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THE EXPLORATION OF CHARACTERISTICS OF *AKKERMANSIA MUCINIHPILA*, AND EVALUATION OF ITS PROBIOTIC EFFECTS ON DIABETES BY USING C57BL/6 MOUSE MODEL

by

JIANGQI TANG

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2018

MAJOR: NUTRITION AND FOOD SCIENCE

Approved By:

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Advisor Date

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DEDICATION

This dissertation is dedicated to my families, Jian Tang and Tiandi Jiang for their love and caring through all the years in my life; to Cookie Tang and Mia Tang for their companion through the graduate years. I truly appreciate the suggestions and encouragement from Dr. Catherine Jen and Dr. Kequan (Kevin) Zhou to overcome difficulties in this project. I'm also grateful to my friends and colleagues for fulfilling my days with unforgettable memories, for sharing all the ups and downs with me, and for their generous support whenever I was in pain or in trouble.

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ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Kequan Zhou for providing me the opportunity to explore probiotics as my PhD project and to both Dr. Kequan Zhou and Dr. Catherine Jen for guidance and support during the whole project. I also appreciate Dr. Yifan Zhang and Dr. Patricia Jarosz for serving on my committee. I'm grateful to Dr. Yifan Zhang lab, Dr. Diane Cress lab, Dr. Ahmad Heydari lab for providing equipment and assistance in my experiments. I would also like to thank my colleague Dr. Shi Sun, Dr. Kai Nie, Wenjun Zhu, Paba Edirisuriya, Fei Yang and Qing Ai for all the support and help.

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CHAPTER 1. INTRODUCTION

The incidence and prevalence of Type 2 diabetes (T2D) is climbing steadily across the globe. There are approximately 425 million adults living with diabetes around the world with more than 90% of them having T2D. This prevalence will increase to 629 million worldwide by 2045 (1). The incidence of T2D was most commonly seen in older adults; however, it has been discovered with increasing incidence in younger adults, adolescents and children due to physical inactivity, poor diet and increased incidence of obesity. Despite of the fact that the causes of T2D are not been completely understood, it is revealed to be strongly related to overweight, obesity, ethnicity, family history and increasing age. Additional but modifiable risk factors include: prediabetes, impaired glucose tolerance, excess adiposity, poor nutrition, smoking and physical inactivity (1). Among these factors increasing the risk of T2D, the most influential factors are behaviors related to modern lifestyle, which include unhealthy food choices and more sedentary lifestyle. It has been demonstrated by clinical trials that adopting healthy diet (2-4) and increasing physical activity (5) can prevent or delay T2D.

T2D is characterized by hyperinsulinemia, insulin resistance, and pancreatic β-cell failure. T2D is generally affected by genetic and environmental factors. Recent studies have revealed possible interactions between T2D and the gut microbiome (6, 7). It was revealed that healthy individuals and patients with T2D had different composition of gut microbiome, specifically the population ratio of Bacteroidetes to Firmicutes (8). In addition, a specific strain (*Akkermansia muciniphila*) was reported to have a decreased abundance even prior to the incidence of diabetes and inflammatory bowel disease (9).

Obesity is one of the major factors that contributes to the incidence of T2D by decreasing insulin sensitivity in liver, adipose tissue, skeletal muscle and subsequently induce β-cell function

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(10). The global prevalence of obesity explains the tremendously increased incidence of T2D over the past two decades. Obesity is characterized by low-grade chronic inflammation, and it is known to increase the risk of life-threatening diseases including diabetes, cardiovascular diseases, and nonalcoholic fatty liver disease. Over 600 million people are suffering from obesity and its related comorbidities (11).

The understanding of obesity etiology and developing effective prevention and treatment strategies have been challenging due to various contributing risk factors. Obesity occurs mainly due to unregulated balance between energy intake and expenditure. Increasingly researches have been focused on the regulation of energy balance, including biological, behavioral and environmental interactions from the point of human gut microbiome (12). To reverse the consequence of caloric imbalance, lifestyle intervention has been a focus for its potential benefits. In general, individuals with modified behaviors achieved effective weight loss of 5% – 10% for up to 12 months (13). In addition, the association between obesity and gut microbiome in both mouse and human studies have shown evidence that obesity is associated with altered relative abundance of gut microbiota diversity, such that a higher capacity for harvesting energy from the diet was observed in obese individuals (8). Recent studies have shown evidence that gut microbiota has a causal role in energy balance, obesity development and associated metabolic dysfunctions (14).

Gut microbiota

The human microbiome is a complicated and dynamic ecosystem, which contains trillions of microbes. It is considered a “hidden organ” due to its inhabiting microbes (15) and accounts for approximately 1 kg of body weight (16). It has effects on nutrient absorption, metabolism, immunologic regulation and pathogen resistance (17, 18). Human gut is initially colonized by bacteria starting from the mother and the surrounding environment from the moment of birth.

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Factors contributing to the composition of gut microbiota include infant delivery methods, mode of feeding, antibiotic treatment and environment hygiene. In the first year of age, the infant gastrointestinal tract (GIT) develops extremely dense microbial colonization when influenced by factors including host genotype, gut environment changes and the introduction of solid foods (19). The gut microbiota gradually shapes into a stable and more complex community at approximately 3 years of age (16). In adult individuals, gut microbiota remains relatively stable and most strains are gut colonizers for decades. The early colonizers acquired from parents and siblings have the most possibility to exert physiologic, metabolic and immunologic effects on the host (20). In fact, the ecosystem of gut microbiota is modified by various factors in daily life, including diet alteration and antibiotic exposure. Both the gut bacteria and the host receive corresponding nutrients when various diets are introduced. According to a mouse study, it has been reported that diet alteration accounted for approximately 57% of total structural variation in gut microbiota while genetics explained less than 12%. Furthermore, diet might play a dominating role in shaping gut environment into an entity which would accelerate the development of metabolic syndrome (21). In addition to diet, antibiotic is another factor that significantly disturb the gut microbiota ecosystem. When an individual takes a single dose of an oral antibiotic, it takes the gut microbiota approximately four weeks to resemble the pre-treatment status while some species need significantly longer time such as six months. Repeated antibiotic exposure may also induce a diversity alteration that gut microbiota would not be able to recover to its original status ever. Instead of recovering, it shifts into a new stabilized but altered composition (18, 22).

Evidence showed that healthy adults share most bacterial species, which constitute the concept of “core microbiota”. On the other hand, culture-independent sequencing researches demonstrated a vast gut microbial diversity over time and across populations. There are more than

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a thousand species-level phylotypes in an adult, while most of them belong to a limited number of phyla (15). The two major bacteria divisions that most phylotypes belong to are Bacteroidetes and Firmicutes, fewer phylotypes are members of Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia phyla (23), and others belong to methanogenic archaea, eukarya and viruses (15).

Over the last few decades, the discovery of the composition and functions of the human gut microbiota has increased enormously. The composition of gut microbiota varies depending on pH, temperature, oxygen tension, redox potential, water activity, salinity and light (24). The gut microbiota mainly colonizes in the colon where no digestive enzymes are secreted to process indigestible macronutrients from the ileum. With gut microbial metabolism, oligosaccharides and polysaccharides are processed into short chain fatty acids, and phenolic compounds are processed into bioactive compounds (25). Backhead et al. discovered that the alteration of gut microbiota in germ-free mice with microbiota achieved from conventionally raised, genetically obese mice resulted in an increase of 60% of body fat, and development of insulin resistance in two weeks regardless of a decreased consumption by 29% and an increased activity by 27% when compared to germ-free mice whose gut microbiota composition was not altered (26). Based on these findings in animal studies, clinical trials have been conducted to study the differences of microbiota in the human gut. It was reported that the overweight/obese and lean individuals showed substantial differences in intestinal microbiota composition and their functions, which including decreased microbial richness and increased pro-inflammatory microbial species related to low-grade inflammation and insulin resistance (27, 28). The major contribution of gut microbiota to the host is to prevent the colonization of potential pathogenic microorganisms, including outcompeting invading ecological niches and metabolic substances from pathogens. In addition, gut microbial metabolites are providing up to 50% of the daily energy requirements for colonocytes, which is

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mainly butyrate by fermenting carbohydrates into organic acids. The gut microbiota also play an important role in modulating the host immune system, not only affecting new-borns but also healthy adults (29). In fact, the benefits of gut microbiota can be easily overcome by gastrointestinal infection, chemotherapy and chronic diseases such as inflammatory bowel disease and colon cancer (29).

Probiotics

The concept of probiotic was first described as "substances secreted by one microorganism which stimulates the growth of another" by Lilly and Stillwell in 1965 (30). In the following two decades, the definition of "probiotic" has been continuously revised by Fuller (31) and Havenaar et al. (32). The current internationally accepted definition was refined by Guarner and Schaafsma, which is "living microorganisms, which upon ingestion in certain numbers, exert health benefits to the host beyond inherent basic nutrition"(33). In Expert Panel 2001, the Food and Agriculture Organization of the United Nations and the WHO (FAO/WHO) gave probiotics the definition as, "living microorganisms that, when administered in adequate amounts, confer a health benefit on the host", which has been most widely adopted and accepted worldwide since then. In the following year, FAO/WHO Working Group produced guidelines to help with interpretation of the original document (34).

The effects of probiotics cannot be generalized since the effects are very strain specific. And a single probiotic strain might present various benefits when used in combination or used individually. The benefits may also vary when used among different individuals (35). Bifidobacterium, Lactobacillus and Saccharomyces are extensively studied and commonly used as probiotics in humans and animals (36). Dairy products are the most widely distributed products containing Lactobacillus and Bifidobacteria, which has made consumers worldwide aware of the concept of "probiotics". Some of the benefits of dairy probiotics have been extensively studied,

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such as reducing antibiotic-associated diarrhea (37), relieving seasonal allergies (38), improving symptoms of inflammatory bowel diseases (39), shortening duration of acute respiratory infections in healthy children and adults(40), and lowering blood cholesterol in hypercholesterolaemic adults (41).

Probiotics can interact with the host in multiple ways, which include inhibiting pathogenic effects by producing bactericidal substances to compete with pathogens and toxins for adhering to intestinal epithelial layer; improving epithelial homeostasis by enhancing barrier function, promoting intestinal epithelial cell survival and stimulating epithelial protective responses; promoting immune system by modulating innate immunity and controlling pathogen-induced inflammation (36).

Prebiotics

In addition to probiotics that are beneficial to host health, prebiotics are supplemented to boost effects of probiotics. Prebiotics was first defined by Gibson as non-digestible food ingredients that can benefit the host health by selectively stimulating the growth and/or activity of one single bacterium or a limited number of bacteria in the colon. A food ingredient can be categorized as a prebiotic when it meets all the following requirement: Can be neither hydrolyzed nor absorbed in upper part of GIT; be a selective substrate for one bacteria or a limited number of beneficial bacteria commensal in the colon, which are stimulated to grow and/or are metabolically activated; has the ability to alter colonic microbiota by favoring a healthier composition; be able to induce luminal or systematic improvement on host health. The potential categories of prebiotics include non-digestible carbohydrates, peptides and proteins, and lipids (42).

In 2016, the International Scientific Association for Probiotics and Prebiotics (ISAPP) updated the definition of prebiotic as: a substrate that is selectively utilized by host micro-organisms and confers a health benefit (43). Ingredients that have been reported with

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gastrointestinal health benefit include non-digestible oligosaccharides (NDO), human milk oligosaccharides, and soluble fermentable fibers. NDO are carbohydrates with low molecular weight in-between simple sugars and polysaccharides (44), and the most studied NDO are galactooligosaccharides (GOS), fructooligosaccharides (FOS) and inulin. NDO have been used widely since 1980 for modifying emulsification capacity, gel formation, viscosity, freezing point and coloring in food industry. In addition, NDO show beneficial properties such as moderate sweetness, low calorimetric value and low glycemic index. Dietary fibers cannot be digested by human enzymes but can be fermented by microbes in the GIT. Common substances produced from fermentation are lactic acid, hydrogen, methane, carbon dioxide and short-chain fatty acids including acetate, propionate and butyrate. They are able to increase fecal weight and defecation frequency, to relieve constipation and improve the health of large intestine mucus layers (45).

Numerous studies have focused on improving gut microbial ecosystem using prebiotics by modification of bifidobacterial and lactobacilli colonization (46). Evidence showed that supplementation of prebiotics (a GOS and FOS combination) was associated with reducing early occurrences of atopic dermatitis in infants (47); decreasing incidence of atopic eczema in six month old infants (48); relief of symptoms associated with intestinal bowel disorders and providing positive effects on lowering risk of colon cancer (49); and improving immune responses in elderly people (50).

Food companies started to add prebiotics into probiotic foods in the market, then the term “synbiotic” was defined. Andersson et al. gave synbiotics the definition as: mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the GIT of the host (51). Studies have shown evidencs that

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synbiotics are beneficial for immune system, which include reducing C-reactive protein levels, increasing glutathione levels (52), and suppressing intestinal and systemic inflammation (53).

Akkermansia muciniphila

The probiotic this study focused on was *Akkermansia muciniphila* (*A. muciniphila*). The name Akkermansia was derived from a Dutch microbiologist Antoon Akkermansia for his contribution to the study of microbial ecology. Muciniphila was named after its preference of mucin as an important nutrient. The *A. muciniphila* cells are non-motile, Gram-negative and oval-shaped. The reason for limited studies on *A. muciniphila* is the characteristics of strictly anaerobic. Growth of *A. muciniphila* occurs at temperature of 20 - 40 °C with pH 5.5 - 8.0. It is able to grow on brain-heart infusion, Columbia media, and gastric mucin. *A. muciniphila* has the ability to use mucin as carbon, nitrogen and energy source (54). It is the first isolated intestinal microbial of the Verrucomicrobia phyla (55). It was suggested by Derrien et al that *A. muciniphila* had a higher abundance in the colon than in the ileum, both in mono-colonized and conventionally raised mice (56, 57). The development of *A. muciniphila* in human with different demographics and at various life stages was investigated by Carmen et al.. *A. muciniphila* was detected in the GIT of 1-month old infants and it takes less than one year to reach the abundance level of 10^8 cells/g as in adults while the level decreases significantly (1 logarithmic unit) in elderly subjects (58).

Several clinical studies have been done to reveal abundance of *A. muciniphila* among different target population. Results showed that there is a negative correlation between *A. muciniphila* abundance and BMI, both in children and adults (59-61). Interventions such as weight loss and calorie restriction were able to increase *A. muciniphila* prevalence significantly (62, 63). Dietary ingredients were tested for their benefits on *A. muciniphila* population and relevant improvement on host health status. These ingredients included as FOS (64, 65), oat bran(66), black tea extracted polyphenols (67), grape pomace polyphenols (68), and cranberry polyphenols (69).

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With all the potential health benefits, food manufacturers have been promoting food products containing probiotics and/or prebiotics. However, before largescale production can be achieved, the characteristics of probiotics and optimal culturing conditions should be defined. At the present time, there is not much research that has been conducted in these areas. Therefore, the current research was conducted to collect this information.

Specific aims

The objective of the current study was to improve the growth and survivability of *A. muciniphila* in GIT conditions and to further examine the long term (6-month) dietary effect of *A. muciniphila* supplementation on high-fat diet induced obesity and diabetes in C57BL/6 mice.

Aim 1

The first aim was to optimize the growing conditions of *A. muciniphila*, such as temperature, pH and prebiotics. These would subsequently be used in improving techniques to overcome the issue of culturing difficulties. It is hypothesized that alternative carbohydrate source other than dextrose could also be used as a part of growth medium that may improve the optimal growth condition and eliminate adverse effects on host glucose homeostasis. The selected prebiotics could be incorporated as synbtiotic to further bring out potential benefits.

Aim 2

Aim 2 was to improve sustainability of *A. muciniphila* in GIT by developing new coating technology. From the evaluated properties of *A. muciniphila*, additional obstacles such as adverse environmental conditions from human GIT would be investigated to discover the the ability of *A. muciniphila* to tolerate stressed conditions. Specifically, the significant impact on cell viability from human GIT such as the acidic stomach environment and the drastic change in pH post stomach digestion with the digestive enzymes would be examined. The improvement of encapsulation was hypothesized to enhance cell viability throughout the passage of GIT where

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harsh environment would significantly diminish cell count post digestion without encapsulation. Thus, various frequently used encapsulation methods would be evaluated to determine the potential optimal technique in protecting *A. muciniphila* viability and availability. The optimal encapsulation method would ensure the delivery of coated *A. muciniphila* cell through GIT digestion with minimal loss of viability and consequently reaching favorable condition in the large intestines.

Aim 3

Aim 3 was to determine long term effect of *A. muciniphila* dietary supplementation on high-fat diet induced obesity and diabetes. Continuous supplementation of *A. muciniphila* was hypothesized to maintain C57BL/6 mice glucose homeostasis, reduce body weight and improve body composition. It was expected that high-fat fed mice treated with *A. muciniphila* would maintain consistent weight gain similar to that of mice fed the control low-fat diet, and maintain normal fasting glucose levels via an altered energy absorption. This aim would focus on evaluating potential beneficial effects of *A. muciniphila* supplementation in order to improve the understanding of its sustainability and dosage effect over long time of supplementation.

