


2022

Finding the Balance: The Effects of α -Cyclodextrin, 2-Hydroxypropyl- β -Cyclodextrin, and Cholesterol on *Bacteroides Vulgatus* and *Clostridium Bolteae*

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Weaver, Bethany (2022) "Finding the Balance: The Effects of α -Cyclodextrin, 2-Hydroxypropyl- β -Cyclodextrin, and Cholesterol on *Bacteroides Vulgatus* and *Clostridium Bolteae*," *ELAIA*: Vol. 4, Article 13. Available at: <https://digitalcommons.olivet.edu/elaia/vol4/iss1/13>

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Cover Page Footnote

This research was made possible by the Honors Program, the Pence-Boyce summer research program, and Catalyst. I am grateful to have also received funding from the Department of Biological Sciences and the Department of Chemistry and Geosciences. I would like to thank Dr. Himes for all his work as my research mentor. I would also like to thank Dr. Sharda and Dr. Heyen for their contributions. I want to extend a thank you to my peers, from Honors and from the Pence-Boyce summer research program, for all the encouragement.



**Finding the Balance: The Effects
of α -Cyclodextrin, 2-Hydroxypropyl-
 β -Cyclodextrin, and Cholesterol on
Bacteroides Vulgatus and *Clostridium Bolteae***

Bethany G. Weaver

ACKNOWLEDGEMENTS

This research was made possible by the Honors Program, the Pence-Boyce summer research program, and Catalyst. I am grateful to have also received funding from the Department of Biological Sciences and the Department of Chemistry and Geosciences. I would like to thank Dr. Himes for all his work as my research mentor. I would also like to thank Dr. Sharda and Dr. Heyen for their contributions. I want to extend a thank you to my peers, from Honors and from the Pence-Boyce summer research program, for all the encouragement.

ABSTRACT

Atherosclerosis is a cardiovascular disease characterized by the hardening of arteries through the formation of cholesterol plaques. Cyclodextrins have the potential to treat atherosclerosis by shrinking plaques. These cyclic oligosaccharides are known to make complexes with cholesterol and have potentially dangerous side effects such as cytotoxicity and gut microbiome changes. This study looked for potential negative effects of cyclodextrins and cholesterol on gut bacteria. It was hypothesized that *Bacteroides vulgatus* will have decreased growth when grown in broth with cholesterol. In contrast, it was hypothesized that *Clostridium bolteae* will have decreased growth when grown in broth with cyclodextrins. Due to the fact that these bacteria are anaerobic, *Clostridium bolteae* and *Bacteroides vulgatus*, were grown using Gifu Anaerobic Broth under CO₂. Data was collected by using a spectrophotometer to measure changes in bacterial growth throughout the growth cycle. Each bacteria was treated with one of three chemicals at one of three concentrations to make a total of 9 different conditions. α -cyclodextrin, 2-hydroxypropyl- β -cyclodextrin, and cholesterol were used to treat the bacteria at concentrations of 1 mM, 10 mM, and 100 mM. The slopes of the log phase of bacterial growth were compared using a two tailed t-test with $\alpha = 0.05$. Growth of *Clostridium bolteae* was significantly inhibited for most of the cyclodextrin treatments and all the cholesterol concentrations. *Bacteroides vulgatus* growth was inhibited by 100 mM concentrations of cholesterol and α -cyclodextrins. Interestingly, *Bacteroides vulgatus* growth was significantly increased when grown with 1 mM and 10 mM concentrations of cholesterol. These results demonstrate that cyclodextrins are associated with inhibited growth for these two prototypic gram negative and gram positive gut bacteria. Expansion of this study to other gut bacteria is key for a deeper understanding of the impact that cyclodextrins would have on the gut microbiome as a whole.

