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Optimization Of Growth Conditions And Characterization Of Properties On Christensenella Minuta

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OPTIMIZATION of GROWTH CONDITIONS and CHARACTERIZATION of PROPERTIES on CHRISTENSENELLA MINUTA

by

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THESIS

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DEDICATIONS

This thesis is dedicated to my parents, Qishuang Ai and Xiujiang Shen for their love, support and endless encouragement. I am grateful for Dr. Gong, who is my professor in my bachelor degree, for inspiring me to study further and imparting me the basic knowledge of nutritional science. I also wish to express gratitude to all my friends and colleagues for helping and improving my project.

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LIST OF ABBREVIATIONS

WHO: World Health Organization

BMI: Body Mass Index

C. Minuta: Christensenella minuta

SCFAs: Short Chain Fatty Acids

RCM: Reinforced Clostridium Medium

CFU: Colony-forming unit

PBS: Phosphate Buffer

Min: Minute

GI tract: Gastrointestinal tract

INTRODUCTION

Obesity

More and more people are developing obesity, especially in the USA. As of 2014 over one third (36.5%) of the American adults and youth are obese.[1]. Obesity, defined by the WHO (World Health Organization), is measure of body mass index (BMI) over 30 kg/m^2[2]. There are three major reasons causing obesity, including obesogenic environment[3], genetics, and medical influences. First, two aspects are involved in obesogenic environment, which include dietary intake and physical activities. The urban environment, including urban design, transportation systems, and land use, could increase our sedentary lifestyle[4, 5]. Moreover, the unhealthy dietary habits and easier access to fast food, highly processed and sugar-laden foods, and soda drinks can also promote obesity. A recent study reported that the sugar consumption in USA has been increased from 150 to 170 pounds/year/per person on average[3]. Overall, an obesogenic environment being a reason for causing obesity occurs because energy intake exceeds energy expenditure with reduced physical activities. Recent studies also showed that genetics also play an important role in developing obesity[6, 7]. Some genetic disorders such as Bardet-Biedl syndrome and polycystic ovary syndrome have been associated with the development of obesity[8]. However, the genes interact with environmental factors, including physical activity, healthy dietary habits, level of education, age, gender and ethnicity[7]. Finally, medical influences are also another major reason causing weight gain. A study noticed that a majority of psychiatric medications result in weight gain which is caused by metabolic syndrome or metabolic disorders[9]. Except psychotropic prescribing, some other diseases related to hormones may also contribute to weight gain, including hypothyroidism[10] , and Cushing's syndrome[11].

The consequences of obesity are diverse. For pediatric obesity, it is more likely to lead to adult obesity and higher risks of obesity-related issues. These can include cardiovascular disease, dyslipidemia, impaired glucose homeostasis, metabolic syndrome, pulmonary comorbidities, gastrointestinal comorbidities, orthopedic complications, and psychosocial and neurocognitive issues[12]. For obese adults, there are also numerous problems related to obesity, including elevated for the risk of various cancers, such as colon, breast, pancreas, and kidney. Obese adults also have an increased risk of type-2 diabetes, coronary artery disease and hypertension, decreased years of life, osteoarthritis and other health issues[3, 4].

Gut microbiome and Obesity

Researchers are attempting to find solutions to decrease the rate of obesity occurrences. Since the greatest density and numbers of bacteria are in our gut, an increasing number of studies focus on gut microbiome, which has attracted researchers' interests in finding out the relevant associations with obesity. As crucial factors of the microbiota ecosystem are in the human gut, the gut microbiome and the host live in a symbiotic manner[13]. The composition of the gut microbiome differs significantly based on nutrient status or metabolism, suggesting there may be a connection between the gut microbial profile and the risk of developing obesity[4, 13]. There are predominantly two phylums of bacteria in the human gut possibly associated with obesity, Firmicutes and Bacteroidetes.[14] Studies pointed out that obese mice had 50% fewer Bacteroidetes, and correspondingly more Firmicutes, than their lean siblings[15, 16]. Furthermore, Turnbaugh *et al.* discovered that the gut microbiome in obese mice had a greater ability to store energy for their host from their diets than those in lean mice. [17] In another human study, by transferring the intestinal microbiome from a lean donor to a male recipient with metabolic syndromes, Vrieze *et al*. showed improvement in insulin sensitivity that could be

a beneficial effect for diabetic patients.[18] These results further indicate that the dysbiosis of intestinal bacteria could lead to metabolic dysfunction and elevate the risk of diabetes.[19]

