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Development Of Gi Sustainable Probiotic Beads Using Microencapsulation

Jiarun Cui
Wayne State University,

DEVELOPMENT OF GI SUSTAINABLE PROBIOTIC BEADS USING MICROENCAPSULATION

by

JIARUN CUI

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DEDICATION

This thesis is dedicated to my parents Qinghai Cui and Liping Chen for their support and love. And also I appreciate to all my friends' inspiration and support for helping me to complete my master research.

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I'm grateful Dr. Kequan Zhou's great aid and supporting during the period of my master study in Wayne State University. Also, I want to say thank you to Wenjun Zhu for selflessly teaching me lab techniques and sharing his valuable experience about research with me. I appreciate every lab member in Dr. Kequan Zhou's lab for helping me and providing me a great academic environment to do my study. I also would like to thank Dr. Yifan Zhang and Dr. Heydari Ahmad for serving on my graduate committee.

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LIST OF ABBREVITIONS

BMI: Body Mass Index

C. minuta: Christensenella minuta

GOS: Galactooligosaccharides

GIT: Gastrointestinal tract

RCM: Reinforced Clostridial Medium

DI: demineralization

PBS: Phosphate-buffered saline

CFU: colony forming unit

INTRODUCTION

Obesity and Diabetes

Recent statistics show that obesity (BMI over 30kg/m^2) has become one of the most serious global health problems (1). The situation is even worse in America, where more than 30 percent of the adult population is obese, and the trend is alarming (2). Since obesity is considered to be a key risk factor in various conditions, such as cardiovascular diseases, hypertension, arthritis and diabetes, more researchers are investigating means to prevent and treat obesity (3).

Diabetes is also a severe disease which threatens people's heath. Statistics from the World Health Organization showed that the global prevalence has reached up to 10%, the number is even higher in some specific areas (4). The origin of diabetes can be many different factors such as genetic, environmental factors that related to diet, life-style, and lack of exercise, and intestinal microbiota condition. Researchers also have reported that the inflammatory stress may lead to insulin resistance (5).

Probiotics and Prebiotics

Scientists are putting more and more attentions on probiotics for obesity treatment and diabetes prevention in recent years due to the incredible benefits displayed by probiotics. Probiotics are live microorganisms which can provide a health benefit for the host when its amount is sufficient (6). Particularly, some probiotics have the ability to alter the gut microbiota for regulating inflammatory which can reduce potential insulin resistance caused by the stress of

inflammatory. And some probiotics have the ability to contribute to host strain and improve the metabolism system in human body. In another word, there are different health benefits, which mainly depend on the specific strain and specific amount (7). Therefore, gut microbiome could largely affect a person's health status (8).

The probiotics of interest for our study is Christensenella minuta (C. minuta), C. minuta was first found in the human gut as a Gram-negative, strictly anaerobic bacterium (9). A recent study related to gut microbiome has shown that C. minuta reduced body weight gain in the recipient mice (10), suggesting that C. minuta as a gut microbiota may provide potential benefits related to obesity treatment by modulating the host microbiome system (11).

Prebiotics are non-digestible carbohydrates that can be selectively fermented by the gut bacteria. The process could have a beneficial effect on metabolism and gut health (12). Several recent researches have reported that prebiotics have the ability to alter the gut microbiota (13). Furthermore, current diet, especially western-type diet, contains limited amount of prebiotics (14). Prebiotics were also investigated and applied during our study for promoting the growth of growth of C. Minuta. Galactooligosaccharides (GOS) was added during C. Minuta microsphere-making process due to its stability in acidic conditions and specific promotion of these probiotics (15).

Encapsulation

The ability of probiotics to survive during storage (in food products) and gastrointestinal digestion is extremely important for them to exert any beneficial activities. The gastrointestinal

tract, which is the first physiological step of digesting and absorbing nutrients from diet, will kill most of probiotics due to the harsh acidic condition (16). Moreover, it is indicated that food containing probiotic bacteria once consumed by humans should contain at least 10^9 live microorganisms per g or mL in order to exert its protective effects as recommended (17). As we mentioned before, the probiotic that we are focusing on is anaerobic and has a low survive-rate after GIT. Therefore, a way to protect the probiotic from being killed and maintaining its viability during the storage and GI digestion is needed.

Encapsulation techniques are considered to be one of the most effective techniques to help bacteria go through GIT without significant loss in cell viability and prevent possible interaction during the storage and process by providing a physical barrier (18). Furthermore, encapsulation allows bacteria to be released in the intestinal environment and bypass the GIT altogether (19).