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CHAPTER 2. OPTIMIZATION OF THE GROWTH CONDITIONS FOR *A. MUCINIPHILA*

The determination of optimal growth condition is fundamental but critical because the information will be used to further enhance culturing techniques, optimize manufacturing processes, determine storage methods and evaluate the necessity of additional protection for delivering through human GIT. Temperature and pH are the two most important factors for growing microorganisms; therefore, tests were designed to determine the optimal temperature and pH for supporting the optimal growth of *A. muciniphila* within tolerable ranges of temperature and pH. To stimulate the growth of *A. muciniphila* as a probiotic, several well-known prebiotic ingredients were compared as part of growth media to create a potentially superior synbiotic formula. After achieving an optimal growth condition for *A. muciniphila* on a small scale, the next test was set to find the proper and cost-efficient media supplement for manufacturing *A. muciniphila* for achieving a larger quantity, which could be further utilized when preparing *A. muciniphila* culture for mouse studies and human clinical trials.

In animals and humans, most of the gut microbiota confronts large amounts of bile salts. Bile acids are synthesized mainly from cholesterol in the liver, conjugated with taurine or glycine, stored in gall bladder and released into duodenum upon the need in fatty acid digestion. The presence of bile salts is continuous through enterohepatic circulation (70). Besides the fact that bile salts are natural emulsifiers in the intestines, they also function as a microbial detergent. Some microorganisms possess the ability to modify bile salts biochemically to resist the detergent property. Therefore, bile salt tolerance (carried out by bile salt hydrolase (BSH) activity) is desirable for probiotics (71). In the small intestine, microbial bile salt hydrolase produces unconjugated bile acids by removing taurine or glycine molecules (72). Released bile acids are

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absorbed back to the enterohepatic portal system via terminal ileum, while part of them enters large intestine to be metabolized into secondary and tertiary bile acids (73). These freed bile acids are less soluble thus less are reabsorbed by intestinal lumen when compared to conjugated bile acids. Consequently, these free bile acids are excreted in feces from human body. This could promote higher metabolism of cholesterol and lower serum cholesterol subsequently. Based on this mechanism, the deconjugation of bile salts by BSH is ubiquitous within healthy gut microbiota (74, 75). Probiotics with BSH activity have been proved to improve LDL-cholesterol and subsequently bile acids level in clinical study (75). A few microorganisms have been discovered with BSH activities, such as Bifidobacterium spp., Lactobacillus spp., Bacteroides fragilis subsp. fragilis, Enterococcus spp., Clostridium perfringens, and Listeria monocytogenes (70, 76, 77). Inspired by discovery of BSH activity in probiotics like Bifidobacterium spp., Lactobacillus spp, BSH activity of *A. muciniphila* was an evaluation objective for its potential benefits to the host health in the current study.

In addition to discovery of optimum culture and manufacture conditions and potential BSH activity of *A. muciniphila*, the last aim was to evaluate the efficacy of proper storage method for maintaining viability of *A. muciniphila* during short and long-term storage.

Methods

Anaerobic culture medium preparation

Dehydrated Brain-heart Infusion (BHI) medium (Bioworld, Dublin, OH) was dissolved in distilled water with concentration of 37 g/L and 0.1% w/v resazurin solution (Thermofisher Acros Organics, Morris Plains, NJ) was supplemented afterwards in the medium. After boiling, BHI medium was cooled to room temperature while sparging with 100% nitrogen, then dispensed into anoxic Hungate-type tubes with a volume of 10 mL each under the same gas atmosphere. Filled

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hungate tubes were then autoclaved at 121 °C for 15 min, cooled to room temperature, and stored at 4 °C for further culture if not used immediately.

Resuspension and culture of *A. muciniphila*

The procedure of resuspension and culturing requires to be under oxygen-free environment. The purchased *A. muciniphila* came in a double-layered vacuum ampoule. One cannula was prepared to supply nitrogen to the ampoule containing freeze-dried *A. muciniphila* powder. With gassing cannula inserted in the ampoule, 0.5 mL of BHI medium was added to the vial and the cell pellet was resuspended completely. The cell suspension was transferred into a prepared hungate tube using a 1 mL syringe with hypodermic needle, which was also flushed with nitrogen. It is possible that certain ingredients of the freeze-dried pellet might inhibit growth in the first hungate tube, therefore additional two hungate tubes were used to make diluted culture of 1:10 and 1:100 in order to guarantee successful resuscitation.

Handling and transferring of actively growing *A. muciniphila* cultures

When culturing *A. muciniphila* in small quantity, hungate tubes were used to culture and transfer at all times. The surface of hungate tube cap had to be sanitized before and after inoculation and transfer. The butyl rubber septum and screw cap were sterilized over flaming. Since microbial growth induces overpressure in hungate tubes, excess gas was removed by puncturing the septum with a sterile injection needle. Then a sterile anoxic, disposable 1 mL syringe with a 25 G hypodermic needle was used to withdraw and transfer cultures.

Strain verification

Contamination quick screening

Gram staining technique was used for quick screening of cross-species contamination. *A. muciniphila* culture sample was heat-fixed on slide, then stained as manufacturer instructed.

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Stained slides were viewed under light-microscope (Fisher scientific, Hampton, NH) with oil-immersion at 100/1.25 lens to check color and shape of cells.

General PCR verification

DNA of *A. muciniphila* sample was extracted using QIAamp DNA Mini Kit (Qiagen, Germantown, MD) according to manufacturer's instructions. Extracted DNA was stored at – 20 °C for future tests. Two specific primers were used to verify the sample DNA, 16s rDNA forward primer: 5' CAG CAC GTG AAG GTG GGG AC 3', 16s rDNA reverse primer: 5' CCT TGC GGT TGG CTT CAG AT 3' (78). The PCR mixture was prepared with final volume of 25 µL, which contained 12.5 µL of 2 x PCR Sigma ReadyMix RedTaq PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO), 0.1 µM forward primer, 0.1 µM reverse primer, 2 µL template DNA, 8.5 µL nuclease-free water. Eppendorf Mastercycler EP S Thermal Cycler (Eppendorf, Hamburg, Germany) was used to for PCR with procedure of initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, primer annealing at 60 °C for 40 seconds, extension at 72 °C for 30 seconds, and final extension at 72 °C for 5 minutes. 1.5% agarose gel was prepared with 1 x Tris-Borate-EDTA (TBE) buffer for gel electrophoresis. PCR product was compared to low range DNA ladder with range 25 – 700 bp (Thermo Fisher Scientific, Ann Arbor, MI). The gel containing separated PCR products was visualized by SYBR Green staining under Bio-rad Imager (Bio-rad, Hercules, CA).

Turbidity verification

Fresh *A. muciniphila* culture was used from the original *A. muciniphila* stock, then a ten-fold serial dilution was made. Duplicated hungate tubes were prepared for each diluted concentration. *A. muciniphila* original culture and all diluted cultures were incubated at 37 °C for 10 hours. 500 µL of each culture was transferred using an anoxic syringe into 48-well plate and optical density (OD) values were measured at 595 nm using Epoch 2 spectrophotometer (Biotek,

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Winooski, VT). At the same time, each diluted culture was tested for colony forming unit (CFU) using pour-plating technique with ten-fold dilution (79). BHI agar was prepared by dissolving 1.6 % agar and 3.7 % BHI medium in distilled water. 10 µL of each dilution was pipetted onto the bottom of petri dish (60mm x 15mm), then 15 mL BHI agar was poured into the petri dish and swirled for even distribution. All petri dishes were placed in anaerobic jar for 24 hours at 37 °C. Standard curve was established based on OD values and their corresponding CFU/mL.

Exponential growth rate calculation

Growth curve under each condition was established with OD values at each time point. Based on the equation achieved by turbidity verification, CFU of each time point could be determined by corresponding OD value. The first step was to identify the start and end points of exponential phase based on the growth curve. The following formula was adopted to calculate growth rate. X_0 implies the initial CFU, X_t implies the final CFU and "log" is the logarithm of the base (80).

$$\text{Growth rate}_{\text{generation/hour}} = \frac{2.303\times (\log X_t - \log X_0)}{\text{Duration time (hour)}}$$

Optimal and tolerable temperature test

Fresh *A. muciniphila* culture was used as original culture stock, from which 0.1 mL was inoculated into each anaerobic BHI tube. Duplicate hungate tubes were cultured for testing under each temperature. Then each sets of tubes were incubated for 10 hours in incubators with temperature set up to 25 °C, 35 °C, 37 °C, 40 °C, 43 °C and 46 °C respectively. 500 µL of *A. muciniphila* culture was transferred from each hungate tube into 48-well microplate using anoxic syringe and OD values were measured at 595 nm at hourly interval. Consequent growth curves, growth rate and final viabilities were generated and calculated.

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Optimal and tolerable pH test

After hungate tubes were filled with 10 mL BHI medium, pH value was adjusted to 2, 3, 4, 5, 6, 6.5, 7, 7.5, 8, 9 with Na_2CO_3 and HCl using pH meter (Oakton, Vernon Hills, IL). Duplicate hungate tubes were cultured for testing at each pH level. All tubes were purged with nitrogen, autoclaved and cooled down to room temperature before inoculation. Actively growing *A. muciniphila* was used as the original *A. muciniphila* stock, then each hungate tube prepared of varied pH value was inoculated with 0.1 mL of original *A. muciniphila* stock. All tubes were incubated for 10 hours at 37 °C in incubator. 500 µL of *A. muciniphila* culture was transferred using anoxic syringe from each hungate tube into 48-well and OD values were measured at 595 nm. Consequent growth curves, growth rate and final viabilities were generated and calculated.

Prebiotics selection

Duplicate hungate tubes were used for testing with each prebiotic. After filled with 10 mL BHI without dextrose medium, each hungate tube was supplemented with 0.2% w/v prebiotic ingredients including: isomaltooligosaccharide (IMO), inulin (INU), fructooligosaccharide (FOS), galactooligosaccharide (GOS), guar gum (GG), acacia gum (AG), karaya gum (KG), tragacanth gum (81), and potato starch (PS). All tubes were purged with nitrogen, autoclaved and cooled down to room temperature before inoculating. Actively growing *A. muciniphila* culture was used as the original *A. muciniphila* stock, then each supplemented hungate tube was inoculated with 0.1 mL of orginal *A. muciniphila* stock. 500 µL of each culture was transferred by anoxic syringe into 48-well plate, and the plate was incubated at 37 °C for 10 hours. OD values were measured at 595 nm every 30 min after a 5-second orbital swirl for the most consistent reading. Subsequent growth curves, growth rate and final viabilities were generated and calculated.

Prebiotic ingredients were purchased from the following companies. GG: Bulk supplements.com, Henderson, NV; IMO: FiberYum, Hawthorne, NY; INU and TG: MP

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Biomedicals, Solon, OH; FOS and GOS: Quantum Hi-Tech Biological Company, Guangzhou, China; AG: Heather's Tummy Fiber, Seattle, Washington; KG: Alfa Aesar, Haverhill, MS; PS: Bob's Red Mill, Milwaukie, OR.

Upscale supplement selection

The 0.2% w/v dextrose was replaced using five different sugars with the same concentration. The supplement options included fructose, galactose, lactose, sucrose and maltose. Same volume of *A. muciniphila* was inoculated into each medium and incubated at 37 °C for 10 hours. Consequent growth curves, growth rate and final viabilities were generated and calculated. After the best three supplement ingredients were selected, the next step was to explore the concentration that favored the growth of *A. muciniphila* most. The concentration levels being tested were adjusted to 0.1%, 0.2%, 0.5%, 1% and 2%. Procedure was the same as sugar selection. Final viabilities were calculated and compared.

Quantitative bile salt hydrolase activity

BSH activity was determined by measuring the amount of amino acid released from conjugated bile salts by selected probiotics (82). *A. muciniphila* culture pellet was collected by centrifugation and transferred into 2 mL microtubes. PBS (pH 7.4) buffer was used twice to wash off BHI medium from *A. muciniphila* culture collect at centrifuge speed of 12,000 rpm for 1 minute. Supernatant from washing was discarded after centrifuge, then the pellet was resuspended with 100 µL of PBS buffer and 100 µL of 1% bile salts (LP0055 OXOID, Ontario, Canada) and was incubated at 37 °C for 75 minutes. After being centrifuged at 13,000 rpm for 5 minutes, 100 µL supernatant was transferred into 1.5 mL microtube and 400 µL of 2% ninhydrin was added. Microtubes were heated for 2 minutes in a 90 °C waterbath, and subsequently OD values of all culture were measured at 570 nm. One unit of BSH activity (U/g) was defined as the amount of enzyme liberating 1mmol of amino acid from the substrate in one minute. The concentration of

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protein was measured using Bradford method with albumin as standard (Thermo Fisher Scientific, Ann Arbor, MI). In addition to *A. muciniphila*, another four probiotic strains were prepared with the same steps as comparisons to *A. muciniphila*. The strains included *Lactobacillus acidophilus* (Swanson, Fargo, ND), *Lactobacillus reuteri* (Nature's Bounty, Bohemia, NY), *Lactobacillus plantarum* (Swanson, Fargo, ND) *and Bacillus coagulans* (Sundown Naturals, Bohemia, NY). All samples were triplicated in this study.

Storage test

Storage glycerol solution (50% v/v) was autoclaved and stored at 4 °C for future use. *A. muciniphila* culture was centrifuged, and the pellet was suspended with sterile 50% glycerol solution with a ratio of 1:1. The *A. muciniphila* glycerol mixture was transferred into microtubes, flash frozen with liquid nitrogen and stored at - 80 °C. Frozen *A. muciniphila* glycerol mixture was tested for CFU after 1-month, 2-month and 6-month of storage.

Statistics

Data were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) with a Tukey post-hoc test using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA). Results were considered statistically significant at $p < 0.05$.

Results

Verification of strain *A. muciniphila* was performed using PCR and gel electrophoresis imaging. The band appeared with 327 bp was considered as positive result. The verified *A. muciniphila* culture was used for all further tests.

The correlation of *A. muciniphila* culture OD value and its corresponding CFU was established as the following equation: $y = 1E + 07\ x^{1.2956}$ (Figure 1).

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Figure 2 presented the growth of *A. muciniphila* during an incubation time of 10 hours by showing its growth curve at temperature 25, 35, 37, 40, 43 and 46 °C. These growth curves showed that temperatures ranging from 35 °C to 43 °C had similar growth curves while 46 °C reduced cell growth and cells barely grew under 25 °C. Growth rate (Figure 3) of cells incubated at 35 °C, 37 °C, 40 °C and 43 °C showed no significant difference. Growth rate at 46 °C was at an intermediate level, which was not significantly different from growth rates at all other temperatures. Growth rate at 25 °C was significantly slowed down when compared to 35 °C and 37 °C ($p < 0.05$), while no difference was found when compared to growth rates at 43 °C and 46 °C. Figure 4 presented the effects of temperature on final culture viability (CFU x 10^6/mL) of *A. muciniphila*. Viabilities were similar at 35 °C, 37 °C and 40 °C while viabilities at 43 °C and 46 °C was significantly lower when comparing to 37 °C ($p < 0.05$). 25 °C supported the growth of *A. muciniphila* at the lowest level, whose viability was significantly less than viabilities at all other temperatures ($p < 0.0001$).

Growth curves of *A. muciniphila* in BHI medium with adjusted pH level of 2, 3, 4, 5, 6, 6.5, 7, 8 and 9 at 37 °C during an incubation time of 12 hours were presented in Figure 5. The results showed that pH 6.5 was the best pH promoting growth of *A. muciniphila*, followed by pH 6, pH 7 and pH 7.5. Further calculation confirmed that pH 6.5 was the best pH level with the fastest growth rate (Figure 6), which was significantly faster than growth rates at any other temperature ($p < 0.0001$). Growth rates at pH 6, 7 and 7.5 were at similar level ranging with no significant difference among these three levels. Growth of *A. muciniphila* at pH 4 and pH 5 were significantly slower when compared to pH 6.5 ($p < 0.0001$) while nearly no growth was detected in medium adjusted to pH 2, 3, 8 and 9. The final viability of *A. muciniphila* at each pH level was presented in Figure 7. Medium with pH adjusted to 6.5 promoted the highest final viability, which was

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significantly higher when compared to the cells grew under all other pH levels ($p < 0.0001$). Medium with pH level at 6 and 7 showed final viabilities at a similar level with no significant difference. The viability at pH 7.5 was significantly lower than the viability at pH 7 ($p < 0.01$) and the viability dropped to around 4×10^6 CFU/mL in medium at pH 5. Medium with pH adjusted to 2, 3 and 4 was not able to promote the growth of *A. muciniphila*, whose final viabilities were around half of the viability in medium with pH 5. Growth in medium adjusted to 8 and 9 was shown with the least viability, which was significantly lower than viabilities at all other pH levels ($p < 0.0001$)

The effects of prebiotics on growth of *A. muciniphila* was presented as Figure 8. IMO and GOS had the best two growth curves among the nine selected prebiotics. In comparison, medium with GG, FOS, INU, AG, KG and TG resulted in reduced cell growth while PS was shown with the least growth of *A. muciniphila*. Further calculation of growth rate (Figure 9) showed that all nine prebiotics were able to promote the growth of at a similar growth rate with no significant difference. There was no difference found when compared the growth rates in medium adjusted with prebiotics to medium with dextrose, except for PS ($p < 0.05$). Figure 10 presented the final viabilities of *A. muciniphila* in medium modified with each prebiotic. IMO promoted the highest viability, which was significantly higher than GOS as the second highest ($p < 0.05$). FOS, GG, AG and INU showed approximately half viabilities of IMO while KG and TG had comparable final viabilities as BHI medium with no dextrose. PS showed a significantly lower level of viability comparing to all other prebiotics ($p < 0.0001$).