A potential new probiotic-Christensenella minuta (C. Minuta)

C. Minuta, discovered in 2012, was isolated from the feces of healthy Japanese males. It is an anaerobic and Gram-negative bacterium, with a straight rod shape[20]. C. Minuta also produces short chain fatty acids (SCFAs) as major end products of the metabolism of glucose, including acetic and butyric acid[20]. These SCFAs are associated with overfeeding, obesity, and the metabolic syndrome[21, 22]. A recent study showed that C. Minuta abundance was increased after dietary supplementation with a resistant starch type 4 supplement (q=0.038)[23]. Furthermore, oral intake of C. Minuta has been shown to significantly reduce body weight gain in mice[20, 24]. C. minuta is also inheritable and able to affect the host phenotype[24].Interestingly, the abundance of fecal C. Minuta was lower in obese/overweight German twins as compared lean pairs(BMI<25)[24]. Collectively, these data suggest that C. Minuta may be a potential novel probiotic that can help to reduce risk of obesity.

OBJECTIVE

The objective of this study is: to characterize properties of C. Minuta, including generation time, DNA identification, and growth curve; to optimize its growing conditions; and to check its survivability in simulated gastrointestinal tract.

MATERIALS AND METHODS

Materials

The bacterium, Christensenella minuta (C. Minuta), was purchased from DSMZ (Leibniz Institute DSMZ, Germany). Reinforced clostridium medium (RCM) was purchased from Becton, Dickinson and Company (Sparks, MD). Resazurin was purchased from ACROS (New Jersey). QIAamp DNA Mini Kit (Qiagen, CA, USA), Sigma ReadyMix™ RedTaq© PCR Reaction Mix (Foster, CA, USA), SYBR© Green (Carlsbad, CA, USA), Thermo Scientific© GeneRuler ultra low range DNA ladder (Carlsbad, CA, USA), SIGMA-ALDRICH sodium carbonate (St. Louis, MO, USA), and Fisher Science Education bile salts (Nazareth, PA, USA) were purchased from Fisher Scientific website.

Methods

Routine Culturing of Christensenella minuta

Routine culturing of this bacterium was done in RCM medium, using Hungate tube method and under N2 gassing. A Hungate tube is an anaerobic tube with butyl rubber stopper and screw cap, which can prevent air from going inside while transferring substances[25] . Anaerobic indicator resazurin, 0.5-1mg/L was added to growth medium to ensure the anaerobic condition. Bacteria were incubated for 24 h at 37 ℃ in a Hungate tube. To check the growth of bacteria, turbidity was measured hourly by SIEMENS© turbidity meter (West Sacramento, CA, USA), with brief vortexing prior to each reading.

Characterization of C. Minuta

DNA Extraction

C. Minuta DNA was extracted using QIAamp DNA Mini Kit (Qiagen, CA, USA) according to manufacturer's instructions. DNA is stored at -20℃ after extraction.

PCR Amplification

Oligonucleotide primers were designed according to C. Minuta's 16s rDNA. The sequences of primers used in this study are given in Table 1. The PCR reactions running by Eppendorf© thermocycler (USA) were carried out in a total volume of 25 µl containing 12.5 µl of 2× PCR Sigma ReadyMix™ RedTaq© PCR Reaction Mix, 2 µl of DNA template (5ng), 0.1µM of each primer, rest was of PCR water. The denaturation step was carried out at 95˚C for 5 min, followed by 30 amplification cycles of 95°C for 15 secs, 55°C for 20 secs, and 72°C for 45 secs, with the final extension step of 3 min at 72°C. 1.5% agarose gel prepared in 1 x Tris-Borate-EDTA (TBE) buffer was used for gel electrophoresis. PCR product Thermo Scientific© GeneRuler ultra low range DNA ladder (10-300bp) was used as molecular weight standard for comparison. Final gel containing separated PCR products was visualized by SYBR© Green staining under Bio-rad© Imager (USA).