The current main techniques for microencapsulation of probiotics are listed as the following: spray-drying, freeze-drying, emulsion, coacervation, and extrusion. When the active ingredient can be dissolved in the encapsulating agent which lead to emulsion or suspension, drying is used as an encapsulation method (20). Spray-drying is mainly used for probiotics production due to its ability for the large scale production (21). During spray drying, the atomization of solution which contains an active ingredient transports from drying chamber to the cyclone powder collector with heated air or gas stream. Finally, by a rapid evaporation of the solvent, the dried powder is collected in the collector (22). Spray-drying is considered the most economic and effective drying technique in probiotics industry. However, not only is spray-

drying unsuccessful in promoting a high survival rate because of heating and drying, but also often fails to provide enough protection to the probiotics during storage (23). The process of spray-cooling is somewhat similar comparing to spray-drying. The main difference during the process is the carrier material and conditions of working. Spray-cooling is also not popular on probiotics encapsulation due to the thermal environment (24).

Freeze-drying is one of the most widely used encapsulation technique for drying probiotics (25). The principle of dehydration process works by freezing the product and decreasing the pressure of the environment which can make the frozen water to sublimate from solid to gas (26). The water phase transition and avoided oxidation environment are one of the most important advantages of freeze-drying technique. However, most freeze dry techniques can only provide protection for the storage of probiotics, the freeze dried probiotics could be still vulnerable during digestion. Thus, the freeze-drying technique often needs to be combined with other encapsulating methods (27).

Emulsion-based technique is considered to be an effective method that helps the microorganism cells to keep a high survival rate after passing the harsh conditions of the stomach (28). Coacervation process involves a precipitation of polymers by separating the phase. Coacervation is also a dehydration process which can turn the liquid phase to solid droplets to form encapsulation (29). Generally, the coacervation technique is used for flavor microencapsulation. Some probiotic bacteria are also rely on coacervation to provide protection by using water-in-oil emulsion as medium (30).

Extrusion are techniques that encapsulate probiotics in microspheres. The principle is to produce probiotics encapsulation with microspheres. Basically, the process can be separated to 2 crucial steps: 1) the probiotics were dispersed to create small drops; 2) Gelatin or formation will turn the drops to solid by forming membrane in the surface of the drops (31). The size of the drops is determined by the size of nozzle and the speed of dropping. There have been many studies use extrusion techniques for probiotic encapsulation (32).

Among the several encapsulated techniques, extrusion method shows the features that fit our objective the most. Not only because the process of extrusion technique is simple to operate, but also because the well protection it provides to the type of probiotic we are focusing on (33). The polymer formed during encapsulation should be food-grade, non-toxic, non-antimicrobial. Therefore, alginate and chitosan are considered to be two of the most widely used material for extrusion encapsulation (34). Chitosan-based extrusion can improve the survival rate of the probiotics during the process of GI tract and storage. However, due to the antibacterial activity of chitosan polymer, chitosan is mainly used as the surface shell in capsules (35). Alginate is a popular material for coating which can cross-link $CaCl_2$ solution. The process is simple, safe and cost (36). Furthermore, alginate-made capsule is biodegradable and biocompatible, which means alginate-based extrusion encapsulation is an effective way to delivery viable probiotics to the intestine (37).

The Application of Probiotics

Many different probiotic bacteria have been successfully applied on several food industry areas in recent years (38). Bifidobacterium and Lactobacillus are the most widely used probiotics in varieties of food and dietary supplements. The commercial interest and market shares of probiotic foods are increasing in recent years (39). More and more new benefit strains were discovered and more and more new technology was using on probiotics application (40). Generally, probiotics were mostly used in fermented dairy food. Although there are some nondairy food products in the market, there is still lots of potential for nondairy food product development (41). In the future, probiotics food will definitely play an important role in human's daily life.

Objective

The objective of this study is to develop an encapsulation technique which can protect C. minuta and maintain its viability during storage and GI digestion. We also plan to develop specific C. Minuta-based functional food products. Our ultimate goal is to develop effective make C. minuta-based nutritional products that can help combat the global obesity and diabetes challenges.

MATERIALS AND METHODS

Culturing and Viability Test of Freeze Dried Powder

C. Minuta was bought from DSMZ (Leibniz Institute DSMZ, Germany). Routine culturing of this bacterium was in Reinforced Clostridial Medium (RCM Sparks, MD), using Hungate tube

method and under N_2 gassing flowing (42). Then incubated at the temperature of 37°C for one night.

Freeze-drying technique was applied to get the bacteria powder with sucrose (New jersey, USA) as the washing buffer. Weigh optimized amount of freeze dried powder into RCM. After the solution was completely mixed, dilute the solution to several different concentrations with selected pipettes. Then pour-plating method was used to check the viability of the powder, and each diluted concentration was plated duplicated, All the plates were incubated at 37°C for one night and the CFU was calculated depended on the colonies checked at the next day.

