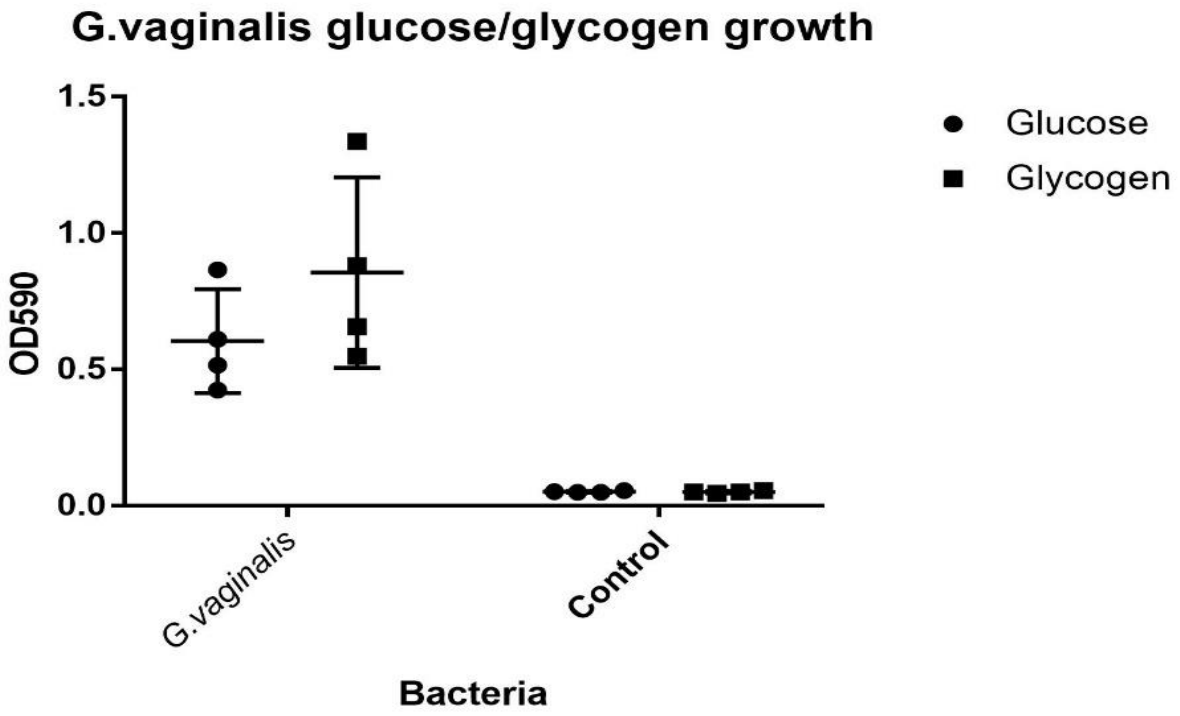


Figure 6: G. vaginalis final OD growth for 26h on either 5 g/L glycogen or 5 g/L glucose (mean glycogen = 0,86 ± 0.35 versus mean glucose = 0.60 ± 0.19, p = 0.27). Every data point represents an experiment. The horizontal line represents the mean, while the vertical lines represent the standard deviation. Two cell free controls were used with 5 g/L glucose or 5 g/L glycogen.

G. vaginalis glucose/glycogen usage remains unclear

During the growth experiments it was noticed that *G. vaginalis* grows to a higher final OD on glycogen than on glucose. This could indicate that glycogen acts as a preferred carbon source for the growth of *G. vaginalis.* Next, we wanted to explore whether glycogen is the first carbon source that is used for growth by *G. vaginalis*. To investigate the carbon source usage, *G. vaginalis* was grown on 2 g/L glucose and 2 g/L glycogen. GOPOD was used to investigate the utilization of these carbon sources. Figure 7 shows the growth curve on double carbon sources (fig 7A) and the glucose and glycogen usage of *G. vaginalis* (fig 7B). Between 7 and 24 hours, *G. vaginalis* is able to grow and during this time both glucose and glycogen are almost completely depleted. However, in this period there was no measurement, so with GOPOD it could not be determined whether *G. vaginalis* starts to grow first on glucose or glycogen.

A **B**

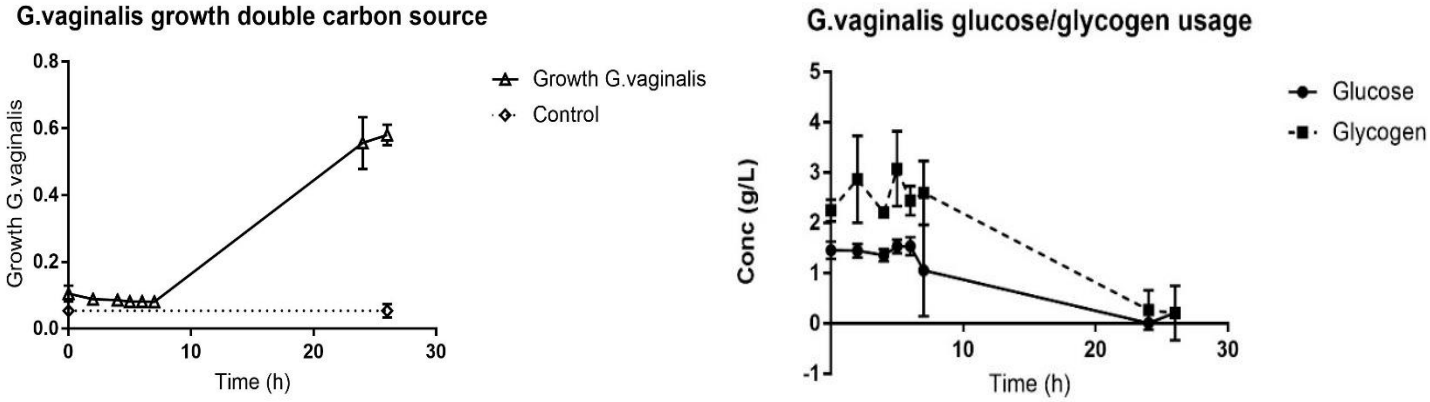


Figure 7: Glucose and glycogen usage *G. vaginalis*. **A** Growth curve *G. vaginalis* on double carbon sources. A cell free control with 2 g/L glucose and 2 g/L glycogen was used. **B** Glucose and glycogen usage of *G. vaginalis*. This experiment was carried out in triplicate. The vertical lines represent the standard deviation.

Acarbose inhibits growth on glycogen of both *L. crispatus* and *G. vaginalis*

In the previous sections it was noticed that *L. crispatus 10* shows a preference for glucose while *G. vaginalis* seems to have a preference for glycogen. This could be important for novel treatments that can target glycogen. In this section it was investigated whether acarbose is a potential novel treatment against BV. As mentioned in the introduction acarbose is an α- glucosidase inhibitor that can reversibly inhibit the conversion of glycogen to glucose. *L. crispatus 10* and *G. vaginalis* were both grown on either 2 g/L glucose or 2 g/L glycogen. In both cases either no acarbose or 100 µM acarbose was added. Figure 8 represents the growth of both L. crispatus 10 and *G. vaginalis*. Growth of *L. crispatus 10* is not significantly influenced by acarbose when grown on glucose as carbon source (mean - acarbose = 0.39 ± 0.05 versus mean + acarbose = 0.38 ± 0.05, p = 0.74). However, there is a significant difference in growth

on glycogen between without acarbose and growth with 100 µM acarbose (mean - acarbose = 0.29 ± 0.05 versus mean + acarbose = 0.13 ± 0.02, p = 0.02) (figure 9A). It was also studied what the effect of different concentrations of acarbose was on the growth of *L. crispatus 10*. *L. crispatus 10* grown on 2 g/L glycogen was subjected to different concentrations of acarbose (0 µM, 25 µM, 50 µM and 100 µM). It was shown that the higher the concentration of acarbose, the higher the impact on *L. crispatus 10* growth (figure S4). Due to time restraints we were unable to perform this experiment multiple times and to investigate whether this result would be the same for *G. vaginalis*.

Figure 8B shows the influence of acarbose on the growth of *G. vaginalis*. This experiment was carried out twice, which also meant that it could not be determined yet whether there is a significant difference in growth when acarbose is added. However, based on these results it is suggested that acarbose has an impact on growth of *G. vaginalis* when grown on either glucose or glycogen. *G. vaginalis* was able to grow to a higher OD on glucose when no acarbose was added, while the OD was lower when 100 µM acarbose was added (mean - acarbose = 0.34 ± 0.004 versus mean + acarbose= 0.21 ± 0.006). *G. vaginalis* was unable to grow on glycogen when 100 µM acarbose was added(mean - acarbose = 0.42 ± 0.03 versus mean + acarbose = 0.15 ± 0.001).