Table 1 C. Minuta Oligonucleotide Primers Design

Primer name	Primer Sequence	Position on rRNA gene sequence (bases)
16s rRNA Forward	CTGGAACTGAGACACGGTCC	316-335
16s rRNA Reverse	CACGTAGTTAGCCGGAGCTT	495-476

The Product length is 180.

Correlation of Turbidity and CFU numbers

After bacteria was cultured for 24 hours at 37 °C, turbidity was measured by a turbidity meter. The original tube was diluted by 2 times, 4 times, 8 times, 10 times, and 16 times. After

dilution, the turbidity absorbance of the different diluted tubes was recorded. CFU (colony-forming unit) of the original bacterial tube was counted from growth on poured diluted plates with 100uL inoculation of each diluted culture after one day anaerobic incubation at 37 °C.

Generation Time

According to the growth curve generated from temperature test and pH test, the CFU of each point can be calculated by the equation ($y = 348.7x^4 - 389.53x^3 + 217.96x^2 + 48.128x + 3.181$), which is from the turbidity verification. After knowing the CFU at each point, the exponential phase needed to be decided since generation time is the rate of exponential growth of a bacterial culture. The formula below was used to calculate the generation time. Xt implies final concentration of CFU, X_0 implies initial concentration of CFU, and "log" is the logarithm of the base.[26, 27].

$$\text{Tgeneration time} = \frac{\text{duration} * \log(2)}{logXt - logX0}$$

Optimization of Growth Conditions

Temperature

C. Minuta live culture were inoculated into different Hungate tubes and placed under varying temperatures, 25℃, 35℃,37℃, 40℃, and 45℃ separately. Turbidity absorbance of bacteria growth for 15 hours was recorded hourly.

pH Effect on the Growth

C. Minuta was incubated for 15 h at 37 ℃ in a microplate reader in different pH conditions: pH 5, pH 6, pH 7, pH 8, and pH 9, which was adjusted with concentrated hydrochloric (1M) acid and 5% w/v sodium carbonate solution and using OAKTON® pH 700 meter (Singapore) The optical density readings were measured hourly at 595 nm using the Perkin

Elmer bioassay reader HTS7000 (Norwalk, CT), with low speed shaking for 5s prior to each reading.

Survivability of C. Minuta

Against Different pH

Bacteria were cultured in RCM broth at 37°C overnight, and subcultured in 10 ml of fresh RCM broth, which had already adjusted to different pH values (2,3,4,5,6,7) with concentrated hydrochloric acid (1M) and 5% w/v sodium carbonate solution. The initial bacterial concentration was over 10^6 CFU/mL and was checked by turbidity verification. After incubation for 1 h at 37°C, samples were serially diluted 10-fold with PBS (phosphate buffer) (0.1 M, pH 6.2). The residual viability count was determined by dilution and plate counting on RCM agar after 24 hour of incubation.

Survivability against Bile Salts

For bile salts, fresh 10 mL RCM media was prepared in Hungate tubes with adjusting solid Fisher Science Education bile salts (Nazareth, PA, USA) in different concentrations: 0.1g/L, 0.5g/L, 1g/L, 2g/L, 4g/L and 6g/L. The initial bacterial concentration added in each Hungate tube was over 10^6 CFU/mL and was checked by turbidity verification. After incubation for 2h at 37°C, samples were serially diluted 10-fold in PBS. Plate count method was used to determine CFU.

Statistics Analysis

Collected data were analyzed using Excel (Microsoft Corp., Redmond, WA). The OD values, turbidities and standard deviations were calculated from duplicate samples.

RESULTS and DISCUSSIONS

Characterization of C. Minuta

Gel Electrophoresis

As seen in Figure 1, the No.1 well was a 300bp ladder, No. 2 was non-template control, and No.3 was the C. Minuta PCR product. The No. 2 well did not show any bright bands in the line, which means no contamination was caused by the reaction mix or primers. The bright band shown in No. 3 was between 150bp and 200bp, which is in accordance to product length. In the figure, there were two blurred bands at the bottom of No. 2 and No. 3, which is caused by primer dimer. Therefore, the DNA extracted from cultured bacteria is C. Minuta's DNA.