Figure 11 presented the effects of sugars on growth of *A. muciniphila*. There were four candidates showing similar growth curves, which were fructose, dextrose, sucrose and lactose. Fructose and dextrose had same sharp exponential phase, and sucrose had similar OD value as

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fructose during lag phase. *A. muciniphila* cultured in lactose medium showed longer time in exponential phase with lower final viability when compared to cultures in fructose, sucrose and dextrose medium. Maltose medium was able to support the growth of *A. muciniphila*, which presented a good exponential slope while the final OD value was less than half of values measured in top four sugar media. Culture in galactose medium revealed nearly the same growth curve as control, which was BHI medium without dextrose. Calculation of growth rate (Figure 12) reported that growth rates of *A. muciniphila* in fructose, sucrose and maltose reached to a similar growth rate as in dextrose with no significant difference noted. Growth in lactose and galactose was significantly slower than the growth in fructose and dextrose ($p < 0.01$). The final cell viabilities suggested that *A. muciniphila* achieved the highest viability in medium fortified with fructose, which was significantly higher than that in dextrose ($p < 0.05$). The second highest viability was observed in sucrose, which promoted the viability of *A. muciniphila* with no significant difference from the viability in dextrose. Lactose was able to promote the viability of *A. muciniphila* at an intermediate level, which was significantly lower than fructose and sucrose ($p < 0.05$) but significantly higher than maltose and galactose ($p < 0.0001$). It was clear that *A. muciniphila* could grow in medium adjusted with maltose and galactose; however, the viabilities were at the same level as in control medium with no significant difference, which were only around one third of the viability in fructose medium (Figure 13).

The effects of sugar concentrations on *A. muciniphila* final culture viabilities were showed in **Figure 14**. A similar trend was found among all three figures, which showed that medium with 0.5, 1.0 and 0.2% of sugar were the best three concentrations with higher level of final culture viabilities than at other two concentrations. In medium adjusted with fructose, 0.5% fructose showed a significantly high level of viabilities when compared to 1.0% ($p < 0.01$) and 0.2% ($p <$

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0.001). Dextrose with concentrations of 0.5% and 1.0% promoted the highest viabilities, which were significantly better than viability achieved at concentration of 0.2% ($p < 0.01$). In medium adjusted with sucrose, viabilities were similar at concentrations of 0.2, 0.5 and 1%, which were significantly higher than at other concentrations ($p < 0.01$).

BSH activity of strains *A. muciniphila*, *L. acidophilus*, *L. reuteri*, *L. plantarum* and *B. coagulans* were compared in

Table **1**. To appropriately compare their BSH activity, all probiotics strains were adjusted to start with a similar total protein content of approximately 356.5 (μg/mL). Results showed that *L. reuteri* and *L. plantarum* had significantly higher BSH activity levels than all the other strains ($p < 0.0001$). *L. acidophilus*, *A. muciniphila* and *B. coagulans* presented a similar level of BSH activity without significant differences.

The efficiency of glycerol stock in maintaining viabilities of *A. muciniphila* was presented in Figure 15. Results showed that *A. muciniphila* was able to be recovered successfully with less than 0.1 Log number of viability lost after 1, 2 and 6 months of storage.

Discussion

Routine verification of *A. muciniphila* was tested using PCR and gel electrophoresis imaging. Cultures with positively verified *A. muciniphila* were used for all further tests. Thus there should be no concern regarding contamination of all the test samples.

In order to culture sufficient amount of *A. muciniphila* for discovering its characteristics and preparing for in-vivo mouse supplementation study, it is necessary to identify the best growth conditions for culturing *A. muciniphila*. Based on previous findings, *A. muciniphila* was able to grow within 20 - 40 °C with pH range of 5.5-8.0 (54). The growth of bacterial can be largely characterized by three fundamental growth constants, which are exponential growth rate, lag time and total growth (83). Due to the shape of standard curve, the determination of lag time is difficult

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to guarantee the precision. Therefore, growth rate and final cell viability were used to determine the optimal growth conditions for *A. muciniphila*. It was found that *A. muciniphila* was able to growth within the temperature range of 25 – 46 °C. Based on calculated growth rates and final viabilities, temperature from 35 – 43 °C could promote the growth with no significant difference among the different temperatures. Temperature at or below 25 °C and temperature at or above 46 °C were not appropriate temperatures for *A. muciniphila* to grow. The next growth condition being evaluated was pH tolerance range, which helps to determine optimal growing pH and also important for exploring the survival rate of *A. muciniphila* under various pH environment in GIT. To exert health benefits, dietary probiotics have to survive through GIT (stomach acids) and colonize in the gut (84). Therefore, the growth of *A. muciniphila* were compared in culture medium with adjusted pHs including pH 2, 3, 4, 5, 6, 6.5, 7, 7.5, 8, and 9. The results showed that the optimal pH range for culturing *A. muciniphila* should be controlled within 6 – 7.5 where pH 6.5 was the best pH level, which was consistent with the findings from previous study (54). The minimal cell growth at pH 2, 3, 4, 5, 8 and 9 indicated a low survival probability of *A. muciniphila* when administered orally. In short conclusion, *A. muciniphila* showed similar growth under a range of temperature; however, it was highly sensitive to different pH levels. Therefore, the control of culture pH is the most critical step to guarantee the optimal growth of *A. muciniphila*.

The gastric pH values of healthy adults are within the range of pH 2 – 3 under fasting conditions. While during gastric emptying procedure, pH level changes from highly acidic environment in stomach to nearly neutral environment in duodenum (pH 6 – 6.5). In small intestines, pH shifts gradually in different locations as well: pH value is 7.4 in terminal ileum, 5.7 in cecum and 6.7 in rectum (85). Based on these conditions and the results, the in-vivo delivery of probiotics through stomach is critical since the strongly acidic condition would significantly

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diminish the viability of *A. muciniphila*. Therefore, an effective protection method is necessary to maintain the integrity of probiotics through GIT.

Limited studies have been done to investigate proper prebiotics for promoting growth of *A. muciniphila*. A previous study showed that supplementation of FOS restored the decreased *A. muciniphila* population of 10^7/g feces back to 10^9/g feces induced by obesity in DIO mice (78). Similar promoting benefit was reported by Everard et al. and Reid et al. as well (65, 86). Although FOS has been widely investigated as a common prebiotic, there are a number of potential prebiotic ingredients haven't been evaluated for the strain of *A. muciniphila*. Therefore, nine prebiotic ingredients including IMO, INU, FOS, GOS, GG, AG, KG, TG and PS were evaluated. To compare the potential effects of prebiotics on *A. muciniphila* growth, 0.2% w/v dextrose as a carbohydrate source in BHI medium was replaced by each of the individual prebiotic at the same concentration and subsequently being inoculated with *A. muciniphila*. Based on the comparison of exponential growth rates of *A. muciniphila*, it was concluded that IMO and GOS were able to support the growth of *A. muciniphila* at the best efficiency when compared to all other prebiotics. The potential factor for their superior bioavailability to *A. muciniphila* might be due to the simple structures of sugar molecular composition as compared to other sugar molecules. Viabilities of *A. muciniphila* in medium with other prebiotics suggested that these prebiotics were not favoring ingredients for *A. muciniphila* growth, which could be explained by their long and complex structure branch-chained with extra sugar or sugar acid units such as mannose, rhamnose and galacturonic acid. In conclusion, IMO was the best prebiotic option in promoting growth of *A. muciniphila* in regard to growth rate and final viabilities. A synbiotic formula of *A. muciniphila* with IMO would be able to deliver additional viabilities and its related benefits.

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In addition to optimum growth condition in terms of temperature and pH, optimization of cost-efficient and effective ingredients was also investigated for finding better method to promote productivity in scaling up *A. muciniphila* production. According to results of prebiotics test, we've demonstrated that dextrose was the best ingredient that showed the outstanding growth rate and the highest culture viability as expected. Additionally, dextrose is less expensive as compared to all other types of prebiotics. Inspired by this finding, six common carbohydrates were tested, which included dextrose, galactose, lactose, fructose, sucrose and maltose. Final culture viabilities indicated that fructose and sucrose promoted the most growth of *A. muciniphila*. In conclusion, fructose was the best sugar for culturing *A. muciniphila*. Dextrose was another candidate that could assure fast growth rate while sucrose was the candidate to guarantee a better culture viability.

Before starting to produce large quantity of *A.* muciniphila for animal study, it was necessary to determine the optimal concentrations for the selected top three sugars. Results have shown that 0.5% of sugar supplementation promoted the best growth of *A. muciniphila*, which was significantly higher than the growth in BHI culture medium with an original sugar concentration of 0.2%. A sugar concentration higher than 1.0% was shown to lower viability of *A. muciniphila* suggesting that the osmotic pressure balance might be disturbed by higher concentration of sugar. In conclusion, the optimal concentration for culturing *A. muciniphila* was 0.5% of fructose, sucrose or dextrose.

Inspired by evidences that Lactobacillus strains having varied level of bile salt hydrolase activity (87, 88), the BSH activity of *A. muciniphila* was also investigated. *L. acidophilus, L. reuteri, L. plantarum* and *B. coagulans* were compared with *A. muciniphila*. *L. acidophilus* has been reported to present hydrolase performance for sodium glycocholate and sodium taurocholate in a previous study (89). *B. coagulans* was lately shown to have deconjugation ability on bile salts

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(90) while its BSH activity level has not been quantified. In this study, *L. acidophilus*, *B. coagulans* and *A. muciniphila* were shown to have a similar level of BSH activity with no significant difference observed among these three strains. These results indicated that *A. muciniphila* would have a good survival rate due to its BSH activity, which could protect probiotics against the detergent effects of bile salts in the gut.

Following optimizing the culturing conditions of *A. muciniphila*, its stability and potential functions were evaluated as well. Before moving forward, an effective storage method had to be evaluated and confirmed. Glycerol stock is the solution normally used for long-term bacteria storage. To ensure *A. muciniphila* with reliable quality and quantity during storage, the efficiency of 50% glycerol stock has been confirmed in this study. The viability of strictly anaerobic strain *A. muciniphila* could be guaranteed with minimal loss of viability after being stored within glycerol stock in short-term and long-term storage.

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CHAPTER 3. ENHANCE THE SUSTAINABILITY OF *A. MUCINIPHILA* IN GIT USING ENCAPSULATION TECHNOLOGY

Preserving the sustainability of probiotics is extremely important to convey potential benefits to the host. Probiotics, especially a large number of anaerobes, are fragile cells which could be affected by a wide range of environmental factors including atmosphere, pH, temperature, acidification during fermentation and hydrogen peroxide production. In the current market besides capsulated supplements, probiotics are widely incorporated in dairy products. Meanwhile, the variety of non-dairy food products is increasing rapidly as well, such as energy bars and beverages (91). However, the survival of probiotics in those products has been reported to be low (92). Therefore, it is important to design in vitro studies to investigate the ability of probiotic strains survivability through harsh environment such as GI tract. Resistance to high acidity in stomach and to high concentration of bile components in proximal intestine are critical selection criteria for probiotics. Since *Lactobacillus* and *Bifidobacterium* have been widely incorporated in food products, their tolerance to low pH and bile salts were extensively studied. Evidences showed that popular strains such as *Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium animalis, Bifidobacterium lactis* showed minimal survival rate under these stress factors (93). For spore-forming lactic acid producing bacteria, very few strains (*Bacillus laevolacticus* and most *Sporolactobacillus* strains) were tolerant to acidic environment while only *Bacillus racemilacticus* and *Bacillus coagulans* were tolerant to bile salts (94).

Based on previous evidence that most probiotic strains would lose viability in simulated stomach and intestine conditions, protecting viable quantity and maximizing health benefits of those probiotics have drawn extensive research interest (95). When incorporating microbial species in foods or supplements, a functional dose is required. Health Canada accepted that when

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Bifidobacterium and/or Lactobacillus is delivered orally, the dose level should be at one billion (1 x 10^9) CFU per serving. The Italian Ministry of Health has regulated that the number of viable cells administered per day should be 1 x 10^9 CFU minimum (34). The efficacy of added probiotic strains depends on the dosage and the viability throughout the digestive tract after consumption. Dairy companies have modified their formulations to ensure sufficient viability in their probiotic products, such as using freeze-dried powders, or packaging with sachet and stick forms (96).

Nowadays, encapsulation has been used for maintaining probiotic viability in food products. It is a process of utilizing a material or system to coat or entrap one or a mixture of materials. The coating material is called wall material or shell and the material coated is called core material or internal phase. The wall material is designed to protect the core material against environmental stresses including oxygen, acidity, enzyme reactions (97). Encapsulation techniques are utilized to produce efficient delivery vehicle to target locations within GIT, which have been extensively utilized in food industry. The current family of encapsulation includes emulsification, coacervation, spray-drying, freeze-drying, and extrusion (92).

Spray-drying is one of the most widely utilized encapsulation technologies in commercial food processing. It is considered as an inexpensive, fast and consistently reproducible procedure. The principle of the procedure is dissolving the core material in a dispersion of matrix material. The dispersion is atomized by heated air to remove water quickly, and then separate powdered particles at a lower temperature. The major disadvantage is the high temperature could be detrimental to temperature and oxygen sensitive constituents, especially probiotic culture (98).

On the other hand, freeze-drying is an alternative to spray-drying for probiotics that are sensitive to heat or oxygen. The lyophilization procedure produces a vaccum-drying condition under very low temperature, which significantly preserves viability of microorganism during

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process. The process is beneficial for oxygen sensitive strains whose viability would be dramatically damaged during spray-drying. However, commercial application of freeze-drying is limited due to its higher cost and longer processing time when compared to spray-drying (99).

Extrusion is another encapsulation technology which is less hazardous when compared to spray-drying. It is a procedure of producing small droplets of an encapsulated material using pressure to force solution through openings in droplet-generating device or nozzles. The size of droplets depends on diameter of openings or nozzle. This technology is relatively gentle and it could be operated under aerobic or anaerobic environment (92). Syringe-extrusion is one of the common used process, which is typically used to produce alginate beads. An alginate solution containing bioactive core is extruded in calcium chloride solution to form droplets (99). For the basic protection against acidity, alginate beads have been proven to maintain viability throughout GIT and can be suitable for long-term storage (100).

There is no study showing evidence regarding the tolerability of *A. muciniphila* to high level of acid and bile salt. In current study, the first objective was to investigate the survival rate of *A. muciniphila* under stressed environment. The second objective was to evaluate the best encapsulation methods to preserve the viability of *A. muciniphila*; in addition, the ability of encapsulation in protecting the bioactivity of *A. muciniphila* was further investigated using a simulated digestion method. It was hypothesized that a significantly improved viability would be observed using encapsulation method when compared to delivering probiotics with no protection.

Methods

Acid tolerance

A. muciniphila cells were harvested by centrifugation and were washed three times using PBS. Washed pellet was suspended with 1 mL of BHI. Fresh simulated gastric solution was prepared daily by mixing 3 g/L pepsin in 0.9% w/v saline, and the pH was adjusted to 2.0. The

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suspended *A. muciniphila* was mixed with 5 mL of simulated gastric solution and 1.5 mL of 0.9% w/v saline (87). The mixture was incubated at 37 °C for 3 hours, during which 0.1 mL aliquots were drawn to determine viability at constant intervals of 0, 1, 2 and 3 hours.

Bile salts tolerance

Bile salt medium was prepared by suspending 0.3% w/v bile salts (LP0055 OXOID, Ontario, Canada) in BHI medium. *A. muciniphila* cells were harvested by centrifugation and were washed twice using 0.9% saline (87). Washed pellet was suspended in 1 mL bile salt medium and inoculated into bile broth and incubated at 37 °C for 3 hours, during which 0.1 mL aliquots were drawn to determine viability at constant intervals of 0, 1, 2 and 3 hours.

Spray-drying

A. muciniphila culture was centrifuged to form *A. muciniphila* pellet, which was suspended with PBS to prepare for spray-dry. The Yamato GB210A spray dryer (Yamato Scientific America, Santa Clara, CA) was used in current study. Inlet temperature was set to 120 °C and outlet temperature was 55 – 60 °C. Speed was controlled at 1.75 mL/min, and pressure was 0.15-0.20 Mpa. Dried *A. muciniphila* powder was sprayed into the collector and then stored in 50 mL tubes at – 20 °C for testing. Viability of *A. muciniphila* stock prepared for spray-drying and viability of spray-dried *A. muciniphila* powder were compared using pour-plating method to evaluate the loss of cell viability during spray-drying procedure. All samples were tested in duplicates.

Freeze-drying

A. muciniphila culture was centrifuged to form *A. muciniphila* pellet, which was suspended with sterile 10% sucrose BHI solution in 50 mL tube to prepare for freeze-dry. The *A. muciniphila* and sucrose BHI mixture was flash frozen with liquid nitrogen and stored at – 80 °C overnight. In the next day, the 50 mL tube was uncapped, sealed with gauze and was freeze-dried for 48 hours using FreeZone Triad Freeze Dryer (Labconco, Kansas City, MO). Freeze-dried *A. muciniphila*

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was gently grinded into powder and stored in – 20 °C freezer. Viability of *A. muciniphila* stock prepared prior to freeze-drying and viability of freeze-dried *A. muciniphila* powder were compared by pour-plating method to evaluate the loss of cell viability during freeze-drying procedure. All samples were tested in duplicate.

Extrusion

Freeze-dried *A. muciniphila* powder was dissolved in 1% sterile sodium alginate solution (Sigma-Aldrich, St. Louis, MO) at 5% (w/v). A homogenizer (IKA-labortechnik, Wilmington, NC) was used to homogenize *A. muciniphila* powder with the solution. Once the mixture was fully homogenized, it was transferred into a 60 mL syringe with a 22 G needle to extrude droplets into sterile 0.1 M $CaCl_2$ solution (Thermo Fisher Scientific, Ann Arbor, MI). Droplets of *A. muciniphila* formed solid sphere beads after a 30-minute swirl in the $CaCl_2$ solution. All the beads were drained from the solution and washed twice with sterile deionized water to get rid of excessive solution. Washed beads were dehydrated in the dehydrator (Excalibur, Sacramento, CA) at room temperature for 48 hours, and stored at room temperature after fully dehydrated. Viability of *A. muciniphila* stock prepared for extrusion and viability of dehydratedd *A. muciniphila* beads were compared using pour-plating method to evaluate the loss of cell viability during extrusion procedure. All samples were duplicated in the test.

