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INTERACTION BETWEEN DIETARY COMPONENTS AND GUT MICROBIOTA AND ITS IMPLICATION IN BIOACTIVITIES AND METABOLISM IN COLON

A Dissertation Presented

by

MIN GU

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2018

The Department of Food Science
INTERACTION BETWEEN DIETARY COMPONENTS AND GUT MICROBIOTA AND ITS IMPLICATION IN BIOACTIVITIES AND METABOLISM IN COLON

A Dissertation Presented

by

MIN GU

Approved as to style and content by:

Hang Xiao, Chair

David A. Sela, Member

Klaus Nüsslein, Member

Eric A. Decker, Department Head
Department of Food Science
DEDICATION

I dedicate this thesis to

my beloved families and friends

for their constant support and unconditional love

and to my respected professors

for their encouragement and guidance
ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my advisor, Dr. Hang Xiao, for providing me an opportunity to pursue my Ph.D. study in his lab and for his continuous support, his trust, his encouragement, his patience and motivation. He is always like a lighthouse and a model to guide me to the right direction in both my research work and my daily life. Without his help and guidance, I cannot complete this work. Also, I would like to thank my committee members, Dr. David A. Sela, and Dr. Klaus Nüsslein, for their insightful feedback and encouragement.

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ABSTRACT

INTERACTION BETWEEN DIETARY COMPONENTS AND GUT MICROBIOTA AND ITS IMPLICATION IN BIOACTIVITIES AND METABOLISM IN COLON

SEPTEMBER 2018

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The microbial cells that colonize the human body, including mucosal and skin environments, are at least as abundant as our somatic cells and certainly contain far more genes than our human genome. Gut microbiota dysbiosis is associated with the onset and progress of several diseases, like diabetes, obesity, inflammatory bowel disease. One of the environmental factor-diet intervention can modulate the compositions of gut microbiota, which in turn may contribute to altered health outcomes such as changes in the risks of chronic diseases. Orange peel has been a traditional herb in China, and some
of its compounds has shown health benefits. However, to our knowledge, limited information is available about the interaction between bioactive dietary compounds in orange peel and gut microbiota. Firstly, we aim to study the metabolic fate of limonin and how it will modify the gut microbiota in mice.

Unchallenged mice were fed a diet containing limonin (0.05% w/w) for 9 weeks. Limonin distribution analysis revealed that most limonin was unabsorbed and persisted to colon. During the 9-week feeding time, mice gut microbiota profile was altered continuously. After 9 weeks’ dietary intervention, diversity of the mouse gut microbiota was significantly enhanced compared with control group. In the meantime, the gut microbiota community structures were markedly distinct between the two groups. Limonin treatment significantly unregulated the abundance of 27 genera and downregulated the level of 5 genera. Based on the analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) database, 11 functional pathways were predicted to be upregulated while 9 functional pathways were downregulated dramatically. Specifically, the functional pathways related to the production of bacterial toxin and the Staphylococcus aureus infection were suppressed dramatically with limonin treatment.

Here we observed that gut microbiota was changed by limonin supplementation. We hypothesized that nobiletin, another major compound from orange peel, could alter mice gut microbiota, which might contribute to host health benefits of nobiletin. In this study, we determined the effects of dietary nobiletin intervention dextran sodium sulfate (DSS)-induced colitis as well as the composition of gut microbiota in mice. The results revealed that nobiletin treatment significantly decreased the severity of colonic inflammation in DSS-treated mice, evidenced by the reduced production of pro-
inflammatory cytokines (i.e., GM-CSF, INF-γ, IL-1β, IL-2, IL-6, KC/GRO, and TNF-α) in the colonic mucosa, increased colon length, and decreased disease activity index and histologic score of inflammation (p<0.05) by nobiletin treatment. Results from next generation sequencing of the fecal microbiota showed that DSS treatment significantly altered the microbial structure of fecal microbiota in mice, and dietary nobiletin treatment trended to reverse these alterations. It was also demonstrated that DSS treatment decreased the relative abundance of Bifidobacterium and Lactobacillus compared with control (unchallenged) group. nobiletin treatment partially reversed the DSS-induced decrease in these two genera in DSS treated mice. DSS treatment significantly increased the abundance of Sutterella and Bilophila, while nobiletin effectively reversed DSS-induced increases in these two genera. Overall, our results for the first time suggested that modification of gut microbiota by dietary nobiletin might contribute to its inhibitory effects against the development of colitis in mice.