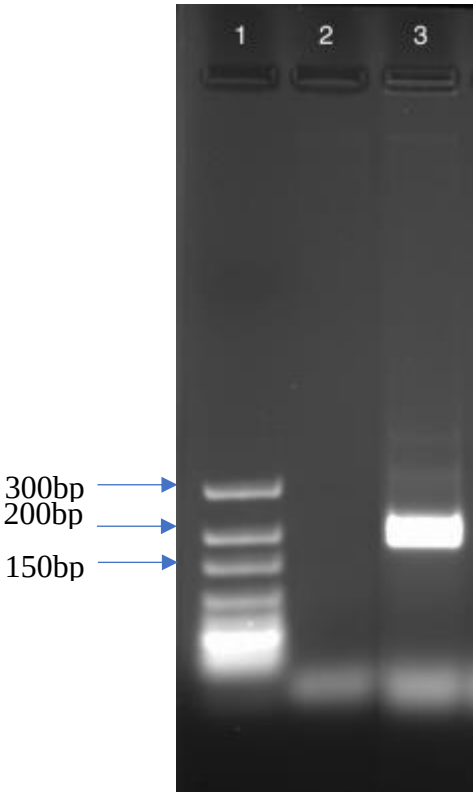

Figure 1 DNA Agarose Gel
The DNA agarose gel demonstrating PCR samples for amplification of the 16S rDNA of the C. Minuta isolates.

Correlation of Turbidity and CFU numbers

The relationship between CFU numbers and turbidity was determined in overnight growth of C. Minuta and CFU numbers were plated-counted. The R squared value of the equation is equal to 0.99931. Turbidity measurement is more convenient than plate-counting method for evaluating CFU numbers in the samples.