These results suggest that acarbose is able to inhibit growth on glycogen of both *L. crispatus 10* and *G. vaginalis.* Furthermore, we saw that *G. vaginalis* grew to a lower final OD on glucose when acarbose was added.

A **B**

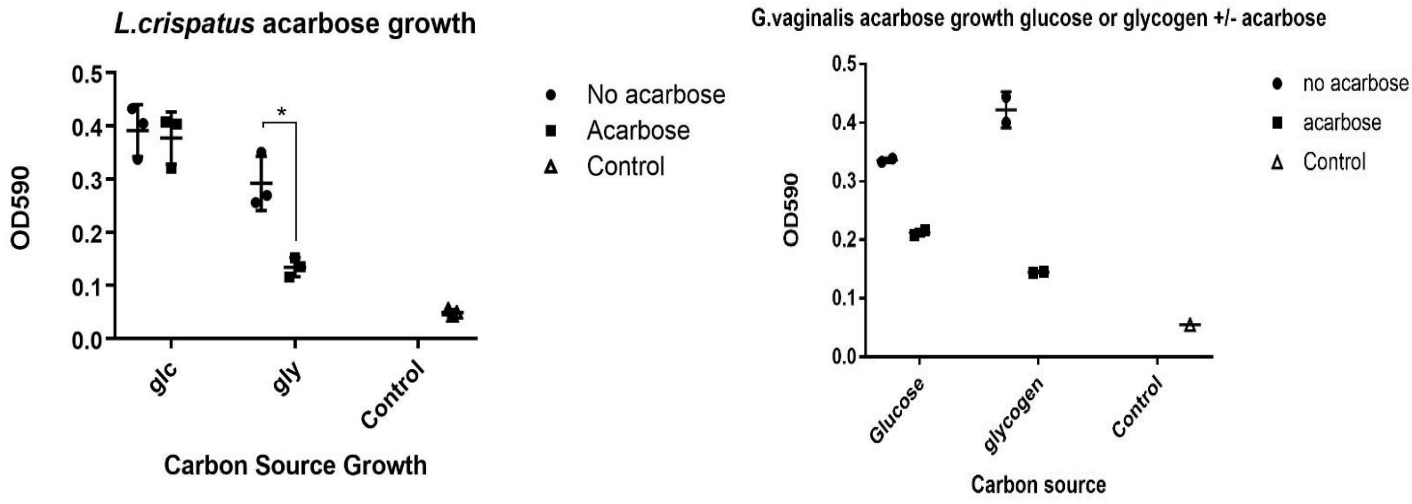


Figure 8: Growth of *L. crispatus* and *G.vaginalis* with and without acarbose. **A** *L. crispatus 10* final OD growth for 26 h on 2 g/L glucose with or without 100 µM acarbose (mean - acarbose = 0.39 ± 0.05 versus mean + acarbose = 0.38± 0.05, p = 0.74) or 2g/L glycogen with or without addition of 100 µM acarbose (mean - acarbose = 0.29 ± 0.05 versus mean + acarbose = 0.13 ± 0.02, p = 0.02) **B** *G. vaginalis* growth on 2 g/L glucose with or without 100 µM (mean - acarbose = 0.34 ± 0.004 versus mean + acarbose = 0.21 ± 0.006) or 2 g/L glycogen with or without addition of 100 µM acarbose (mean - acarbose = 0.42 ± 0.03 versus mean + acarbose = 0.15 ± 0.001). Every data point represents an experiment. The horizontal line represents the mean, while the vertical lines represent the standard deviation. In both *L. crispatus* and *G.vaginalis* experiments a cell free control with 2 g/L glucose or 2 g/L glycogen and 100 µM acarbose was added.

G. vaginalis growth is slightly more affected when grown on double carbon source and acarbose in contrast to *L. crispatus 10*

In the previous section it was described that acarbose inhibits the growth of both *L. crispatus 10* and *G. vaginalis* when grown on only glycogen. Now we want to know whether acarbose reaches the same inhibitory effect when the bacteria are grown on double carbon sources because it could be that the growth on glucose diminishes the inhibitory effect of acarbose. In this section both bacteria were grown on double carbon sources and with or without the addition of 100 µM acarbose. There was no significant difference in growth of *L. crispatus* with or without addition of acarbose (mean - acarbose = 0.52 ± 0.06 versus mean + acarbose = 0.49 ± 0.07, p = 0.54). Growth of *G. vaginalis* was slightly impaired with the addition of acarbose, although not significantly (mean - acarbose = 0.58 ± 0.02 versus mean + acarbose = 0.39 ± 0.09, p= 0.06) (fig 9A). However, growth of *G. vaginalis* without acarbose shows a slightly higher OD than growth with acarbose in every measurement, suggesting that acarbose might have a greater influence on the growth of *G. vaginalis* than of *L. crispatus 10* (figure 9B).

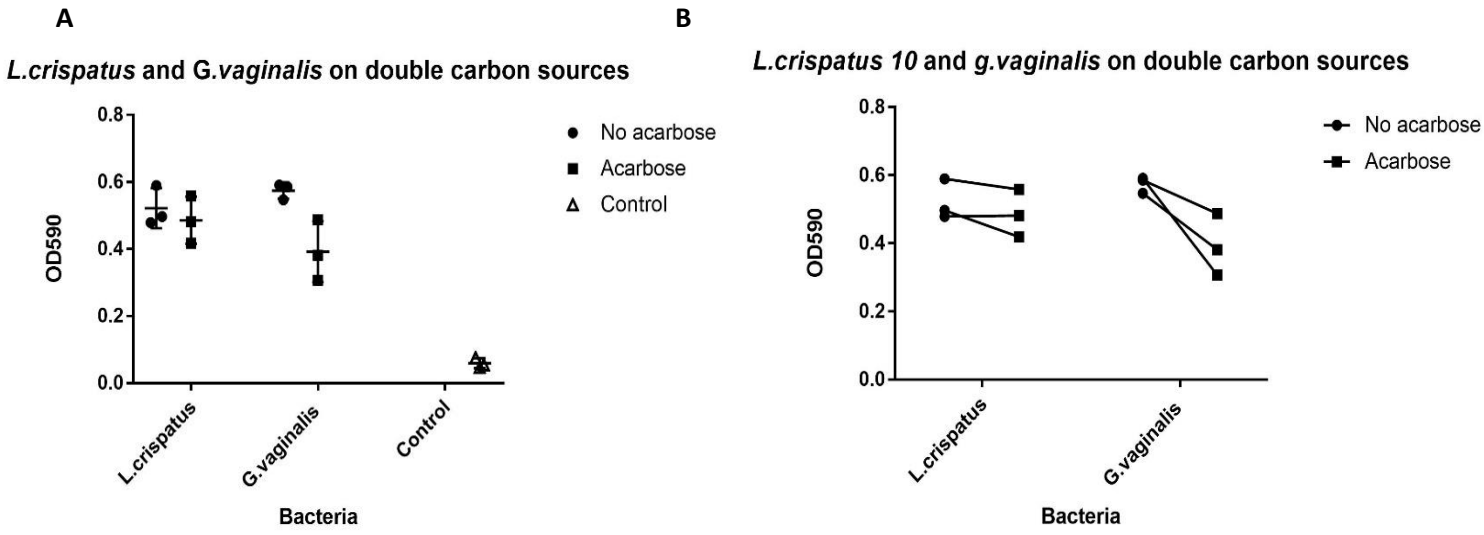


Figure 9: Final OD Growth for 26h of *L. crispatus* and *G. vaginalis* on 2 g/L glucose and 2 g/L glycogen with or without addition of 100 µM acarbose. **A** Final OD growth for 26h of *L. crispatus 10* with or without acarbose (*mean* + acarbose *= 0.49 ± 0.07 versus mean - acarbose = 0.52 ± 0.06, p = 0.54). G.vaginalis* final OD growth for 26h with or without acarbose *(mean - acarbose = 0.58 ± 0.02 versus mean + acarbose = 0.39 ± 0.09, p= 0.06).* Every data point in the figure represents an experiment. The horizontal line represents the mean, while the vertical lines represent the standard deviation. During the experiments a cell free control with 2 g/L glucose, 2 g/L glycogen and 100 µM was added. **B** Relation glucose/glycogen between every experiment. The lines indicate that these measurement belong to the same experiment. Every data point represents an experiment.

