Unraveling the dynamics of glycogen metabolism of *Lactobacillus crispatus* – a prominent member of the vaginal microbiome

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Abbreviations

MRS, Man, Rogosa and Sharpe; NYCIII, New York City III; OD, optical density; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; GLC, glucose; GLY, glycogen; MT, maltose; MTT, maltotriose; GAL, galactose; cre, catabolite response element; CcpA, Catabolite control protein A.

Abstract

A vaginal microbiome dominated by lactobacilli (including *Lactobacillus crispatus*) provides protection from pre-term delivery in pregnant women, decreases the chance of acquiring sexually transmitted diseases and increases the success rate of IVF treatments. Glycogen in the vaginal epithelium is thought to support lactobacilli colonization in vivo, a recent study by Hertzberger & Brandt confirmed growth of *L. crispatus* on glycogen-supplemented growth media. Here we studied the regulation of glycogen metabolism of *L. crispatus*. We used *L. crispatus* strain 9 and 10, conducted growth experiments on various carbon sources and performed enzymatic assays using starch as a proxy for glycogen. The most important results outline that *L. crispatus* strain 10 can grow on glycogen and produce lactate. This supports the novel theory that lactobacilli can utilize glycogen to acidify the vagina. Starch assays illustrated that after growth on glycogen or galactose, *L. crispatus* strain 10 can break down starch. Strain 9 cannot breakdown starch regardless of the carbon source present during growth. The presence of glucose, maltose and maltotriose during growth inhibited starch degradation activity of *L. crispatus* strain 10. These results imply that glycogen metabolism of *L. crispatus* might be regulated by carbon catabolite repression. This entails that *L. crispatus* uses the carbon sources that are most easily accessible and allow for fastest growth while the synthesis of enzymes involved in the metabolism of secondary carbon sources are inhibited.

Introduction

The vaginal milieu is highly dynamic. Sexual interaction, hormonal changes, menstruation, giving birth and use of bathing products for hygiene are all factors that affect the vagina. Yet, the vaginal microbiome shows a remarkable lack of biodiversity, which contrasts with microbiomes of the mouth, the skin and the gut (Human Microbiome Project, 2012). Most vaginal microbiomes of reproductive-age women have a dominance of only a handful of closely related lactic acid bacteria: *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus jensenii* or *Lactobacillus gasseri* (Ravel et al., 2011). A key characteristic of colonization by lactobacilli is the relatively low pH in the female genital tract (pH ≤ 4.5) as a result of the ability of lactobacilli to produce lactic acid (Boskey, Cone, Whaley, & Moench, 2001; Boskey, Telsch, Whaley, Moench, & Cone, 1999).

A subset of women may encounter a decline in vaginal acidity, an overgrowth of a variety of mostly anaerobic bacteria and a reduction in lactobacilli over time. This is commonly recognized as bacterial vaginosis (O'Hanlon, Moench, & Cone, 2011). Some women with this dysbiosis are asymptomatic but others experience symptoms such as vaginal malodor, unusual discharge and genital itchiness (Klebanoff et al., 2004). There are more serious health effects associated with bacterial vaginosis. These include an increased risk of pre-term delivery in pregnant women (Hay et al., 1994; Petricevic et al., 2014), an increased risk of acquiring STDs including HIV (Gosmann et al., 2017), chlamydia and gonorrhoeae (Wiesenfeld, Hillier, Krohn, Landers, & Sweet, 2003), and a lower chance of success in in vitro fertilization treatments (Haahr et al., 2016).

All resources necessary to support vaginal bacterial communities are ultimately derived from the host (Nunn & Forney, 2016). Glycogen is an abundant vaginal source of carbohydrates (Mirmonsef et al., 2016). A positive correlation exists between the levels of free glycogen and the abundance of vaginal lactobacilli (Mirmonsef et al., 2014). The general assumption in the field is that lactobacilli can break down glycogen. However, multiple international studies have tried growing lactobacilli on glycogen but failed (Martin et al., 2008; Spear et al., 2014; Wylie & Henderson, 1969). In contrast to these results, a study completed by Hertzberger recently confirmed the hypothesis and showed that various strains of *L. crispatus* can grow on glycogen (Hertzberger & Brandt, 2018). Comparative genomics analyses on *L. crispatus* strains by Ojala and van der Veer found several strains carrying a putative type I pullulanase gene (Ojala et al., 2014; van der Veer et al., 2018). Type I pullulanase catalyzes hydrolysis of α-(1,6) linked branches in glycogen, amylopectin and other starch-derived glycans (Moller et al., 2017). This enzyme might be of importance in glycogen degradation by *L. crispatus* in the vagina.

Lactobacilli have a protective role in the vagina by maintaining an acidic environment through the production of lactic acid that inactivates the bacteria associated with bacterial vaginosis (O'Hanlon et al., 2011). Unfortunately, over the past 20 years, no significant improvements in cures for bacterial vaginosis have been made (Bradshaw & Brotman, 2015). Approaches that aim to restore and sustain a vaginal microbiome dominated by lactobacilli are essential to prevent bacterial vaginosis and its negative health outcomes. Further research is important to develop pre- and probiotics to maintain and support a *Lactobacillus*-dominated vaginal microbiome in women.

This study builds on the finding that *L. crispatus* can grow on glycogen found by Hertzberger & Brandt (2018). Here we studied the regulation of glycogen degradation activity of *L. crispatus*. We hypothesized that glycogen metabolism in *L. crispatus* is dependent on carbon sources present during growth. Growth experiments on multiple carbon sources were conducted and enzymatic assays using starch as a proxy for glycogen were performed.

Results

L. crispatus grows faster in the Anaerobic Jar than in Eppendorf tubes

Here we compared two methods of anaerobic growth to determine which method is most suitable to grow *L. crispatus*.