Extrusion efficacy test

The efficacy of encapsulated *A. muciniphila* beads was evaluated using simulated gastrointestinal digestion fluids to investigate the protective effect of encapsulation on viabilities. PBS with 3 mg/mL pepsin (Sigma, St Louis, MO) was adjusted to pH 2 and enteric digestion fluid (EDF) were used to simulate pH and digestive enzymes as human GIT. EDF was prepared with the following formula: 0.4% w/v pancreatin (Sigma, St Louis, MO), 1.5% w/v bile salt (LP0055 OXOID, Ontario, Canada), 0.5% w/v amylase (Sigma, St Louis, MO), 0.1% w/v trypsin (Sigma,

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St Louis, MO), and 0.5% w/v lipase (Sigma, St Louis, MO) (81). *A. muciniphila* beads were weighed first and then added into pepsin-PBS for an incubation of 1 hour at 37 °C. All liquid was discarded after incubation. Then EDF was added and incubated with *A. muciniphila* beads at 37 °C for 1.5 hours. After incubation, *A. muciniphila* beads were drained from excessive liquid and washed with sterile deionized water. Washed *A. muciniphila* beads were added into sterile PBS and homogenized. Serial dilution and pour-plating method were used to test the viability of beads. At the same time, the same amount of original *A. muciniphila* beads were processed with same procedure and tested for viability as untreated control.

Statistics

Data were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) with a Tukey post-hoc test using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA). Results were considered statistically significant at $p < 0.05$.

Results

Figure 16 showed the effects of low pH and pepsin on viability of *A. muciniphila*. It was clear that *A. muciniphila* was intolerant to this stressed condition. One hour of incubation resulted in loss of 3.7 Logs of CFU ($p < 0.0001$), while in the following two-hour incubation, the cell viability was maintained at a relatively stable status with no significant difference in the following hours.

The tolerance ability of *A. muciniphila* to bile salts was presented in Figure 17. *A. muciniphila* was observed with a significant decreased level of viability (1 Log number) within the first hour ($p < 0.0001$). The viability was maintained at a similar level with no significant difference in the following two hours.

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Table 2 listed the loss of viabilities after the procedure of encapsulation. Freeze-drying method showed the minimal loss of cell viability and extrusion method presented an intermediate preservation. Among three methods evaluated, viability of *A. muciniphila* had the most loss during the procedure of spray-drying. Consequently, freeze-drying and extrusion methods were selected for testing protection efficacy through simulated GIT. The results noted that freeze-dried *A. muciniphila* without protection encountered a tremendous decrease of viability while extruded beads were able to maintain *A. muciniphila* viability to the maximum level with barely no loss.

Discussion

The significant loss of CFU in both simulated stomach and proximal intestinal conditions indicated that *A. muciniphila* was not able to survive through acidic environment as in a stomach. This finding was consistent with previous test in exploring optimal pH for promoting growth of *A. muciniphila*, which reported that *A. muciniphila* was sensitive to low pH level (pH < 5) and was not able to survive. When testing its tolerance to bile salt, there was only 1 Log number of decrease found in its cell viability, which suggested that *A. muciniphila* had better tolerance to bile salts when compared to acids. The previous data reported that *A. muciniphila* showed a good BSH activity, which helped with bile salts deconjugation and elimination of the detergent effect of bile salts on *A. muciniphila.* In conclusion, it was necessary to provide additional protection for *A. muciniphila* in order to guarantee sufficient and viable cells through human GIT.

The determination of proper encapsulation method is extremely critical for incorporating probiotics in food products with adequate amount of living cells. In this study, three widely used encapsulation methods were tested to determine the optimal vehicle for protection and the potential delivery of *A. muciniphila*. The results showed consistency as hypothesized that freeze-drying method maintained the highest viability of *A. muciniphila* while extrusion method showed an higher viability loss, which was possibly due to steps as washing, drying and dehydration

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additional to the steps of freeze-drying. The largest viability loss during spray-drying process might be caused by high temperature and potential air exposure. Although spray-drying is the most time and cost saving method among the three options, result in this study suggested that spray-drying would not be a proper encapsulation method for processing *A. muciniphila* especially in large quantity production. Based on this comparison, freeze-drying and extrusion were reliable encapsulation methods to minimize the loss of *A. muciniphila* cells during process. The next step was to test the efficacy of freeze-drying and extrusion in protecting *A. muciniphila* from low pH and enzyme activities through simulated gastrointestinal digestion. The results showed that *A. muciniphila* beads produced from extrusion method were effective in maintaining viabilities under simulated stressed environment. The minimal loss in CFU has proved that *A. muciniphila* beads could highly preserve the cell viability while the coating served anti-acid and non-enzymatic reactive purposes. In comparison, the huge viability loss in freeze-dried *A. muciniphila* powder indicated that *A. muciniphila* cells barely survived with sucrose solution as the only coating material. In conclusion, extrusion would be the best encapsulation technique that could be used to incorporate *A. muciniphila* in food product to minimize cell loss during processing and GIT digestion. This efficient protection could guarantee bioaccessibility and bioavailability of *A. muciniphila* for subsequent beneficial function in GIT.

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CHAPTER 4. LONG-TERM DIETARY EFFECTS OF *A. MUCINIPHILA* SUPPLEMENTATION ON HIGH-FAT INDUCED OBESITY AND DIABETES

In-vivo study is useful to better understand the effect of gut microbiota on health and disease. Various studies have been focused on individual biomarkers or key attributes for certain adverse physiological conditions (101); however, very few studies suggested any longer-term effect for *A. muciniphila* administration in an obese mouse model. Current study was designed to attempt to evaluate weight loss potential and glycemic control, and to observe possible sustainable effects of *A. muciniphila* supplementation over a significantly longer period in a diet-induced obese mouse model. It was hypothesized that supplementation of *A. muciniphila* for six months would decrease body weight gain and improve glucose homeostasis. Mouse body weight was monitored regularly in this study; additionally, individual mouse body composition was measured at the end of the study to further investigate the effects of long-term *A. muciniphila* supplementation. Glucose homeostasis was also evaluated to understand the effect of *A. muciniphila* on parameters including fasting blood glucose, glucose tolerance and insulin resistance. It was intended to further explore the potential anti-obesity mechanism of *A. muciniphila*. Energy balance including food intake, fecal energy loss and basal energy expenditure were measured to provide valuable information on energy absorption and metabolism. In addition, supplementation safety of *A. muciniphila* oral administration was evaluated for the intention of its future potential human study and food product development as a probiotic additive. The results achieved from this mouse study would be informative and inspiring for future investigation of *A. muciniphila* in clinical studies.

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Methods

Experimental animals

6-8 week old male C57BL/6 mice (Charles River Laboratoreis, Wilmingtong, MA) were housed in Biological Science Building within a controlled environment: 12-hr day/night cycle, room temperature kept at 72 - 77 °F, and moisture of 35 - 40%. Mice were randomly grouped as six mice per cage with food and water ad libitum. Sani-chip bedding was provided in each cage to avoid possible consumption of bedding materials. Upon reception, mice were acclimated for three days with control diets. Grouped mice were assigned to three groups of 12 including control diet (CD), high-fat diet (HF) and *A. muciniphila* supplemented high-fat diet (A. m). The energy content of CD diet was 3.85 kcal/gram, which contained 10% of calories from fat and 70% from carbohydrate (D12450J, Research Diets Inc, New Brunswick, NJ). The HF diet had 5.24 kcal/gram, with 60% energy from fat and 20% from carbohydrates (D12492M, Research Diets Inc, New Brunswick, NJ). All diets were stored at 4 °C until use. Mice in group A. m had daily oral administration of *A. muciniphila* solution with a CFU of approximately 10^{10}. *A. muciniphila* solution was prepared daily by washing frozen *A. muciniphila* glycerol stock twice with sterile water for the purpose of getting rid of any excess storage media solution with glycerol. The final *A. muciniphila* culture pellet was resuspended in sterile water and fed to mice within 30 min after preparation via gavaging. Mice in group CD and HF were orally administered daily with equivalent volume of sterile water. Treatments lasted for six months. Food intake and body weight were recorded weekly. Mice in groups HF and A. m were transferred to Wayne State University iBio facility in the fifth month of treatment. After three-weeks' stay in iBio facility, mice returned to Biological Science Building.

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Glucose homeostasis analysis

Fasting glucose was measured monthly and the last measurement was performed prior to sacrifice. In each of the monthly blood collection, mice were fasted for six hours with only water provided. Mouse was restrained in the restraining device with the tail exposed. A small drop of blood was obtained from the tail vein and placed on the test strip of blood glucose meter. Accu-check glucometer was used in this study (Roche, Indianapolis, IN).

Glucose tolerance test was performed in the fifth month of treatment. Glucose solution (10%) was prepared and subsequently filtration sterilizated using a 0.2 μm syringe filter. Following the same fasting procedure as above, fasting blood glucose of each mouse was measured as the first time point. Each mouse was then gavaged with a glucose solution with a dose of 2 mg/g of body weight. Blood glucose levels of each mouse were measured at 15, 30, 60 and 120 minutes time points. Areas under the curve were calculated using standard trapezoid method (102).

Fecal energy test

Fecal samples were collected in the fifth month of treatment. Fecal pieces from each individual cage were collected for a 24-hour period with food and water provided. Samples were air-dried and stored in - 20 °C for further analysis. Bomb Calorimeter (Parr, Moline, IL) was used to measure fecal energy content. In order to produce sufficient energy output, duplicated fecal samples of 0.5 g each from each cage was weighed and burnt in the calorimeter to measure energy released from the samples by measuring the quantity of heat produced. The fecal energy (kcal/g) was calculated based on the actual amount of fecal content combusted, which was pre-test fecal sample weight with post-test non-combustible residue weight subtracted. In addition, fecal energy output/ energy intake ratio was calculated using the following formula: output ratio = total feces energy/ total energy intake × 100%.

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Body composition analysis (BCA) and energy expenditure analysis

Mice of group HF and A. m were transferred to Wayne State University iBio facility at the end of fifth month. After a three-day acclimation, lean mass and fat mass of each mouse were determined using EchoMRI-100 analyzer (EchoMRI, Houston, TX). Mouse was placed in the holder tube without anesthetization, and the holder was inserted into the MRI analyzer. It took 2 - 3 minutes to finish the measurement of one mouse. In this study, each mouse was measured consecutively three times to minimize free water error during measurement.

After BCA was performed, each mouse in groups HF and A. m was caged individually for five days in TSE PhenoMaster home cage system (TSE systems, Chesterfield, MO). Activity level of each mouse was measured by light beam interruptions recorded by activity monitor embedded in the metabolic cage. Records were generated automatically to report the accumulation of distance travelled.

Blood collection and serum sample preparation

After each mouse was euthanized by CO_2 exposure, approximately 1 to 1.5 mL of blood was collected from each mouse using heart puncture method. Cervical dislocation was performed afterwards to make sure mouse was deceased. The blood sample collected was transferred into a 1.5 mL microtube and left undisturbed at room temperature for approximately 30 min. Blood samples were then centrifuged at 1000 g for 10 minutes at 4 °C and the resulting supernatant was aliquoted immediately into microtubes for different tests in order to avoid freeze-thaw cycles. All microtubes were stored in a - 80 °C freezer for future testing. Any hemolyzed samples were excluded from all tests.

Homeostatic model assessment of insulin resistance (HOMA-IR)

Fasting insulin was measured as manufacturer instructed using Ultra sensitive mouse insulin ELISA kit (#90080 Crystal Chem, Doners Grove, IL). OD values were measured within

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30 minutes using plate reader at 450 nm and 630 nm. Insulin standard curve was generated to quantify sample fasting insulin levels. OD values of mouse serum samples were interpolated using the mean absorbance value of each sample and the standard curve. Fasting glucose and fasting insulin results were used to calculate HOMA-IR using the following equation: HOMA-IR = 26 x fasting glucose level (mg/dL) x fasting insulin level (ng/mL) / 405 (103).

Toxicity evaluation

Serum alanine transaminase Assay

Serum alanine transaminase (ALT) concentration was measured as manufacturer instructed using ALT assay kit (#EALT-100, BioAssay Systems, Hayward, CA). OD values were measured at 5 minutes and 10 minutes at 340 nm. ALT activity was determined using the equation: $ALT\ (U/L) = 381 \times \frac{\Delta OD_S - \Delta OD_{NADH}}{OD_{STD} - OD_{BLK}}$. ΔOD_S was calculated by subtracting each sample OD at 10 minutes from the OD at 5 minutes. ΔOD_{NADH} was calculated by subtracting NADH standard OD at 10 minutes from the OD at 5 minutes. OD_{STD} and OD_{BLK} were OD values of NADH standard and blank at 340 nm at 5 minutes.

Aspartate transaminase assay

Serum aspartate transaminase (AST) concentration was measured as manufacturer instructed using AST assay kit (#EASTR-100, BioAssay Systems, Hayward, CA). OD values were measured at 5 minutes and 10 minutes at 340 nm. ALT activity was determined using the equation: $AST\ (U/L) = 388 \times \frac{\Delta OD_S - \Delta OD_{NADH}}{OD_{STD} - OD_{BLK}}$. ΔOD_S was calculated by subtracting each sample OD at 10 minutes from the OD at 5 minutes. ΔOD_{NADH} was calculated by subtracting NADH standard OD at 10min from the OD at 5 minutes. OD_{STD} and OD_{BLK} were OD values of NADH standard and blank at 340 nm at 5 minutes.

γ-Glutamyltransferase activity assay

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Serum γ-Glutamyltransferase (GGT) activity was measured as manufacturer instructed using GGT activity calorimetric assay kit (MAK089 Sigma-Aldrich, St. Louis, MO). OD values were measured every 5 minutes when incubated at 37 °C until the OD value was greater than the OD value of the highest standard concentration. The final measurement $(A_{418})_{final}$ for calculating the enzyme activity was the penultimate reading and the time of the penultimate reading was T_{final}. The first step of calculations was to establish a standard curve with initial measurement of pNA standards. Change in measurement from $T_{initial}$ to T_{final} was calculated as $\Delta A_{418} = (A_{418})_{final} - (A_{418})_{initial}$, which was compared to the standard curve to determine the amount of pNA generated (B) between $T_{initial}$ and T_{final}. GGT activity was determined using the equation: $GGT\ (mU/mL) = \frac{B \times sample\ dilution\ factor}{(reaction\ time) \times sample\ volume}$, reaction time = $T_{final} - T_{initial}$.

Blood urea nitrogen assay

Urea concentration in blood was measured as manufacturer instructed using QuantiChrom urea assay kit (DIUR-100 BioAssay Systems, Hayward, CA). OD values were measured at 520 nm and the urea concentration was determined the equation: $[Urea]\ (mg/dL) = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times n \times [STD]$, n was the dilution factor, [STD] was 50. Conversion of BUN and Urea was: BUN (mg/dL) = [Urea]/ 2.14.

C-reactive protein assay

C-reactive protein concentration was measured as manufacturer instructed using Mouse C-reactive protein (CRP) ELISA kit (#80634 Crystal Chem, Elk Grove Village, IL). The final OD values were measured within 30 minutes at 450 nm and 630 nm. CRP calibration curve was plotted using OD values and their corresponding CRP concentrations. The CRP concentration of serum sample was interpolated using the calibration curve and mean OD value of each sample.

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Statistics

Data were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) with a Tukey post-hoc test using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA) after a significant difference was identified by ANOVA. Results were considered statistically significant at $p < 0.05$.

Results

After six months of treatment, body weight trends of the three groups were presented in Figure 18. The rate of body weight gain of CD group was the slowest, in which CD mice gained about 8 g in the last thirty days. The group with the fastest gaining trend was HF group, and A. m group showed a body weight gain between CD and HF groups, which had a rate of weight gain faster than CD but slower than HF. The weight gain in A. m group was not significantly different from weight gain in HF group, except a significant lower level of weight gain in A.m group was observed within a period of 26 days (day 46 to day 72) when compared to HF group ($p < 0.05$).

Food intake of grouped mice was measured every week. Food intake of CD group was significantly higher than that of HF and A. m groups ($p< 0.01$), while no significant difference was found between HF and A. m groups (Figure 19). Energy intakes were calculated based on the caloric content in foods, which was 3.9 kcal/g for CD diet and 5.2 kcal/g for HF/A. m diet. During six-month of treatment, mice in CD, HF and A. m groups consumed similar level of energy with no significant difference among the three groups (Figure 20).

Fasting blood glucose of each mouse was measured on monthly basis, which corresponding to first, second, fourth, fifth and sixth month. Figure 21 clearly pictured that fasting blood glucose of HF group mice was at the highest level among the three groups with a peak at 232 ± 39 mg/dL after four months of treatment. It was noted that CD and A. m group had relatively stable fasting blood glucose during all six months. There was no significant difference found between CD and

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A. m group during all six months treatment except for the third month ($p < 0.05$); however, mice in HF group showed significantly higher fasting blood glucose when compared to CD group along the treatment period ($p < 0.01$).