Discussion

Bacterial vaginosis can be defined as a shift in the bacterial composition of lactobacilli to various anaerobic species including *G. vaginalis*. There is currently not much known about the growth of these bacteria which resulted in inefficient treatments with antibiotics. In this study it was investigated what the growth is of *L. crispatus 9, 10* and *G. vaginalis* on glucose or glycogen and glucose and glycogen together. This question was answered by carrying out growth experiments on glucose and glycogen and enzymatic assays to investigate glucose/glycogen usage of these bacteria. Furthermore, it was studied whether acarbose was able to inhibit the growth of *L. crispatus 10* and *G.vaginalis* by growing these bacteria on glucose and glycogen without or with acarbose. It was hypothesized that *L. crispatus 10* uses glucose as a preferred carbon source for growth, while *G. vaginalis* is able to use both carbon sources at the same time.

	L. crispatus 9	***L. crispatus 10***	***G. vaginalis***
Glucose	✓	✓ (preferred)	✓
Glycogen	✗	✓	✓

Table 1: Key findings growth carbon sources

	***L. crispatus 10* + glucose**	***L. crispatus 10* + glycogen**	***L. crispatus 10* + glucose + glycogen**
No acarbose	✓	✓	✓
100 µM acarbose	✓	✗	✓

Table 2: Key findings *L. crispatus 10* with or without 100 µM acarbose

	***G. vaginalis* + glucose**	***G. vaginalis* + glycogen**	***G. vaginalis* + glucose + glycogen**
No acarbose	✓	✓	✓
100 µM acarbose	✓ (impact on growth)	✗	✓ (impact on growth)

Table 3: Key findings *G. vaginalis* with or without 100 µM acarbose

This study had several important findings (summarized in Table 1,2 and 3) that bring us closer to understanding the growth of *L. crispatus* and *G. vaginalis*. In summary, we saw that *L. crispatus 9* was unable to grow on glycogen and that *L. crispatus 10* grows to a higher OD on glucose than on glycogen. Furthermore, we saw that *G. vaginalis* grows to a higher OD on glycogen then on glucose. With the GOPOD assay we established that *L. crispatus 10* starts growing on glucose and starts using glycogen after 2-3 hours (fig 6). Although we did not perform a GOPOD assay on *L.crispatus* 9, it is assumed that it follows the same usage for glucose, but that glycogen is not processed and stays at the same concentration level based on the final growth OD. With this assay we were not able to find which carbon source *G. vaginalis* uses to start growing (fig 8). Lastly, we saw that acarbose was able to inhibit the growth of both bacteria when grown on only glycogen. However, when grown on both glucose and glycogen, it did not have an impact on final OD in case of *L. crispatus 10*, while on *G. vaginalis* it seemed to affect growth (Table 2 and 3).

L.crispatus 9, 10 show preference for glucose, *G.vaginalis* grows better on glycogen

L. crispatus 9 was unable to grow on glycogen, while it was able to grow on glucose (table 1). This is consistent with other studies and literature, because they showed that *L.crispatus 9* contains a deletion in the putative pullulanase type I gene that is involved in conversion of glycogen to glucose[10]. We showed here that *L. crispatus 10* is able to grow to a higher final OD on glucose than on glycogen. This was a remarkable observation because we would have expected, even after a slower start, that *L. crispatus 10* grows to the same OD on glycogen as on glucose. Mirmonsef *et al* (2016) speculated that lactobacilli might not be able to breakdown all glycogen into glucose, maltose or maltotriose[13]. Glycogen consists of both α-(1,6) linked branches and α-(1,4) linked branches and it is thought that *L. crispatus 10* has a type I pullulanase that is only able to cut α-(1,6) linked branches, so glycogen might not be fully cut into smaller units by *L. crispatus 10*[16]. This could explain the reason why *L. crispatus 10* grows to a lower OD on glycogen then on glucose. Hypothetically, both non- glycogen degrading (including *L. crispatus 9*) and glycogen degrading (lincluding *L. crispatus 10*) lactobacilli strains can be isolated from the same patient. This suggests that glycogen presence is not a prerequisite for growth of these bacteria. It could be that non- glycogen degrading lactobacilli strains can survive based on the glucose products produced by lactobacilli strains that can degrade glycogen in a sort of wild type helper effect.

Lastly, we noticed that *G. vaginalis* grows to a higher final OD on glycogen than on glucose. It has been suggested that *G. vaginalis* contains an α-1,4 glucan phosphorylase[18]. It could be that adding a phosphate group is beneficial for *G. vaginalis* survival because *L. crispatus* might not be able anymore to take up glucose with a phosphate group from outside the cell. It has also been demonstrated that the *G. vaginalis* genome contains domains that express pullulanases, which indicates that these bacteria could have a second option to cut glycogen into glucose and other oligosaccharides[19]. Furthermore, one study demonstrated that pH is negatively correlated with the amount of glycogen present in the vagina[13]. A low pH protects the vaginal microbiome against colonization of anaerobic bacteria including *G. vaginalis*. It could be that *G. vaginalis* uses glycogen more efficiently than *L. crispatus* that can result in an increase in pH. The pH increases because the lactobacilli have less glycogen to convert into lactic acid.

The strength of our growth method was that we were now able to grow our bacteria over time in the anaerobic chamber. Due to the anaerobic nature of our bacteria this increased their viability and gives a more trustworthy result than growing it partly anaerobic and then slightly aerobic in the plate reader with a sealed 96 wells plate. However, one of the limitations was that the plate reader was not

situated in the anaerobic chamber. This meant that we had to transfer samples from the anaerobic chamber to the plate reader and back every hour. This is time consuming because this also meant that every hour we had to pipet sample in a 96 wells plate to measure the OD. This also resulted in a higher chance of pipetting errors, especially after 24h because of the pellet forming that was not sufficiently solved in the sample. We accounted for this by doing additional OD measurements at later time points to check whether our 24h measurement was correct.

Although we found important results, there are still some questions open. For future studies it is recommended to look at what happens with the products that arise from glycogen degradation. It could be that they are simply used for growth, but it can also be that they are used for the production of other metabolites. This could be done with a High Performance Liquid Chromatography (HPLC) that can identify various compounds in a mixture (for example supernatant). During the growth experiments each hour or every two hours the supernatant can be acquired for the HPLC. With this method you can identify the compounds for every measurement and how this relates to the growth.

On double carbon sources *L. crispatus 10* uses glucose for start growth, *G. vaginalis* remains unknown

We were not only interested in the growth of *L. crispatus* and *G. vaginalis* on glucose or glycogen, but also when these bacteria start using these carbon sources. With the GOPOD assay we saw that *L. crispatus 10* immediately uses glucose when it grows, while glycogen only began to decrease after approximately 2 hours. This indicated that *L. crispatus 10* has a preference for glucose as carbon source for growth, which was what we hypothesized. In the case of *G. vaginalis* we were unable to determine which carbon source it starts to use first for growth. The main reason was because of the long lag phase of *G. vaginalis*, which meant that it started growing between 7h and 24h where no measurement was taking place. At 24h we noticed that both glucose and glycogen were already depleted and the exponential phase already happened.

This study is one of the first that uses the GOPOD assay to identify both glucose and glycogen usage of *L. crispatus 10* and *G. vaginalis.* One of the strengths of the GOPOD assay is that we could identify the use of glucose and glycogen of *L. crispatus* and *G. vaginalis* with only a small amount of sample. Furthermore, this assay is faster than other techniques such as the HPLC to measure the glucose and glycogen concentrations. A HPLC needs a half hour for 1 sample, while with GOPOD we could simultaneously measure all samples in one 96 wells plate. Unfortunately, one of the limitations of this assay was the ability of GOPOD to measure the exact amount of glucose and glycogen. Especially determining the amount of glycogen proved to be difficult. However, we were still able to identify the usage of glucose and glycogen over time quantitavely of *L. crispatus 10* and *G. vaginalis*.

Future studies have to discover the time that *G. vaginalis* starts to grow exponentially. When this is known, it can be used to investigate whether on glucose or glycogen *G. vaginalis* starts to use first for growth. We saw that *G. vaginalis* started to grow between the 7^{th} and 24^{th} hour, the period that no measurement took place. We suggest that either a lower amount of *G. vaginalis* sample is inoculated, that results in expanding the lag phase, or inoculating a higher amount of *G. vaginalis,* that results in shortening the lag phase to identify the start of the exponential growth phase.