L. crispatus strain 9 was cultured in New York City (NYC) III medium supplemented with glucose (4.8 g/L) in an Anaerobic Jar or in Eppendorf tubes with minimal headspace to reduce oxygen concentrations to a minimum. The culture grown in the Anaerobic Jar reached an approximate optical density (OD) of 2.5 in about 20 hours while the Eppendorf tubes needed about 50 hours of growth to reach an OD of 2.5 (figure 1). We conclude that the Anaerobic Jar is a better method to grow *L. crispatus* and therefore we proceded growth of *L. crispatus* in the Anaerobic Jar for further experiments.

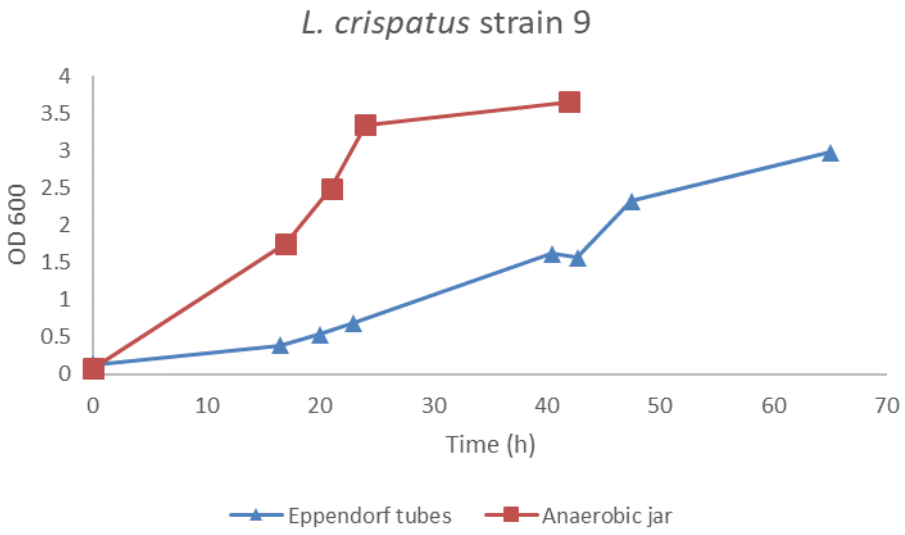

Figure 1: The OD at 600 nm was measured of *L. crispatus* strain 9 grown in NYCIII medium supplemented with glucose (4.8 g/L) using an Anaerobic Jar or Eppendorf tubes. Each method was run in duplicate, a representative is shown here. File 'Anaerobic_growth'

L. crispatus strain 10 can growth on glycogen

To successfully dominate the vaginal mucosa, it may be of importance for *L. crispatus* to efficiently utilize available nutrients in the vagina. Glycogen is an abundant vaginal source of carbohydrates (Mirmonsef et al., 2016). A positive correlation exists between the levels of free glycogen and the abundance of vaginal lactobacilli (Mirmonsef et al., 2014).

We compared growth of *L. crispatus* strain 9 and 10 on NYCIII medium supplemented with glycogen to growth on NYCIII medium supplemented with glucose (positive control) and water (negative control). Glucose and glycogen were both supplemented with a final concentration of 4.8 g/L. In

addition, we looked at lactate concentrations in the supernatants of *L. crispatus* after growth on glycogen, glucose and water.

Results present that *L. crispatus* strain 9 cannot grow on glycogen as the OD remains the same as the OD of water over time (figure 2). *L. crispatus* strain 10 grown on glycogen shows a similar OD as growth on glucose over time (figure 2). To summarize, *L. crispatus* strain 9 cannot grow on glycogen and strain 10 can. When we look at lactate concentrations, we see that strain 9 grown on glycogen has a similar lactate concentration as growth on water (figure 3). For *L. crispatus* strain 10 we see comparable concentrations of lactate between growth on glycogen and glucose (figure 3). Since the medium contains very small amounts of lactate, we can conclude that *L. crispatus* produces lactate after growth on glucose (strain 9 and 10) or glycogen (strain 10) (figure 3).

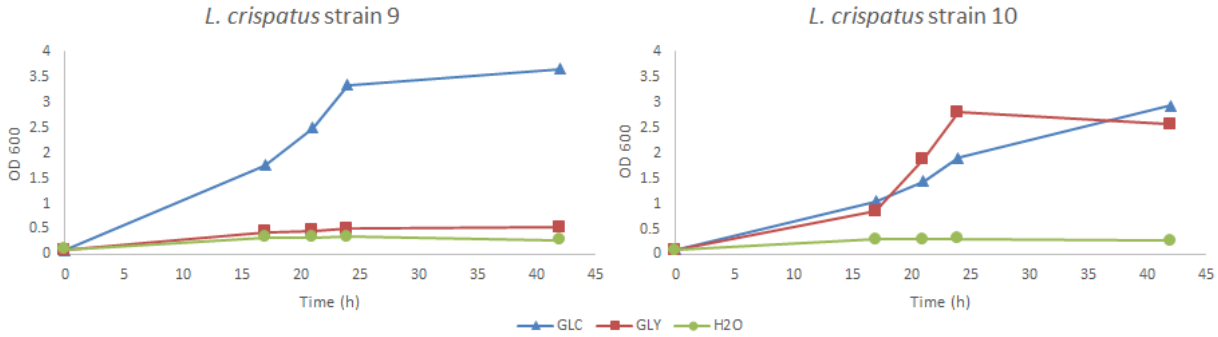

Figure 2: The OD at 600 nm was measured of *L. crispatus* strain 9 and 10 grown in NYCIII medium supplemented with glucose (4.8 g/L), glycogen (4.8 g/L) or demi water. Experiment was run in duplicate; a representative is shown here. Abbreviations: NYC, New York City; OD, optical density; GLC, glucose; GLY, glycogen. File 'Anaerobic_growth'