INTRODUCTION

Atherosclerosis is a cardiovascular disease with one of the highest mortality rates in the world. It is characterized by the formation of cholesterol-filled plaques that are chronically inflamed and continue to develop until they occlude the artery or break off causing a stroke or heart attack. Statins are the current standard treatment for atherosclerosis and for the precursor to atherosclerosis, high cholesterol. These drugs lower the levels of unhealthy lipoproteins to slow the progression of plaques (Lusis 2000). However, there remains a gap in medical treatment for a way to reduce inflammation and remove cholesterol from plaques to lower the risk for heart attacks and strokes. Cyclodextrins are oligosaccharides that have been found to regress atherosclerosis through the potential mechanism of decreasing inflammation and shrinking plaques by extracting cholesterol from membranes (Zimmer et al. 2016). Using a computer simulation, it was demonstrated that the mechanism for this removal may be that cyclodextrins form dimers to pull out cholesterol molecules, forming 2:1 cyclodextrin-cholesterol complexes (López et al. 2011).

The most widely studied cyclodextrin, 2-Hydroxypropyl- β -cyclodextrin (HP β CD), has been shown to solubilize cholesterol and have minimal cytotoxic effects on human cells (Szente et al. 2018). Indeed, HP β CD was studied in clinical trials for the treatment of Niemann-Pick C, a fatal hereditary disease that can be characterized by the buildup of cholesterol in lysosomes (Singhal et al. 2018). Theoretically, HP β CD promotes the removal of cholesterol from these lysosomes in a similar way to atherosclerotic plaque regression. HP β CD is a frontrunner in atherosclerosis research because of its success in Niemann-Pick C clinical trials (Singhal et al. 2018). Another commonly studied cyclodextrin for atherosclerosis treatment is α -cyclodextrin (α CD). α CD was found to inhibit inflammatory pathways better than HP β CD (Pilely et al. 2019). In one study, α CD was also found to decrease obesity by improving lipid metabolism in mice (Nihei et al. 2018).

A study with human erythrocytes and a Caco-2 cell line showed that α CD was cytotoxic to these cells; however, once a complex was formed with cholesterol, the cytotoxicity of α CD decreased. This same study proposed that α CD could also form complexes with phospholipids (Roka et al. 2015). The mechanism for cyclodextrin cytotoxicity has not been confirmed but is speculated to be related to the removal of cholesterol from membranes. However, the direct effect of cyclodextrins on the cytotoxicity of gut bacteria has not been assessed.

The microbiome has become the focus of research for a variety of diseases because of its connectivity to human health. Atherosclerosis is no exception; distinct changes in the human gut microbiome have been noted in patients with atherosclerosis (Jie et al. 2017). Interestingly, changes in gut microbiomes of mice with atherosclerosis have also been studied. These mice were fed high fat diets (HFD), which affected their gut microbiome; then, other mice were fed the same diet with α CD and had another large change in bacterial composition. There was a decrease in the *Bacteroides* genus in mice fed a HFD, but they rebounded in mice that were treated with α CD along with their HFD. On the other hand, *Clostridium* cluster XIVa was increased in mice that were fed a HFD but drastically decreased when α CD was added (Nihei et al. 2018). These results indicate that α CD and cholesterol are impacting the growth of the gut microbiome. Cyclodextrins are not absorbed but are fermented by the gut bacteria, as evidenced by their absence in fecal analysis, suggesting direct contact between the bacteria and these molecules (Amar et al. 2016). Analysis of these changes of bacteria found that inflammation increased when certain strains of bacteria were decreased *in vivo* (Yoshida et al. 2018). *Clostridium bolteae* and *Bacteroides vulgatus* are anaerobic bacteria found in the human gut microbiome (Maier et al. 2018). Each has its own unique properties and benefits for overall health. *B. vulgatus* was studied specifically in mice with induced atherosclerosis and was found to have anti-inflammatory effects (Yoshida et al. 2018). This is an important piece of information because the genus, *Bacteroides*, was found to be less prevalent in the gut of mice that were fed a high fat diet. On the other hand, *C. bolteae* is a member of *Clostridium* cluster XIVa, which was found to be increased in mice that were being fed a high fat diet (Nihei et al. 2018). *Clostridium* bacteria have been found to both be proinflammatory and to help regulate inflammation depending on the species (Barnes and Powrie 2011). A healthy

balance of gut bacteria is key to a healthy host. It is vitally important to be aware of how different treatments affect microbiome health and to ensure that they are as beneficial as possible and do not have unforeseen side effects. It is important to see if these bacteria are being negatively affected by cyclodextrin treatment. The human microbiome plays an important role in atherosclerosis, so the health of these bacteria is vital and should be considered when testing possible treatments.