Besides, we found that gut microbiota in the colon involved the metabolism of nobiletin and contributed to the anti-inflammation effect of nobiletin. Therefore, we isolated and identified the bacteria with nobiletin metabolizing capacity. Bacteria Isolate G7 was the potential one belong to species Bifidobacterium pseudocatenulatum, which is commonly regarded as probiotic. Except the metabolizing ability, its secretion has strong anti-inflammation effect by blocking the NF-κB pathway in Raw 264.7 cells. Isolate G7 secretion performed strong anti-cancer effect on human colon cancer cell HCT116 by promoting cell apoptosis, arrest cell cycle at G2/M phase and downregulate the gene expression of wnt signaling pathway.
The oral delivery of probiotics to the colon is often difficult because they lose viability when exposed to the harsh conditions in the upper gastrointestinal tract, such as the highly acidic gastric fluids. Properly designed encapsulation technologies can be used to protect probiotics during their transit through the human gut. In this study, an anaerobic probiotic (*Bifidobacterium pseudocatenulatum* G7 or BPG7) was encapsulated within alginate microgels that also contained antacid agents to control their internal pH within the stomach. Probiotic-loaded microgels were exposed to a simulated gastrointestinal tract (GIT) model to establish the impact of gastric and small intestinal conditions on their physicochemical properties and cell viability. In the absence of antacid, no live probiotic cells were detected in the microgels after exposure to gastrointestinal conditions. Conversely, in the presence of antacid, there was only a 1.5 log CFU decrease in cell viability after incubation in simulated gastric fluids for 2 h. After the antacid microgels were then incubated in simulated intestinal fluids, viable probiotic cells were still detected when CaCO$_3$ was used as an antacid but not when Mg(OH)$_2$ was used. Overall, these results indicate that alginate microgels containing CaCO$_3$ as an antacid were the most efficient at protecting the probiotic during passage through the upper GIT. This novel encapsulation technology may be useful for the oral delivery of probiotics to the gut in the form of functional foods or supplements.
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CHAPTER 1
INTRODUCTION

Over millions of years, microbes that have colonized and coevolved with humans have gradually come to help regulate nutrition and metabolism processes and train both innate and adaptive immune responses. are at least as abundant as our somatic cells [1] and certainly contain far more genes than our human genome. An estimated 500–1,000 species of bacteria exist in human body at any one time during the lifespan [2]. Considering the huge number and cohabitation with human, microbe has been recognized as a new organ [3]. Researches have indicated that gut microbiota dysbiosis has been associated with chronic diseases, including obesity[4], diabetes[5, 6], cardiovascular disease [7, 8], inflammatory bowel disease (IBD) [9, 10] and cancer [11]. It’s the lifestyle rather than genetic modulated the microbiota more significant [12]. Among the lifestyle related factors, diet intervention is an important and easy controlled method to help people preserve health or inhibit the onset or attenuate the progress of disease.

Citrus fruit is common on people’s table in North America, and orange skin has been a traditional herb in China. Limonin and nobiletin are major compounds belong to classes furanolactones and flavonoids. Limonin exerted anti-cancer [13-15] and anti-HIV effects [16]. Nobiletin was studied for its anti-cancer, anti-inflammatory effects. While limited microbiota study has been included in neither of the two compounds.

In this dissertation, we studied the metabolic fate of limoin to confirm whether it
can reach colon in rodent animals, and studied the microbiota composition and function to test our hypothesis that dietary limonin intervention could modify mice gut microbiota and help to preserve host wellness. Nobiletin has been proved for the beneficial effects on obesity associated with inflammation in mice [17], while anti-inflammation effect in colitic mice model was rare. Therefore, we studied the anti-inflammatory effect of nobiletin in DSS-induced colitis in mice model. Besides, microbiota was observed to investigate its role in nobiletin’s anti-inflammation effect.

On one hand, dietary component could modify gut microbiota structure, and on the other hand dietary component can be metabolized by gut microbiota residing in gut. Xenobiotic metabolism by gut microbiota will activate, deactivate or toxify the compound. Our lab has found that gut microbiota in the colon could deconjugate the nobiletin metabolite conjugates formed by Phase II metabolism in liver. What’s more, the free form metabolites have distinctively stronger anti-inflammation activity than their parent compound [18], indicating that gut microbiota metabolism activate the activity of nobiletin. For the metabolic ability of gut microbiota is largely unknown. We’re interested in figuring out the bacteria with the deconjugation capacity. Besides, residing bacteria can contribute to host health by its secretion, like anti-inflammation[19].

Our **long-term goal** is to develop diet-based strategies that are safe and cost-efficient alternative approaches to improve human gut health. To reach that goal, the **overall objective** of this project is to study the interaction between gut microbiota and dietary component. Our **central hypothesis** is dietary component could modify gut microbiota composition and function, which might benefit host health. Moreover, nobiletin will be metabolized by specific bacteria living in colon. Our hypothesis has
been cultivated on our lab’s preliminary data regarding the anti-inflammatory efficacy of nobiletin, and stronger anti-inflammation effect of metabolites on cell RAW 246.7.