Acarbose shows potential as an inhibitor for growth *G. vaginalis*

Although we were not able to determine whether *G. vaginalis* uses glucose and glycogen first as carbon source for growth, there are some indications that *G. vaginalis* prefers glycogen as a carbon source for growth. First of all is that it grows to a higher OD on glycogen (Fig 6). Secondly, *G. vaginalis* on double carbon sources grew to a lower final OD when acarbose has been added (fig 8). Another remarkable finding was that *G. vaginalis* grew to a lower OD on glucose as a single carbon source in addition with acarbose (fig 9A). As mentioned in the introduction acarbose is an α- glucosidase inhibitor. It inhibits the degradation of glycogen into glucose molecules. An argument for that *G. vaginalis* grows to a lower OD with acarbose on glucose is that *G. vaginalis* has a longer lag phase then *L. crispatus 10* and that it requires more energy to start growing when acarbose is present (fig 7A). It could also be hypothesized that an α- glucosidase of *G. vaginalis* not only serves to degrade glycogen into smaller saccharides but also as transporter to transport the saccharides inside the cell. In case of *L. crispatus* we saw that the OD with or without acarbose was approximately the same when grown on double carbon source. Moreover, acarbose did not show to have an impact on growth on glucose. In gut lactobacilli it was proposed that the pullulanase type I enzyme is bound to the outside layer of the cell and that the glucose molecules are transported via a different selective channel inside the cell[16]. This could explain why the growth on glucose is not affected in the case of *L. crispatus* 10. That growth on double carbon sources is not significantly altered could be explained because *L. crispatus* first starts to use glucose and then glycogen for growth. By first growing on glucose it is hypothesized that there are enough bacteria present to diminish the effect of acarbose. Furthermore, it could be that because there is only a certain amount of acarbose present some bacteria can still use glycogen for growth. This was also noticed when we added a lower dose of acarbose to *L. crispatus* which still allowed the bacteria to grow to a higher OD on glycogen (fig S5).

This experiment was one of the first to investigate whether acarbose could be a potential novel treatment against BV. However, multiple studies have performed acarbose experiments with lactobacilli that are present in the gut microbiome. One of these studies showed that acarbose decreased the abundance of lactobacilli in the gut microbiome in mice[26]. This study was performed with an extremely high dose of 1550 µM acarbose. Another study treated mice with either a high dose of acarbose or a low dose of acarbose and showed that a low dose of acarbose did not have a significant effect on growth of lactobacilli in the gut, while a high dose acarbose significantly decreased the abundance of lactobacilli[27]. These results correspond with our result that the growth of *L. crispatus 10* depends on the dose of acarbose and that a higher dose has a bigger effect on the growth than a lower dose (fig S5). Based on our results and the results from other studies you could argue that at a low dose acarbose could be a potential novel treatment.

Our *G. vaginalis* growth experiments on single carbon source with or without acarbose were performed twice on the same day with two different precultures from the same plate. It could be argued whether this is truly a biological replicate. In our opinion it is, because not all colonies on the plate are the same and different amount are scraped from the plate to make a preculture. However, to be absolutely sure it is better to redo this experiment to validate the results.

For potential future use in the clinic acarbose has to have a limited effect on the growth of lactobacilli and a higher impact on the growth of *G. vaginalis.* A possible solution to maintain a microbiome dominated by lactobacilli is a combination of acarbose and prebiotics. Prebiotics are defined as substrates that are used by beneficial host microorganisms selectively to obtain an advantage against pathogenic bacteria[28]. A recent study identified the prebiotic lactulose that was able to stimulate various

Lactobacilli including *L. crispatus*, while not affecting the growth of various BV causing species including *G. vaginalis*. More importantly they also showed that in case of *L. crispatus* the stimulation was not strain specific, which indicates that this prebiotic could be beneficial for many women[29].

Future studies need to investigate further the effect of acarbose on *G. vaginalis* growth. We saw that different doses of acarbose have a different impact on *L.crispatus*, so further investigation is needed to test whether this is the same for *G. vaginalis*. Furthermore, future studies could involve a competition experiment where *L. crispatus* and *G. vaginalis* are grown together with lactulose and with or without acarbose. This could be carried out by a quantitative PCR to identify the abundance of these bacteria in combination with these compounds to investigate whether *L. crispatus* is able to outcompete *G. vaginalis* with these resources.

Conclusions

To conclude, this study provided novel insights in the growth of *L. crispatus* and *G. vaginalis* that could be introduced as novel targets against BV. lactobacilli are important for vaginal health, so the notion that *L. crispatus* grows better on glucose and *G. vaginalis* possibly better on glycogen suggests that bacterial glycogen metabolism could be a new target for treatment. This was supported by our finding that acarbose seems to have a higher impact on the growth of *G. vaginalis* than on the growth of *L. crispatus.* Future studies are needed to identify whether *G. vaginalis* prefers glucose or glycogen and to identify how the bacteria behave in competition with each other with or without the addition of acarbose. With these extra information it can be investigated whether acarbose is truly a novel treatment against BV.

Acknowledgements

First of all I would like to thank dr. Rosanne Hertzberger for providing me this internship and guiding me through the process of becoming a researcher. Thank you for providing me with feedback and suggestions (sometimes from a distance). I admire your enthusiasm for your subject and how you are able to work independently without being on a payroll to work on what you most believe in. Although the internship ended a little bit abruptly due to the Covid-19 crisis, I enjoyed it and learned a lot of skills that I can use for the next internships and even after my study.

Secondly, I would like to thank dr. Jurgen Haanstra for providing me with feedback and suggestions during the student meetings. Your feedback provided me with a solid background in how I can present my results the best way. Furthermore, I would like to thank you for taking over the feedback duty of my first draft, due to the other obligations Rosanne had.

I would like to thank my fellow interns, especially Luuk, Job, Senin, Lucas and Ellen. You provided me with valuable suggestions and feedback throughout my internship. I will miss the talks that we had during the lunch or at the coffee machine.

Last but not least I would like to thank the Systems Biology department for making me feel me welcome in the group.

References

1 Bradshaw, C. S. & Brotman, R. M. Making inroads into improving treatment of bacterial vaginosis - striving for long-term cure. *BMC Infect Dis* **15**, 292-292, doi:10.1186/s12879-015-1027-4 (2015).

2 Koumans, E. H. *et al.* The Prevalence of Bacterial Vaginosis in the United States, 2001–2004; Associations With Symptoms, Sexual Behaviors, and Reproductive Health. *Sexually Transmitted Diseases* **34**, 864-869, doi:10.1097/OLQ.0b013e318074e565 (2007).

3 Peebles, K., Velloza, J., Balkus, J. E., McClelland, R. S. & Barnabas, R. V. High Global Burden and Costs of Bacterial Vaginosis: A Systematic Review and Meta-Analysis. *Sex Transm Dis* **46**, 304-311, doi:10.1097/olq.0000000000000972 (2019).

4 Borgdorff, H. *et al.* The association between ethnicity and vaginal microbiota composition in Amsterdam, the Netherlands. **12**, e0181135, doi:10.1371/journal.pone.0181135 (2017).

5 Witkin, S. S. *et al.* Influence of vaginal bacteria and D- and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: implications for protection against upper genital tract infections. *MBio* **4**, e00460-00413, doi:10.1128/mBio.00460-13 (2013).

6 Goldenberg, R. L., Hauth, J. C. & Andrews, W. W. Intrauterine Infection and Preterm Delivery. *New England Journal of Medicine* **342**, 1500-1507, doi:10.1056/NEJM200005183422007 (2000).

7 Bitew, A., Abebaw, Y., Bekele, D. & Mihret, A. Prevalence of Bacterial Vaginosis and Associated Risk Factors among Women Complaining of Genital Tract Infection. *Int J Microbiol* **2017**, 4919404-4919404, doi:10.1155/2017/4919404 (2017).

8 Nunn, K. L. & Forney, L. J. Unraveling the Dynamics of the Human Vaginal Microbiome. *Yale J Biol Med* **89**, 331-337 (2016).

9 Witkin, S. S. *et al.* Vaginal Biomarkers That Predict Cervical Length and Dominant Bacteria in the Vaginal Microbiomes of Pregnant Women. *MBio* **10**, e02242-02219, doi:10.1128/mBio.02242-19 (2019).

10 van der Veer, C. *et al.* Comparative genomics of human Lactobacillus crispatus isolates reveals genes for glycosylation and glycogen degradation: implications for in vivo dominance of the vaginal microbiota. **7**, 49, doi:10.1186/s40168-019-0667-9 (2019).

11 Gosmann, C. *et al.* Lactobacillus-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased HIV Acquisition in Young South African Women. *Immunity* **46**, 29-37, doi:https://doi.org/10.1016/j.immuni.2016.12.013 (2017).

