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The Potential Stimulation Of C. Minuta Growth By Various Prebiotics, And Its Sensitivity To Various Antibiotics

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THE POTENTIAL STIMULATION OF *C. MINUTA* GROWTH BY VARIOUS PREBIOTICS, AND ITS SENSITIVITY TO VARIOUS ANTIBIOTICS

by

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DEDICATION

I would like to dedicate my work to my parents, Dr Hanna Hakim and Mrs. Katia Hakim. You have been my constant and solid support, and have always believed in me. Everything you do is for your children, and for that I thank you and love you unconditionally.

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GIT: gastrointestinal tract

GID: gastrointestinal disorder

IBD: inflammatory bowel disease

IBS: irritable bowel syndrome

TIID: type 2 diabetes

WHO: world health organization

UC: ulcerative colitis

LPS: lipopolysaccharide

GOS: galacto-oligosaccharide

FOS: fructo-oligosaccharide

AG: acacia gum

GG: guar gum

IMO: isomalto-oligosaccharide

RCM: reinforced Clostridial medium

Abx: antibiotics

CHAPTER 1: INTRODUCTION

The intestinal epithelium is a natural barrier of the gastrointestinal tract (GIT), providing defense against extrinsic invasions. There has been increasing evidence that disruption of the epithelial barrier integrity is one of the major aetiological factors involved in several gastrointestinal disorders (GID), including pathogenic infections, obesity and diabetes, enterocolitis, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) (Everard, 2013).

At birth, the human intestine is rather sterile, and with time, it develops and becomes influenced by factors including gut flora, use of antibiotics, diet, genetics and other environmental aspects. The normal human intestinal tract is colonized by a variety of gram positive and gram negative bacteria belonging - but not limited to - the genera *Lactobacillus, Streptococcus, Bifidobacterium, Peptostreptococcus, Clostridium, Eubacterium, Bacteroides, Veillonella, Fusobacter* and *E. coli* – largely in the colon (Greene and Klaenhammer, 1994).

Intestinal microbiota play a crucial role in nutritional, physiological, immunological and protective functions of the host (Kareem *et al*, 2016). Resident microflora has shown involvement in preserving the balance of intestinal microbiota, reducing the colonization and invasion of pathogens, retaining the epithelial integrity (Belkaid *et al*, 2014), and the production of essential nutrients - namely vitamin K menaquinone and biotin. Recent evidence suggests that gut microbiota is involved in the control of body weight, energy homeostasis, and inflammatory responses; thus, playing a role in the pathophysiology of obesity (Kobyliak and Virchenko, 2016). In addition, there has been evidence indicating associations between diseases and a host's microbial composition, including - but not limited to - metabolic syndrome, diabetes (Carlson *et al*, 2016), non-alcoholic steatohepatitis (Everard, 2013), and inflammatory bowel diseases; among many other gastrointestinal

diseases. Various studies have suggested that gut microbiota play a crucial role in the development of fat mass and altered energy homeostasis. Supporting evidence has been demonstrated in studies conducted on germ-free mice (mice raised in absence of microorganisms), in which the former were leaner compared to mice harboring microbiota since birth. One proposed mechanism is that gut microbiota have the ability to increase energy gathered from the diet and modify host signaling pathways associated with energy balance and metabolism. Other studies have hypothesized that gut microbiota play an important role in the onset of insulin resistance; thus, type II diabetes (TIID) (Everard, 2013).

Probiotics

One major contributor to the balance of gut microbiota is probiotics. The World Health Organization (WHO) regards probiotics as living microorganisms which have beneficial health effects when administered in appropriate amounts (Morelli and Capurso, 2012). Many of the probiotic bacteria are lactic acid bacteria, and are useful in the treatment of dysfunctions which disturb intestinal microflora and abnormal gut permeability (Lee and Salminen, 1995).

The Western Civilization has seen an increase in gut-related health problems, such as autoimmune and inflammatory diseases, antibiotic-induced diarrhea, *C. difficile*-induced colitis, urinary tract infections, etc.… Randomized double-blind studies have shown the effectiveness in the treatment, management, and/or prevention of the former, by providing protective barriers, enhancing immune responses, and clearing pathogens in the gastrointestinal - as well as urinary - tract.

In diarrhea-related diseases, probiotics may induce a general immune response, in addition to increasing IgA. In inflammatory-related diseases, probiotics are thought to decrease disease activity and promote remission. Decreasing inflammation is thought to occur

by decreasing pathogenic bacterial growth through the enhancement of barrier functions which prevents the invasion of tight junctions, by reducing gut pH and stimulating non-specific and specific immune responses. A study showed that the administration of Lactobacillus salivarius, E. coli Nissle, and S. boulardii resulted in fewer relapses and steroid use among IBD patients who received these probiotics. Other studies revealed that patients with IBS showed lower amounts of Lactobacillus and Bifidobacterium colonies, and an increase in anaerobic Clostridium spp which has taken place of Bifidobacterium spp and Bacteroides spp. A study conducted in Italy (Palumbo *et al*, 2016) with the aim to investigate the potential benefits of probiotics administration with drug therapy on ulcerative colitis (UC) patients found that over a course of 2 years, patients treated with the combination of drug therapy and probiotics showed overall better results and better improvements of general clinical conditions than patients who were administered the drug therapy alone.

Several studies have shown that both diet-induced and genetic obesity and TIID mice displayed change in gut microbiota; mostly reductions in Bifidobacteria, which has been associated with improvement in mucosal barrier in mice. One study aimed to determine if there is an association between Bifibacterium spp and metabolic endotoxemia – defined as increased plasma concentrations of the bacterial endotoxin lipopolysaccharide (LPS) – in high-fat-diet-induced diabetic mice. Metabolic endotoxemia has been linked with the onset of high-fat-diet-induced obesity and TIID. In the study, they demonstrated that a high-fat diet significantly decreased Bifidobacteria, and significantly increased endotoxemia. When supplemented with a dietary fiber, Bifidobacteria levels were significantly restored and positively correlated with improved glucose tolerance, glucose-induced insulin secretion, reduced endotoxemia, as well as inflammation (Cani *et al*, 2007); another phenomenon that has been associated with obesity and metabolic disorders (TIID and insulin resistance) (Wellen and Hotamisligil, 2005).

One suggested mechanism is that a high-fat diet may alter the balance in our gut flora, subsequently increasing gut permeability. This results in increased absorption of LPS; giving rise to inflammation. Consequently, a host of dysfunctions arises, falling within metabolic disorders (Cani *et al*, 2008).

Prebiotics

Gibson and Roberfroid define prebiotics as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Kleniewska *et al*, 2016). In simpler terms, prebiotics are considered the nutrients that fertilize probiotics. The most commonly thought of or consumed prebiotics are dietary fibers. Dietary fibers are known to be involved in maintaining healthy body weight, improving cardiovascular health, supporting overall digestive health and overall growth of intestinal microbiota. Several other commonly known and used prebiotics include inulin, guar gum, galacto-oligosaccharide (GOS), and β-glucan etc.…

Although all prebiotics are fiber, not all fibers are considered prebiotic. The major difference between prebiotics and dietary fibers is that the former are selectively beneficial to specific probiotics whereas the latter generally benefit a variety of gut microbiota. To be classified as a prebiotic, the ingredient must be resistant to gastric acidity and hydrolysis by human enzymes and resistant to absorption in the upper GIT. In addition, classification is based on whether the ingredient is fermented by gut microflora, and whether it has proven to stimulate intestinal bacterial growth associated with health and well-being. To date, all known and studied prebiotics are carbohydrates, primarily oligosaccharides. An oligosaccharide is a molecule containing a small number, usually 2 to 10, monosaccharides connected by glycosidic bonds. GOS is an oligosaccharide comprised of mainly galactose,

and some lactose and glucose. It is produced commercially from lactose by β-galactosidase. FOS is an oligosaccharide fructan naturally occurring in plants or commercially synthesized from sucrose. Acacia gum – also known as gum Arabic – is a naturally occurring gum consisting of the hardened sap of various species of the acacia tree. Compared with the negative controls, the number of Bifidobacteria and Lactobacilli after 4 weeks of consumption of AG were significantly enhanced (Camale *et al*, 2008).