Despite the promising data suggesting that cyclodextrins may help to regress atherosclerotic plaques, the specific, direct effects of cyclodextrins on the gut bacteria *B. vulgatus* and *C. bolteae* are not clear. *In vivo* studies are plagued by complex variables that are difficult to control. Isolating individual bacteria for testing can negate these variables. *B. vulgatus* was decreased in both human and mouse studies when under atherosclerotic conditions, whereas *C. bolteae* was decreased when α CD was introduced into the diet of the mice. This leads to the central questions being addressed in this current proposal: what are the effects of cyclodextrins and cholesterol on these bacterial species? Based on the changes that occur in vivo the following hypotheses were developed:

1. *Bacteroides vulgatus* will have decreased growth when grown in broth with cholesterol.
2. *Clostridium bolteae* will have decreased growth when grown in broth with cyclodextrins.

METHODS

Materials

Bacteria were purchased from ATCC, *Clostridium bolteae* and *Bacteroides vulgatus*. Cyclodextrins were purchased from Fischer Scientific, α CD 98% (100 g) and HP β CD (100 g). 92.5% cholesterol was purchased from Sigma Aldrich. Gifu Anaerobic Broth was purchased from Thomas Scientific. Olivet Nazarene University provided 12mL Falcon tubes and 1 mL, 5 mL, and 10 mL sterile single use pipets.

Equipment

All equipment was provided by Olivet Nazarene University: UV/VIS spectrophotometer, shaking water bath incubator and a carbon dioxide gas tank.

Methods

Bacteroides vulgatus was revived from frozen stock in Gifu Anaerobic Broth (GAM) in a shaking water incubator. Growth curves were determined using the same procedure for each trial: two bacterial falcon tubes were made containing 10 mL of fresh broth and 0.5 mL of bacteria-containing broth. These tubes were made anaerobic by filling them with carbon dioxide gas then immediately sealing them. 1 mL samples were taken every hour for 12 hours, alternating between two tubes. Every time a tube was opened it was filled with carbon dioxide again. The absorbance of these samples was immediately measured at 560 nm using the UV/Vis spectrophotometer. The absorbance

and time of each sample were recorded to create the growth curve. Samples were tested at these times: 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, 6 hr, 8 hr, 10 hr. This was found to accurately capture the growth curve of *B. vulgatus* and was used for the experimental trials as well. α CD, HP β CD, and cholesterol were all tested individually at three different concentrations: 1mM, 10mM, and 100mM. Three trials were run for each treatment to limit random error.

Clostridium bolteae was rehydrated from a freeze-dried stock in GAM broth in a shaking water bath, and then frozen stocks were made as previously stated. All methods were the same for *C. bolteae* apart from the times that samples were taken. The absorbances were recorded at these times: 0.5 hr, 1 hr, 2 hr, 2.5 hr, 3 hr, 4 hr, 5 hr, 6 hr.

Growth curves were analyzed by measuring the slope of the log phase growth where the linear upward slope was found. The slopes of each trial were compared to the slopes of the control with a two-tailed t-test. The data is displayed as means \pm standard deviation and P values of less than 0.05 will be used as the threshold for determining statistical significance. Though more rigorous statistical analysis is needed to more accurately understand the data, this was the method developed within the limited timespan.

RESULTS

Bacteroides vulgatus has a standard sigmoidal growth curve with a linear growth period. α CD treatments for *Bacteroides vulgatus* growth was not affected by α CD at lower concentrations. However, *Bacteroides vulgatus* growth was completely inhibited by 100 mM α CD (**Figure 1**).