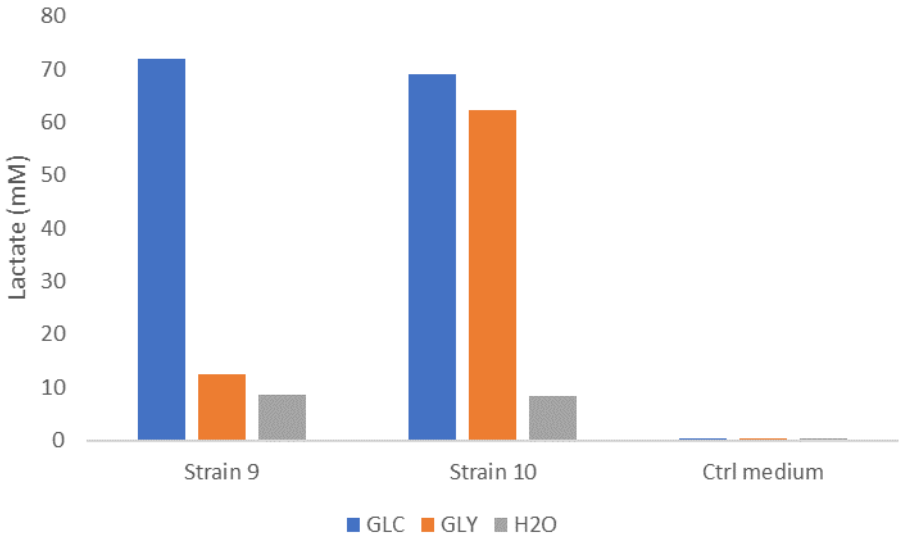

Figure 3: Lactate concentration (mM) in supernatants of *L. crispatus* strains 9 and 10 after 48 hours of growth on NYCIII medium supplemented with glucose (4.8 g/L), glycogen (4.8 g/L) or demi water. Experiment was conducted once. Abbreviations: NYC, New York City; GLC, glucose; GLY, glycogen. File 'Organic_acids_9&10_GLC_GLY_H2O_11-4'

L. crispatus strain 10 can break down starch after growth on glycogen

The pellets and supernatants of *L. crispatus* strain 9 and 10 after growth on glucose, glycogen and demi water were incubated in a starch solution to investigate enzymatic activity. Starch is of interest

because it shows similarities in structure with glycogen. Starch is composed of amylose and amylopectin. Amylose is a linear polymer of glucose units with α,1-4 glycosidic bonds. Amylopectin consists of α,1-4 linked linear chains of glucose units and α,1-6 linked side chains of glucose units (Ganzle & Follador, 2012). Glycogen has a similar structure as amylopectin, but the major difference lies within the side chains: in glycogen, they are shorter and about twice higher in number than in starch (van der Maarel, van der Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002). This essentially means that are more α,1-6 linked glucose units in glycogen.

L. crispatus strain 9 and 10 were cultured in NYCIII medium supplemented with glucose, glycogen and demi water for 48 hours. Glucose and glycogen were both supplemented with a final concentration of 4.8 g/L. After growth, the pellets and supernatants were separated and incubated in a starch solution (7.5 g/L). After 24 hours of incubation, residual starch was measured with an iodine staining and subtracted from the water control to calculate starch degradation (g/L).

Results illustrate that there is minimal starch degradation activity in the pellets and supernatants of *L. crispatus* strain 9 after growth on glycogen or glucose (figure 4). This also applies to the pellet and supernatant of *L. crispatus* strain 10 after growth on glucose. Only the pellet and supernatant of *L. crispatus* strain 10 after growth on glycogen demonstrate starch degradation activity (figure 4). We cannot observe compelling differences in starch breakdown activity between the pellets and supernatants regardless of the carbon source present during growth (figure 4). As *L. crispatus* strain 9 cannot grow on glycogen and shows no starch degradation activity, this strain is excluded in further experiments.

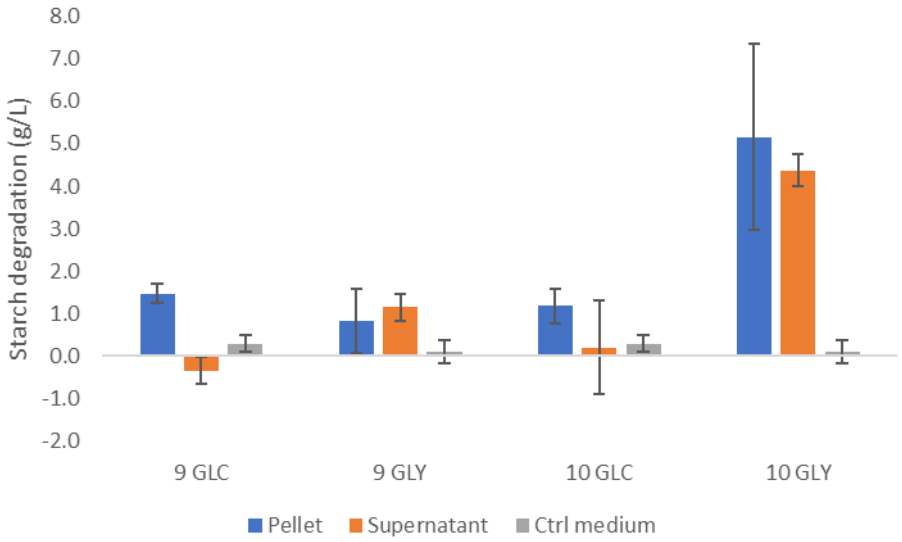

Figure 4: Starch degradation activity of the pellets and supernatants of *L. crispatus* strain 9 and 10 grown in NYCIII medium supplemented with glucose (4.8 g/L), glycogen (4.8 g/L) or demi water and after incubation in a starch solution (7.5 g/L). Experiment was run in duplicate; averages and standard deviations are shown. Abbreviations: NYC, New York City; GLC, glucose; GLY, glycogen. File 'Starch_assay_9&10_GLC_GLY_H2O'

L. crispatus strain 10 can grow on glycogen breakdown products but not on lactulose

Previous results (figure 4) showed that the presence of glycogen during growth of *L. crispatus* strain 10 induces starch breakdown activity. We wish to examine whether other carbon sources can also induce starch breakdown activity of *L. crispatus* strain 10. Here we first analyze if *L. crispatus* strain 10 can grow on other carbon sources besides glucose and glycogen (figure 2). Carbon sources of interest are maltose, maltotriose, galactose and lactulose. Maltose and maltotriose are breakdown products of glycogen. Galactose has the same structural formula as glucose but a different orientation of functional groups around the fourth carbon atom and a different metabolic pathway. Lactulose was considered as a promising prebiotic by Collins et al (2018) to support a *Lactobacillus*-dominated vaginal microbiome.