Human breast milk contains lactose and relatively large quantities of diverse oligosaccharides - including nondigestible oligosaccharides - both of which influence gut microbiota profile, as lactose is a carbohydrate bacteria feed on for energy source. In addition, the oligosaccharide composition of human milk protects against pathogens by stimulating bifidus bacterial growth, inhibiting pathogenic binding to host epithelial cells, and creating an acidic environment that inhibits pathogenic growth. These properties may be attributed to the reduced infection rates in breast-fed infants. Supplementation of low levels of GOS in infant formula seemed to enhanced stool frequency, reduce fecal pH and stimulate intestinal Bifidobacteria and Lactobacilli compared with formula free of GOS (Cai *et al*, 2008).

The mechanism by which prebiotics work and benefit the gut flora can be simplified as follows. The intestinal microflora salvage energy by fermenting carbohydrates that bypass digestion in the upper GIT. This fermentation process produces carbon dioxide, methane, hydrogen, lactate and short-chain fatty acids (SCFA) - mainly acetate, propionate and butyrate. The epithelial colonic cells utilize the produced SCFA - preferentially butyrate - as an energy source for differentiation and proliferation. In addition, generally, the production of SCFA and subsequently their metabolism has been proven to contribute to the reduction of luminal pH, which in turn inhibits the growth of pathogenic bacteria and reduces peptide degradation, resulting in reduction in ammonia formation (Slavin, 2013).

Prebiotics have also shown to be associated with enhanced lipid profiles. Rats fed a diet supplemented with oligosaccharides showed lower levels of serum triacylglycerol and phospholipid concentrations (Fiordaliso, 1995). In addition, inulin supplementation may reduce serum cholesterol as it has been associated with enhanced secretion of bile salts and inhibition of HMG CoA reductase activity – a key enzyme in the pathway of cholesterol synthesis (Kim & Shin, 1998; Trautwein *et al*, 1998).

Antibiotics

Antibiotics - also called antibacterials - are a class of medication used to inhibit or slow down bacterial growth. Not all antibiotics work in the same manner; bactericidal antibiotics interfere with either the formation of the bacterium's cell wall or its contents while bacteriostatic antibiotics target bacterial multiplication (Finberg *et al*, 2004). Furthermore, some antibiotics target aerobic bacteria and others work against anaerobic bacteria.

The main classifications of antibiotics include - but are not limited to - β-lactams, fluoroquinolones, macrolides, tetracyclines and aminoglycosides. β-lactams act by hindering the bacterium's ability to form its cell wall. Fluoroquinolones target and inhibit the bacterium's ability to produce DNA, making it difficult to reproduce. Macrolides, tetracyclines and aminoglycosides generally inhibit bacterial protein synthesis (Hooper, 2001). ***Table 1*** illustrates the specific targets involved in bacterial growth, of the different classes of antibiotics.

Christensenella minuta

C. minuta is a novel gram negative, non-motile, strictly anaerobic, non-spore-forming bacterium. *C. minuta* is shaped like a straight rod with tapered ends. The bacterium was given its name *Christensenella* after the late professor Henrick Christensen, for his contributions to bacteriology, and *minuta* refers to its small cell and colony size. It was designated as strain

YIT 12065 and identified based on 16S rRNA gene sequence. The bacterium belongs to the family *Christensenellaceae* fam. nov. within the order *Clostridiales* of the phylum *Firmicutes* (Morotomi *et al*, 2012).

There has been belief that *C. minuta* gut-profile is highly heritable and promotes a lean host phenotype of which the biological mechanism is yet to be determined. *C. minuta* was isolated in human feces, and found to be abundant in lean individuals versus obese individuals. A study was conducted with the aim to demonstrate the role of the human genome in shaping the gut microbiome, in which they found that the Christensenellaceae family was the most heritable taxon, and was co-related in a hub of heritability with another family of bacteria belonging to the phylum Firmicutes; Dehalobacteriaceae. They found a negative relation between the families Bacteroidaceae and Bifidobacteriaceae. When they studied BMI profiles, Christensenellaceae family was significantly associated with lean BMI; as well as Dehalobacteriaceae. Within the study, twin participants were selected as gut microbiome donors; one twin was lean and the other obese. They transplanted the gut microbiome from the lean twin into a group of germ-free mice and found that after 21 days, the mice became lean. In contrast, when the gut microbiome of the obese twin was transplanted into a group of germ-free mice, the mice showed obese characteristic features. Interestingly, when they also transplanted the gut microbiome from the obese twin into germ-free mice and amended with Christensenella, the mice showed lean characteristic features. They found that after 21 days, the group of mice transplanted with the obese twin's microbiome had higher percent body weight change and percent total adiposity compared to the reduced percent weight change and total adiposity demonstrated in the group of mice that received the obese twin's microbiome amended with Christensenella (Goodrich *et al*, 2014). However, this study was carried out on germ-free mice, a phenomenon that does not represent reality; especially in the human host. Nonetheless, it is intriguing as to whether *C.*

minuta may play a role in the pathophysiology of obesity; thus, suggesting it as a potential probiotic for the treatment and management of chronic diseases associated; specifically, those associated with obesity.

Knowledge and evidence concerning whether *C. minuta* gut profile is truly associated with obesity is lacking. In addition, the mechanism behind this possible association is intriguing, and yet to be discovered. The factors that characterize and contribute to *C. minuta* and its activity are crucial to better understand how the profile of this bacterium may be linked to the onset of obesity and diabetes, as well as balance in the gut microbiome.

CHAPTER 2: OBJECTIVE OF THE STUDY

The aim of the current study was to identify specific prebiotics that can stimulate growth of *C. minuta* and to determine their optimal conditions for growth stimulation. This study also sought to determine the sensitivity to various antibiotics including sulfamethoxazole, azithromycin, trimethoprim, ciprofloxacin, erythromycin, and oxytetracycline.

CHAPTER 3: METHODOLOGY

Culture medium

Reinforced Clostridial Medium (RCM) was the medium of choice for C. minuta culture. To make 1L of medium, 38g of the already-packaged powder is added to distilled water; thus, measurements for all experiments conducted were based on the former. The mixture was then brought to boil – normally around 1 hour or until mixture is well homogenized – and autoclaved for 45-60 minutes to sterilize from any possible contamination. RCM free of dextrose was prepared by mixing all other ingredients; according to their original amounts in the package. Medium was then prepared in the same manner as original RCM.

Prebiotics

Six different prebiotics were used to test their effects on *C. minuta* growth; GG, IMO, GOS, inulin, AG, and FOS. The prebiotics solutions were prepared by weighing and adding 0.45g prebiotics into 45 mL RCM. Concentrations of the saccharides were based on the concentration of dextrose in RCM, as to have comparable results between all the prebiotics and dextrose. The prebiotics' effects were tested in the co-presence and absence of dextrose in the media, under different concentrations of prebiotic: dextrose, illustrated as follows:

A. 0.5% prebiotic

B. 1:1 – 0.25% prebiotic: 0.25% dextrose

C. 1:1 – 0.5% prebiotic: 0.5% dextrose

D. 1:2 – 0.25% prebiotic: 0.5% dextrose

E. 2:1 – 0.5% prebiotic:0.25% dextrose

Control I: RCM (free of saccharide)

Control II: 0.5% dextrose

In total, 62 test tubes were prepared: 6 prebiotics x 5 conditions - carried out in duplicate - and 2 different controls. All conditions were prepared under the presence of N_2/CO_2 to prevent the exposure to O_2. 10^8 cfu *C. minuta* was added into each tube using a syringe, in the presence of a Bunsen Burner. Tubes were then incubated at 37□c, and absorbance was recorded hourly using a plate reader in spectrophotometer.

Antibiotics

Six different antibiotics were used when testing resistance vs sensitivity of *C. minuta*; ciprofloxacin, erythromycin, trimethoprim, oxytetracycline, sulfamethoxazole and azithromycin. The following illustrates how the antibiotics' efficacy were tested:

A. 1μg/mL antibiotic
B. 5μg/mL antibiotic
C. 10μg/mL antibiotic
D. 50μg/mL antibiotic

Control: RCM (antibiotic-free)

In total, 49 tubes were prepared: 6 antibiotics x 4 conditions – each carried out in duplicate – and a control. All conditions were prepared under the presence of N_2/CO_2 to prevent the exposure to O_2. 10^8 cfu *C. minuta* was added into each tube using a syringe, in the presence of a Bunsen Burner. Tubes were incubated at 37□c, and absorbance was recorded hourly using a plate reader in spectrophotometer.