Bacteroides vulgatus cultured with 1 mM and 10 mM cholesterol exhibited a significant increase in growth rate. The 100 mM concentrations of cholesterol and α CD completely inhibited growth (**Figure 2**). These three treatments, along with the 100 mM α CD, are all the statistically significant treatments for *Bacteroides vulgatus*. Growth curves for HP β CD treatments are not depicted due to their lack of significant growth inhibition.

All HP β CD and all cholesterol treatments, along with 1 mM and 100 mM α CD, were shown to inhibit *Clostridium bolteae* growth (**Figure 3**).

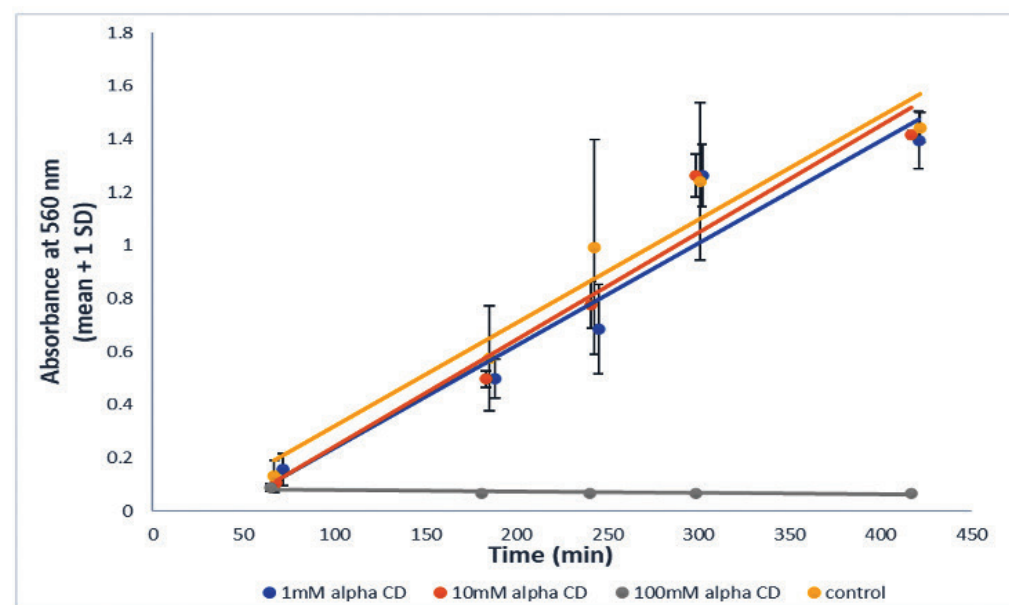
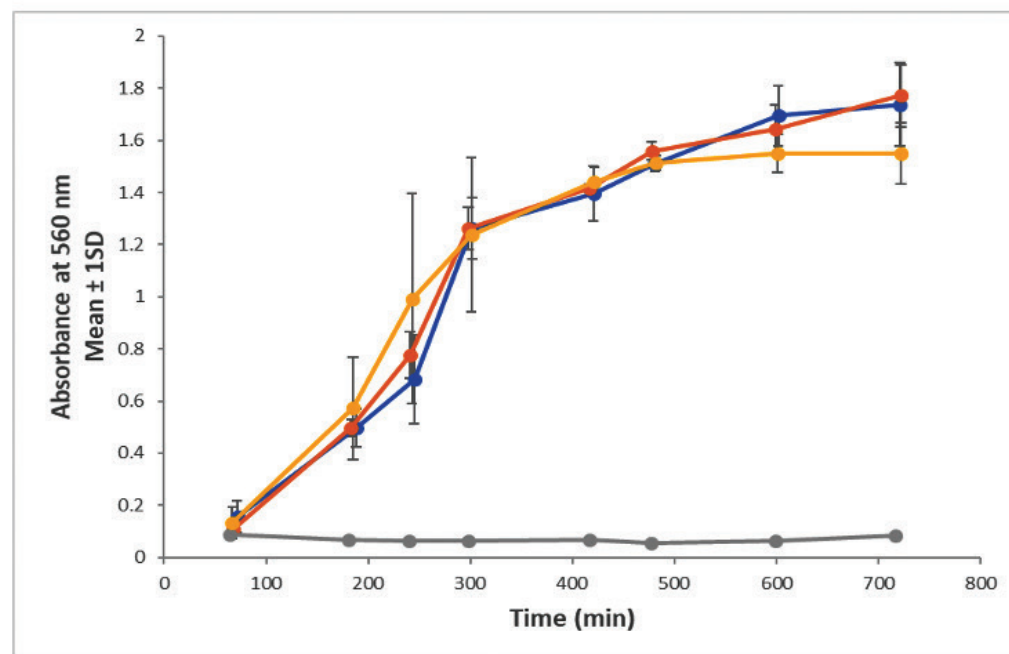