L. crispatus strain 10 was grown in NYCIII medium supplemented with maltose, maltotriose, galactose or lactulose with a final concentration of 4.8 g/L. After 48 hours of growth, the OD was measured. Results were compared to the OD's of glucose (positive control) and demi water (negative control).

The results pose growth of *L. crispatus* strain 10 on all examined carbon sources except for lactulose (figure 5).

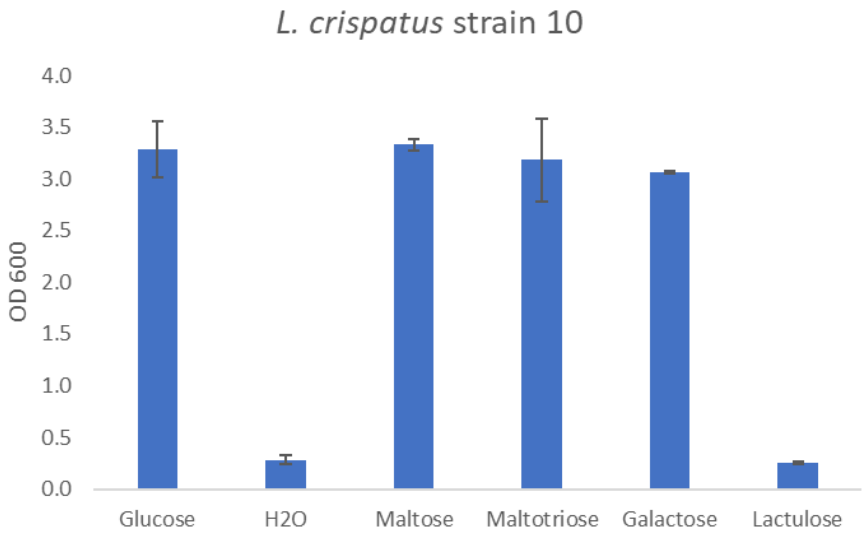

Figure 5: The OD at 600 nm was measured of *L. crispatus* strain 10 after 48 hours of growth on NYCIII medium supplemented with glucose (4.8 g/L), maltose (4.8 g/L), maltotriose (4.8 g/L), galactose (4.8 g/L), lactulose (4.8 g/L) or demi water. Experiment was run in duplicate; averages and standard deviations are shown. Abbreviations: NYC, New York City; OD, optical density. File 'Growth_multiple_carbon_sources'

L. crispatus strain 10 can break down starch after growth on galactose

Previous results (figure 5) showed that *L. crispatus* strain 10 can grow on maltose, maltotriose and galactose. We examined whether the presence of maltose, maltotriose or galactose during growth can induce starch breakdown activity in *L. crispatus* strain 10.

L. crispatus strain 10 was cultured in NYCIII medium supplemented with maltose, maltotriose or galactose with a final concentration of 4.8 g/L for 48 hours. The pellets and supernatants were separated and incubated in a starch solution (7.5 g/L). After 24 hours of incubation, residual starch

was measured with an iodine staining and subtracted from the water control to calculate starch degradation. Results were compared with glycogen and glucose which were included as positive and negative controls.

Results indicate that the pellet and supernatant of *L. crispatus* strain 10 after growth on galactose can break down starch (figure 6). The pellets of *L. crispatus* strain 10 after growth on maltose and maltotriose illustrate minimal starch degradation while the supernatants show an absence of starch degradation (figure 6).

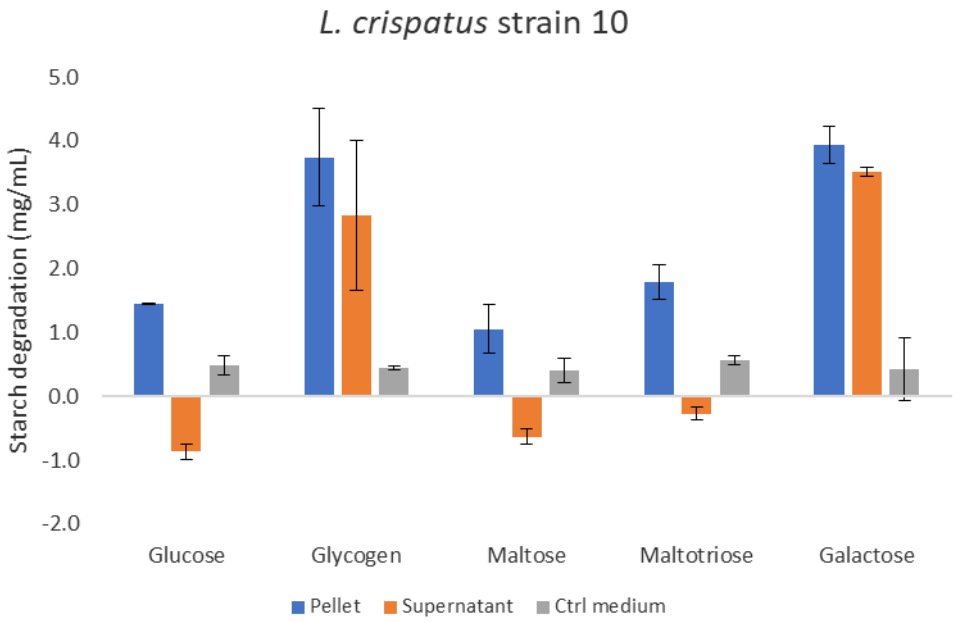

Figure 6: Starch degradation activity of the pellets and supernatants of *L. crispatus* strain 10 grown in NYCIII medium supplemented with glucose (4.8 g/L), glycogen (4.8 g/L), maltose (4.8 g/L), maltotriose (4.8 g/L) or galactose (4.8 g/L) and after incubation in a starch solution (7.5 g/L). Experiment was run in duplicate; averages and standard deviations are shown. Abbreviations: NYC, New York City. File 'Starch_assay_multiple_carbon_sources'

L. crispatus strain 10 breaks down less starch after simultaneous growth on glycogen with either glucose, maltose or maltotriose

Results of the previous experiment (figure 6) lead to the conclusion that the presence of glycogen or galactose during growth induce starch degradation activity of *L. crispatus* strain 10. In contrast, the presence of glucose, maltose and maltotriose during growth repress starch degradation activity of *L. crispatus* strain 10 (figure 6).