CHAPTER 4: RESULTS AND DISCUSSION

Prebiotics

Figure 1 illustrates the growth of *C. minuta* in controls I and II; the former being a condition free of saccharide, and the latter containing the monosaccharide dextrose. There is notable enhancement of *C. minuta* growth seen by the effects of dextrose compared to lack of any saccharide. This shows the significance of the presence of saccharide for optimal *C. minuta* growth.

Condition A: Condition A was carried with the purpose of determining which oligosaccharide showed promise as a potential dietary supplement for possible *in vivo* stimulation of C. minuta. At a concentration of 0.5% prebiotics, and the absence of dextrose, all prebiotics seem to have the same growth stimulation on *C. minuta* up until 6 hours, as noted by the recordings illustrated in ***Figure 2***. After 6 hours, absorbance in the presence of GOS and FOS increases, reflecting better enhanced *C. minuta* growth compared to the other prebiotics; yet slightly less stimulating than dextrose. An explanation could be as simple as the fact that dextrose is a monosaccharide that is easily broken down to provide energy. The sample containing AG records the lowest absorbance rates, reflecting least stimulating effects on *C. minuta* growth; only slightly better than growth in the absence of any saccharide (control I).

Condition B: As seen in ***Figure 3,*** when equal amounts of prebiotic and dextrose are present, ratio of 1:1, 0.25%: 0.25%, all samples have close absorbance up to 10 hours. Interestingly, after 11 hours, absorbance in the presence of AG increases to reach 1.1 at 14 hours, and remains constant throughout. Control II follows suit in stimulating *C. minuta* growth. The prebiotics GOS, FOS, GG, IMO and inulin all appear to record similar absorbance, reflecting similar enhancing effects the growth of *C. minuta*; in contrast to condition A where GOS is the leading prebiotic in stimulating growth, followed by FOS.

Condition C: When equal amounts of prebiotic and dextrose are added at 0.5% each, ratio 1:1, all samples have close absorbance recordings throughout the first 10 hours. After 10 hours, the sample containing GOS notably has increased absorbance recordings in comparison to the GOS, GG, IMO and inulin, and in comparison to condition B in which a ratio of prebiotic: dextrose is also 1:1 but at half the concentration. The AG sample takes the lead in absorbance recording; reflecting optimal *C. minuta* growth stimulation compared to all other samples; reflected in ***Figure 4***. Control II appears to enhance growth better than FOS, GG, IMO and inulin, yet at a slightly lesser extent than does GOS. IMO and FOS reach the lowest absorbance recordings between all prebiotics, with slightly better stimulating activities seen with GG and inulin.

It is interesting that when equal amounts of prebiotic and dextrose are added in condition B – 0.25% each – control II appears to record higher absorbance than GOS sample whereas equal concentrations – 0.5% each - of GOS and dextrose seem to record higher absorbance than dextrose alone (control II). The other 4 prebiotics do not seem to have a big difference in absorbance recorded than that seen in condition B, neither do they appear to optimize *C. minuta* growth as well as AG, or GOS-dextrose mixture. Nevertheless, all samples record absorbance markedly higher than control I, in which no saccharide is present.

Condition D: Represented in ***Figure 5***, in condition D, the amounts of dextrose added was double that of prebiotic: 1:2. The sample containing AG-dextrose shows stimulatory activities to be optimal for *C. minuta* growth; as noted by the absorbance recordings; followed by control II. Interestingly, the sample containing 1:2 GOS: dextrose seems to perform similarly to the samples containing GG, IMO, FOS and inulin.

Condition E: When the amount of prebiotic added is double the amount of dextrose - ratio of 2:1 - the GOS sample leads with an absorbance of 0.55 at 9 hours, and increases to reach 0.9

at 12 hours, after which the AG sample takes the lead with an absorbance of 0.99 at 13 hours, and increases to reach 1.2 after 15 hours and remains constant, during which GOS is second best in stimulating *C. minuta* growth. The other 4 prebiotic samples record absorbance between 0.65 and 0.78 after 14 hours and beyond, with inulin and GG taking the lead among the 4. 0.5 % dextrose (control II) does have an effect significantly higher than those by GG, IMO, FOS and inulin. The stimulatory effect of the AG-dextrose combination, however, does not appear to be significantly greater than the other samples, as seen in conditions B, C, and D; as illustrated by ***Figure 6***.

There appears to be a possible interaction between AG and dextrose, resulting in an optimized stimulation of *C. minuta* growth. When AG is present alone (Condition A), there is little effect on *C. minuta* growth in comparison to control I. However, in all other conditions (B, C, D and E) in the presence of different ratios of AG: dextrose, *C. minuta* growth is optimized and significantly comparable to that of the other 5 prebiotics-dextrose mixtures. In contrast, the co-presence of GOS and dextrose does not enhance *C. minuta* gravely, compared to control II and the AG-dextrose mixture. However, when GOS is the only saccharide present, it is the second best in optimizing *C. minuta* growth following dextrose (control II). These results suggest *C. minuta* may not prefer consuming GOS when dextrose is present, but resorts to its consumption – and grows well – when GOS is the only saccharide available; hence, refuting any theory of an interaction between GOS and dextrose to optimize growth. In addition, results do not indicate any interactions between dextrose and FOS, GG, IMO or inulin.

When ratios of AG: dextrose are 1:1, 1:2 or 2:1, it appears the mixture has similar stimulating effects on *C. minuta* growth. There is a slightly notable enhancing effect of GOS: dextrose at ratios of 1:1 and 2:1 where the concentration of GOS is 0.5%. In general,

increasing the concentrations or changing the ratios of prebiotic: dextrose does not have any significant differences in stimulatory effects on the growth of *C. minuta*. This suggests that ratio does not play a significant role in enhancing the interaction between AG and dextrose, nor does it result in an interaction between dextrose and GOS or the remaining 4 prebiotics. In addition, doubling the concentration from 0.25% to 0.5% of prebiotic or dextrose does not result in significant changes in the stimulatory effects of the prebiotics, nor does it optimize the interaction between AG and dextrose (***Figure 7***); suggesting that the interaction between AG is not induced or formed by changing the concentrations of either saccharide.

A combination of AG and dextrose demonstrates to be an optimal mixture in culturing *C. minuta* as a probiotic supplement. GOS may serve as a promising prebiotic supplement to optimize the growth and activity of *C. minuta in vivo*. Even though the presence of dextrose alone is enough to stimulate and enhance the growth and survival of C. minuta *in vitro*, it is not the preferable supplement. The fact that dextrose is a monosaccharide, *in vivo*, it will be easily digested and broken down by human enzymes and will not reach the colon.

Antibiotics

At concentrations of 1µg/ml, 5µg/ml, and 10µg/ml, sulfamethoxazole does not appear to have any inhibitory activity on the growth and survival of *C. minuta*; represented in ***Figure 8***. Even at a concentration as high as 50µg/ml, *C. minuta* growth is slowed down yet not hindered in the presence of sulfamethoxazole.

Ciprofloxacin at a concentration of 1µg/ml does not show inhibitory activity on *C. minuta* growth; however, growth is comparably lower in the presence of this antibiotic compared to sulfamethoxazole, azithromycin and trimethoprim at 1µg/ml. as concentration increases to 5µg/ml, 10µg/ml, and 50µg/ml – respectively – ciprofloxacin acts on inhibiting *C. minuta* growth; as noted by the near-constant absorbance in ***Figure 9*** at these concentrations.

Similarly to sulfamethoxazole, the activities of azithromycin and trimethoprim (***Figures 10*** and ***11***, respectively) have no effect on inhibiting *C. minuta* growth in concentrations of 1μg/ml, 5μg/ml, and 10μg/ml; however, a concentration as high as 50μg/ml of both azithromycin and trimethoprim work in a similar manner as sulfamethoxazole but only slowing down the growth of *C. minuta*.