In addition, all three groups of mice were tested for glucose tolerance as well. Blood glucose levels were measured at time points of 0, 15, 30, 60, and 120 minutes (Figure 22). Baseline glucose started at a similar level across groups; however, significant difference were observed after 15 minutes when HF group showed higher blood glucose levels as compared to both CD and A. m groups. The blood glucose of CD and A. m groups decreased to baseline level 60 minutes after glucose intake. This was not observed in HF group, where average blood glucose level was decreasing very gradually in a span of 120 minutes before returning to baseline level. HF group reached to the peak at 15 minutes time point, when A. m group showed a significantly lower level ($p < 0.001$). After another 15 minutes, no obvious decrease was found in HF group while A. m group had a significant drop of blood glucose ($p < 0.0001$). At the 60-minute time point, blood glucose level of HF group slowly decreased, which was still significantly higher than the level of A. m group ($p < 0.001$). At the end of 120 minutes, A. m group showed the lowest blood glucose when HF showed a significantly higher level ($p < 0.001$).

Subsequently, area under curve (AUC) based on the results of glucose levels in GTT was calculated, which is depicted in Figure 23. Significant difference was discovered in AUC among CD, HF and A. m groups. HF group was shown with a significantly higher level of AUC when compared to CD and A. m groups ($p < 0.0001$) while there was no significant difference between CD and A. m groups. Fasting insulin and glucose at month six were used to calculate HOMA-IR. Insulin results showed that while CD and HF groups had the lowest and highest respectively, there was no significant difference among CD, HF and A. m groups (Table 3). Fasting blood glucose of

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HF group was shown significantly higher than that of the CD group ($p < 0.01$). The calculation of HOMA-IR confirmed that HF group had a significantly higher level when compared to CD group ($p < 0.01$) while no difference was observed between A. m and CD groups.

Figure 24 compared the fecal energy content of the three groups. The results showed that CD group had the highest and A. m group had a lower fecal energy content but no significant difference between these two groups. HF group had the lowest fecal energy content, which was significantly lower than CD group ($p < 0.01$). Figure 25 showed that group A. m and HF had significantly higher fecal energy output ratio than that of the CD group ($p < 0.05$), while no significant difference was observed between A. m and HF groups.

Body composition of mice in HF group and A. m group was measured after five months of *A. muciniphila* administration. No significant difference was observed between HF and A. m groups in both lean mass percentage and fat mass percentage. Data collected from metabolic cages showed the distance travelled of each mouse accumulated in one day. HF group and A. m group showed a similar level of average distance with no significant difference, which were 25431.7 ± 9387.4 cm/day per mouse and 23681.6 ± 7181.2 cm/day per mouse respectively.

Safety of *A. muciniphila* administration was evaluated by measuring liver and kidney toxicity. Serum AST, ALT and GGT of mice in CD, HF and A. m groups were compared and presented in **Table 5**. No significant difference was noted among three groups in AST, ALT and GGT as liver toxicity indicators. CD, HF and A. m groups showed a similar level of serum BUN ($p > 0.05$) in kidney function test.

CRP levels of mice in three groups were tested at the end of 6-month treatment. Figure 26 showed that there were no differences among CD, HF and A. m groups.

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Discussion

In the beginning of this study, mice were randomly assigned to groups with control diet, high-fat diet and high-fat diet with *A. muciniphila* administration. When comparing body weight of mice in HF group and A. m group, it was obvious that mice treated with *A. muciniphila* had lower body weight and slower body weight gain. These two groups started with no difference of body weight gain in the first two months, while the difference gradually increased and became significant after 46 days. However, the significance between HF and A. m groups disappeared during the last three months of this study. This finding indicated that the supplementation of *A. muciniphila* had ability to reduce body weight gain, but the benefit might be short-lived and may be reversed by other factors. Schneeberger et al. reported that abundance of *A. muciniphila* in DIO mice decreased significantly with time. The HF diet lowered *A. muciniphila* population by about 10,000 folds than its initial population. In addition, *A. muciniphila* population also had a decrease of 100 folds in mice fed with CD diet for 4 months (104). Thus, it was hypothesized that population of *A. muciniphila* in GIT reduced slowly with time. Furthermore, a diet with high fat level damaged *A. muciniphila* abundance even more. Aging may be another factor that affected the abundance as well. When *A. muciniphila* was first fed to the mice, it had a consistent viability of 10^9 CFU, which could be enough to induce beneficial effects to control body weight gain in mice; however, the benefits were reduced because this viability was not sufficient enough to compensate for the significant loss of *A. muciniphila* abundance induced by long-term high fat feeding and aging. As a result, it suggested the dosage of *A. muciniphila* is highly important in delivering and preserving the benefits of *A. muciniphila* to the host. Moreover, it was found that mice had varied responses when supplemented with *A. muciniphila*. The body weight gain in A. m group had large variation: the lowest body weight gain was 16.54 g while the highest was 30.06 g. Therefore, it indicated

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that the effect of probiotic supplementation varied individually and other genetic or environmental factors may play a role in determining the beneficial effects of probiotic supplementation.

Food intakes (grams) of grouped mice showed that mice in HF and A. m groups were similar, which suggested that *A. muciniphila* had no influence on mice's appetite for food. After further calculation of calories intake, it was shown that mice in CD, HF and A. m groups consumed similar calories each day. These results suggested that mice did not adjust the energy content of food by consuming less volume of HF food, and HF intake was not affected by *A. muciniphila* consumption.

Mice in CD group had the lowest level of blood glucose while mice in A. m group presented lower values when compared to mice in HF group during the whole period of treatment. Additionally, blood glucose responses in GTT demonstrated the ability of A. m to improve glucose tolerance and consequently bring blood glucose back to normal level. This result supported the findings reported by Everard et al. (78). The improvement was further confirmed by calculation of AUC based on glucose tolerance test results. AUC of A. m group was similar to that of the CD group, while AUC of HF group was at a significantly higher level comparing to both CD and A. m groups. Inspired by this significant difference of AUC between A. m and HF groups, HOMA-IR was calculated by using fasting insulin and fasting blood glucose values. HOMA-IR of HF group was significantly higher than CD group while the significance was diminished after supplementation of *A. muciniphila* for six months. In conclusion, the administration of A. m was able to lower fasting blood glucose and reduce insulin resistance when compared to HF group. An increase of *A. muciniphila* dosage might be able to further improve on blood glucose, insulin levels and reduce insulin resistance.

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Fecal energy output, body composition, and activity levels were analyzed to evaluate the possible beneficial effects of *A. muciniphila* treatment. It was expected to see higher fecal energy content from mice in A. m group. However, the calculated energy per gram of fecal sample only showed a difference between CD and HF group. Therefore, the energy intake/ output ratio was determined as a better expression of actual energy utilization. Results revealed a significant difference between CD and both HF and A. m groups, but A. m and HF groups were not different. The trend of higher output ratio in A. m group could indicate the effect of A. m in altering energy absorption and excretion. However, it was suspected that 24hr fecal sample may not be sufficient to obtain a statistical significance in fecal energy measurement. In addition, mouse fecal pieces were collected per cage instead of individually, which might have contributed to inaccurate representation of output ratio for each mouse based on intake. Further collection of larger quantity of fecal sample, such as 48-hr samples collection, from each mouse individually could provide a better estimate of fecal energy output.

In order to investigate whether *A. muciniphila* administration with a HF diet would positively affect body composition, body composition of mice in HF and A. m groups were measured at the fifth month of treatment using echo MRI machine. The similar percentage of lean and fat content between HF and A. m groups indicated that the administration of *A. muciniphila* was not able to alter mouse body composition. Energy expenditure of mice were measured using metabolic cages during the fifth month of treatment. The metabolic cages were able to record the activity level in term of distance traveled of each mouse throughout housing period, which provided more information regarding whether activity level could correlate with different treatment in an indirect manner. Results indicated that mice in A. m group had a non-significantly

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lower level of daily physical activity when compared to mice in HF group. However, the 2000 cm per day difference could form into a significant difference after a long-term treatment of A. m.

Evaluating the safety of A. m consumption on animal models is crucial for establishing safe administration in human consumers. At the end of 6-month treatment, liver and kidney toxicity biomarkers AST, ALT, GTT and BUN were measured to ensure safety and any possible unknown adverse reaction (105-107). The results showed that mice fed with control diet, high-fat diet and A. m fortified high-fat diet had no significant differences in AST, ALT and GTT concentrations, and no difference was found in BUN concentration as a kidney function parameter as well. These results demonstrated the safety of *A. muciniphila* consumption at the concentration administered to mice. Whether this concentration is also safe for human consumption requires further study with humans.

CRP level is associated with insulin resistance (108) and diabetes mellitus (109). In insulin-resistant obese individuals, the elevated CRP level parallel with insulin resistance and reduction in CRP level is associated with weight loss, but independent of body mass (110). The concentration of CRP is also considered as an important nonspecific biochemical marker for inflammation, thus CRP has been used as an indicator for the development of chronic diseases and for monitoring responses to treatment for inflammation and infection (111). Results in this study indicated that there was no significant difference among the three groups. Additional tests with higher concentration of *A. muciniphila* administration or for a longer period of time is warranted to further investigate the inflammation conditions.

Conclusion

The current study demonstrated that there were beneficial effects of *A. muciniphila* administration on glucose homeostasis on high fat induced obesity. With six months feeding of a

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HF diet with added *A. muciniphila* intervention, there was no alteration of body weight and body composition, although there was a significant effect on reduced body weight gain at the earlier stage of treatment. The diminishing effect was clearly observed as mice aged and consumed high fat diet continuously. This led to a speculation that aging and high fat feeding had more negative effects than what *A. muciniphila* could improve with current dosage. The most consistent effect was observed in improving fasting blood glucose and glucose tolerance. A. m group had lower average levels of these parameters when compare to HF group in a consistent manner. It was demonstrated that *A. muciniphila* could maintain the long-term sustainable effect of improving glucose tolerance as oppose to the suppression of weight gain at the end of sixth month. Furthermore, GTT test and HOMA-IR as indicators for pancreatic function, did show significant improvement in *A. muciniphila* treated group, which had similar observation as low fat diet control group. This further shows plausible long-term effect on improving glucose homeostasis by *A. muciniphila*. In addition, the safety of *A. muciniphila* supplementation to the mice at current concentration has been demonstrated in this study. These results would provide guidelines to future studies with human subjects.

Future direction

In current study, the administration of *A. muciniphila* showed a slower weight gain trend when compared to HF group, even though a significant difference was only maintained for approximately one month. For future studies, energy intake and exertion, physical activity levels and basal metabolic rate during the time when body weight difference is significant should be measured in order to identify the factors that contribute to the difference. There were evidences showing a dramatic decrease of *A. muciniphila* population associated with high fat feeding and aging; therefore, future study should modify the dosage or frequency of *A. muciniphila* supplementation. The dosage might be adjusted depending on the on-set of body weight gain and

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age in order to reverse the loss of *A. muciniphila* in the host gut. This dosage information may be extrapolated to human studies before a recommendation for human supplementation can be reached.

Based on energy expenditure recorded in metabolic cages, mice in A. m group had lower activity level when compared to mice in HF group regarding their distance travelled in 24 hours. When less physical activity was accumulated for months, it might diminish the benefits of *A. muciniphila* consumption. This might be one of the factors contributed to the disappearance of significant difference of body weight between the two groups and similar body composition during the later period of treatment. If mice in A. m group could increase their activity level to the level of mice in HF group, it was spectulated that the effects of *A. muciniphila* would be more significant. Thus, it is hypothesized that increasing physical activity could enhance the beneficial effects of probiotic supplementation. This hypothesis should be examined in future studies. In addition, the future use of metabolic cages for each individual mouse and for the entire study period could provide information to reveal different reaction of individual mouse to probiotic supplementation, including difference in food intake, energy expenditure and fecal energy content.

The next area to focus is regarding the formulation and delivery of strain *A. muciniphila*. According to prebiotic screening test, it was clear that the incorporation of IMO as prebiotic significantly promoted the growth of *A. muciniphila.* This finding could be used to adjust formulation of potential *A. muciniphila* supplementation products. The mixture of IMO and *A. muciniphila* could easily enhance its viability, which will be more cost-effective instead of manufacturing *A. muciniphila* with high CFU. In addition, this strain requires additional encapsulation in order to guarantee sufficient cell viability through GIT.

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Since the safety of consuming *A. muciniphila* for six months has been demonstrated, the future study should evaluate the benefits of *A. muciniphila* supplementation in human subjects. Human gut has different microbiome composition from research animals, and more environmental factors could be involved in the balance of gut microbiome. Thus, clinical trials with human volunteers are warranted to examine effects of probiotic intake on host health. Clinical studies on *A. muciniphila* are still very limited. This study provided evidences that supplementation of *A. muciniphila* may help with body weight control and better improvement on glucose homeostasis. In future clinical studies, it is recommended to encapsulate *A. muciniphila* with IMO with extrusion method to make sure minimal viability loss through GIT. The mechanism of *A. muciniphila* affecting the host could be further studied using different targeted groups by consuming *A. muciniphila* with customized dosages, such as obese individuals, patients with T2D or patients with metabolic syndrome. The possible interactions of *A. muciniphila* with microbiome of the host could be further analyzed, such as comparing the alteration of gut microbiome, screening strains or metabolites for possible symbiotic effects, evaluating benefits of weight management through energy balance, lipid metabolism and nutrient harvest, investigating benefits of insulin and glucose homeostasis, analyzing improvement on gut barrier function such as intestinal permeability and metabolic endotoxemia.

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FIGURES AND TABLES

Table 1. Quantitative determination of BSH activity of five selected probiotic strains

Probiotic strain	Total protein (μg/mL)	Total activity (U/g total protein)
A. muciniphila	356.5 ± 24.8	32.3 ± 4.7[c]
L. acidophilus	334.5 ± 48.0	33.9 ± 4.2[c]
L.reuteri	364.0 ± 33.8	143.6 ± 7.4[a]
L. plantarum	351.5 ± 69.0	96.3 ± 15.2[b]
B. coagulans	376.1 ± 61.0	20.9 ± 4.0[c]

(Values with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.)

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Table 2. Loss of viability after encapsulation procedure and bypass simulated GIT

Loss during encapsulation			Loss bypass GIT	
Spray-dried	Freeze-dried	Extruded	Freeze-dried	Extruded
2.58 ± 0.20 Logs	0.32 ± 0.03 Logs	1.13 ± 0.03 Logs	4.75 ± 0.02 Logs	0.10 ± 0.03 Logs

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Table 3. HOMA-IR of groups CD, HF and A. m after 6-month of treatment

	Fasting blood glucose (mg/dL)	Fasting insulin (ng/mL)	HOMA-IR
CD	161.2 ± 8.9^{a}	2.2 ± 0.8^{a}	22.5 ± 8.6^{a}
HF	217.4 ± 30.2^{b}	4.6 ± 1.1^{a}	64.3 ± 15.5^{b}
A. m	177.2 ± 12.9^{ab}	3.9 ± 2.0^{a}	44.5 ± 22.7^{ab}

(Values in each column with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.)

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Table 4. Body composition of mice in groups HF and A. m

	Fat (g)	Lean (g)	Fat (%)	Lean (%)
HF	21.8 ± 2.0	22.9 ± 2.2	45.1 ± 3.6	47.9 ± 4.6
A. m	21.3 ± 2.9	21.5 ± 2.0	45.3 ± 2.8	46.0 ± 2.6

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Table 5. Safety of *A. muciniphila* administration by evaluating liver and kidney toxicity

	Liver			Kidney
	AST (U/L)	ALT (U/L)	GGT (U/L)	BUN (mg/dL)
CD	4.9 ± 1.4	61.6 ± 21.8	1.7 ± 0.3	19.8 ± 2.1
HF	9.6 ± 5.6	42.6 ± 40.9	1.7 ± 0.9	26.3 ± 5.3
A. m	6.4 ± 3.5	78.4 ± 14.7	1.7 ± 0.4	21.3 ± 1.6

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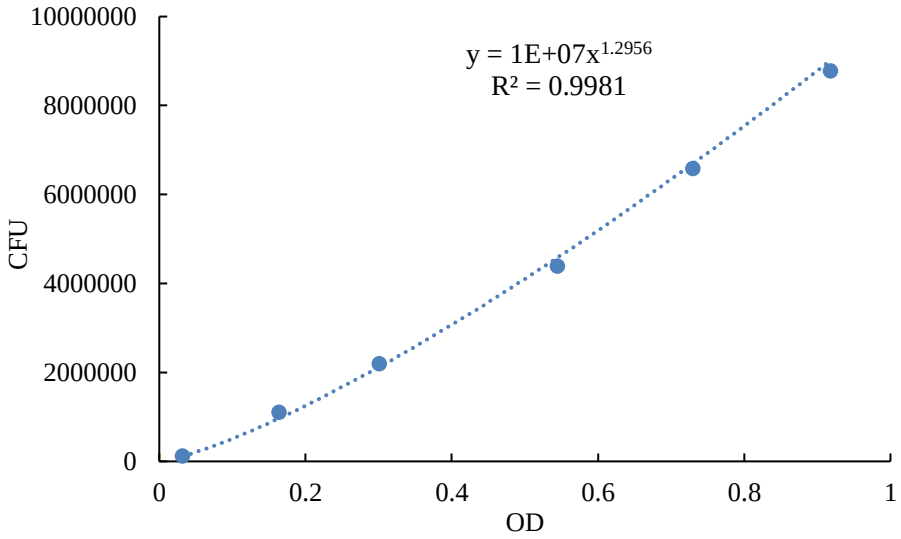

Figure 1. Turbidity standard curve determination. *A. muciniphia* cultures were incubated at 37 °C for 10 hrs, and final optical density (OD) values were measured at 595 nm using spectrophotometer. Colony forming unit (CFU) was evaluated using pour-plating method at the same time. The verification equation was established based on OD values and their corresponding CFU/200 uL.