Here we examined starch degradation activity after growth on two carbon sources simultaneously, glycogen with either glucose, maltose, maltotriose or galactose. This experiment may identify if the addition of glucose, maltose and maltotriose to glycogen represses starch breakdown activity in *L. crispatus* strain 10. Moreover, we can study if the addition of galactose to glycogen enhances starch breakdown activity in *L. crispatus* strain 10.

L. crispatus strain 10 was grown in NYCIII medium supplemented with two carbon sources (glycogen with either glucose, maltose, maltotriose or galactose) with a final concentration of 4.8 g/L of the two carbon sources combined. After 48 hours of growth, the pellets and supernatants were separated and incubated in a starch solution (7.5 g/L). After 24 hours of incubation, residual starch was measured with an iodine staining and subtracted from the water control to calculate starch degradation. Results were compared with glycogen and glucose which were included as positive and negative controls.

Results show that the pellets and supernatants of *L. crispatus* strain 10 after growth on glycogen with additional glucose, maltose or maltotriose breaks down less starch compared to the pellet and supernatant of *L. crispatus* strain 10 after growth on solely glycogen (figure 7). Starch degradation activity of the pellet and supernatant of *L. crispatus* strain 10 grown on glycogen with added galactose is similar to the starch degradation activity of the pellet and supernatant *L. crispatus* strain 10 on glycogen as the sole carbon source during growth (figure 7).

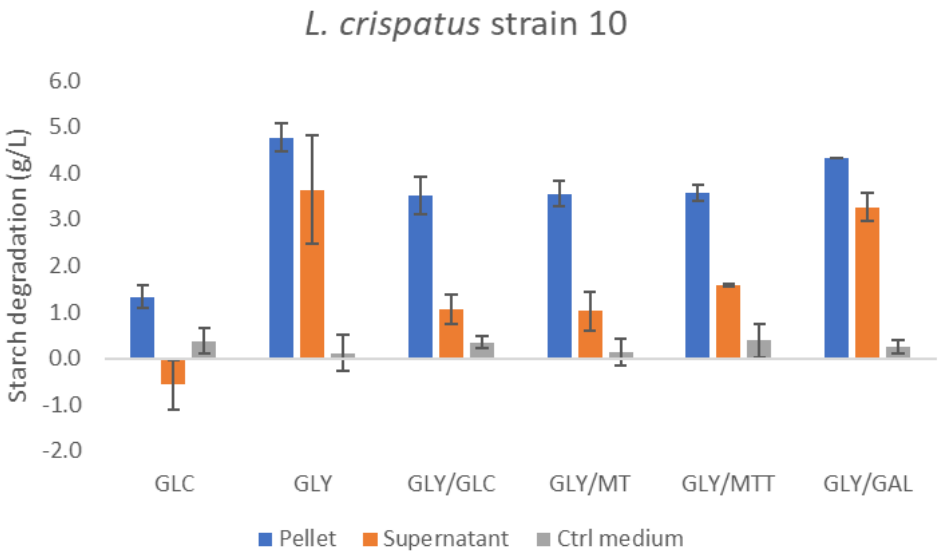

Figure 7: Starch breakdown activity of the pellets and supernatants of *L. crispatus* strain 10 grown in NYCIII medium supplemented with glycogen and either glucose, maltose, maltotriose or galactose with a final concentration of 4.8 g/L of the two carbon sources combined and after incubation in a starch solution (7.5 g/L). Experiment was run in duplicate; averages and standard deviations are shown. Abbreviations: NYC, New York City; GLC, glucose; GLY, glycogen; MT, maltose; MTT, maltotriose; GAL, galactose. File 'Starch_assay_double_carbon_sources'

All *L. crispatus* strains have a palindromic cre site in the promoter

A study completed by van der Veer et al (2018) found that several *L. crispatus* strains, including strain 10, carry a putative type I pullulanase gene. Type I pullulanase can catalyze the hydrolysis of α-(1,6) linked branches in glycogen, amylopectin and other starch-derived glycans (Moller et al., 2017). This enzyme could possibly be responsible for the degradation of starch in *L. crispatus* strain 10.

Results of previous experiments delineate that the presence of glucose, maltose or maltotriose during growth repress starch degradation activity of *L. crispatus* strain 10 (figure 6 and 7). This might be an indication for carbon catabolite repression. This implies that *L. crispatus* preferentially uses the

carbon sources that are most easily accessible and allow for fastest growth while the synthesis of enzymes involved in the metabolism of secondary carbon sources are inhibited. Therefore, we speculate that the putative type I pullulanase in *L. crispatus* strain 10 suspected to be responsible for the starch degradation activity might be regulated at DNA level. A highly conserved regulator of carbon catabolite repression in Gram-positive bacteria is the catabolite control protein A (CcpA) (Lorca et al., 2005). The CcpA forms a complex with a phosphorylated corepressor and has an increased affinity for a cis acting DNA motif, described as a catabolite response element (cre) (Lu et al., 2018). The binding of this complex to the cre site can repress or enhance gene expression depending on the position of the cre site in the promotor (Lu et al., 2018). The cre site has a palindromic nucleotide motif in *Lactobacillus plantarum* (TGWAANCGNTNWCA, where N = any base; W = A or T) (Lu et al., 2018).