Represented in ***Figure 12***, when 1μg/ml of erythromycin were used in order to test *C. minuta* survival, growth was not hindered, but rather slowed down in comparison to sulfamethoxazole, azithromycin and trimethoprim. The use of 5μg/ml of erythromycin had similar effects as 1μg/ml, by which *C. minuta* growth was reduced compared to 5μg/ml of sulfamethoxazole, azithromycin and trimethoprim, yet not inhibited. As concentration was increased to 10μg/ml and 50μg/ml, respectively, the activity of erythromycin appeared to hinder growth.

Figure 13 illustrates the survival of *C. minuta* in the presence of oxytetracycline. At all 4 concentrations, 1μg/ml, 5μg/ml, 10μg/ml, and 50μg/ml, oxytetracycline works on inhibiting *C. minuta* growth from the start; as seen in the near-constant absorbance; suggesting *C. minuta* has high susceptibility to the mode of action of oxytetracycline.

The increasing absorbance rates noted in the higher concentrations of sulfamethoxazole, trimethoprim and azithromycin indicate possible resistance by *C. minuta* to these antibiotics. On the contrary, even at the lowest concentration of 1μg/mL, oxytetracycline shows inhibitory effects on the growth of *C. minuta*. As concentration increases, ciprofloxacin appears to target the growth of *C. minuta*. The results are in line with data concerning the activity of these antibiotics. Sulfamethoxazole and trimethoprim generally target aerobic bacteria. *C. minuta* - being an anaerobic gram negative bacterium - is not a preferential target for these antibiotics. Ciprofloxacin – belonging to the class of fluoroquinolones – targets a

broad-spectrum of gram positive and gram negative bacteria. Similarly, oxytetracycline's activity is broad and acts on the inhibition of both gram positive and gram negative bacteria. Erythromycin and azithromycin belong to the same class of antibiotics, macrolides; azithromycin being a derivative of the former. It is intriguing as to why azithromycin had no inhibitory effects on growth of *C. minuta* whereas erythromycin succeeded in slowing down growth. A possible explanation may be that although erythromycin generally works against gram positive bacteria, higher dosages have proven to be effective against certain susceptible gram negative bacteria; however, the true mechanisms by which erythromycin and azithromycin are still under study. The unknown mechanisms may serve as an explanation to the differences in survival of *C. minuta* – as well as other bacteria – by the action of both antibiotics.

CHAPTER 5: CONCLUSION

Christensenella minuta is a novel bacterium with a heritable gut profile, that may be associated with a lean host and the pathophysiology of obesity. *C. minuta* grows well in the presence of the monosaccharide dextrose, as well as in a combination of AG and dextrose; a mixture for optimal *C. minuta* culturing. The oligosaccharide GOS appears to have great enhancing effects – similar to that of dextrose – when it is the only saccharide present for use; thus, suggesting it as a promising supplement to stimulate *C. minuta* growth and activity *in vivo*. The survival of *C. minuta* is hindered by the inhibitory activities of oxytetracycline, at concentrations as low as 1μg/mL. Growth is also hindered by activities of ciprofloxacin, and slowed down or reduced by erythromycin. *C. minuta* appears to be resistant to the inhibitory activities of sulfamethoxazole, azithromycin and trimethoprim, at concentrations as high as 50μg/mL.

Future implication: Further research is needed in order to better understand and determine the stimulating effects of different oligosaccharides on *C. minuta* growth *in vivo.* Future *in vivo* experimental trials on the efficacy of *C. minuta* against obesity are crucial, and may give insight as to the development of *C. minuta* as a probiotic supplement to prevent and/or manage chronic diseases associated with obesity; as the one study conducted on germ-free mice does not reflect reality. Furthermore, the true mechanism by which different antibiotics work or fail to work on hindering the growth of *C. minuta* is yet to be determined. In addition, information concerning the susceptibility of *C. minuta* to beta-lactams is needed; specifically, penicillin, as it is one of the most common and broad-spectrum antibiotics used.

TARGET	MODE OF ACTION	CLASS/SUBCLASS
Cell wall synthesis	Cell wall components/cell membrane components	B-lactams Cephalosporin Vancomycin Bacitracin
Nucleic acid synthesis	Folate synthesis	Sulfamethoxazole Trimethoprim
	DNA synthesis	Quinolones (ciprofloxacin)
	RNA synthesis	Rifampin
Protein synthesis	50S subunit	Macrolides (azithromycin, erythromycin) Clindamycin
	30S subunit	Tetracyclines (oxytetracycline) Aminoglycosides

Table 1 Target of different classes and sub-classes of antibiotics on bacterial inhibition.

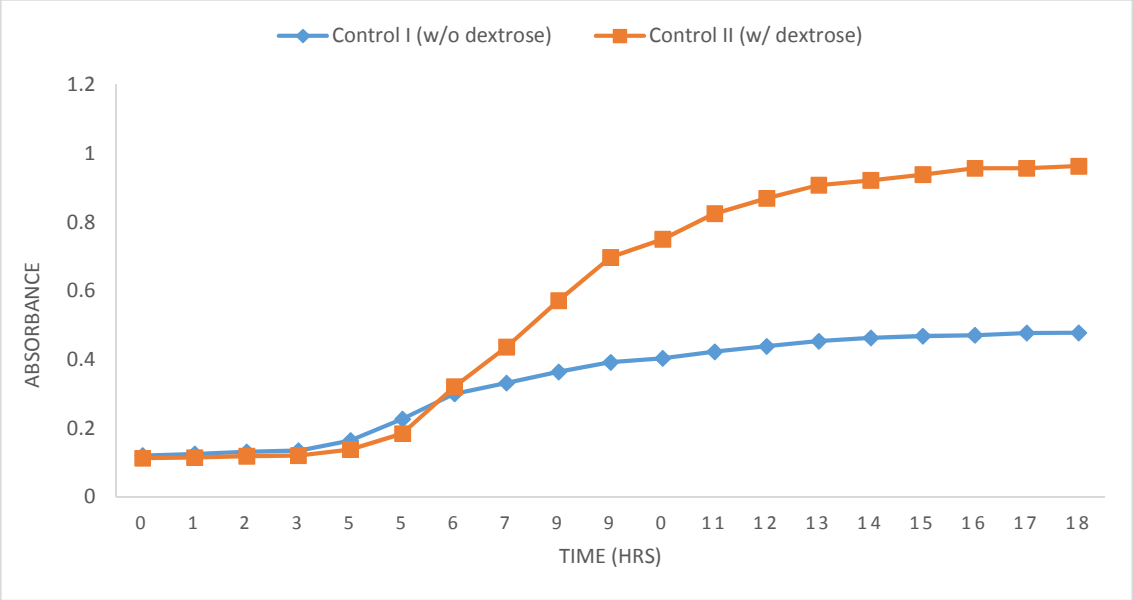

Figure 14 Absorbance recorded by two sample controls; control I in the absence of saccharide, control II contains dextrose