Microencapsulation

Alginate-based extrusion encapsulation technique was applied in the study. In order to encapsulate the bacteria with alginate cross-linking to form stable microbeads, optimized amount of freeze dried C. minuta powder and GOS were added into Sterile Sodium Alginate solution (New jersey, USA). Then a homogenizer (IKA, Germany) was used to mix the powder and the solution. Once the solution was completely mixed, a 10 mL syringe needle (30G, 1/2) was used to extrude the droplets into sterile $CaCl_2$ (New jersey, USA) solution. The probiotic beads were left in $CaCl_2$ solution for 30 minutes at room temperature after extrusion. Then all the beads were taken out from the solution and separated to two groups, one group were put into the dehydrator (Excalibur, USA) at room temperature. Once the beads were completely dried, these beads were transported into a centrifuge tube and store at room temperature for further viability test analysis and GIT. Another group of beads was used in double encapsulation.

Double Encapsulation

To improve the protection of encapsulation and prevent the probiotic b`eads from growing early before delivering, one group of alginate $CaCl_2$ cross-linking beads from last step were put into sodium alginate solution again. After covering by sodium alginate solution, the beads were dipped into $CaCl_2$ solution to form the second encapsulated layer. The double encapsulated beads were taken out from $CaCl_2$ solution and put into the dehydrator at room temperature. Once the beads were completely dried, a centrifuge tube was used to store the beads at room temperature until viability test and GIT.

Gastrointestinal Tract

The purpose of the encapsulation technique is to help C. minuta to achieve a high survival rate after GIT. Therefore, GIT is a crucial step of our study. The dried C. minuta beads were weighted before putting into a 2mL sterile centrifuge tube. HCl (Gibbstown, NJ) was loaded into a beaker, a pH meter and phosphate-buffered saline (PBS) was used to adjust the pH to reach pH=2 which closing to the environment of human stomach. Then the adjusted HCl and Pepsin (200mg/10mL, St louis, MO) were added into the tube. The tube was incubating at 37°C for 1 hour. After incubating, the liquid was discarded. Enteric digestion formula solution (0.12g/10mL) was added into the tube to simulate intestine environment at 37°C for 1 hour. Single and double encapsulated C. minuta beads and freeze dried C. minuta powder were all gone through GIT tests following the same step during the study.

Viability Test of the Beads after GIT

The original dried C. minuta beads and the C. minuta beads after GIT were homogenized by a homogenizer. Then the solution was diluted to several different concentrations with sterile PBS. Pouring-plating technique was used to test the viability. During our study, the dilution factor is 10^{-7} to 10^{-1} which increased by 10^{-1} at each concentration. And each concentration is duplicated plated. All the plates were put into an incubator for overnight incubating at 37°C. The viability is valued by calculating the CFU which depends on the number of colonies counted at the next day.

Extrusion Process Improvement

Although the process of extrusion worked well in the study, we still need to figure out a faster way to make large amount of beads. Three sterile tips were used to extrude the alginate solution instead of 10 mL syringe needle. A pump was used to extract the alginate solution via a silicon tube (**Figure 1**).

Food Product Development

In order to develop microbeads on application, we decided to use jelly as the food vehicle to make coconut jelly with C. minuta beads. Gelatin (St louis, MO) solution was made and autoclaved at 121°C for 15 minutes and cool down to 40°C at room temperature. Then optimized amount of coconut water (Monrovia, CA) was added into gelatin solution as the main flavor of the jelly on a hot plate. The solution was put into a refrigerator for 30 minutes to solidify the solution at 4°C. Once the jelly is completely solidified, it was stored in a plastic plate at room temperature.

Drinkable jelly is also considered as a good food product to carry microbeads. New formula C. minuta beads were made during this step. Optimized freeze dried C. minuta powder, coconut water and casein (New jersey, USA) were added into sterile sodium alginate solution (w/v) to make the extrusion solution. Then the new formula C. minuta beads were made by following the encapsulation pathway with a 10 mL syringe and a sterile tip (**Figure 2**). Glucomannan (Bloomingdale IL) and Kappa Corrageenan (York, ME) were added into demineralization (DI) water with the homogenizer to mix the solution. Then the solution was heated to 80°C and cool down at the room temperature. Once the temperature reached 40°C, the new formula C. minuta beads were put into the solution and put the solvent into a vacuum food-grade pack at a 4°C refrigerator for storage.

Long Term Beads Stability Test

In order to figure out whether the probiotic beads are stable in room temperature for long period. Dried C. Minuta beads were put into 3 centrifuge tubes at room temperature for 8 weeks, the viability of the beads was checked every 4 weeks. The long term stability of C. Minuta beads in coconut jelly was also tested in a period of 2 weeks at room temperature.