Figure 1. 100mM alpha cyclodextrin (α CD) inhibits *Bacteroides vulgatus* growth. The data displayed were from mean values with error bars showing 1 standard deviation. Top graph depicts full growth curve for *Bacteroides vulgatus* under all three treatments of α CD along with a control curve. Bottom graph depicts cropped graphs to display the linear growth segment that was analyzed. These two graphs display the same set of data. *Bacteroides vulgatus* was grown in GAM broth under anaerobic conditions with appropriate treatment mixed into broth. The line of best fit was found for this segment. Slopes were compared using a two-tailed t-test.

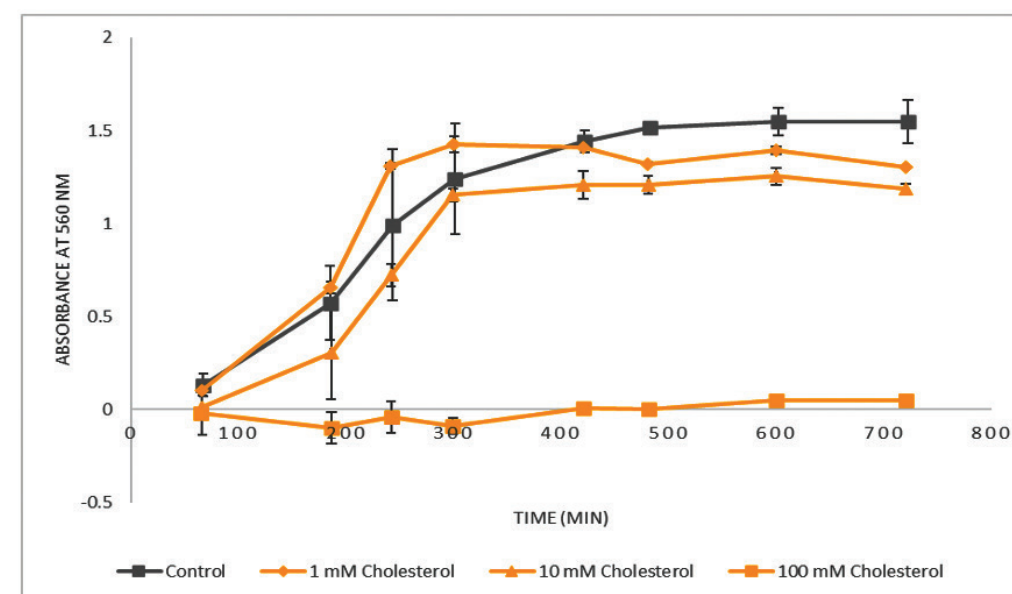


Figure 2. All the cholesterol treatments for *Bacteroides vulgatus* affect growth. *Bacteroides vulgatus* was grown in GAM broth under anaerobic conditions with appropriate treatment mixed into broth. All three treatments were statistically significant. t-tests were run to compare the slopes. Statistical significance was defined as $P < 0.05$.