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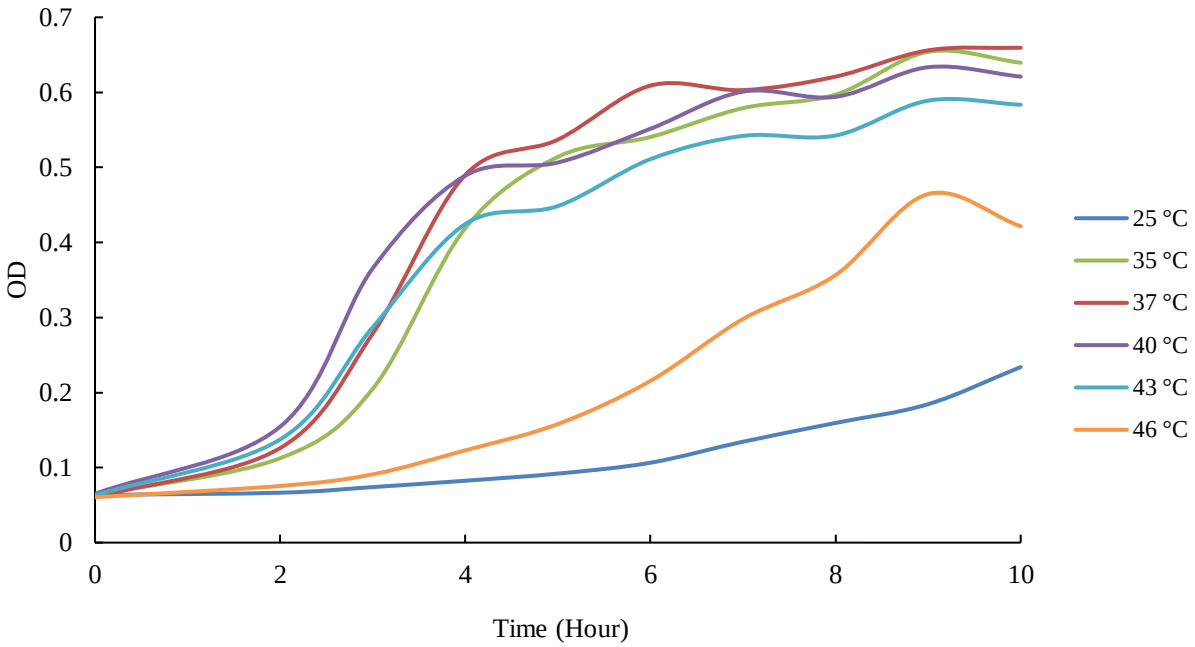

Figure 2. Growth curves of *A. muciniphila* under various temperatures. Cultures with same concentration of *A. muciniphila* were incubated for 10 hrs in incubators set with temperature at 25 °C, 35 °C, 37 °C, 40 °C, 43 °C and 46 °C. Final culture OD values were measured at 595 nm at hourly interval.

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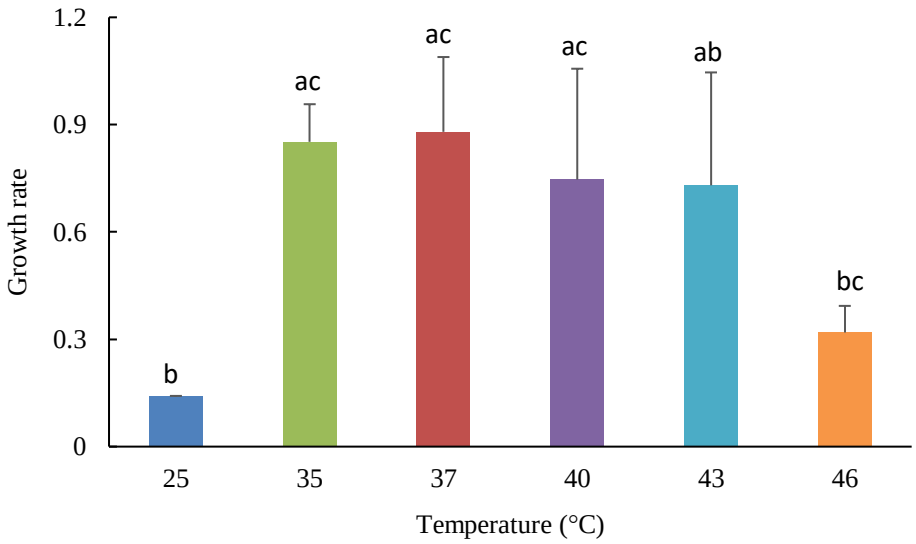

Figure 3. Effects of temperature on exponential growth rate of *A. muciniphila*. Cultures with *A. muciniphila* were incubated at various temperatures for 10 hours.Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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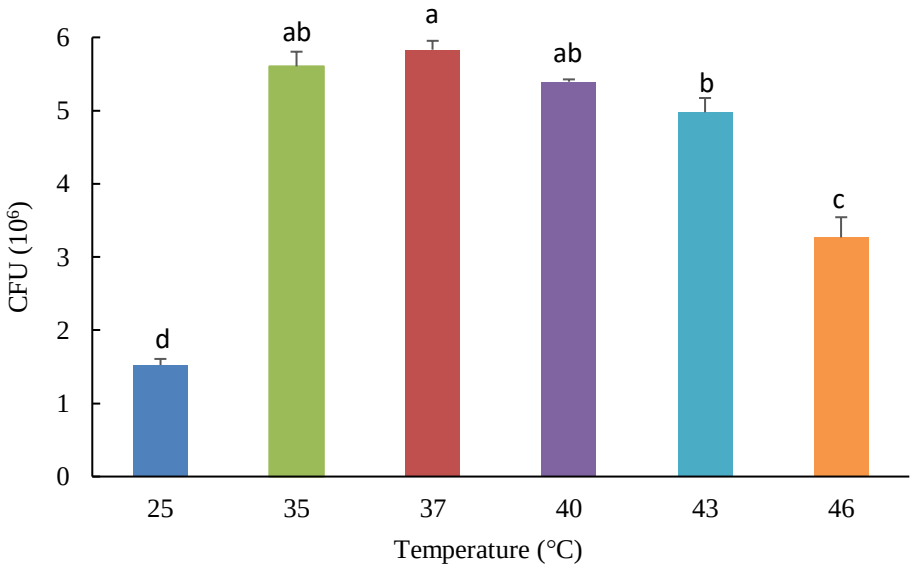

Figure 4. Effects of temperature on final viabilities (CFU/mL) of *A. muciniphila*. Cultures with *A. muciniphila* were incubated at various temperatures for 10 hours.Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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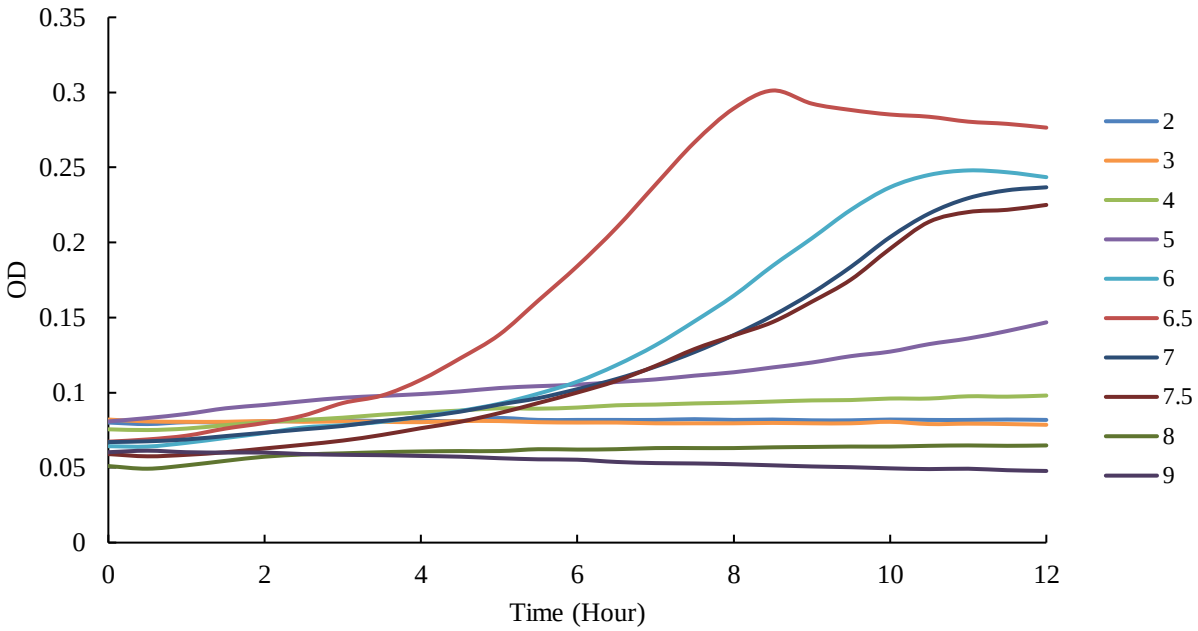

Figure 5. Growth curves of *A. muciniphila* at various pH levels. Tubes with medium adjusted to pH level of 2, 3, 4, 5, 6, 6.5, 7, 7.5, 8, and 9 were inoculated with same concentration of *A. muciniphila*. Cultures were incubated for 12 hrs at 37 °C and final culture OD values were measured at 595 nm at hourly interval.

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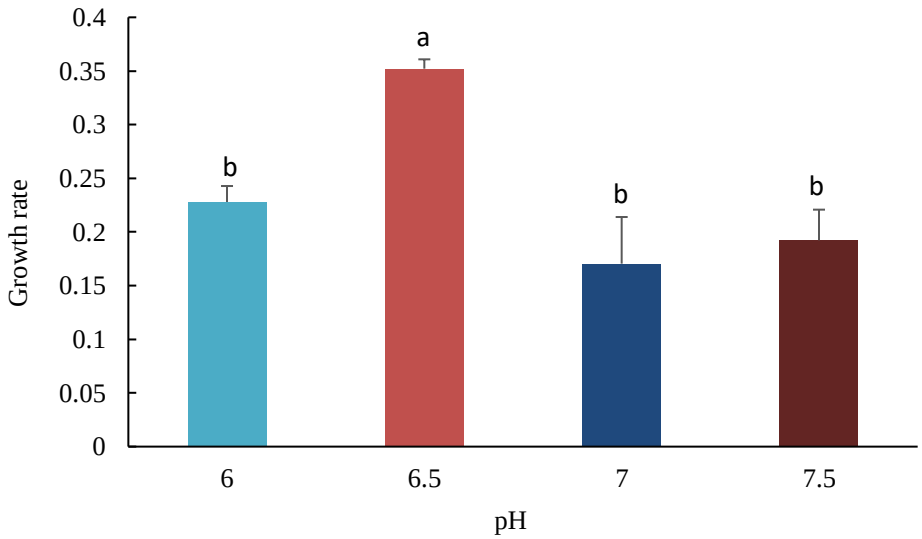

Figure 6. Effects of pH levels on exponential growth rate of *A. muciniphila*. Cultures with *A. muciniphila* were incubated at 37 °C for 12 hours Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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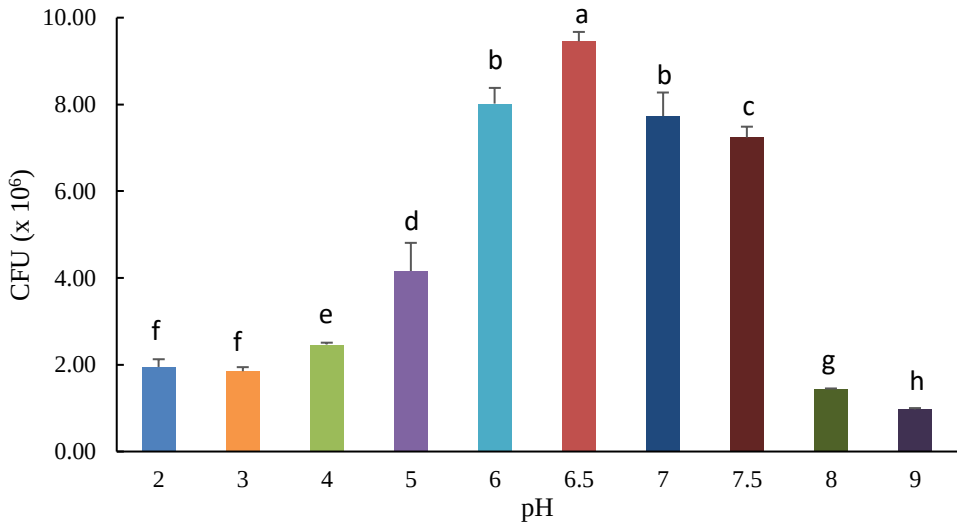

Figure 7. Final viabilities of *A. muciniphila* (CFU/mL) at various pH levels. Cultures with *A. muciniphila* were incubated at 37 °C for 12 hours.Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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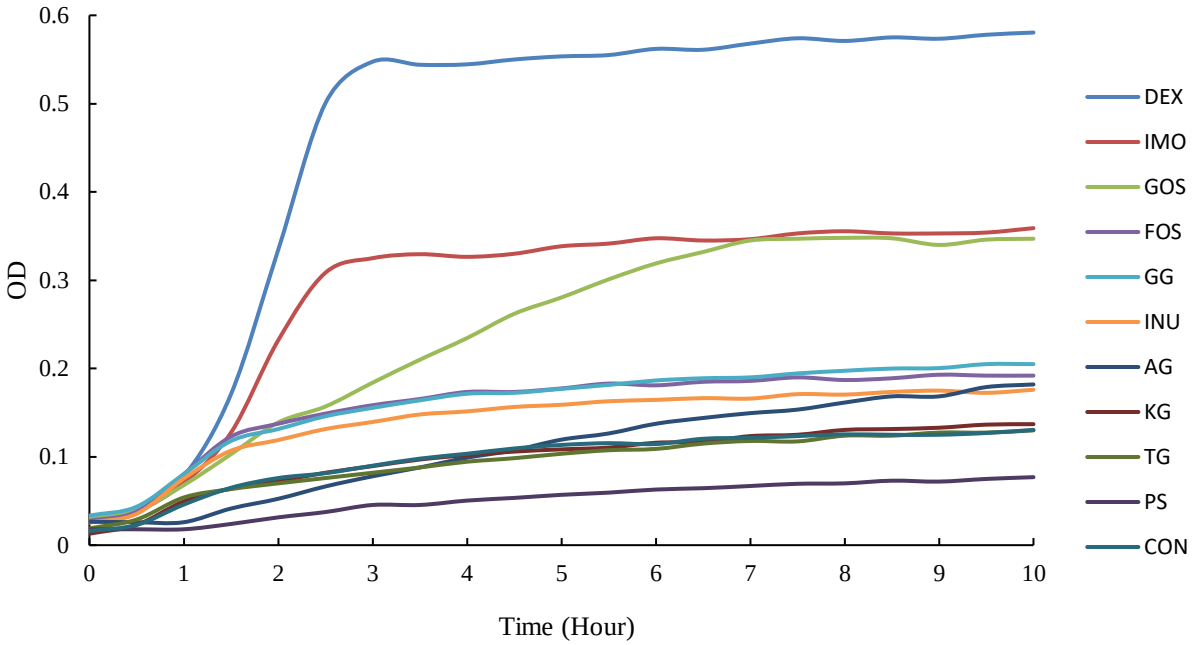

Figure 8. Growth curves of *A. muciniphila* in medium adjusted with prebiotics. Tubes with medium adjusted by 0.2% w/v prebiotic ingredients including: isomaltooligosaccharide (IMO), inulin (INU), fructooligosaccharide (FOS), galactooligosaccharide (GOS), guar gum (GG), acacia gum (AG), karaya gum (KG), tragacanth gum (81), and potato starch (PS) were inoculated with same concentration of *A. muciniphila*. Cultures were incubated for 10 hrs at 37 °C and final culture OD values were measured at 595 nm at hourly interval.