RESULTS AND DISCUSSION

Encapsulation of C. Minuta Strain

The C. minuta freeze dried powder was encapsulated using an alginate-based extrusion technique, in order to protect C. minuta from being killed by the harsh conditions found in the GIT. The C. minuta beads are soft globoid balls which have the size of d=3mm (**Figure 3**). The

size of the dried beads was reduced a great deal, to d=1.6mm (**Figure 4**). The dried beads also became much harder than the original beads when dried. After arriving in the GIT, the dried beads absorbed water to expand and become soft again (**Figure 5**).

Viability Test of Single and Double Encapsulated C. minuta Beads and Freeze Dried C. minuta Powder.

Pour-plate methods were applied during the viability test of C. minuta beads and powder. The viability of freeze dried C. minuta powder was $10^{8.9}$ CFU/mg. From the results shown in the figure (**Figure 6**). we found that the viability of both single and double encapsulated beads were decreased, compared with that of the freeze dried powder. That was probably due to the influence of oxygen and higher temperature during the homogenization. Nevertheless, double encapsulated C. minuta beads presented a higher viability than single encapsulated beads, which is $10^{7.95}$ CFU/mg to $10^{7.72}$ CFU/mg. Thus, double encapsulation provides a more effective protection to reduce the viability lost during the process of encapsulation.

Viability Test After Gastrointestinal Tract

The Gastrointestinal track (GIT) is considered as a way to check the for the effect of encapsulation. We set up the GIT experiment within three different groups: single encapsulated beads, double encapsulated beads, and freeze dried powder. Then the viability of the three groups' subjects was tested by the pour-plate method (**Figure 6**). First, the viability of freeze dried powder dropped from $10^{8.9}$ CFU/mg to $10^{4.17}$ CFU/mg which indicates that freeze dried C. minuta powder cannot reach a high survival rate without any encapsulation. Second, either the

single encapsulated beads or the double encapsulated beads, displayed great protection to the viability of C. minuta after entrance to the GIT. Particularly, the viability of double encapsulated beads achieved a higher survival rate, $10^{7.95}$ CFU/mg to $10^{7.84}$ CFU/mg, than the single encapsulated beads did, $10^{7.72}$ CFU/mg to $10^{7.42}$ CFU/mg. Thus, we obtained the result that double encapsulated C. minuta beads had more effective protection during time spent in the GIT, than single encapsulated C. minuta beads. Overall, the C. minuta freeze dried powder needs to be encapsulated, if it is to safely go through the GIT without losing a lot viability. Alginate-based extrusion encapsulation method can successfully provide C. minuta enough protection to maintain the viability with a high survive-rate after time in the GIT. Double encapsulation of C. minuta beads presented a more effective protection than single encapsulation according to the viability test. However, that's not the only reason why we chose double encapsulation as the main encapsulation method. Another necessary reason is to avoid the early growth of probiotics, before reaching the intestinal environment. Although the probiotics are covered by a shell during the encapsulation process, it still has contact with the external environment. Through the process of double encapsulation, the probiotics are definitely better protected and completely separated from the outer environment. So the double encapsulation was used as the main method to encapsulate the probiotics of the study.

Long Term C. minuta Beads Stability Test

Although we had already figured out that alginate extrusion-based double encapsulation is very effective in protecting C. minuta probiotics, we still do not know the shelf-time of the

encapsulated C. minuta beads. And the shelf-time is considered to be an extremely important feature, which can determine the aspects of our further probiotic food product development. Hence, we designed an experiment of long term C. minuta beads stability test. The result was shown in Table 1. Then, we made a survival curve with the data collected in Table 1 (**Figure 7**). During the 8-week period of storage, the viability of double encapsulated beads was tested every 4 weeks by the pour-plate method. According to the number of viability presented in Table 1 and the survival curve shown in Figure 7, the double encapsulated C. minuta beads can maintain the viability in the first 4 weeks almost perfectly, which was $10^{8.0}$ CFU/mg, and the viability reduced a little bit from $10^{8.0}$ CFU/mg to $10^{7.6}$ CFU/mg in the next 4 weeks. The result indicated that the shelf-time of the double encapsulated C. minuta beads is at least 2 months, which provides a large potential for the future application of C. minuta beads related food products.

C. minuta Beads Application: Coconut Jelly, Drinkable Jelly, and The Stability of C. minuta Beads in The Jelly

Since the encapsulation technique provides good protection to C. minuta beads, we decided to develop some non-dairy food products with the probiotic beads. Coconut products were reported to have the ability to help humans to lose weight in recent studies (43). In addition, the gelatin-based jelly is also considered as a kind of healthy food, with having no sugar added (44). Therefore, coconut jelly was chosen as the food vehicle to carry C. minuta beads in the study (**Figure 8**). The stability of the beads in coconut jelly was tested by an experiment which lasted for 2 weeks. During the experiment, the viability of the beads in coconut jelly was tested every 2

days, started from the first day. The data was collected and is presented in Table 2. Then a survival curve was made in Figure 9. According to the data in the table, and the trend of the curve, it is easily claimed that the C. minuta beads are stable and the viability did not drop significantly. The aforementioned means that the C. minuta beads in coconut jelly have the ability to maintain their viability for at least two weeks at room temperature, which provides a huge potential for the application of C. minuta beads products.