12 Huttenhower, C. *et al.* Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207-214, doi:10.1038/nature11234 (2012).

13 Mirmonsef, P. *et al.* Glycogen Levels in Undiluted Genital Fluid and Their Relationship to Vaginal pH, Estrogen, and Progesterone. *PLOS ONE* **11**, e0153553, doi:10.1371/journal.pone.0153553 (2016).
14 France, M. T., Mendes-Soares, H. & Forney, L. J. Genomic Comparisons of Lactobacillus crispatus and Lactobacillus iners Reveal Potential Ecological Drivers of Community Composition in the Vagina. *Applied and Environmental Microbiology* **82**, 7063, doi:10.1128/AEM.02385-16 (2016).
15 Spear, G. T. *et al.* Human α-amylase Present in Lower-Genital-Tract Mucosal Fluid Processes Glycogen to Support Vaginal Colonization by Lactobacillus. *The Journal of Infectious Diseases* **210**, 1019-1028, doi:10.1093/infdis/jiu231 (2014).
16 Moller, M. S. *et al.* An Extracellular Cell-Attached Pullulanase Confers Branched alpha-Glucan Utilization in Human Gut Lactobacillus acidophilus. *Appl Environ Microbiol* **83**, doi:10.1128/aem.00402-17 (2017).
17 Schwebke, J. R., Muzny, C. A. & Josey, W. E. Role of Gardnerella vaginalis in the Pathogenesis of Bacterial Vaginosis: A Conceptual Model. *The Journal of Infectious Diseases* **210**, 338-343, doi:10.1093/infdis/jiu089 (2014).
18 Zevin, A. S. *et al.* Microbiome Composition and Function Drives Wound-Healing Impairment in the Female Genital Tract. *PLoS Pathog* **12**, e1005889-e1005889, doi:10.1371/journal.ppat.1005889 (2016).
19 Ma, B. *et al.* A comprehensive non-redundant gene catalog reveals extensive within-community intraspecies diversity in the human vagina. *Nature Communications* **11**, 940, doi:10.1038/s41467-020-14677-3 (2020).
20 Bradshaw, C. S. & Sobel, J. D. Current Treatment of Bacterial Vaginosis—Limitations and Need for Innovation. *The Journal of Infectious Diseases* **214**, S14-S20, doi:10.1093/infdis/jiw159 (2016).
21 Workowski, K. A. & Bolan, G. A. Sexually transmitted diseases treatment guidelines, 2015. *MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports* **64**, 1-137 (2015).
22 Laube, H. Acarbose. *Clinical Drug Investigation* **22**, 141-156, doi:10.2165/00044011-200222030-00001 (2002).
23 Rosak, C. & Mertes, G. Critical evaluation of the role of acarbose in the treatment of diabetes: patient considerations. *Diabetes Metab Syndr Obes* **5**, 357-367, doi:10.2147/DMSO.S28340 (2012).
24 Dols, J. A. M. *et al.* Molecular assessment of bacterial vaginosis by Lactobacillus abundance and species diversity. *BMC Infectious Diseases* **16**, 180, doi:10.1186/s12879-016-1513-3 (2016).
25 Greenwood, J. R. & Pickett, M. J. Transfer of Haemophilus vaginalis Gardner and Dukes to a New Genus, Gardnerella: G. vaginalis (Gardner and Dukes) comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **30**, 170-178, doi:https://doi.org/10.1099/00207713-30-1-170 (1980).

26 Smith, B. J. *et al.* Changes in the gut microbiome and fermentation products concurrent with enhanced longevity in acarbose-treated mice. *BMC Microbiology* **19**, 130, doi:10.1186/s12866-019-1494-7 (2019).

27 Baxter, N. T., Lesniak, N. A., Sinani, H., Schloss, P. D. & Koropatkin, N. M. The Glucoamylase Inhibitor Acarbose Has a Diet-Dependent and Reversible Effect on the Murine Gut Microbiome. *mSphere* **4**, e00528-00518, doi:10.1128/mSphere.00528-18 (2019).

28 Gibson, G. R. *et al.* Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology* **14**, 491-502, doi:10.1038/nrgastro.2017.75 (2017).

29 Collins, S. L. *et al.* Promising Prebiotic Candidate Established by Evaluation of Lactitol, Lactulose, Raffinose, and Oligofructose for Maintenance of a Lactobacillus-Dominated Vaginal Microbiota. *Applied and environmental microbiology* **84**, e02200-02217, doi:10.1128/AEM.02200-17 (2018).

Supplements

Supplement 1: Growth Bicarbonate concentration

To investigate whether the CO_2 concentration in the anaerobic chamber was sufficient for anaerobic growth, *L. crispatus 10* was subjected to different concentrations of bicarbonate or no bicarbonate. It was noticed that *L. crispatus 10* without bicarbonate had almost the same OD590 as with *L. crispatus 10* with the highest amount of bicarbonate concentration. Also the growth between different bicarbonate concentrations did not differ much. This indicated that the anaerobic chamber consisted of enough CO_2 on his own for sufficient growth of our bacteria.

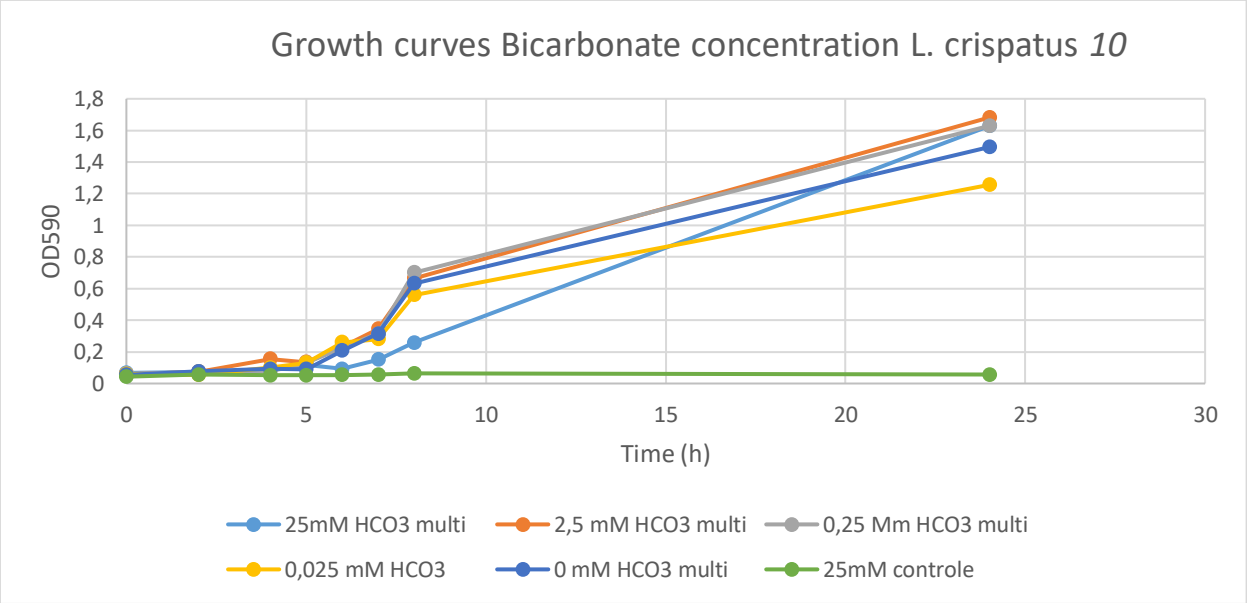


Figure S1: L. crispatus 10 growth on glucose with different bicarbonate concentrations, N = 1. This was a single experiment.

Supplement 2: Growth curve L. crispatus 9 and 10

We compared growth between *L. crispatus 9* and *L. crispatus 10*. Both bacteria were grown with either 5 g/L glucose or 5 g/L glycogen. Figure S2 shows that *L. crispatus 9* cannot grow on glycogen, while it has a shorter lag phase on glucose compared to *L. crispatus 10*. It could be argued that having a pullanase type I gene is also a disadvantage, that require energy to activate. This could explain why *L. crispatus 10* has a longer lag phase compared to *L. crispatus 9* when grown on glucose. Both strains can be isolated from the same patient, so it could be hypothesized that there is a sort of wild type helper effect that *L. crispatus 9* uses the ability of *L. crispatus 10* to cut glycogen into glucose, maltose and maltotriose. These products can also be used by *L. crispatus 9* for growth.