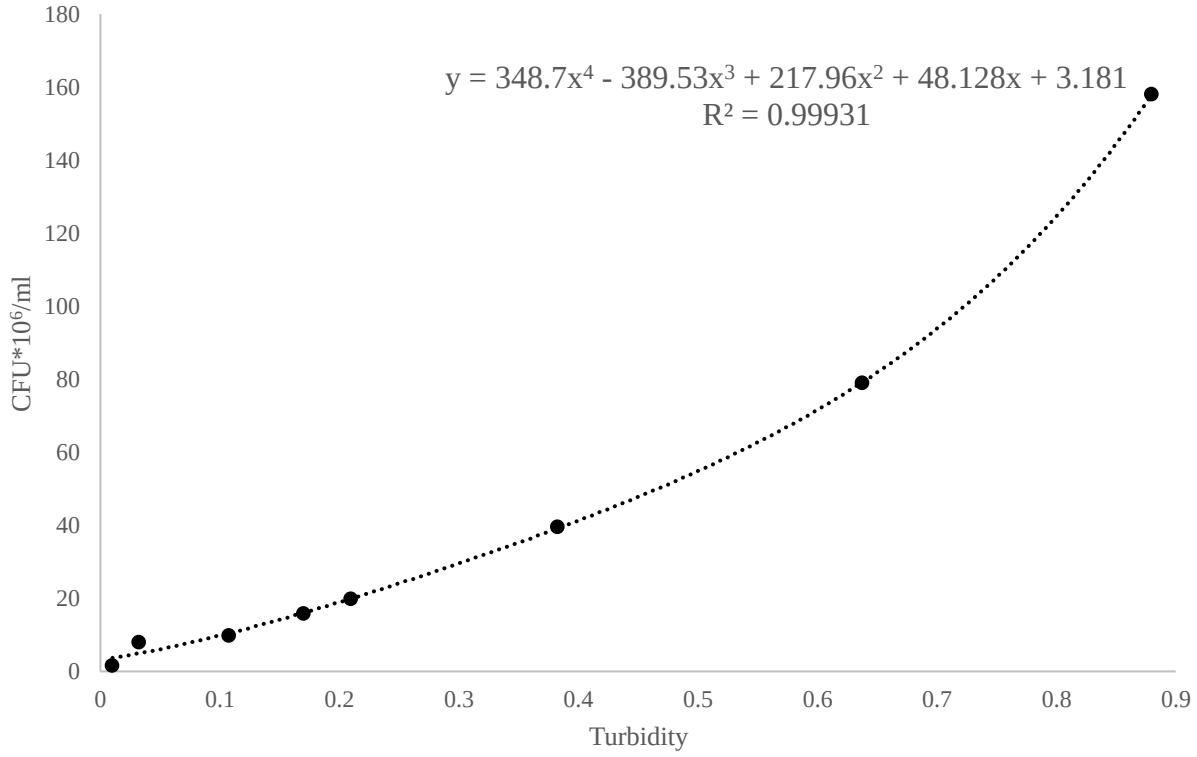

Figure 2 Relationship between turbidity and CFU numbers

Optimization of Growth Conditions

Temperature

As usual, the growth curve of any bacterium had three phases, which include the lag phase, exponential phase, and stationary phase. Figure 3 showed the growth curve of C. Minuta

at different temperatures. The highest line is the curve at 40ºC with exponential growth from 3-hours to 6-hours. It appeared that C. Minuta at 35 ºC, 37 ºC and 40 ºC showed the optimal growth. In addition, for 37 ºC curve, the exponential phase is from 3-hours to 7-hours in comparison to 35 ºC curve whose exponential phase is from 4-hours to 7-hours. All four curves show growth after 2 hours; still, the bacterium showed the slowest growth at 25 ºC and 50 ºC. After turbidity was transferred to CFU number according to the turbidity verification equation ($y = 348.7x^4 - 389.53x^3 + 217.96x^2 + 48.128x + 3.181$), the generation time of each curve was calculated (Table 2). It showed that bacteria growing at 37 ºC had the shortest generation time at 69.87 min. The next two are bacteria growing at 40 ºC and 35 ºC, which their generation time was found to be 71.82 min and 82.67 min, respectively. Bacteria growing at 45 ºC and 50 ºC showed similar generation time, which is 137.45 min and 137.13 min, respectively. The longest generation time was found to be the bacteria growing at 25 ºC, which was found to be 183.43 min.

Therefore, from these results, it showed that C. Minuta has the ability to grow from 25 ºC to 50 ºC, in comparison to the previous study, which pointed out that C. Minuta grows at 25-43 ºC[20]. Although the curve at 45 ºC is higher than the curve at 37 ºC, the generation time of the two curves are almost the same, around 70min. Moreover, the growth rate of the curve at 37 ºC is even shorter than the one at 40 ºC. The potential reason why the curve at 45 ºC is higher could be because the bacteria in the 45 ºC Hungate tube had a higher population to that of the 37 ºC Hungate tube. Since a syringe is used to add the bacteria, discrepancies in total CFU can occur. Bacteria growing at 25 ºC and 50 ºC had longer lag phases. Overall, 37 ºC had the shortest generation time during exponential phase, and is the optimal temperature for C. Minuta.