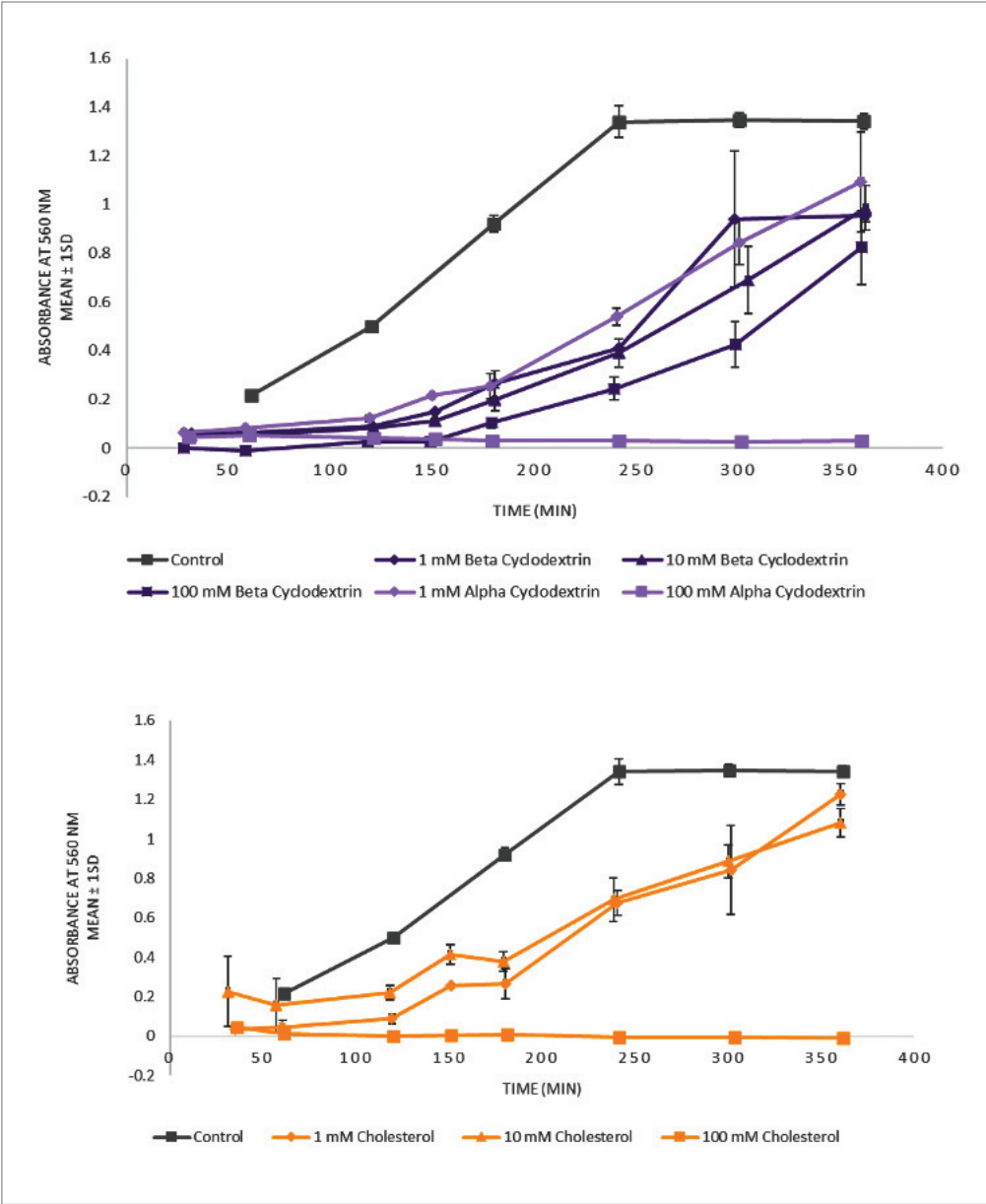


Figure 3. Eight out of nine treatments inhibit *Clostridium bolteae* growth. Top graph depicts cyclodextrin treatments that inhibited *Clostridium bolteae* growth. Bottom graph depicts all cholesterol treatments that inhibited *Clostridium bolteae* growth. *Clostridium bolteae* was grown in GAM broth under anaerobic conditions with appropriate treatment mixed into broth. T-tests were run to compare the slopes of the log phase and significance was defined as $P < 0.05$. All the concentrations shown were inhibiting growth.