We assume that *L. crispatus* might have a similar palindromic nucleotide motif in the promotor as *L. plantarum*. Consequently, we examined the upstream region of the putative type I pullulanase gene of various *L. crispatus* strains to search for a palindromic cre site. Only strains that were sequenced by Dols et al (2016) and had no mutations in the putative type I pullulanase gene (van der Veer et al., 2018) were included.

All examined strains including strain 10 of *L. crispatus* have a palindromic cre site (TGTTATCGATAACA) in the upstream region of the putative type I pullulanase gene (figure 7).

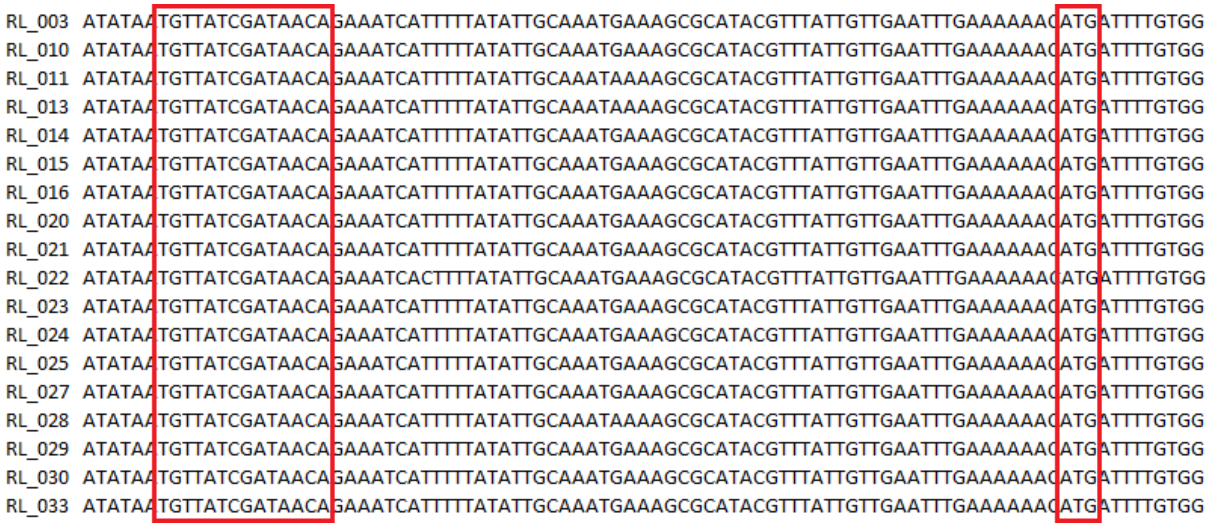

Figure 8: Sequence alignment of the upstream region of the putative type I pullulanase gene in various *L. crispatus* strains. The red boxes indicate the palindromic cre site and the start codon. Abbreviations: cre, catabolite response element. File 'Palindromic_cre_sites_29-4'

Discussion

Key findings of this study

The research question of this study was how glycogen metabolism of *L. crispatus* might be regulated. Here we studied *L. crispatus* strain 9 and 10, conducted growth experiments on various carbon sources and performed enzymatic assays using starch as a proxy for glycogen. The most important results outline that *L. crispatus* strain 10 can grow on glycogen and lactate is produced after growth on glycogen or glucose (table 1 and 2). Starch assays illustrated that after growth on glycogen or galactose, *L. crispatus* strain 10 can break down starch (table 3). Strain 9 cannot breakdown starch regardless of the carbon source present during growth. The presence of glucose, maltose and maltotriose during growth inhibited starch breakdown activity of *L. crispatus* strain 10 (table 3 and figure 7). These results imply that glycogen metabolism in *L. crispatus* might be regulated by carbon catabolite repression.

	L. crispatus strain 9	*L. crispatus* strain 10
Glucose	✓	✓
Demi water	✗	✗
Glycogen	✗	✓
Maltose	-	✓
Maltotriose	-	✓
Galactose	-	✓
Lactulose	-	✗

Table 1: Growth of L. crispatus strain 9 and 10 on various carbon sources

	L. crispatus strain 9	*L. crispatus* strain 10
Glucose	✓	✓
Demi water	✗	✗
Glycogen	✗	✓

Table 2: Lactate production of *L. crispatus* strain 9 and 10 after growth on various carbon sources

	L. crispatus strain 9	*L. crispatus* strain 10
Glucose	✗	✗
Glycogen	✗	✓
Maltose	-	✗
Maltotriose	-	✗
Galactose	-	✓

Table 3: Starch degradation activity of *L. crispatus* strain 9 and 10 after growth on various carbon sources

Putative type I pullulanase of *L. crispatus*

Results in this study demonstrate that *L. crispatus* strain 10 can grow on glycogen while strain 9 cannot (table 1). These results match with research completed earlier by Hertzberger and Brandt (2018). A possible explanation for the lack of growth of strain 9 on glycogen might be the frameshift mutation in the N-terminal region of the putative type I pullulanase gene (van der Veer et al., 2018). This putative type I pullulanase gene was disclosed by Ojala et al (2014) and van der Veer et al (2018) in several strains of *L. crispatus*. Type I pullulanase catalyzes hydrolysis of α-(1,6) linked branches in glycogen, amylopectin and other starch-derived glycans (Moller et al., 2017). The results in this study do not provide enough evidence that the putative type I pullulanase is truly a pullulanase as it could also be an amylase. Articles by Spear and Nasioudis already found amylase in the vagina, this could possibly be of bacterial origin (Nasioudis et al., 2015; Spear et al., 2014). To get more clarity on the enzymatic properties of the putative type I pullulanase of *L. crispatus*, other substrates such as amylose and pullulan can be examined. Amylose is a linear polymer of glucose unites that are α-(1,4) linked and pullulan is a polysaccharide with repeating units of maltotriose that are α-(1,6) linked (van der Maarel et al., 2002).