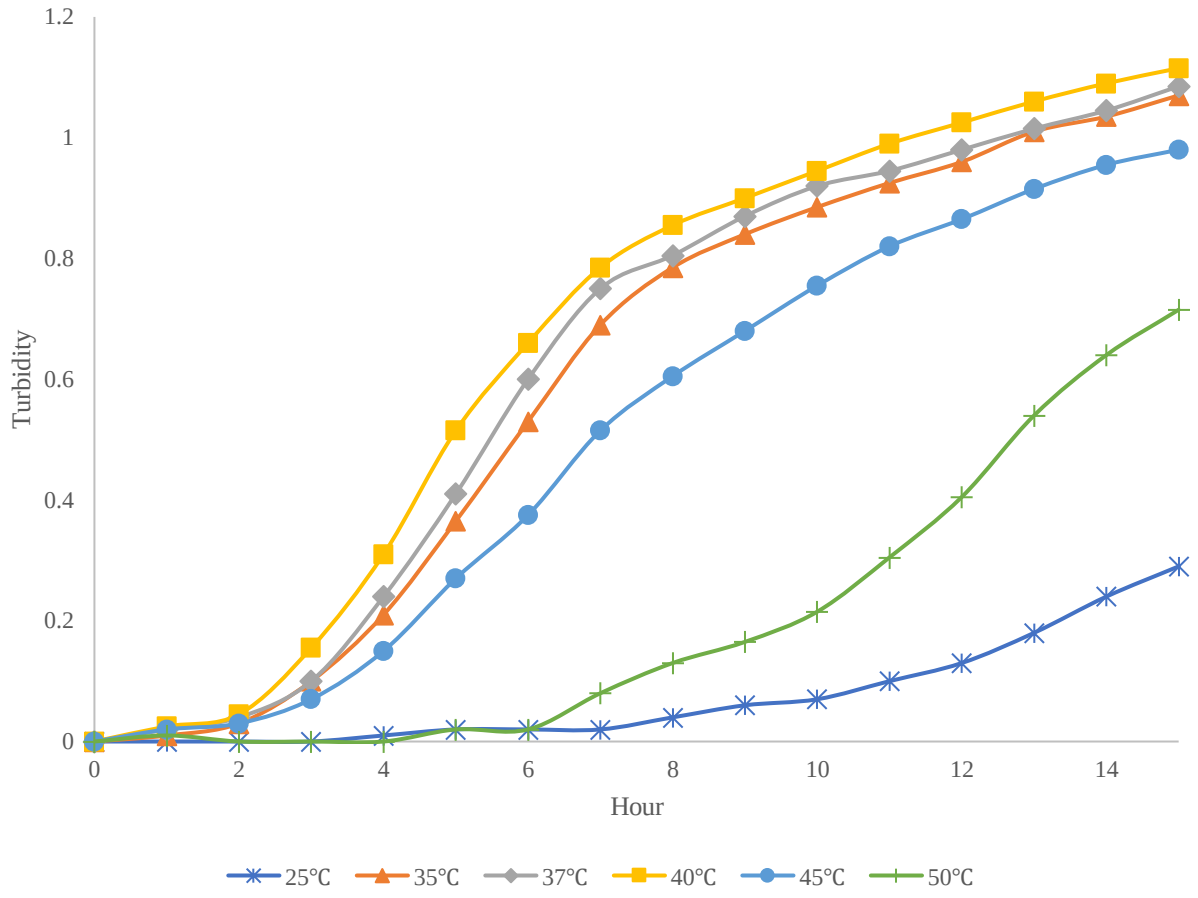

Figure 3 The Growth of C. Minuta on Different Temperatures

Table 2 Generation Time of C. Minuta at Different Temperatures

	25 ºC	35 ºC	37 ºC	40 ºC	45 ºC	50 ºC
Exponential Phase(hour)	8-13	4-7	3-7	3-6	5-9	8-12
Generation time (minute)	183.43	82.67	69.87	71.82	137.45	137.13

pH Effect on the Growth

Figure 4 shows the growth of C. Minuta at different pH. Two curves at pH 9 and pH 10 showed no increase in turbidity, suggesting that the bacteria did not grow under the pH conditions. The bacterium is able to grow at pH 5-8 where pH 7 promoted the most growth. Under pH 6 and 7, the exponential phase started at 7-hours. While, the exponential phase delayed to 8-hours at pH 5 and to 13-hours at pH 8.

According to our results, the higher pH the longer the lag phase was among the six pH values. In the figure, the starting points for different pH were different, which occured because that pH affected the color of the solution. A previous study claims that the pH range of C. Minuta growing at 37 ºC is from pH 6.0 to pH 9.0[20], however these findings in this study suggest that C. Minuta has a lower range of pH balance. It cannot grow at pH 9.0; instead, it was able to grow at pH 5. Based on the results, pH 7 is the optimal pH for C. Minuta growing at 37 ºC, followed by pH 6.