Drinkable jelly is a popular food in some Asian countries, such as China, Japan, and Korea. Therefore, we developed the coconut drinkable jelly by adding coconut water to both the alginate extrusion solution, and the drinkable jelly. The casein, which was added in the new formula beads, helps the beads to be suspended in the drinkable jelly (**Figure10**).

We chose to use the vacuum food-grade pack to store the drinkable jelly product, and also it was easy to drink by opening the side cap (**Figure 11**). Overall, the food product development of C. minuta beads were successful, and showed many possibilities for the probiotics food product industry.

CONCLUSION

Based on the results and analysis of our study, the double encapsulation of C. minuta successfully helped C. minuta to go through the GI tract within a high survival rate. However, the technique still needs to be improved upon to avoid the viability lost during the process of encapsulation. We have already tried some methods to reduce the viability lost, such as using ice

water to rapidly cool down the blender part of the homogenizer, in an effort to avoid the thermal condition.

Particularly, the prebiotics added during encapsulation is a new idea for not only improving the survival rate during the process of passage through the gastrointestinal tract, but also improving the growth condition after the probiotics arrival in the intestinal environment. Currently, we have just finished study of the in-vitro effects of C. minuta encapsulation. The further in-vivo study is still in process of completion. Mice were selected as the object of animal study. The impact of adding prebiotics will be tested in future in-vivo studies.

The application of probiotics has become more and more prevalent in recent years. There are also a lot of new probiotic foods and probiotic dietary supplements available in the supermarket, such as probiotic beverages, probiotic chocolates, and probiotic capsules. Bifidobacterium and Lactobacillus are still the most commonly used probiotics in food product application. However, the production of other, different, featured probiotics will change the situation sooner or later.

Claimed from the results of the C. minuta beads stability test, the long shelf-time provides a lot of possibilities for developing related products. Moreover, The C. minuta beads-based coconut jelly, has great potential to become a successful probiotic food product. More flavors could also be applied during the product development, to fit more people's preferences. The drinkable jelly also provides a more convenient and acceptable way to increase daily probiotic intake, than traditional probiotic supplements.

With the development of microbiology, more and more new probiotics will be discovered, and significantly more opportunities in preventing and treating certain kinds of diseases will be available. Encapsulation will keep playing a key role in protecting the probiotics, and delivering them to those whom imbibe them.

Overall, we have already started our first step to make C. minuta available for obesity prevention and treatment. Future aspects of the study will focus on animal experimentation, and clinical trials of probiotic beads. Furthermore, other newly discovered bacteria can also be involved as the target encapsulation bacteria. Also, more products can be developed based on the probiotic beads.