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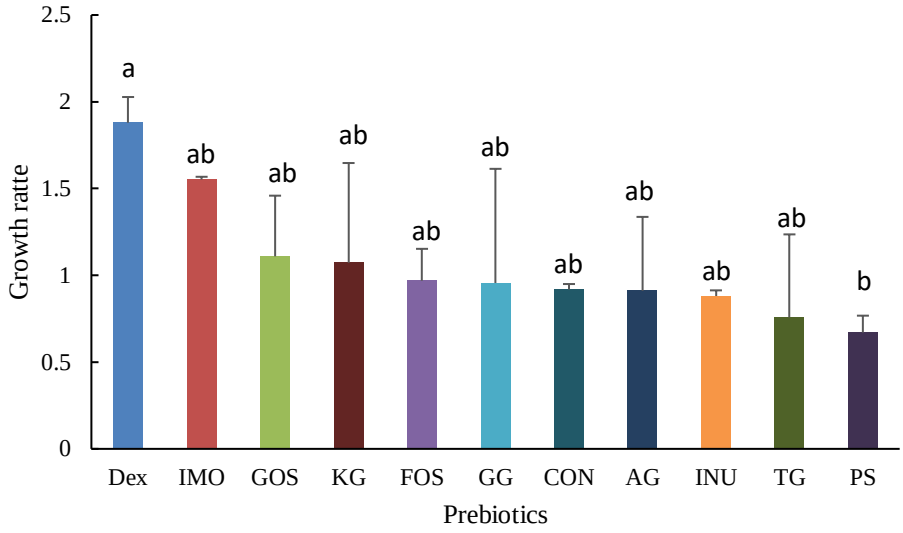

Figure 9. Effects of prebiotics on exponential growth rate of *A. muciniphila.* Cultures with *A. muciniphila* were incubated at 37 °C for 10 hours. Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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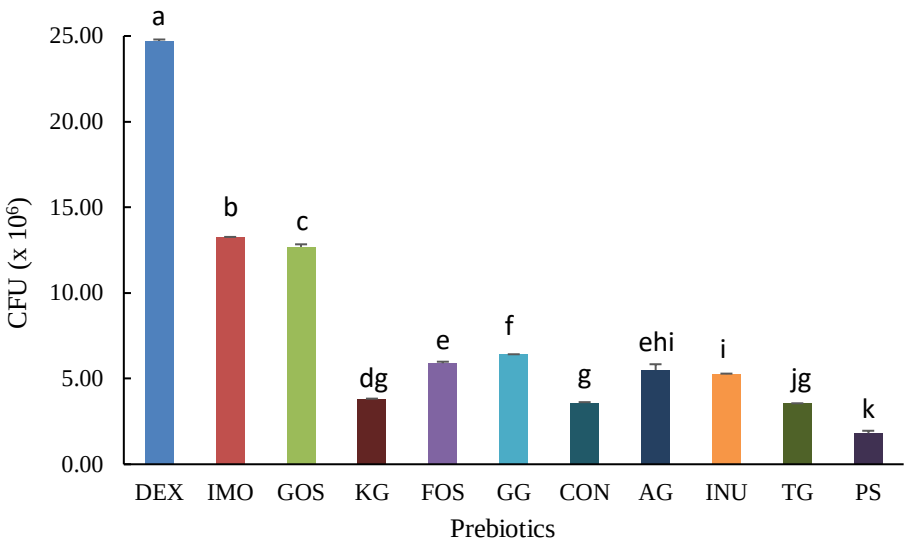

Figure 10. Effects of prebiotics on final viabilities of *A. muciniphila* (CFU/mL). Cultures with *A. muciniphila* were incubated at 37 °C for 10 hours. Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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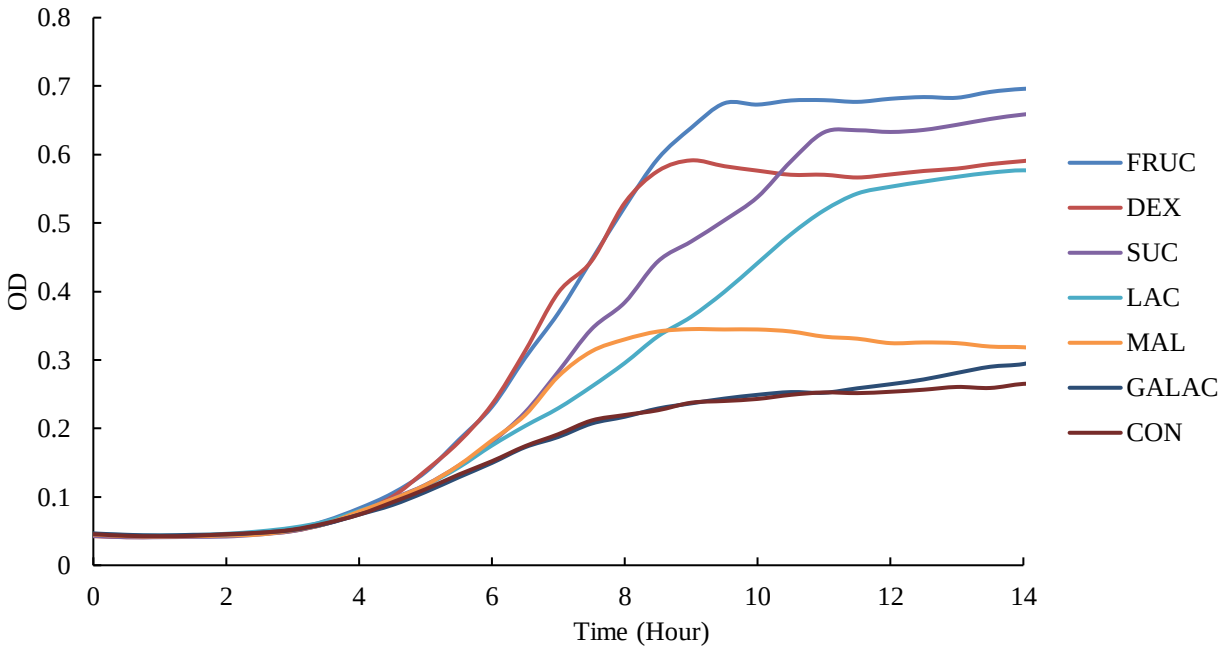

Figure 11. Growth curves of *A. muciniphila* in medium adjusted with sugars. Tubes with medium adjusted by 0.2% w/v sugars including: fructose (FRUC), galactose (GALAC), lactose (LAC), sucrose (SUC), dextrose (DEX) and maltose (MAL) were inoculated with same concentration of *A. muciniphila*. Cultures were incubated for 14 hrs at 37 °C and final culture OD values were measured at 595 nm at hourly interval.

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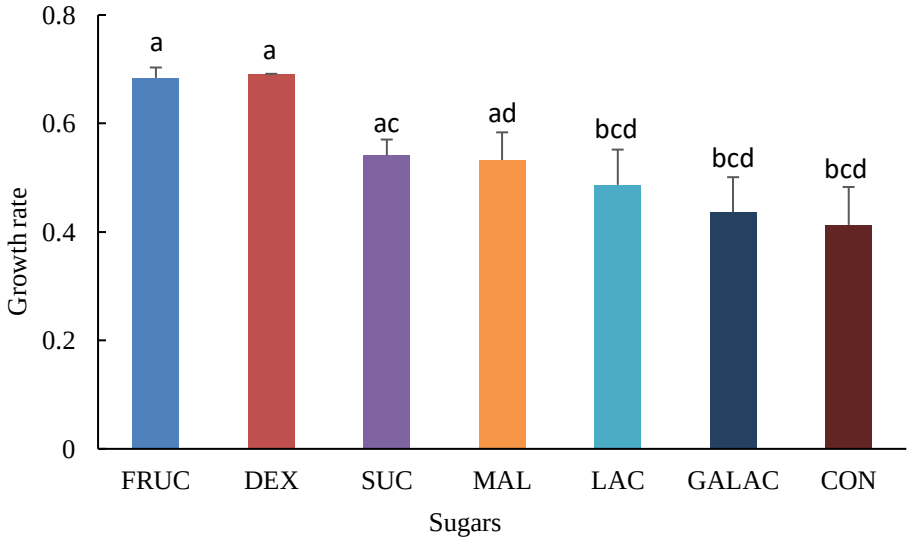

Figure 12. Effects of sugars on growth rate of *A. muciniphila*. Cultures with *A. muciniphila* were incubated at 37 °C for 14 hours. Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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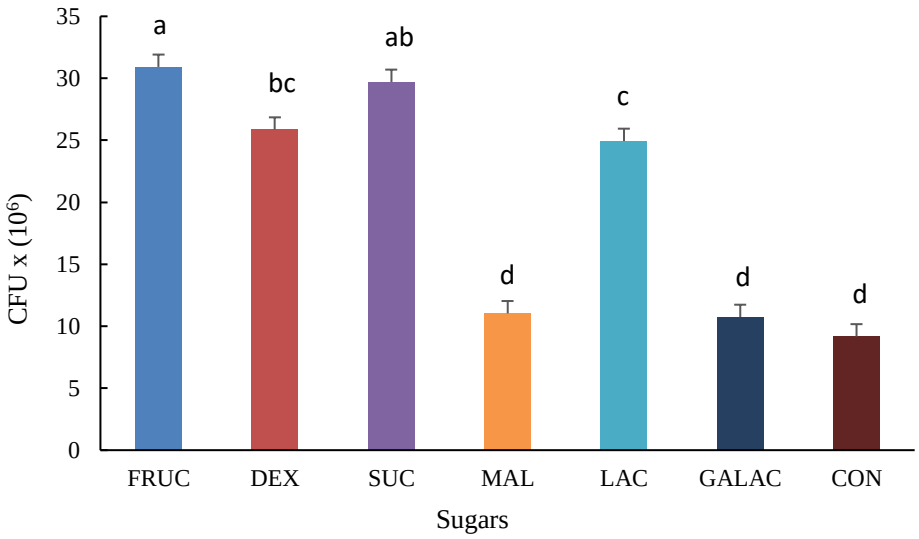

Figure 13. Effects of sugars on final viabilities of *A. muciniphila* (CFU/mL). Cultures with A. muciniphila were incubated at 37 °C for 14 hours.Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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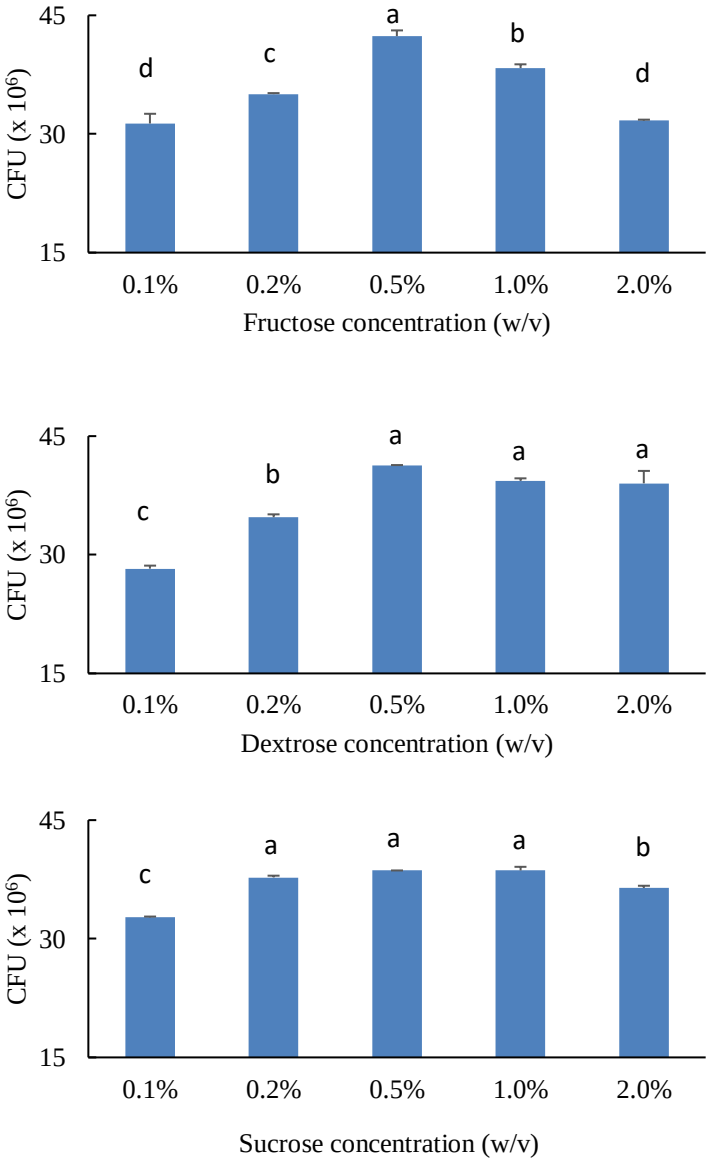

Figure 14. Effects of sugar concentrations on viabilities of *A. muciniphila* (CFU/mL). Tubes with medium adjusted by varied sugar concentrations including: 0.1%, 0.2%, 0.5%, 1.0% and 2.0% were inoculated with same concentration of *A. muciniphila*. Cultures were incubated for 14 hrs at 37 °C and final culture OD values were measured at 595 nm at hourly interval. Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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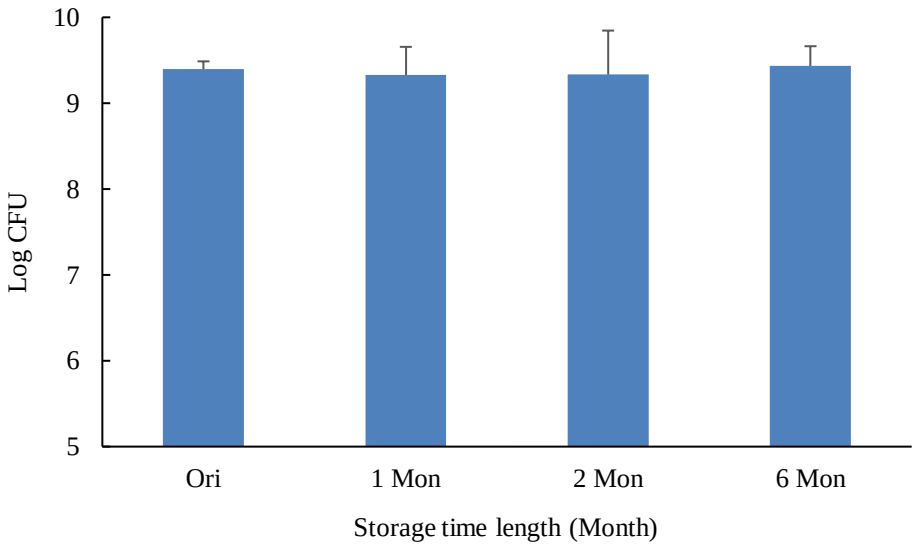

Figure 15. Efficacy of storage method for *A. muciniphila*. *A. muciniphila* was collected from culture medium, suspended and stored in 50% glycerol medium stored for 6 mons. Final CFU was evaluated using pour-plating method.

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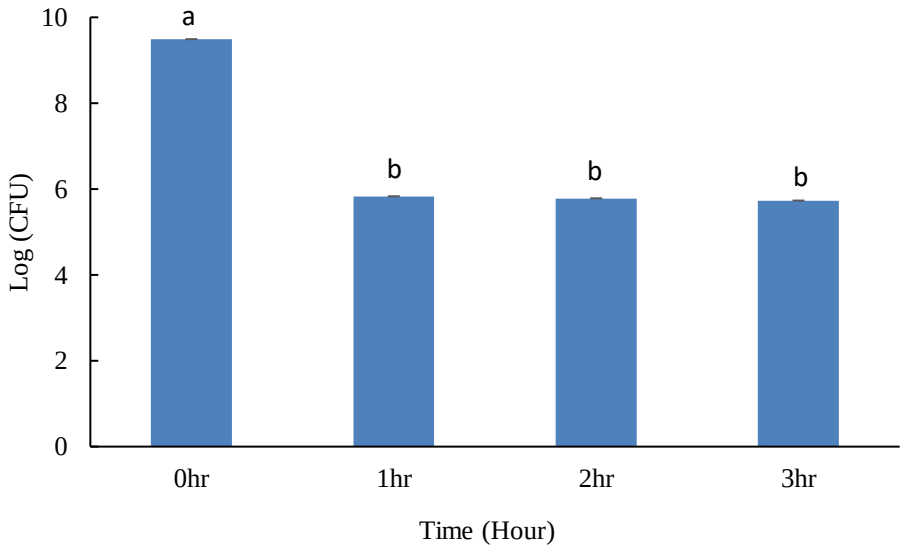

Figure 16. Acid tolerance ability of *A. muciniphila*. *A. muciniphila* was incubated in simulated gastric solution at 37 °C for 3 hrs, and culture CFU was evaluated using pour-plating method at hourly interval. Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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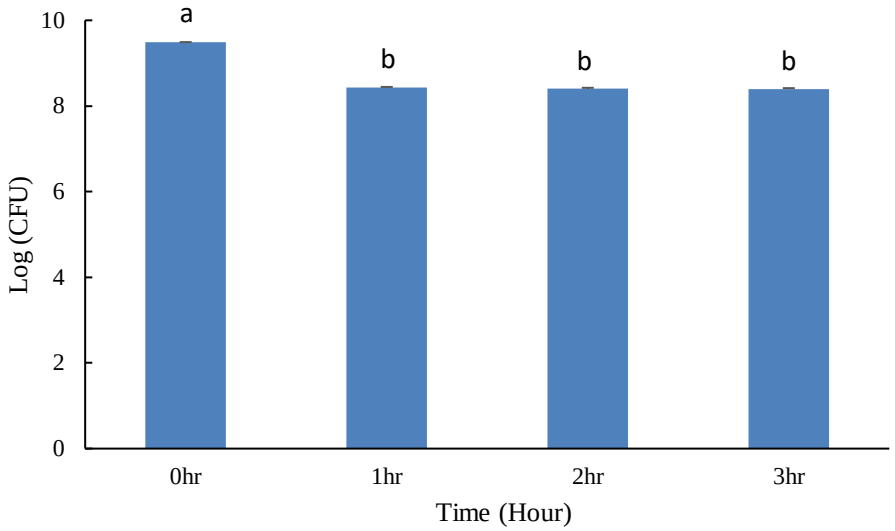

Figure 17. Bile salts tolerance ability of *A. muciniphila*. *A. muciniphila* was incubated in 0.3% bile salts BHI medium at 37 °C for 3 hrs, and culture CFU was evaluated using pour-plating method at hourly interval. Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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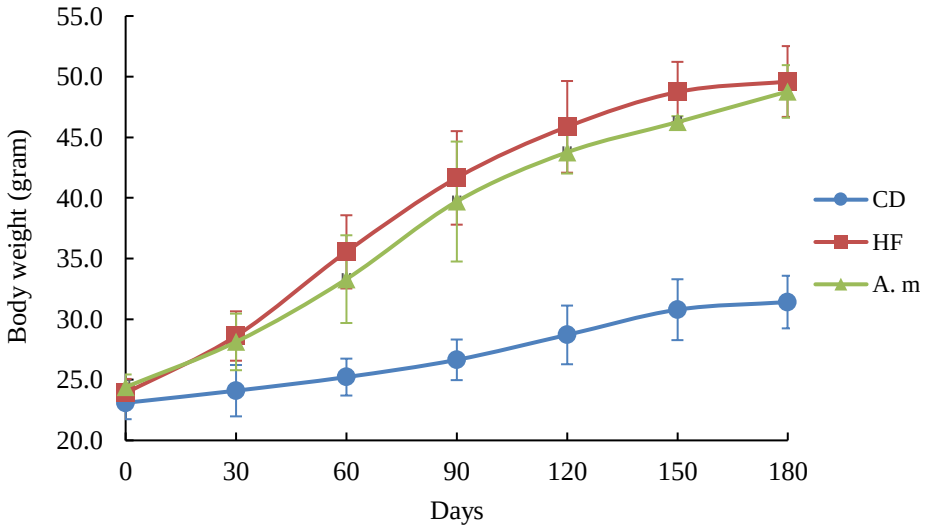

Figure 18. Mice body weight (gram) during 6-month of *A. muciniphila* administration.