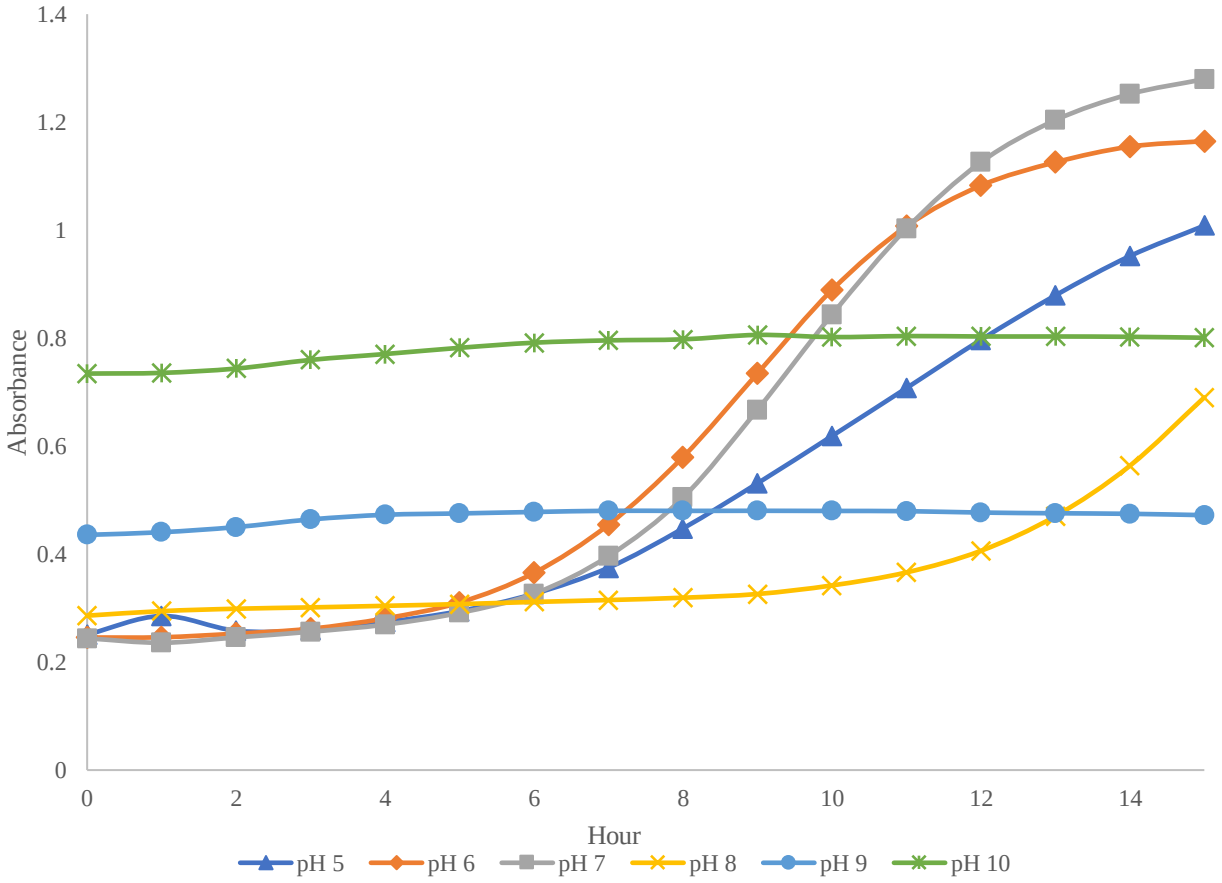

Figure 4 Growth of C. Minuta in meadia with various pH values

Survivability of C. Minuta

Against Different pH

As shown in Table 3, it is evident that the lower the pH value (pH2-7), the less bacterial variability occurred. It appears that the bacterium was able to tolerant pH 5 or higher even with some growth being observed. The bacterium had the highest CFU at pH 7, suggesting the optimal survivability. About 87.83% of the bacteria survived against pH 4, the survival percentage reduced to 7.567% and 1.139% when pH dropped to 3 and 2, respectively.

Compared to the test on optimal pH for growth, the purpose of this tolerance test was to evaluate the survival rate of C. Minuta when mixed with different gastrointestinal pH conditions (pH 2-7). Therefore, the treatments lasted 1 hour. The gastric pH values could be as low as 1 upon an empty stomach and increase to around 5 upon food ingestion. The pH value in esophagus is around 7.0. The pH value is between 5.26 and 6.72 in the ascending colon and 5.20 to 7.02 in the descending colon[28]. Our results showed that the majority of C. Minuta was able to survive against pH 4 or higher. However, gastric fluid with pH lower than 4 killed over 90% of C. Minuta within 1 hour incubation. Hence, if C. Minuta is to be developed as a dietary supplement additional protection in the harsh GI environment is essential to ensure its survival during GI digestion.