Figure 1: Improved Extrusion process of encapsulation

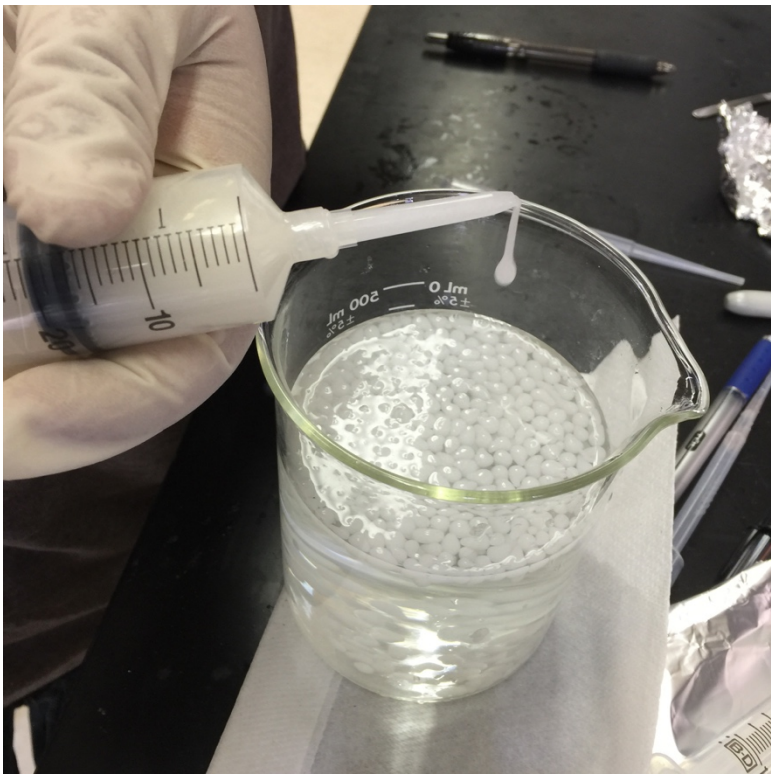

Figure 2: New formula C. minuta beads extrusion

Figure 3: Undried C. minuta beads

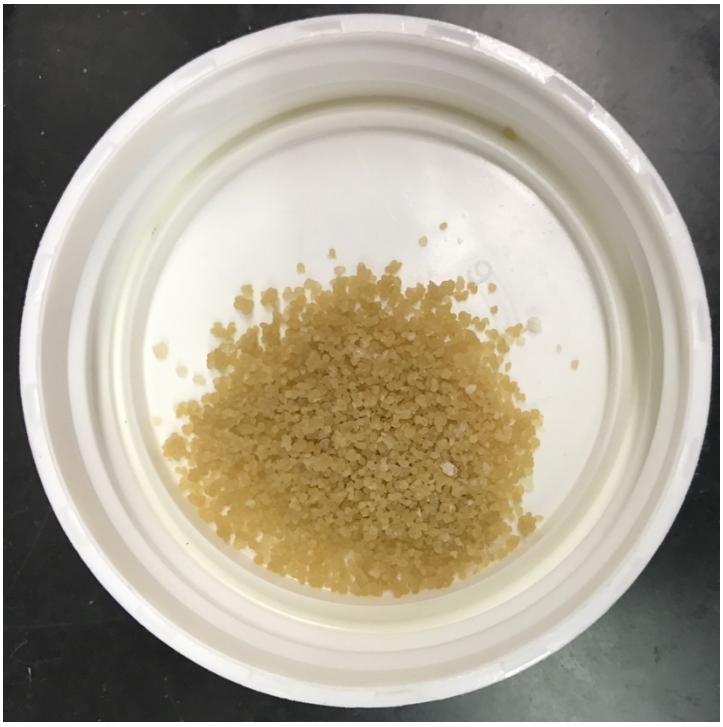

Figure 4: Dried double encapsulated C. Minuta beads

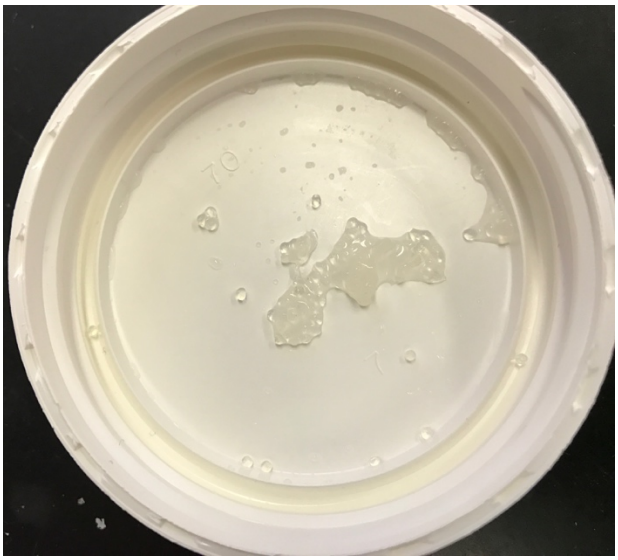

Figure 5: Double encapsulated beads after GIT

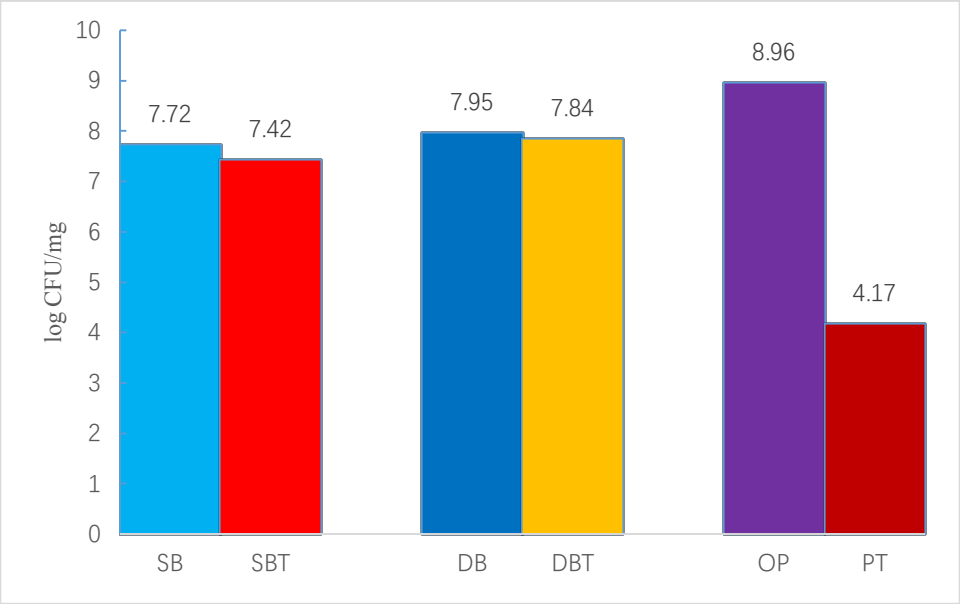

Figure 6: viability test of C. minuta beads before and after GI tract: Comparison of single encapsulated beads (SB), double encapsulated beads (DB) viability, original bacteria powder (OP), single encapsulated beads after GIT (SBT), double encapsulated beads after GIT (DBT), and freeze dried powder after GIT (PT).