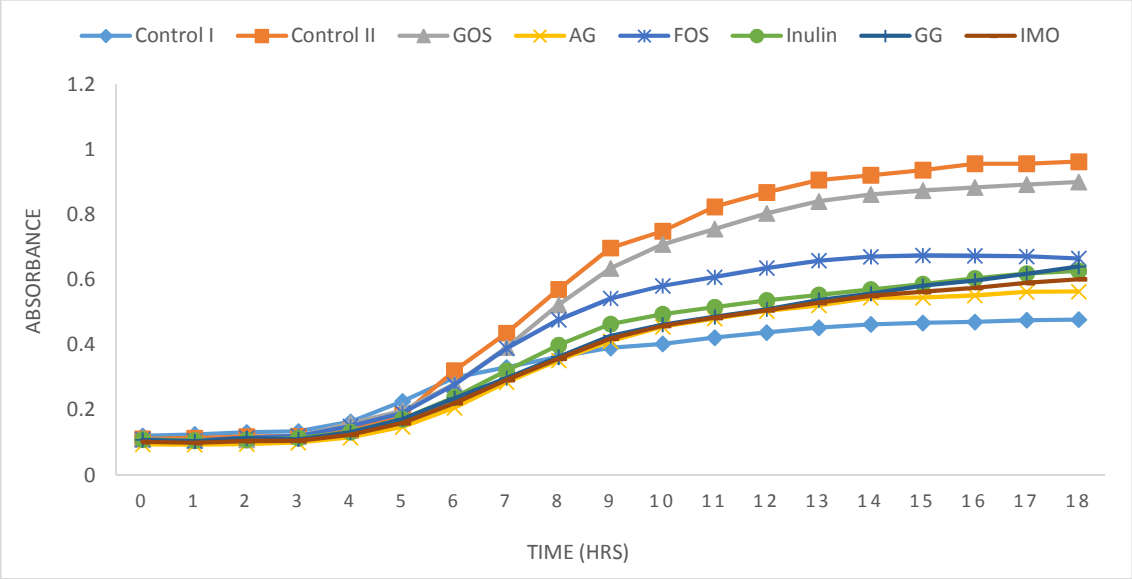

Figure 15 Absorbance recordings of *C. minuta* growth in condition A; 0.5% prebiotic compared to control I and control II; throughout 18 hours

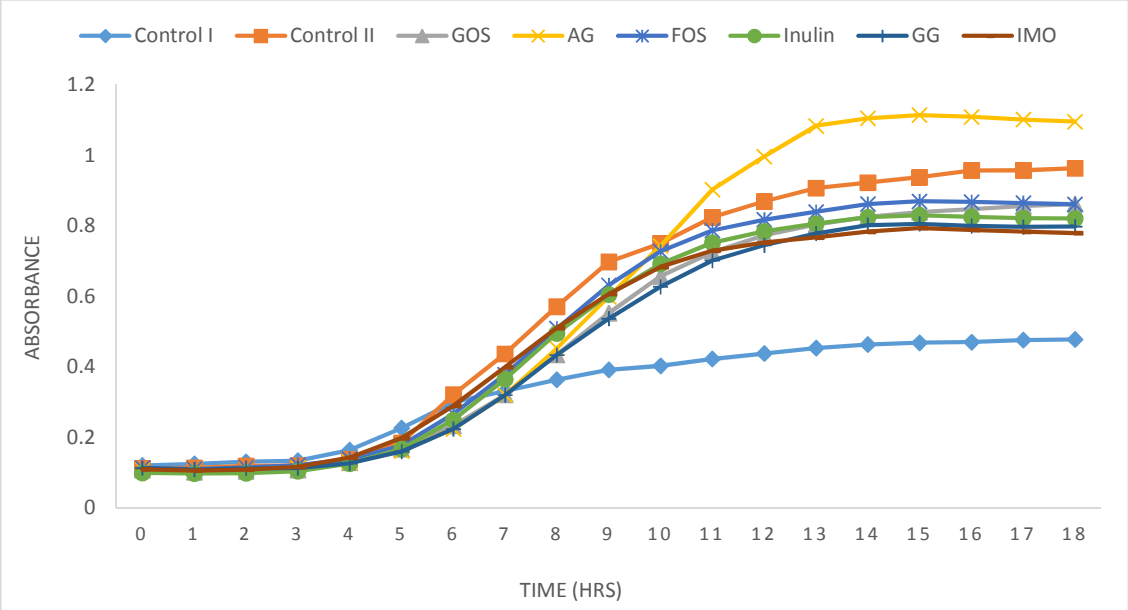

Figure 16 Absorbance recordings of *C. minuta* growth in samples containing a 1:1 ratio of 0.25% prebiotic and dextrose, compared to control I and II, throughout 18 hours (Condition B)

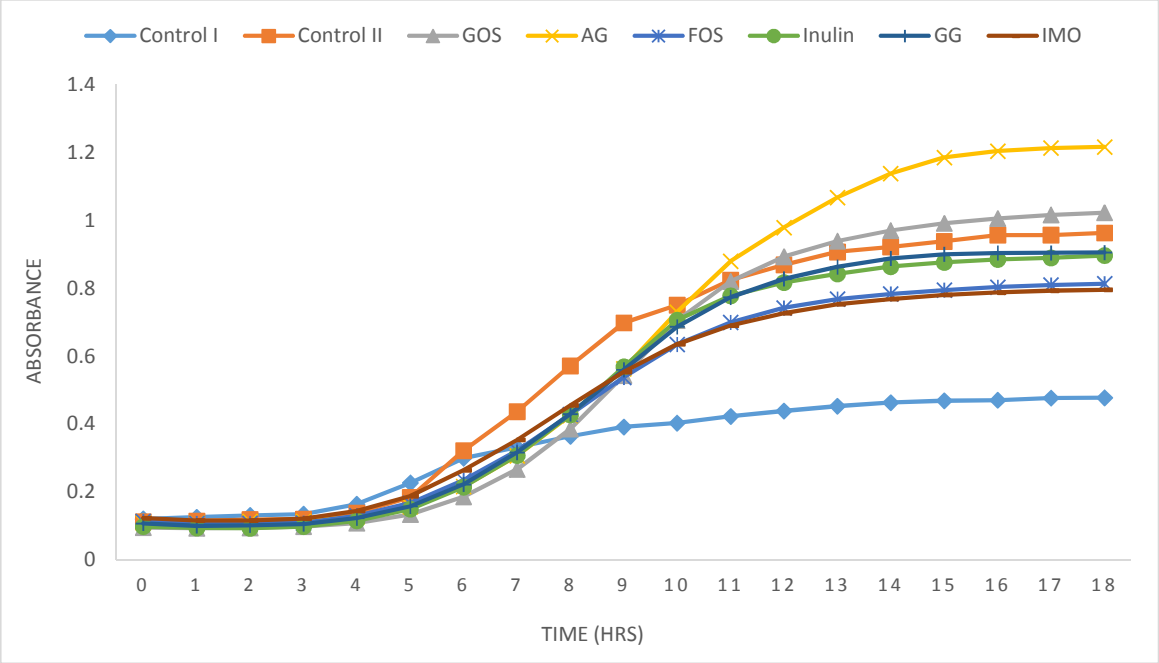

Figure 17 Absorbance recordings of *C. minuta* growth in condition C: a 1:1 ratio of prebiotic: dextrose, at a concentration of 0.5%; compared to control I and II

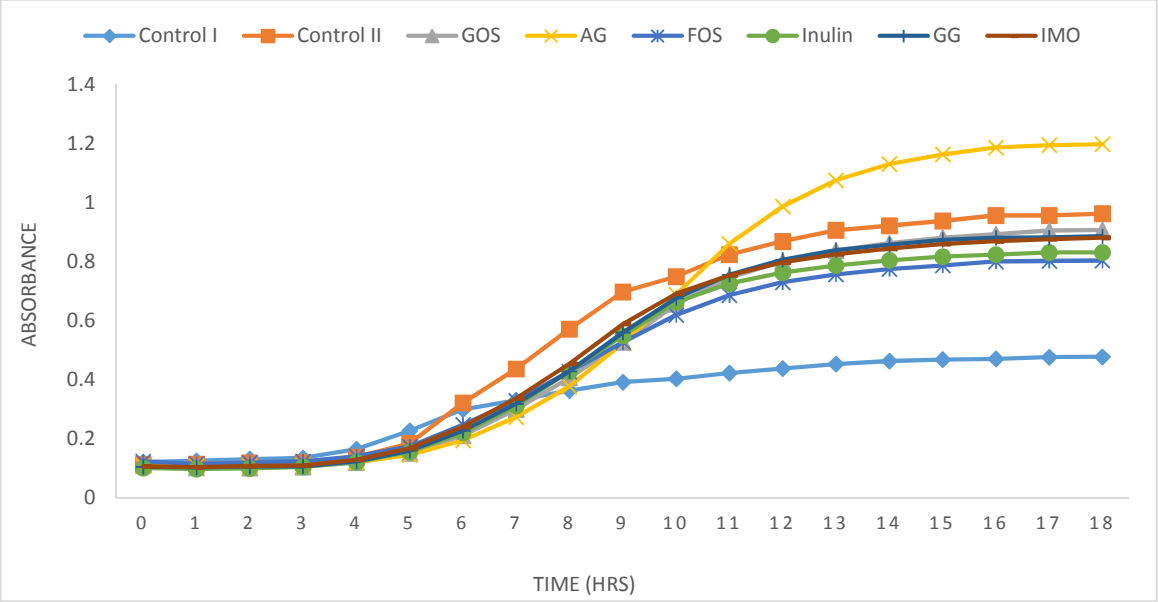

Figure 18 Absorbance recordings of *C. minuta* growth in condition D: samples containing a 1:2 ratio of prebiotic: dextrose (0.25%: 0.5%), compared to control I and II