Table 1 includes all the mean slope values with one standard deviation along with their corresponding p values. $P < 0.05$ indicates statistical significance. There are twelve statistically significant treatments between both bacteria tested. 1 mM, 10 mM and 100 mM concentrations of cholesterol and HP β CD showed statistically significant changes in growth for *Clostridium bolteae*. 1 mM and 100 mM concentrations of α CD also show p values of less than 0.05 for *Clostridium bolteae*. *Bacteroides vulgatus* showed statistically significant changes in growth for 100 mM α CD and 1 mM, 10 mM, and 100 mM concentrations of cholesterol.

TABLE 1: STATISTICALLY SIGNIFICANT CHANGES IN GROWTH FOR *BACTEROIDES VULGATUS* AND *CLOSTRIDIUM BOLTEAE*.

This table displays results from *Bacteroides vulgatus* and *Clostridium bolteae* under varying conditions. Bacteria were grown in GAM broth under anaerobic conditions with appropriate treatment mixed into broth. Slopes from the log phase of graphs for each treatment were analyzed by a two- tailed t-test; p values are shown below. There were varied levels of significance. Values with $p \leq 0.05$ are marked with *, $p \leq 0.01$ is marked with **, and $p \leq 0.001$ ***.

Treatment	Concentration	<i>Bacteroides vulgatus</i>		<i>Clostridium bolteae</i>	
		Slope \pm 1SD	T.TEST p value	Slope \pm 1SD	T.TEST p value
Control		0.0034 \pm 0.00036		0.0052 \pm 0.0002	
Alpha Cyclodextrin	1mM	0.00353 \pm 0.00029	0.6433	0.00403 \pm 0.00058	0.02973*
	10mM	0.00367 \pm 0.000058	0.2746	0.0052 \pm 0.00035	1
	100mM	-0.000053 \pm 0.000021	0.00007785***	-0.000087 \pm 0.000058	0.000001363***
Beta Cyclodextrin	1mM	0.0034 \pm 0.0001	1	0.00423 \pm 0.0004	0.0206*
	10mM	0.00357 \pm 0.000058	0.4734	0.00413 \pm 0.00042	0.01613*
	100mM	0.00303 \pm 0.000058	0.157	0.00357 \pm 0.00071	0.01849*
Cholesterol	1mM	0.00603 \pm 0.00015	0.000311***	0.00463 \pm 0.00012	0.01316*
	10mM	0.0048 \pm 0.00066	0.03166*	0.00353 \pm 0.00029	0.001194**
	100mM	-0.00022 \pm 0.000144	0.00008642***	-0.00005 \pm 0.00001	0.000001407***

DISCUSSION

This study sought to test two hypotheses: *Bacteroides vulgatus* will have decreased growth when grown in broth with cholesterol, and *Clostridium bolteae* will have decreased growth when grown in broth with cyclodextrins. *Bacteroides vulgatus* was treated with nine different treatments. Of those nine treatments, four were statistically significant (**Table 1**). 2-hydroxypropyl- β -cyclodextrin (HP β CD) is specifically designed to be less toxic; the added hydroxypropyl groups decrease the affinity for cholesterol, which has been shown to lower the toxicity (Szente et al. 2018). The data for *Bacteroides vulgatus* is consistent with this purpose; even at 100 mM HP β CD, the growth of this bacteria was not significantly inhibited. The toxic effects of unsubstituted α CD can be seen in the treatment: when *Bacteroides vulgatus* was treated with 100 mM α CD, it was completely inhibited. However, this inhibition is not present at lower

concentrations, suggesting a tipping point of cholesterol regulation for this species. This is consistent with the previous study that α -cyclodextrin is slightly more toxic than 2-hydroxypropyl- β -cyclodextrin. Both are still being studied as treatments for atherosclerosis (Szente et al. 2018).