Table 3 Survivability of C. Minuta against pH on C. Minuta

	pH					
	2	3	4	5	6	7
Before	$3.37*10^7$					
After 1hr CFU	$3.84*10^5$	$2.55*10^6$	$2.96*10^7$	$3.62*10^7$	$4.0*10^7$	$4.15*10^7$
Viability	1.139%	7.567%	87.83%	107.41%	118.69%	123.14%

Survivability against Bile Salts

As shown in Table 4, bile salts significantly reduced the survivability of C. Minuta; the higher concentrations of bile salts, the lower viability. Bile salts at a concentration of 0.1g/L exerted no destructive effect on the bacterium. However, when its concentration increased to 0.5g/L, the viability dropped to 4.7%, which means over 95% of the bacteria were killed. The survivability of the bacteria was reduced further to 1.1%, 0.2% and 0.01% when incubated with 1,2,4 g/L bile salt. The bacteria were completed killed after treatment of 6 g/L bile salt.

Bile acid is derived from cholesterol, which is synthesized by the liver, forming primary bile acid[29]. Bile salts are conjugated bile acid with the sodium and potassium salts[30, 31]. The concentration of bile salts in the intestine could reach 0.3-3g/kg and increases postprandially[32, 33]. The bacteria are in fact very sensitive to bile salts. Since the concentration/secretion of bile salts is increased with food ingestion, it is suggested that C. Minuta should be taken orally on an empty stomachto reduce the risk of the bacterium being killed by high levels of bile salts.

Table 4 Survivability of C. Minuta incubated with various concentraitons of Bile salts

	Bile salts(g/L)					
	0.1	0.5	1	2	4	6
Before	$3.37*10^7$					
After 2hr CFU	$3.57*10^7$	$1.59*10^6$	$3.8*10^5$	$8.5*10^4$	$4*10^3$	≅0
Viability	105.935%	4.718%	1.128%	0.252%	0.012%	≅0%

CONCLUSION

In our study, C. Minuta was investigated for its properties on growth conditions, and survivability in simulated GI conditions. The generation time of C. Minuta was found to be around 70 minutes. The optimal growth conditions are at 37 ºC under pH 7. C. Minuta is sensitive to pH lower than 5 and bile (higher than 0.1g/L). In conclusion, encapsulation or another protection is needed when consuming C. Minuta as a potential probiotic supplement if taken orally in order to maintain the survival rate. Further in vivo experiments are needed to confirm its survivability in GI tract and more importantly its effects on obesity and the associated complications.

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ABSTRACT

OPTIMIZATION of GROWTH CONDITION and CHARACTERIZATION of PROPERTIES on CHRISTENSENELLA MINUTA

by

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05,2017

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MAJOR: NUTRITION AND FOOD SCIENCE

DEGREE: MASTER OF SCIENCE

Currently, increasing numbers of people are suffering from obesity. The gut microbiome becomes a useful approach to control the mechanisms associated with obesity. A novel bacterium, Christensenella minuta, which was discovered in 2012, triggers body weight loss. Our purpose in this study is to further realize characteristics of C. Minuta as a potential probiotic supplement for future obesity studies. This study determines the following characteristics of C. Minuta: DNA identification, turbidity verification, generation time, as well as optimization of pH and temperature. To test the tolerance of C. Minuta as a gastrointestinal treatment, the pH and bile salts concentration in the gastrointestinal tract were mimicked. In this study, the Hungate tube method was involved for anaerobic culture; turbidity absorbance was used to detect bacterial growth; plate-counting method was applied to measure CFU (colony-forming unit). From our findings, the optimal conditions of C. Minuta were found at 37 ºC under pH 7. The generation time of C. Minuta was found to be around 70 min. C. Minuta is sensitive to pH and bile salt, with survivability found at 0.1g/L bile salts and pH 5. In conclusion, encapsulation or another protection is needed when consuming C. Minuta in future animal or human studies.