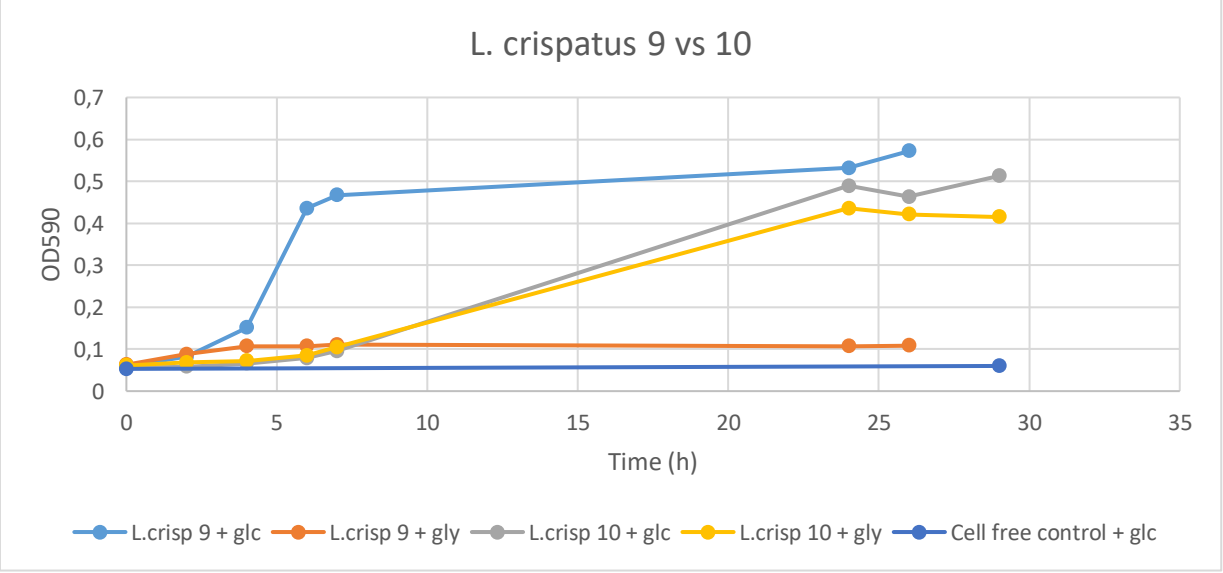


Figure S2: Growth curves of L. crispatus 9 and L. crispatus 10 with either 5 g/L glucose or 5 g/L glycogen. L. crispatus 9 is not able to grow on glycogen, while it starts to grow earlier on glucose then growth on glucose of L. crispatus 10, N = 1. This figure is representative for all three experiments that studied the growth of *L. crispatus 9* and *L. crispatus 10*.

Supplement 3: Temperature experiment and results

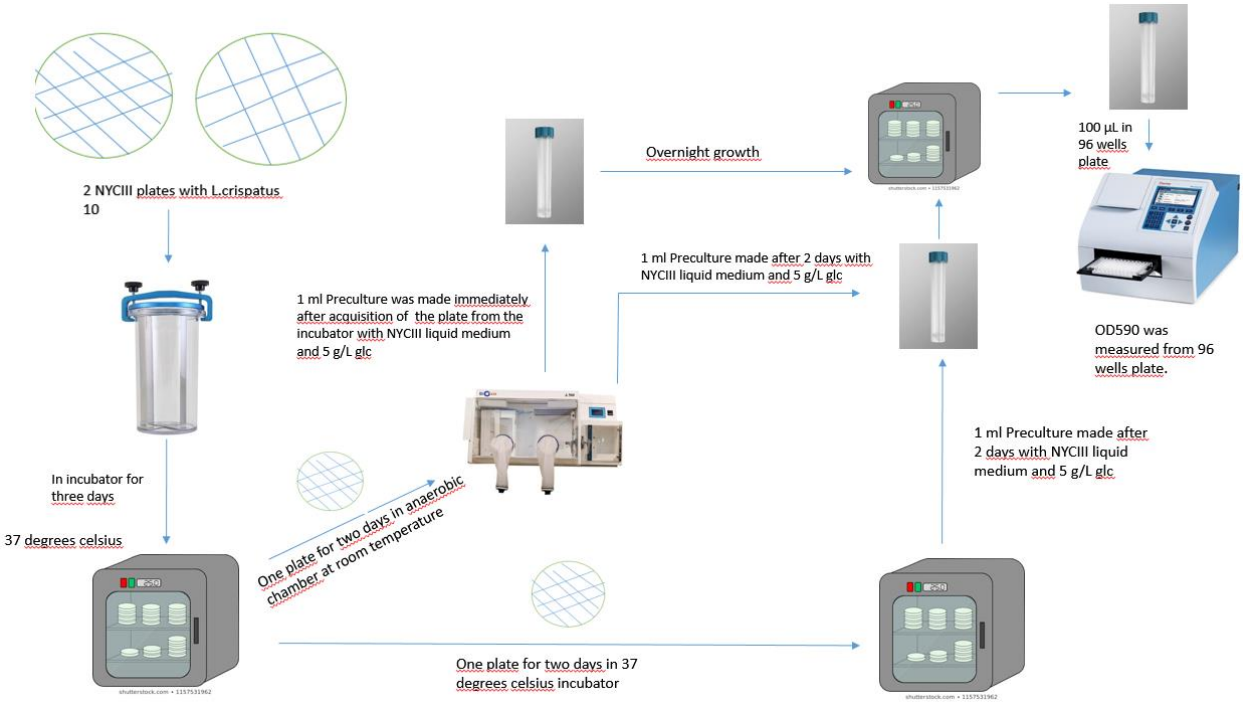


Figure S3: Temperature experiment Scheme

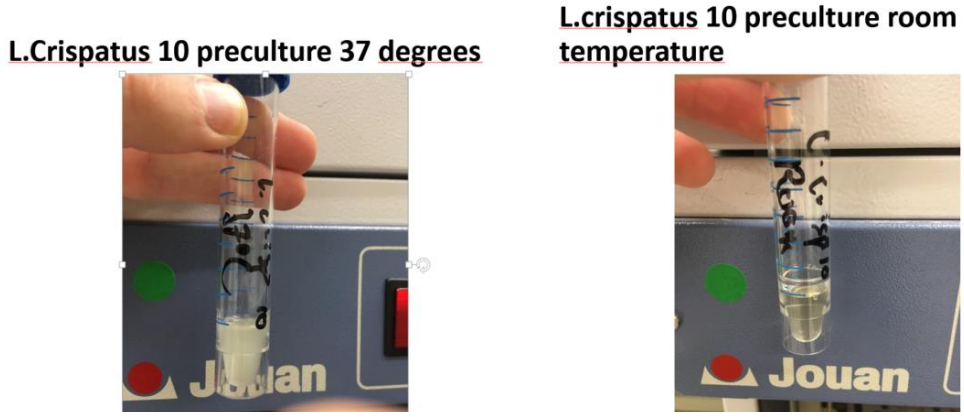


Figure S4: Precultures made from plate situated for 2 days in 37°C (left) and 2 days at room temperature (right). Preculture directly made from plate situated in 37°C showed an OD590 of 0,87 after 1 day of growth while preculture made from plate situated at room temperature for 2 days had an OD590 of 0,20 after 1 day of growth.

Supplement 4: Growth different acarbose concentrations

We wanted to investigate whether a different dose of acarbose results in a different impact on growth of *L. crispatus 10*. *L. crispatus 10* was prepared exactly the same way as previous acarbose experiments (see material and methods) with the exception that we not only used 100 µM acarbose, but also 25 µM and 50 µM acarbose. Growth on 2 g/L glycogen was compared with the different acarbose concentrations. Figure S5 shows the growth with addition of different concentrations acarbose. It is shown that the higher the dose of acarbose, the higher the impact on growth of *L. crispatus 10.* This suggests that the dose of acarbose is important for his inhibitory capacity.