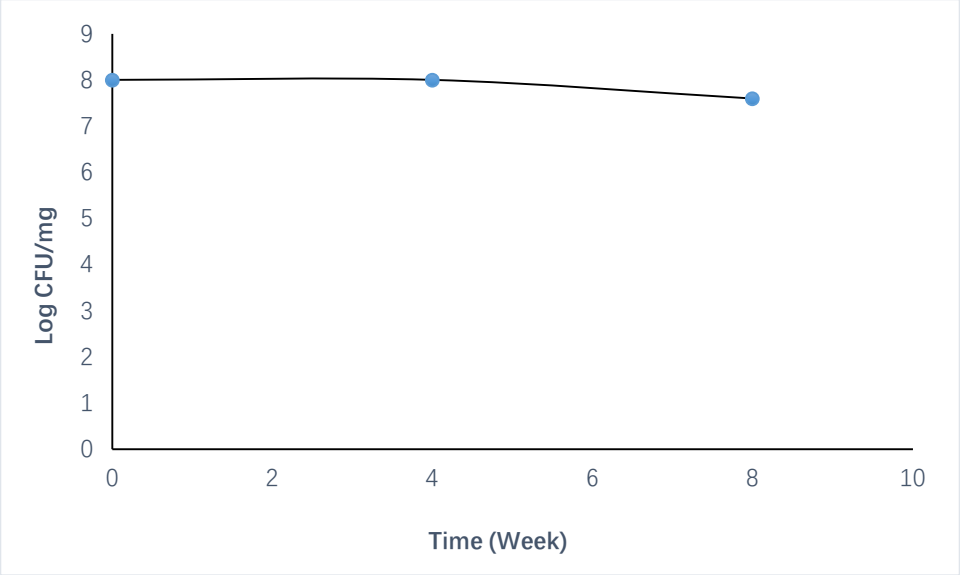

Figure 7: Long term double encapsulated C. minuta beads stability test

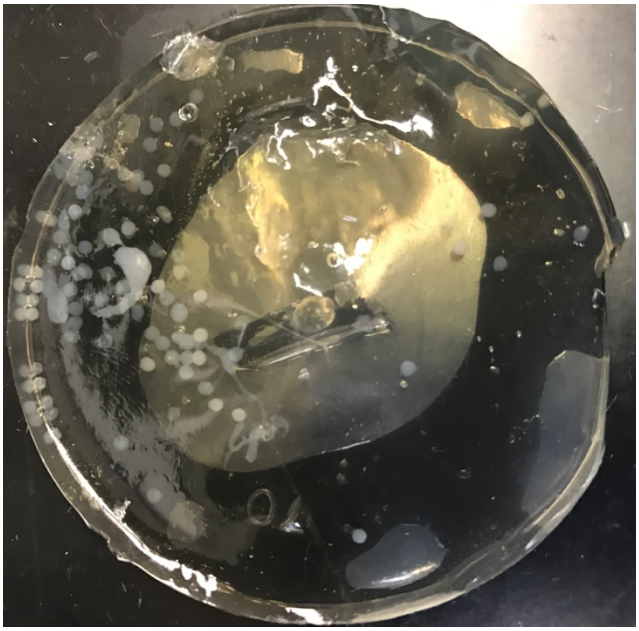

Figure 8: Coconut jelly with C. minuta beads

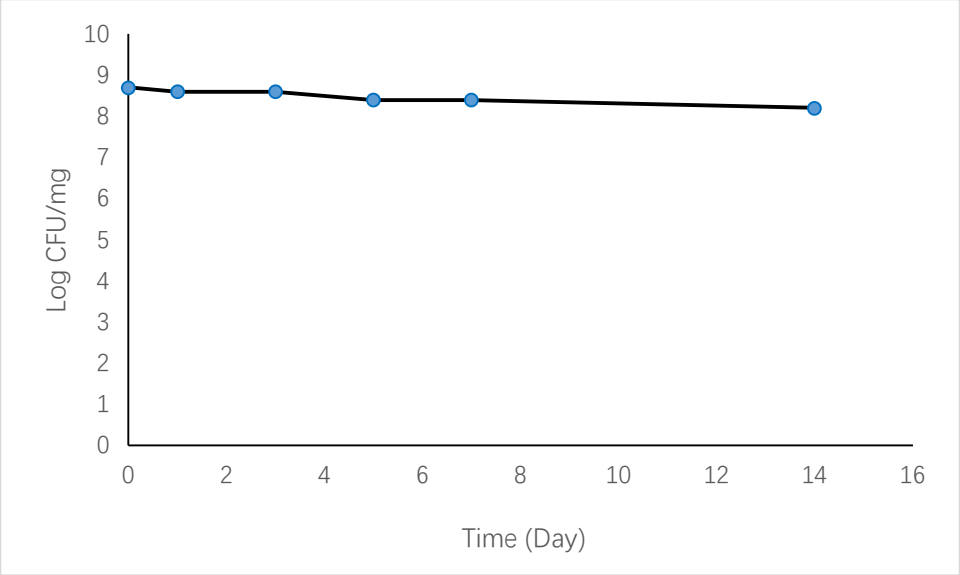

Figure 9: Survive curve of C. minuta beads in coconut jelly for 2 weeks

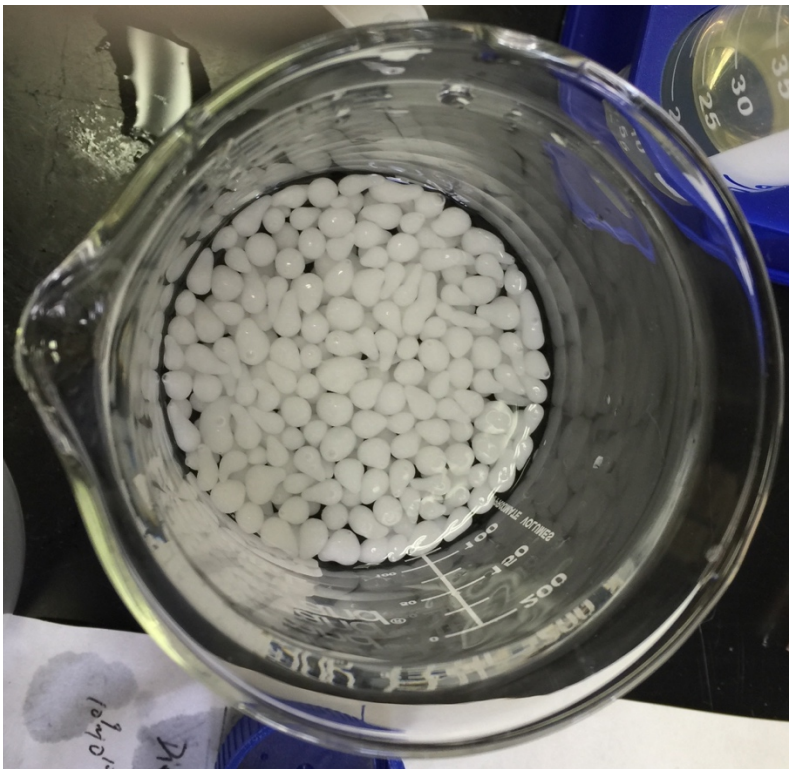

Figure 10: Casein and coconut water added probiotic beads

Figure 11: Drinkable coconut jelly with probiotic beads

Table 1: Long term double encapsulated C. minuta beads stability test at room temperature

Months	viability log (CFU/mg)
0	8.0
1	8.0
2	7.6

Table 2: Two-week stability of double encapsulated C. minuta beads in jelly

Days	viability log (CFU/mg)
0	8.7
1	8.6
3	8.6
5	8.4
7	8.4
14	8.2

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ABSTRACT

DEVELOPMENT OF GI SUSTAINABLE PROBIOTIC BEADS USING MICROENCAPSULATION

by

JIARUN CUI

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Advisor: Dr. Kequan Zhou

Major: Nutrition and Food Science

Degree: Master of Science

Scientists tend to pay a lot attention to probiotics in recent years, due to its health related benefits for humans. Encapsulation is a necessary way to protect probiotics from being killed by harsh conditions of the gastrointestinal tract, and to help probiotics to release in the intestinal environment. This study is mainly focusing on the encapsulation technique of Christensenella minuta, and the food product development of C. minuta beads. The extrusion technique was applied as the encapsulation method, with alginate as the encapsulation material. The effect of single encapsulation and double encapsulation of C. minuta was compared to determine the layers of encapsulation. Long term stability experiments of double encapsulation C. minuta beads indicated that the beads formed by the extrusion technique is stable and maintains the viability, which was $10^{7.95}$ CFU/mg to $10^{7.84}$ CFU/mg. Gelatin jelly and drinkable jelly were developed as potential probiotic food products in the study. Animal experiments and clinical trials are still required to test the feature of C. minuta beads for future study.