Carbon catabolite repression in *L. crispatus*

Results of the starch assays delineate that the presence of glucose, maltose or maltotriose during growth repress starch degradation activity of *L. crispatus* strain 10 (table 3 and figure 7). Carbon catabolite repression may play a major role. This implies that *L. crispatus* uses the carbon sources that are most easily accessible and allow for fastest growth. The presence of a preferred carbon source inhibits the synthesis of enzymes involved in the metabolism of other carbon sources with a lower growth rate (Gorke & Stulke, 2008). The selection of a carbon source is an important determining factor in growth rate and therefore competitive success in the microbiome. Carbon catabolite repression is a common control system of many bacteria that include the lactobacilli such as *L. plantarum (Chen, Lu, Wang, Yu, & Tian, 2018)*. To further study this possible mechanism of regulation in *L. crispatus*, growth rates of various carbon sources need to be examined to determine which carbon sources have the fastest growth rates and may therefore be preferred. We have not been able to calculate growth rates of *L. crispatus* on glycogen and glucose yet as a result of a lack of measurements during the exponential phase of growth. In addition, growth on two carbon sources simultaneously can be further studied whereby the OD is measured over time and supernatant samples are collected to analyze residual carbon concentrations.

Carbon catabolite repression may be regulated at the level of DNA which might involve the catabolite control protein A (CcpA) and potential catabolite response elements (cre) in the DNA. Results in this study describe the presence of cre sites in the promotor of the putative type I pullulanase gene (figure 8). However, this is highly speculative as there is no evidence yet that this gene is truly responsible for the degradation of glycogen in *L. crispatus*. A possibility to elucidate the function of this gene is to use heterologous expression. The type I pullulanase gene can be inserted into another bacterium by recombinant DNA technology and expressed (Binder, Hirokawa, & Windhorst, 2009). This method has already been used to express feruloyl esterase of *L. crispatus* in *E. coli (Xu, Wang, & Zhang, 2019)*.

Starch degradation activity in pellets and supernatants of *L. crispatus*

A large standard deviation can be observed in starch degradation activity of the pellets and supernatants after growth of *L. crispatus* strain 10 on glycogen (figure 4, 6 and 7). Starch degradation differentiated among the experiments, but every experiment always showed a clear difference in starch degradation between the presence of glucose or glycogen during growth of *L. crispatus* strain 10. A possible explanation might be the variations in optical densities after 48 hours of growth on glycogen or the rapid deterioration of the quality of the iodine staining over time.

An increased degradation of starch in the pellets compared to the supernatants of *L. crispatus* strain 10 can be noticed (figure 6 and 7). It could be that the enzyme responsible for glycogen breakdown is located on the cell wall which confirms the increased degradation activity seen in the pellets. This hypothesis matches with the putative type I pullulanase as the gene encodes a bacterial surface layer protein (van der Veer et al., 2018). The presence of this surface layer protein suggests that the enzyme is located at the outermost S-layer of the cell wall (van der Veer et al., 2018). On the other hand, we must take into account that the pellets were resuspended in phosphate-buffered saline (PBS) and the supernatants consisted of NYCIII medium rich in nutrients that may cloud the iodine staining. In addition, the differences in pH during the 24 hours of starch incubation need to be considered as this may affect enzymatic activity. Pellets resuspended in PBS had a pH of 7.4 and the supernatants a pH of about 3-4 as a result of lactic acid production of *L. crispatus*. Moreover, there is no total degradation of starch in the pellets or supernatants of *L. crispatus* regardless of the carbon source present during growth (figure 4, 6 and 7). At the start of incubation, 7.5 g/L starch solution is added. It is conceivable that the enzymes of *L. crispatus* cannot breakdown all the starch as it consists both of α,1-4 and α,1-6 linked glucose or that the pH is of importance for *L. crispatus* to break down starch. To study this, starch assays can be carried out at different pH's and over time to calculate rates of starch degradation.

No growth of *L. crispatus* on lactulose

We were not able to reproduce growth on lactulose that was considered as a promising prebiotic to support a *Lactobacillus*-dominated vaginal microbiome (table 1) (Collins et al., 2018). Possible explanations could be the difference in growth medium and *L. crispatus* strain. In this study *L. crispatus* strain 10 was grown in NYCIII medium supplemented with lactulose (4.8 g/L). The study by Collins et al used *L. crispatus* ATCC 33820 and Man, Rogosa and Sharpe (MRS) medium supplemented with lactulose (5.0 g/L). Further research is needed to exclude that growth on lactulose is strain or medium specific.

Lactate in supernatant of *L. crispatus*

Lactate was found in the supernatant of *L. crispatus* strain 10 after growth on glucose or glycogen and in the supernatant of *L. crispatus* strain 9 after growth on glucose (table 2). Although this experiment was only carried out once, results correspond with the study by van der Veer et al (2018).

For further research glycogen measurements need to be performed to conclusively establish that *L. crispatus* uses glycogen to produce lactate.

Conclusion

The most important results of this study outline that *L. crispatus* strain 10 can grow on glycogen and produce lactate. This supports the novel theory that lactobacilli can utilize glycogen to acidify the vagina. Moreover, this study provided new information on possible carbon catabolite repression in *L. crispatus* but more research is needed to confirm this hypothesis.

For vaginal health, colonization of lactobacilli including *L. crispatus* is important to prevent bacterial vaginosis and its negative health outcomes. Glycogen metabolism of lactobacilli might be a good target for designing pre- or probiotics that support a vagina colonized by lactobacilli. Before we can assess if glycogen metabolism is a good target, we need to know more about glycogen metabolism of other bacteria including bacteria associated with bacterial vaginosis and glycogen abundance in the vagina during bacterial vaginosis.

Materials and methods

Strains

L. crispatus strains 9 and 10 were isolated from vaginal swabs of women which were obtained from the Sexually Transmitted Infections clinic in Amsterdam, The Netherlands, from June to August 2012 (Dols et al., 2016). The strains were stored at -80 ^{o}C in 20% glycerol stocks.