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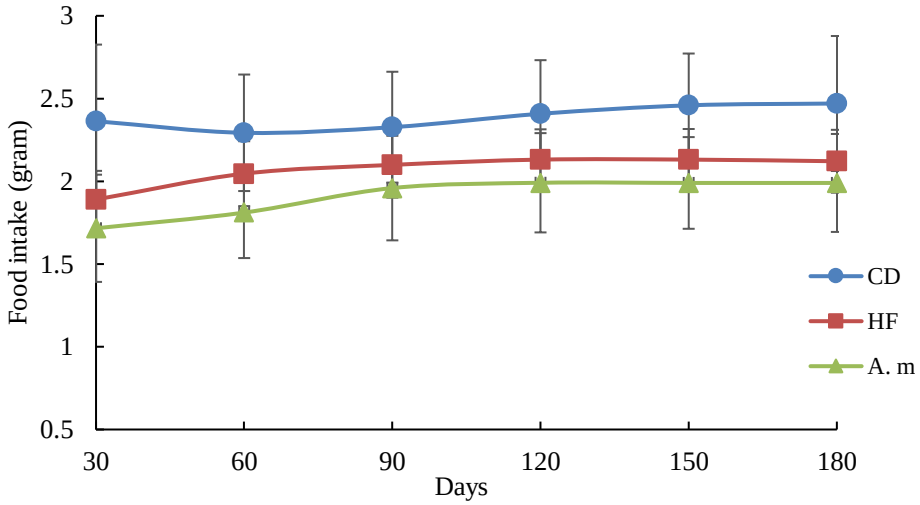

Figure 19. Average food intake (grams/mice) of all groups during 6-month treatment

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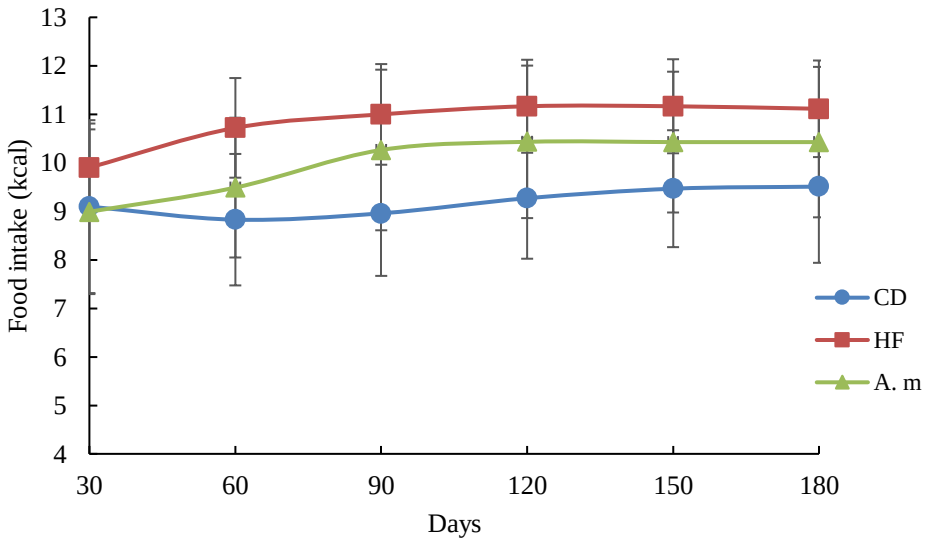

Figure 20. Average food intake (kcal/mice) of all groups during 6-month treatment

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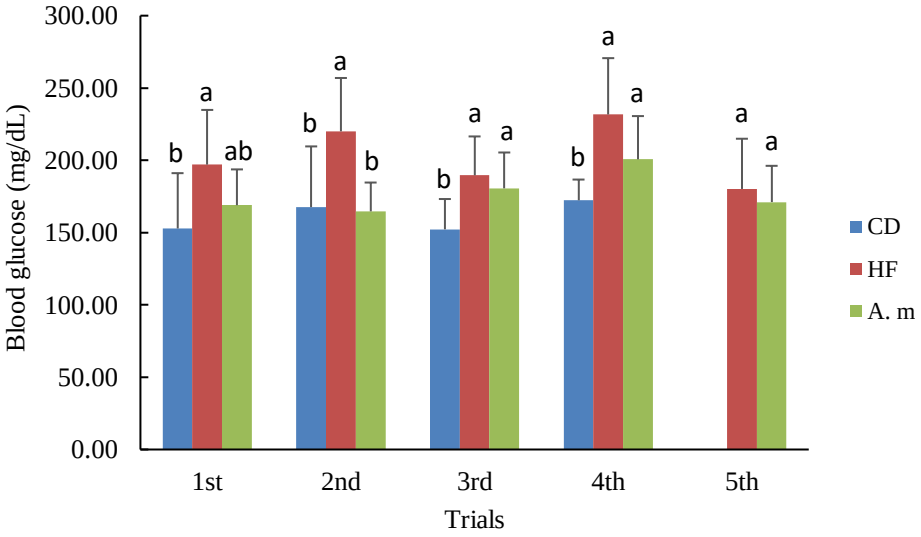

Figure 21. Average fasting blood glucose (mg/dL) of all groups during 6-month treatment. The 1st trial corresponded to the first month, the 2nd trial corresponded to the second month, the 3rd trial corresponded to the fourth month, 4th trial corresponded to the fifth month and the 5th trial corresponded to the sixth month. Statistical significance was analyzed within each trial: bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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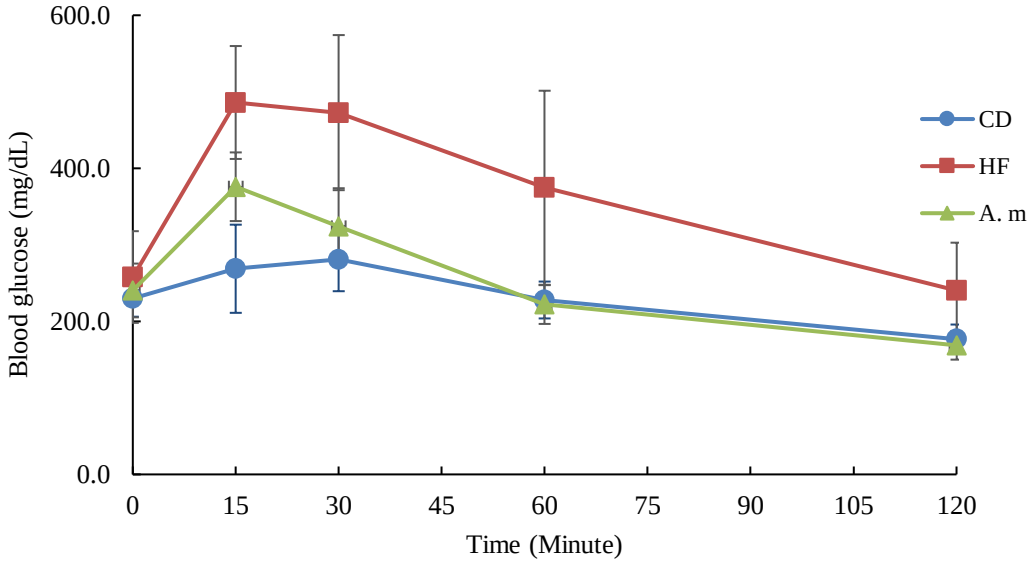

Figure 22. Glucose tolerance test on groups CD, HF and A. m.

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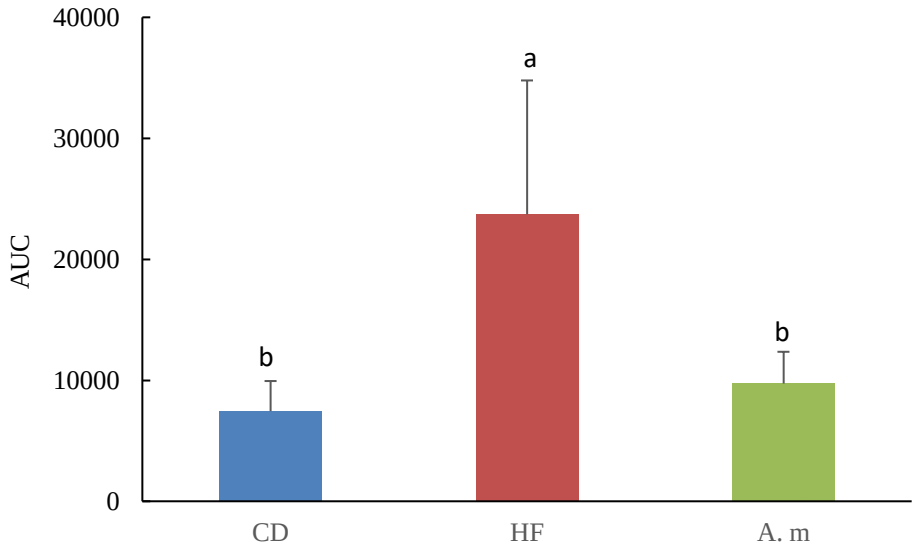

Figure 23. Area under curve (AUC) of glucose tolerance test in groups CD, HF and A. m. Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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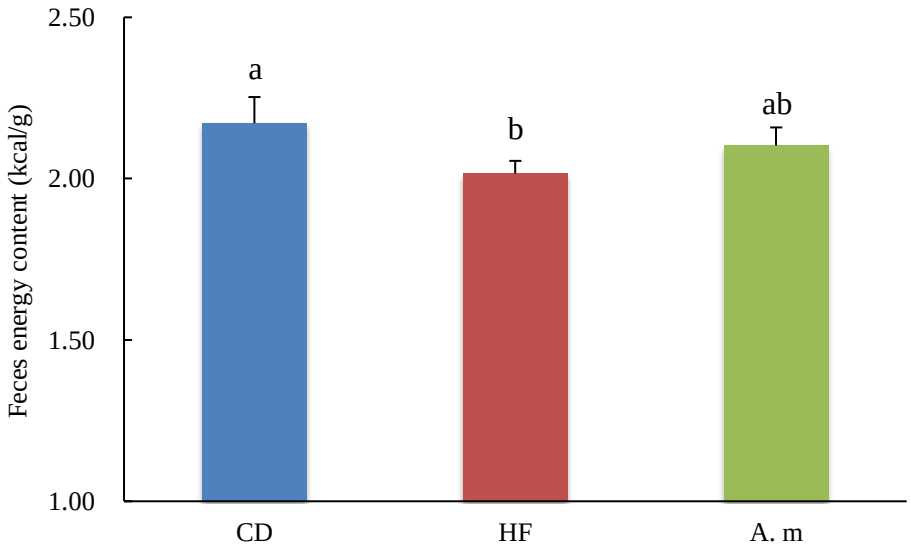

Figure 24. Fecal energy content (kcal/g) of groups CD, HF and A. m. Fecal energy content was measured using Bomb Calorimeter. Each group included two cages, and each cage was measured in duplicates. Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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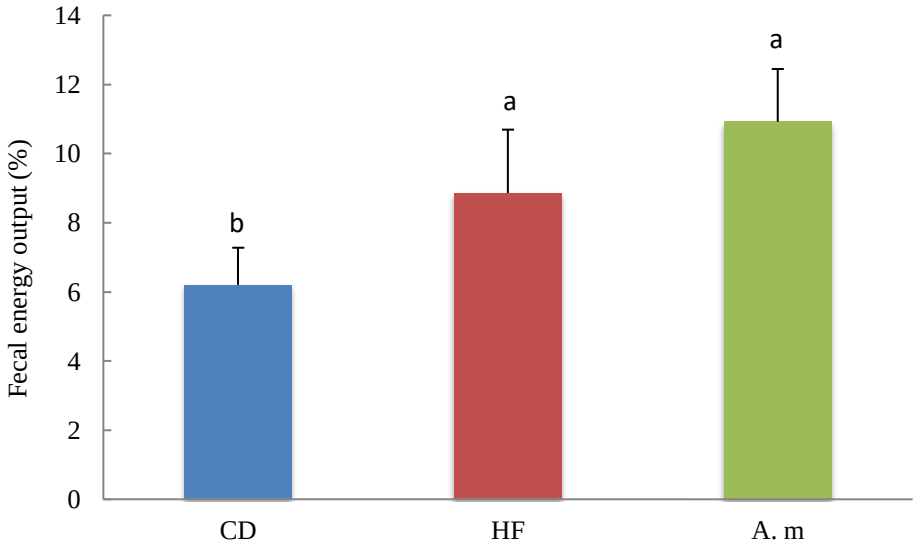

Figure 25. Fecal energy output ratio (%) of groups CD, HF and A. m. Fecal energy output ratio was calculated using formula: output ratio = total feces energy / total energy intake × 100%. Bars with different letters were significantly different from each other at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

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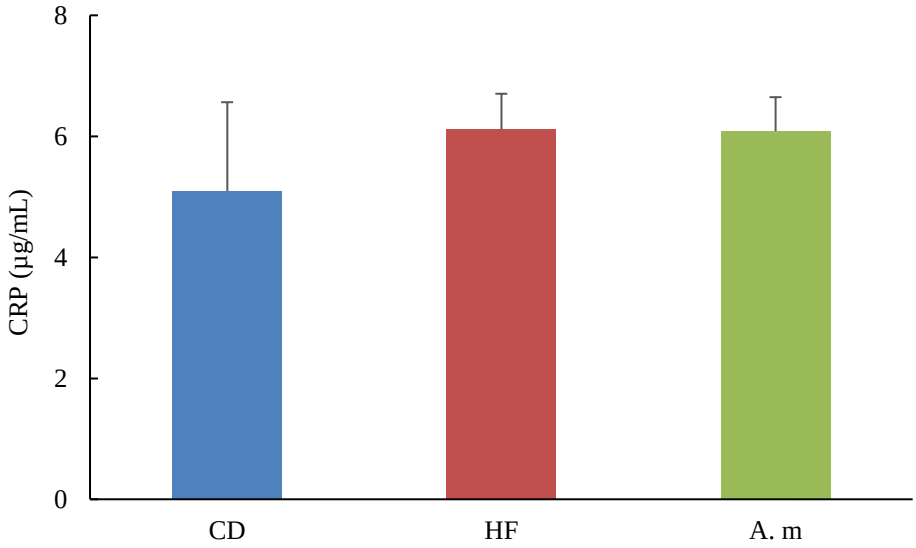

Figure 26. C-reactive protein (µg/mL) of groups CD, HF and A. m. Mouse serum CRP was measured after 6-month of *A. muciniphila* administration

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ABSTRACT

THE EXPLORATION OF CHARACTERISTICS OF *AKKERMANSIA MUCINIPHILA*, AND EVALUATION OF ITS PROBIOTIC EFFECTS ON DIABETES BY USING C57BL/6 MOUSE MODEL

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The prevalence of type 2 diabetes (T2D) is increasing continuously worldwide. The incidence of T2D is highly correlated with poor diet, physical inactivity and occurrence of obesity. Recent studies reported possible interaction between T2D and gut microbiome, which revealed different composition of gut microbiome. A specific strain *Akkermansia muciniphila* (*A. muciniphila*) was reported with significantly lower abundance even prior to the incidence of diabetes. This study was designed to discover the optimal growth conditions based on characteristics of *A. muciniphila* and determine appropriate encapsulation method to ensure sufficient bioactivity through GIT. C57BL/6 mice model was further used to explore in-vivo benefits of *A. muciniphila* supplementation. Results showed medium adjusted to pH 6.5 could promote the best growth of *A. muciniphila* at 37 ℃. Isomaltooligosaccharide was proved to be the best prebiotic ingredient. In order to manufacture in large quantity, fructose, sucrose and dextrose were able to promote the highest viability, especially with the concentration of 0.5%. Bile salt hydrolase activity was discovered in *A. muciniphila* with an intermediate level, which helped to improve its tolerance to digestion system with high concentration of bile salts. Extruded beads of *A. muciniphila* was shown to be an outstanding protection through simulated stomach and GIT.

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After diet induced mice were supplemented with *A. muciniphila* for six months, results showed no significant change in mice body weight or daily food intake; however, improvement on glucose homeostasis was noticed including fasting blood glucose and glucose tolerance. In addition, mice supplemented with *A. muciniphila* showed a higher energy loss in feces while no difference was found in energy expenditure or body composition. *A. muciniphila* has been proved to be safe after a supplementation for six months. Future studies would investigate the benefits of *A. muciniphila* with increased dosage and its possible mechanism in the host.

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