The hypothesis for *Bacteroides vulgatus* was that it would be inhibited when grown in broths with cholesterol. This was based on a study by Jie et al. (2017) on patients with atherosclerosis where their gut microbiome was analyzed for consistent changes occurring in most atherosclerotic patients. The *Bacteroides* genus was decreased in general, and it was suspected that these bacteria grow poorly under high fat conditions that are usually associated with atherosclerosis. This was supported by the study done by Nihei et al. on mice. Cholesterol was tested because of the limited ability to replicate a high fat diet in vitro. This hypothesis is supported at 100 mM cholesterol concentrations where *Bacteroides vulgatus* was significantly inhibited. Surprisingly, 1 mM and 10 mM concentrations of cholesterol significantly increased the growth of *Bacteroides vulgatus*. There was more increased growth in the 1 mM than the 10 mM. Then, at 100 mM, the growth becomes inhibited. The mechanism for this increased growth is unknown. The data presented for the lower concentrations of cholesterol contradict the predicted trend. This may be due to the fact that the studies were using high fat diets, whereas this study looked at cholesterol alone (Jie et al. 2017; Nihei et al. 2018). There is more research to be done to see where the concentrations of cholesterol transition from beneficial to harmful. There is also a need for this study to be replicated to see if similar results are being found. This study focused on only one species in the *Bacteroides* genus, and it would be beneficial to learn if the entire genus reacts similarly to these conditions. It is difficult to draw large conclusions based on a study of a specific bacteria when the gut microbiome is a diverse population. The diversity of the microbiome is key to its function, and a possible unwanted side effect of cyclodextrins could be the destruction of that diversity.

Clostridium bolteae was treated with the same nine treatments as *Bacteroides vulgatus*. This bacteria was more susceptible to inhibition; it was significantly inhibited by eight of the nine treatments. Interestingly, the only treatment that did not show any significant changes in growth was 10 mM α CD. All other data was consistent with previous studies. Similar to *Bacteroides vulgatus*, 100 mM concentrations of cholesterol and α CD inhibited growth significantly. The slopes are nearly zero for these two treatments as it is seen in Table 1. The lower the concentrations, the less significant the growth inhibition. It is interesting that 100 mM α CD has a slope of nearly zero, whereas 100 mM HP β CD still shows growth. This is consistent with the idea presented earlier that HP β CD is less toxic than α CD (Szente et al. 2018).

The hypothesis for *Clostridium bolteae* was that there would be inhibited growth when grown in broth with cyclodextrins. This is supported by results from a study on mice that were treated with cyclodextrins, and they had decreased *Clostridium* populations (Nihei et al. 2018). In general, this is supported with the data from this study, except for 10 mM α CD. It is key to note that *Bacteroides vulgatus* was unaffected by HP β CD but *Clostridium bolteae* had significant growth inhibition at all concentrations. This supports

the hypothesis that *Clostridium bolteae* is specifically inhibited by cyclodextrins, even the one deemed less toxic. *Clostridium bolteae* also showed significant growth inhibition for cholesterol at all concentrations. In Jie's study on atherosclerotic patients, the *Clostridium* genus was increased in these patients, unlike the *Bacteroides* genus. There is not research supporting the decreased growth of *Clostridium bolteae* in cholesterol. *Bacteroides vulgatus* is not entirely representative of the *Bacteroides* genus and the *Clostridium* genus is even more varied (Barnes and Powrie 2011). There is a need for further research on other members of the *Clostridium* genus to explain the increase of the population under atherosclerotic conditions. There is a possibility that *Clostridium bolteae* decreases, but other species thrive. This possibility points to the importance of expanding the study to include a large variety of species of gut bacteria.

CONCLUSION

The results from these two gut bacteria species show that cyclodextrins can inhibit growth of gut bacteria, although further research should be done to see if this inhibition will be universally detrimental to the gut microbiome. The results indicate that there is some dose dependence for the inhibition. Further statistical analysis with a more rigorous method should be used to gain a clearer picture of the data. The health of gut microbiome composition should be monitored in future clinical research. Further research is needed to fully understand the general impact that cyclodextrins would have on the gut microbiome. This study was limited by the small number of bacteria tested along with limited concentration range. The maintenance of an anaerobic environment was difficult to control and though every attempt was made to control the environment, oxygen contamination could contribute to the results. Overall, this research supports cyclodextrins having adverse side effects on gut bacteria while raising new questions for future work.

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