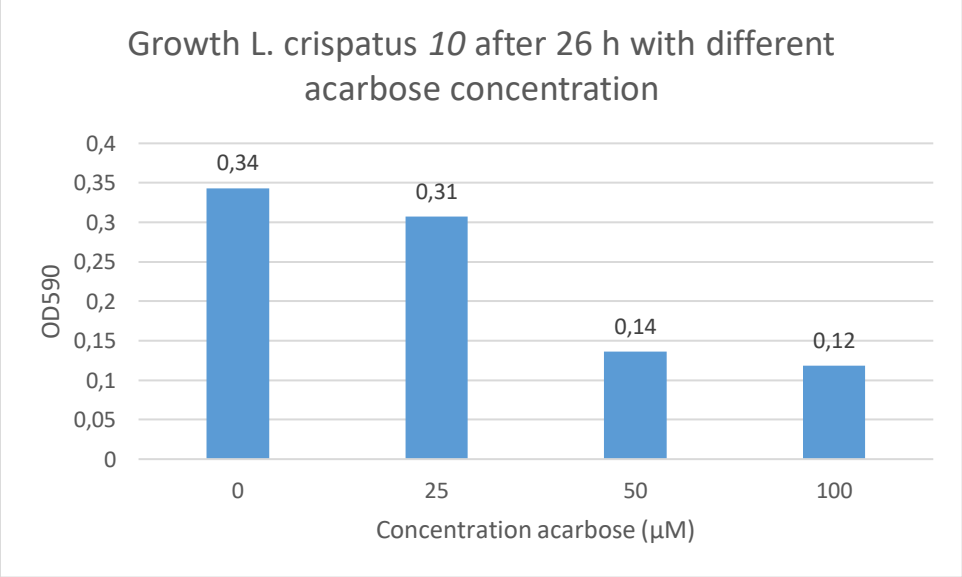
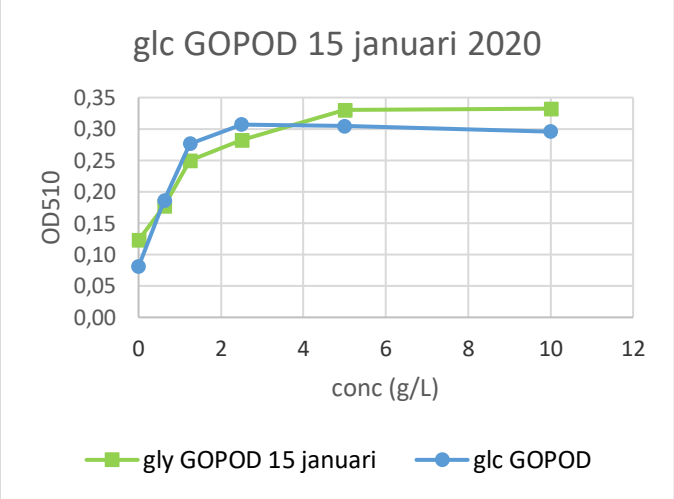


Figure S5: L. crispatus 10 final OD for 26h *on 2 g/L glycogen* and different concentrations of acarbose. The higher the dose of acarbose, the stronger the impact on growth of *L. crispatus 10, N = 1.* This was a single experiment.

Supplement 5: GOPOD assay troubleshooting

This assay required some troublehooting to investigate the usage of glucose and glycogen. The first problem that occurred was the saturation after already 1.25 g/L glucose and glycogen (figure S5A). This meant that the OD590 did not go up proportionally to the amount of glucose or glycogen that was added. The GOPOD reagent consisted of 20 mM amino-antipyrine (AAP), 2 mM 4- hydroxybenzoic- acid (HBA), 5 mg/ml Glucose oxidase (GOX) and 5 mg/ml Horse Radish Peroxidase (HRPO). The amount of AAP and HBA was multiplied by 5. In the mix 0.5 ml AAP, 0.5 ml HBA, 1 ml GOX and 1 ml HRPO were added together and filled to 50 ml with ice cold PBS.
It was investigated whether the ingredients of the GOPOD reagent contained the right amount. It was noticed that adding extra GOX or HRPO did not show a higher amount of color forming in the wells (not shown). However, we did notice that increasing the amount of AAP and HBA also increased the OD value. This resulted in that we made a new GOPOD reagens with the same amounts of ingredients, except that we did not multiply the amount of HBA and AAP by 5 and that we added 2.5 mg extra HBA and 1 ml extra AAP in 25 ml GOPOD reagent.



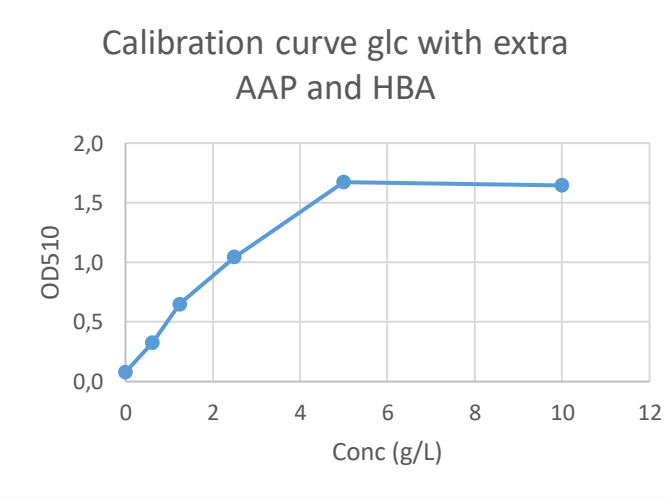


*Figure S5: Calibration curves with old GOPOD (**A**) and new GOPOD (**B**). With old GOPOD saturation occurred after 1.25 g/L glucose or glycogen, while with the new GOPOD saturation occurred at 5 g/L.*

Although the OD510 values corresponded more with the concentration of glucose, a new problem occurred. We discovered that the glycogen calibration curve was always approximately half of the glucose calibration curve (figure S6). For calculations of the glycogen concentration this resulted in a higher amount of glycogen than the amount that we actually added during growth experiments. We explored whether the amyloglucosidase (AMG) was able to convert all the glycogen present in the supernatant to glucose by adding additional amylase. Both AMG and amylase were 100x diluted. It could

be possible that AMG has trouble with cutting multiple glucose molecules into single glucose molecules on his own. By adding extra amylase it was thought that the amylase cuts glycogen into maltose (2 glucose molecules) and maltotriose (three glucose molecules), which would make it more easy for AMG to cut into single glucose molecules. Unfortunately, AMG in combination with amylase did not have a significant better effect on OD values with glycogen (fig S7).

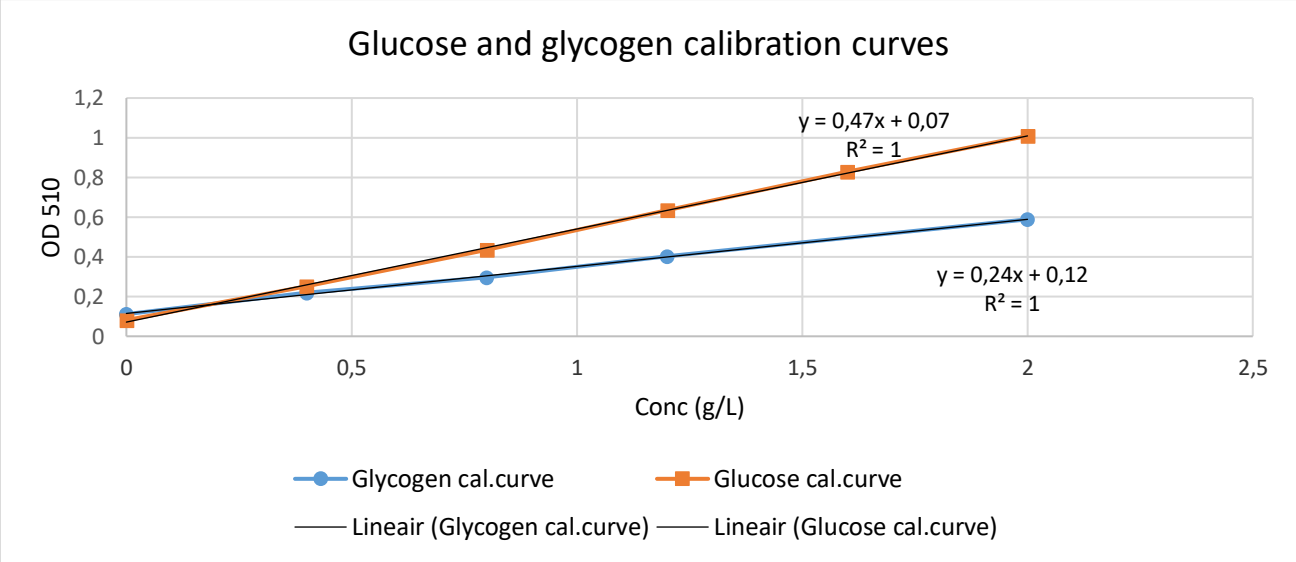


Figure S6: Glucose and glycogen calibration curves after adding 1 ml extra AAP and 2.5 g extra HBA to 25 ml GOPOD reagent. The glycogen calibration curve was approximately 50% lower than the glucose calibration curve.