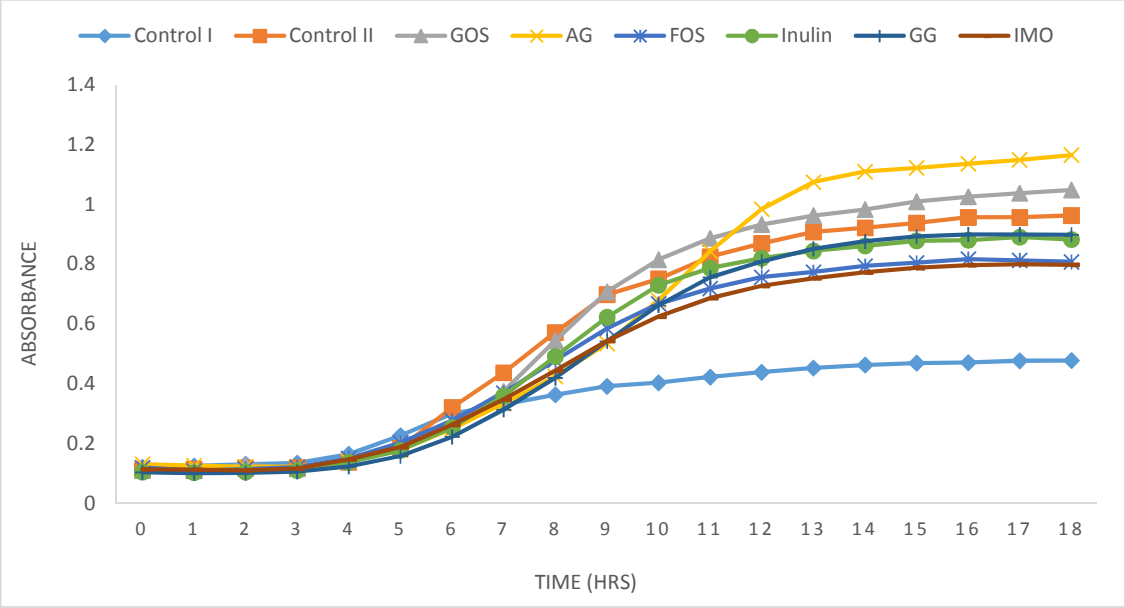

Figure 19 Absorbance recordings of *C. minuta* growth in condition E: samples containing a prebiotic: dextrose ratio of 2:1 (concentrations of 0.5%:0.25%), compared to control I (no saccharide) and control II (0.5% dextrose)

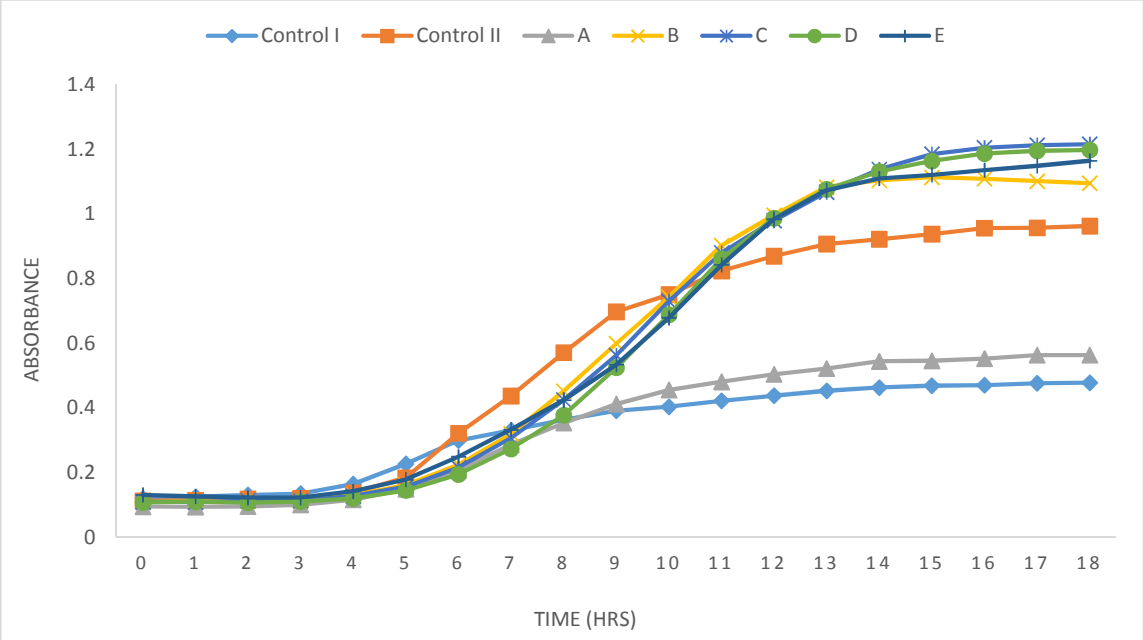

Figure 20 Absorbance recordings of *C. minuta* growth in stimulated by AG throughout 18 hours, in conditions A, B, C, D, and E; compared to control I (no saccharide) and control II (0.5% dextrose)

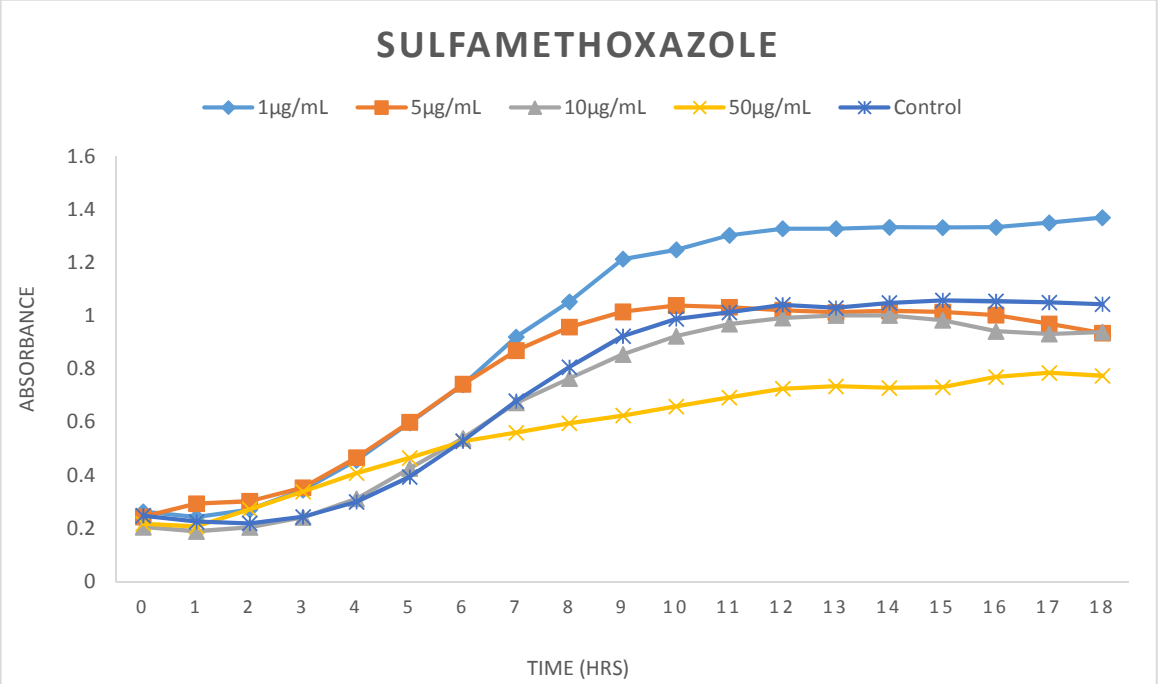

Figure 21 Absorbance recorded reflecting *C. minuta* growth at 4 different concentrations of sulfamethoxazole: 1µg/mL, 5µg/mL, 10µg/mL, and 50µg/mL; compared to control in which no antibiotic was present