Growth media

MRS broth (Sigma-Aldrich, 69966) (51 g), agar (1% w/v) and Tween 80 (1 mL) were dissolved in a liter demi water and autoclaved at 121°C for 20 minutes at 1.1 bar. Petri dishes were poured in a laminar flow after autoclaving when the MRS medium was still hot. The petri dishes were left to dry in the flow cabinet and afterwards stored at 4°C.

A basal NYCIII medium contained HEPES (1.2 g), proteose peptone #3 (Becton Dickinson, 211693) (7.5 g), yeast extract (Oxoid, LP0021) (1.9 g), NaCl (2.5 g), glucose monohydrate (Santa Cruz Biotechnology, 14431-43-7) (2.75 g), heat inactivated horse serum (50 mL) and demi water (450 mL).

1.1× carbohydrate deprived NYCIII medium followed the same recipe as NYCIII medium described above, but glucose monohydrate was left out and 400 mL of demi water was added instead of 450 mL. When preparing cultures, glycogen (Sigma-Aldrich, 9005-79-2), maltose (Sciencelab, 6363-53-7), maltotriose (Santa Cruz Biotechnology, 207511-08-8), galactose (Sigma-Aldrich, G0625) or lactulose (Sigma-Aldrich, 61360-5G) were supplemented to 1.1× carbohydrate-deprived NYCIII medium with a final concentration of 4.8 g/L.

Culture conditions

MRS agar plates were inoculated in a laminar flow with a small scoop of material from -80 ^{o}C 20% glycerol stocks of *L. crispatus* strains 9 and 10 using a disposable sterile inoculation loop. Ice boxes were used to keep the strains frozen and to transfer the tubes from the -80 ^{o}C to the bench and back. Plates were incubated for a minimum of 24 hours and a maximum of 96 hours after which the plates were assessed for homogenous colony morphology. Precultures were made by inoculating a smear of highly populated colonies on the MRS agar plate in NYCIII medium. After a minimum of 24 hours and a maximum of 96 hours of incubation, the precultures were used for experiments.

Plates or precultures were anaerobically grown using an Anaerobic 3.5 Jar (Thermo Fisher) and applying three cycles of: pulling a vacuum and filling with N_2+5%CO_2 gas. The jar was then incubated at 37 ^{o}C without agitation. In some cases, AnaeroGen 3.5L sachets (Thermo Scientific) were used to create an anaerobic environment in the Anaerobic Jar when the gas bottle containing N_2+5%CO_2 gas was not available.

Growth experiment

Cultures were made using 1.1× carbohydrate deprived NYCIII medium supplemented with glucose (4.8 g/L), glycogen (4.8 g/L) or demi water and precultures of *L. crispatus* 9 or 10 were added.

The cultures were anaerobically grown in the Anaerobic Jar or 1.5 mL Eppendorf tubes were used and filled with 1.6 mL to reduce oxygen concentrations to a minimum. The cultures were incubated at 37 ^{o}C without agitation. The optical density (OD) at 600 nm was measured every two hours with the spectrophotometer (Amersham Biosciences, Novaspec III) using a polystyrene cuvette. OD-values exceeding 0.8 were diluted 10x in PBS. For each OD measurement, 1 mL was transferred to a polystyrene cuvet and an Eppendorf tube was taken out of the experiment.

Starch-iodine assay

After 48 hours of growth, the OD was measured and 1 mL of each sample was centrifuged in Eppendorf tubes for 10 minutes at 4 °C at 16350 xg (MIKRO 200R, Hettich zentrifugen). Supernatants were collected and the pellets were resuspended in ice-cold PBS. A flat-bottom transparent polystyrene 96-wells plate (Greiner, 655191) was used. Besides sample (25% v/v), each well contained 7.5 g/L soluble starch (Sigma-Aldrich, S9765) in amylase buffer (100 mM sodium acetate, 5 mM $CaCl_2$) with a pH of 5.8 and 30 µM chloramphenicol in ethanol (100%) resulting in a total volume of 200 µL per well. The well plate was sealed airtight using a sterile covering adhesive film (VWR, 60941-072) and parafilm around the edges. After 24 hours of incubation at 37°C, the plate was examined for condense formation. If condense was present the plate was centrifuged for 2 minutes at 4754 xg (Rotina 420R, Hettich zentrifugen). The seal was removed, and the plate was developed by adding 290 µL iodine working solution (0.078 mM I_2 and 1.20 mM KI in 0.05 M HCl) to 10 µL of sample incubated in starch solution. A calibration curve was made using various concentrations of starch in amylase buffer. The OD at 600 nm was measured on a Multiskan GO plate reader (Thermo Scientific). Remaining supernatants were frozen at -20 °C for future metabolite analysis.

Lactate concentrations

Supernatant samples stored at -20 °C were thawed on ice, 200 µL was taken and ice-cold perchloric acid was added with a final concentration of 3.5%. The mixture was vortexed and put on ice for 10 minutes. 9.5% (v/v) of ice-cold 5M KOH in 0.2M 3-(N-morpholino) propane sulfonic acid was added to the sample, vortexed and put on ice for 10 minutes. The supernatant was centrifuged for 10 minutes at 4°C at 16350 xg (MIKRO 200R, Hettich zentrifugen). The supernatant was filtered through a 0.2 µm filter and put in a vial. Lactate concentrations were analyzed with high-performance liquid chromatography (HPLC) (Shimadzu LC20-AT) using the UV detector and a Rezex (Phenomenex) column at 55 °C. The flow of the eluent (5 mM H_2SO_4 in milli-Q) was 0.5 mL/min.

Palindromic sequences

Genomes of *L. crispatus* strains sequenced by Dols et al that showed no deletions in the putative type I pullulanase gene (van der Veer et al., 2018) were examined for palindromic sequences in the promotor of the putative type I pullulanase gene. Palindromic sequences were found using the online tool palindromic sequence finder by BioPHP. Sequences were only included if they consisted of at least 10 base pairs and if they showed similarity with the following sequence: TGWAANCGNTNWCA, where N = any base; W = A or T (Lu et al., 2018).

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