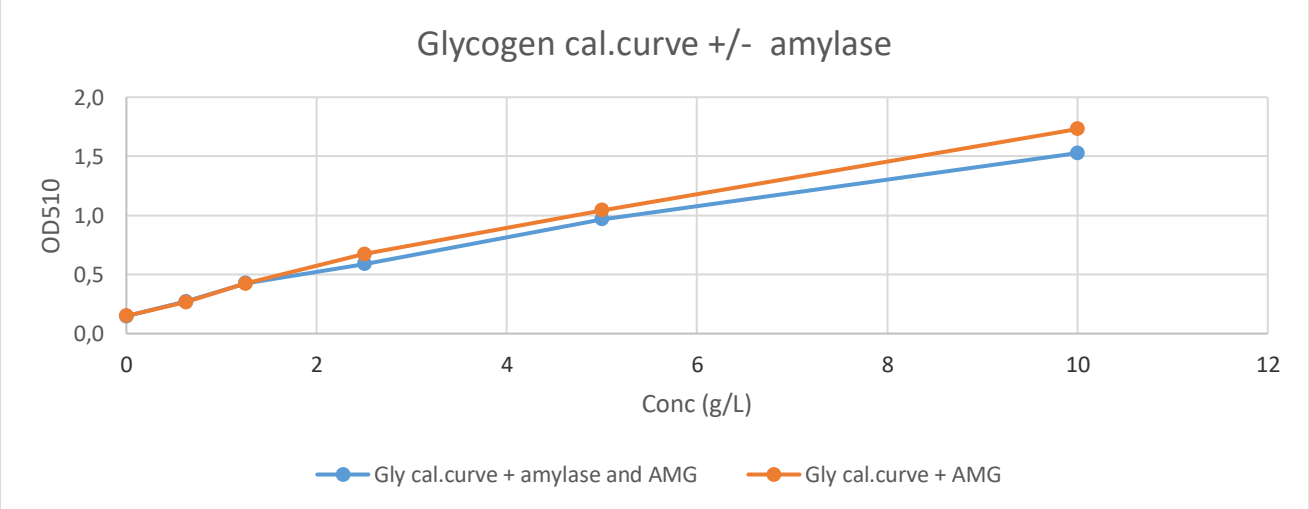


Figure S7: Glycogen calibration curves with or without extra amylase added to AMG. Extra 100x diluted amylase did not result in an increase of optical density.

Subsequently we noticed that the OD510 at 0 g/L glycogen also gave an absorbance signal (S8). We suggested that we had to substract the OD510 of 0 g/L glycogen without AMG from OD510 with AMG and use the result as correction for the glycogen concentration. This meant that we substracted this result together with the OD510 without AMG from the OD510 with AMG to calculate the glycogen OD:

$$glycogen\ OD = OD510\ with\ AMG - OD510\ without\ AMG - correction\ acquired\ from\ calibration\ curve$$

This resulted that the glycogen concentration became closer to 2 g/L, which was the concentration that we actually added (fig S9). However, still the concentration of glycogen was often higher (approx 2.5 g/L) while the concentration of glucose was always lower (approx 1.5 g/L). After this observation we decided that although we cannot measure the glycogen and glucose concentration quantitavely we still can use GOPOD to investigate how the concentration of glucose and glycogen changes over time.

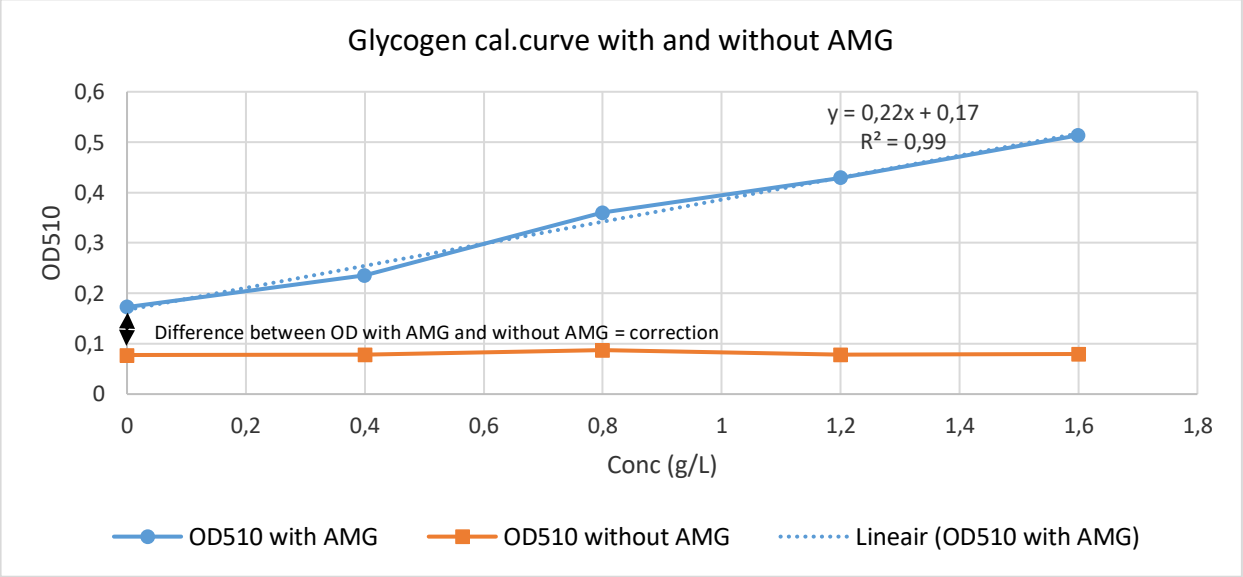


Figure S8: Glycogen calibration curve with and without AMG.

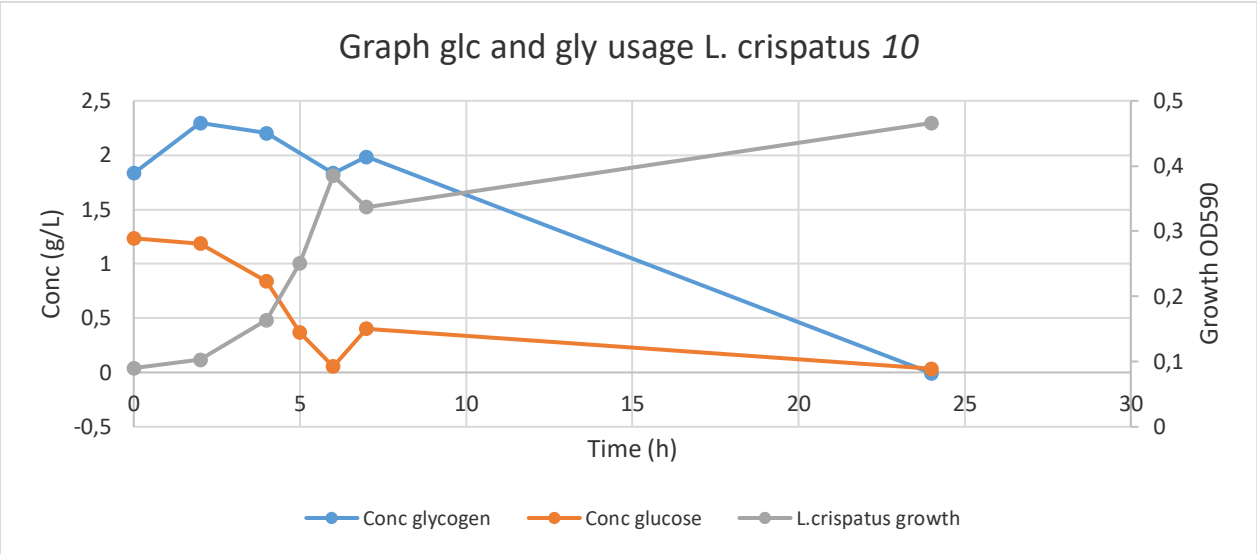


Figure S9: Glycogen and glucose usage after correction. Glycogen concentration becomes closer to 2 g/L after substraction of the correction, while glucose concentration is approximately 1.3 g/L after correction.

Supplement 6: Schedule experiments and strain usage

Experiment	Data used for analysis
Bicarbonate experiment	12/11/20
Growth *L.crisp 9 vs L.crisp 10*	28/11/19, 05/03/20 and 11/03/20
Growth *L.crisp 10* glucose vs glycogen	30/01/20, 06/02/20, 20/02/20
Growth *G. vaginalis*	11/12/19, 19/12/19, 20/02/20 and 11/03/20
GOPOD *L. crispatus 10*	12/02/20, 28/02/20, 06/03/20
GOPOD *G. vaginalis*	05/02/20, 13/02/20, 28/02/20,
Growth acarbose *L. crispatus* glc or gly	30/01/20, 06/02/20, 20/02/20
Growth acarbose *G. vaginalis* glc or gly	11/03/20 (preculture 1 and 2)
Growth acarbose *L. crispatus* double carbon source	06/02/20, 13/02/20, 20/02/20
Growth acarbose *G. vaginalis* double carbon source	06/02/20, 20/02/20, 11/03/20,
Growth for GOPOD *L. crispatus*	06/02/20, 20/02/20, 05/03/20
Growth for GOPOD *G. vaginalis*	30/01/20, 06/02/20, 20/02/20

Bacteria	Stock
L. crispatus 9	Stock 2 (column 2)
L. crispatus 10	Stock 2 (column 2)
G. vaginalis	Original stock (column 2)