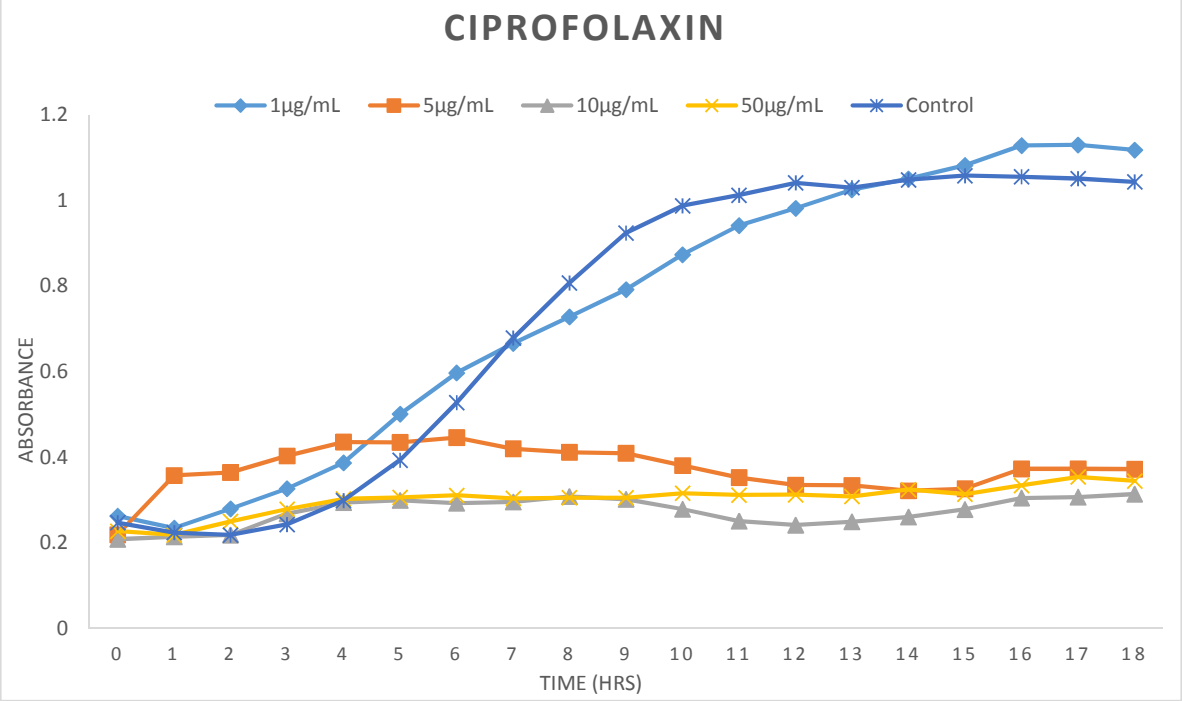

Figure 22 Absorbance recorded reflecting *C. minuta* growth at 4 different concentrations of ciprofloxacin: 1µg/mL, 5µg/mL, 10µg/mL, and 50µg/mL; compared to control in which no antibiotic was present

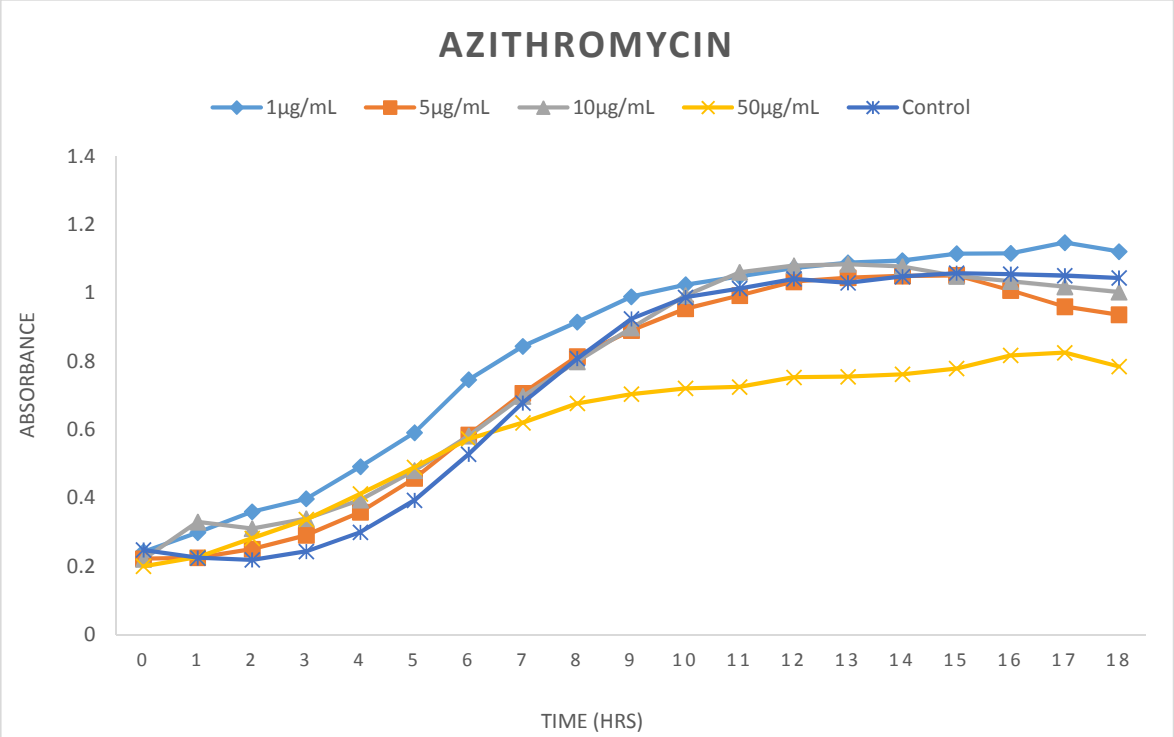

Figure 23 Absorbance recorded reflecting *C. minuta* growth at 4 different concentrations of azithromycin: 1µg/mL, 5µg/mL, 10µg/mL, and 50µg/mL; compared to control in which no antibiotic was present

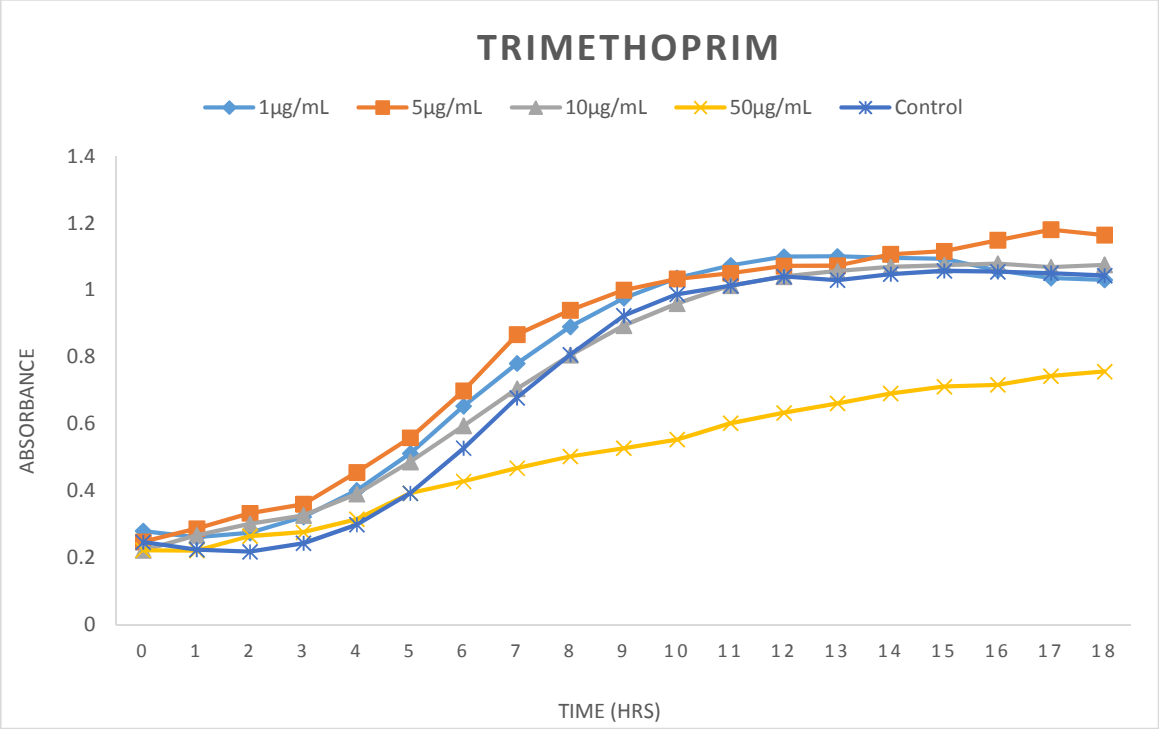

Figure 24 Absorbance recorded reflecting *C. minuta* growth at 4 different concentrations of trimethoprim: 1µg/mL, 5µg/mL, 10µg/mL, and 50µg/mL; compared to control in which no antibiotic was present

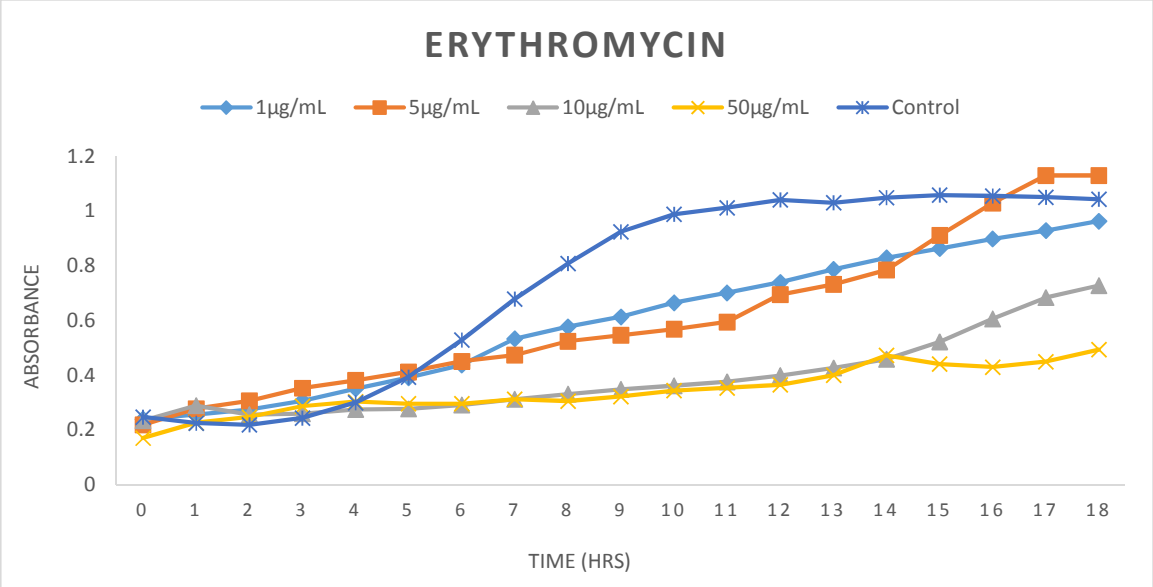

Figure 25 Absorbance recorded reflecting *C. minuta* growth at 4 different concentrations of erythromycin: 1µg/mL, 5µg/mL, 10µg/mL, and 50µg/mL; compared to control in which no antibiotic was present

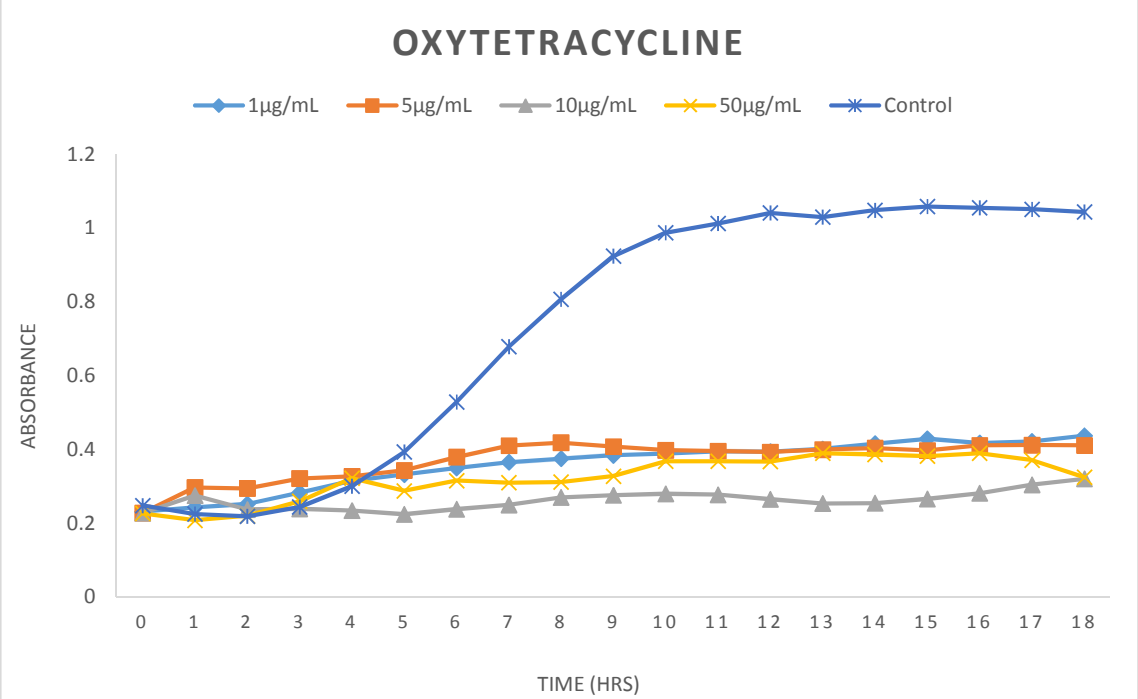

Figure 26 Absorbance recorded reflecting *C. minuta* growth at 4 different concentrations of oxytetracycline: 1µg/mL, 5µg/mL, 10µg/mL, and 50µg/mL; compared to control in which no antibiotic was present

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ABSTRACT

THE POTENTIAL STIMULATION OF *C. MINUTA* GROWTH BY VARIOUS PREBIOTICS, AND ITS SENSITIVITY TO VARIOUS ANTIBIOTICS

by

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Degree: Master of Science

Gut microbiota play a crucial role in maintaining intestinal health and integrity; a feature that is both heritable, as well as affected by the environment and lifestyle. Probiotics are supplements containing live microorganisms that act in a similar manner to gut microflora, and maintain a balance in the latter. *C. minuta* is a novel bacterium in the gut that was found to be associated with reduction in body weight and adiposity. The aim of this study was to determine the possible effects of different prebiotics on *C. minuta* growth, and the survival of *C. minuta* in response to different antibiotics. Six different prebiotics, GOS, FOS, GG, AG, IMO and inulin were added to *C. minuta* culture, and growth was compared in the presence and absence of dextrose; at different ratios. Apart from optimal growth noted in the presence of solely dextrose, the sample containing GOS alone came in second in optimizing *C. minuta* growth. the mixture of AG and dextrose appeared to optimize *C. minuta* growth; in contrast to the slight enhancing activity seen by AG alone. When survival was tested in the presence of six antibiotics at 4 different concentrations, sulfamethoxazole, trimethoprim, azithromycin, erythromycin, oxytetracycline and ciprofloxacin, *C. minuta* showed to be susceptible to

oxytetracycline at a concentration as low as 1μg/mL; and higher concentrations of ciprofloxacin. Erythromycin appeared to slow down or reduce growth compared to sulfamethoxazole, trimethoprim, and azithromycin.

Key words: *Christensenella minuta* - prebiotics - probiotics - antibiotics - gut microflora – obesity