We will test our central hypothesis and achieve our objective by utilizing the following four **specific aims:**

1. **Determine the gastrointestinal fate of limonin and its effect on mice gut microbiota.**

   Mice will be fed a diet containing limonin (0.05% w/w) for 9 weeks and then sacrificed. Limonin distribution in digestive system is determined LC-MS. GC-MS is used to study the SCFAs in mice cecum to investigate the activities of gut microbiota. Microbiota structure in distal colon will be studied by T-RFLP and 16S rRNA gene sequencing. QIIME will be used to analysis the microbiota data to observe the changes of microbiota diversity and compositions led by dietary limonin treatment. LEfSE will be applied to build cladogram figure and figure out the differentiate taxonomic changes of gut microbiota. PiCRUST will be used to study the functional changes of gut microbiota.

2. **Determine the effect of nobiletin on inflammation and gut microbiota in DSS-induced colitic mice.** Mice colon length will be measures and disease activity index will be recorded to study the inflammation degree of mice with the presence or absence of nobiletin treatment. Histological study will be utilized to study the colon mucosa structure. ELISA will be performed to determine the inflammatory cytokines in colon mucosa. Mice feces will be subject to 16S rRNA gene sequencing analysis to study the effect of nobiletin on mice gut microbiota in colitic mice model.
3. **Identify the bacteria with deconjugation capacity and characterize the bioactivities of bacteria secretion.** Human fecal sample will be collected, diluted and spread on GAM agar to form single colonies. The *in vitro* fermentation will be performed between single colonies and small intestinal content from mice fed with nobiletin. The collect fermentation supernatant will be subjected to HPLC analysis to investigate the deconjugation capacity of the isolated single colonies. Active single colony will be subject to Sanger sequencing to identify the bacteria. Bacteria secretion during the 48 h fermentation time will be collect every 12 h to study the anti-inflammation and anti-cancer effect. LPS-stimulated RAW 264.7 cell inflammation will be used to study the anti-inflammation effect. Nitric oxide will be tested and the expression of key enzymes will be analyzed by western blot. Human colon cancer cell HCT116 will be utilized for anti-cancer study. Cell viability will be assessed by MTT, cell cycle and cell apoptosis will be measured by flow cytometry, and the expression of key proteins associated with cell cycle and cell apoptosis will be checked by western blot to study the anti-inflammation effect. Besides, qRT-PCR will be used to measure the gene expression for the wnt signaling in HCT116 with or without bacteria secretion treatment.

4. **Develop an effective oral delivery system for the probiotic isolated.** Probiotic we isolated will be encapsulated in alginate microgels loaded with gastroprotective agent CaCO₃ or Mg(OH)₂. Cryo-SEM will be used to study the internal structure of different encapsulation microgels. Sample before simulated digestion, after simulated gastric digestion and after simulated gastrointestinal digestion will be collect for analysis. Physical properties will be measure, including surface potential and particle size. Live/dead cells will be visualized by Confocal scanning laser
microscopy. Plate culture method will be applied to counting the number of live cells at different stages.
CHAPTER 2
LITERATURE REVIEW

2.1 Overview of Human Gut Microbiota

2.1.1 Introduction to Human Gut Microbiota
The human gastrointestinal (GI) tract represents one of the largest interfaces
(250–400 m²) between the host, environmental factors and antigens in the human body.
In an average life span, around 60 tons of food pass through the GI tract, along with an
abundance of microorganisms from the environment. The collection of bacteria, archaea
and eukaryote inhabiting the GI tract is termed the ‘gut microbiota’ and has co-evolved
with the host over millions of years to form an intricate and mutually beneficial
relationship [20, 21]. The number of microorganisms inhabiting the GI tract has is no less
than human somatic cells, about $10^{14}$ cells [1]. As a result of the vast number of
bacterial cells in the body, host and the microorganisms inhabiting it are often referred as
a ‘superorganism’ [22, 23].

2.1.2 Development of Human Gut Microbiota
It’s generally believed that microbiota colonization starts from birth, while it is
challenged by the detected microbes in womb tissue, such as placenta [24, 25]. After
birth, the GI tract is rapidly colonized by microorganism from environment, with life
events such as illness, antibiotic treatment and diet changes causing chaotic shifts in the
microbiota [25, 26]. The mode of delivery also appears to affect the microbiota
composition, with vaginally delivered infants’ microbiota containing a high abundance of
Lactobacilli during the first few days, reflecting the high dominance of *Lactobacilli* in the vaginal microbial community [24, 27]. Conversely, the gut microbiota of C-section delivered infants is delayed in the colonization of genus *Bacteroides*, but colonized by facultative anaerobes such as *Clostridium* species [28, 29]. The fecal microbiome of vaginally delivered infants resembles that of their mothers with 72%, for C-section delivered babies, the percentage is downregulated to 41%[30]. In early stages of development, the number of microbiota species is relatively low and the species are mainly belonging to phyla, *Actinobacteria* and *Proteobacteria* [25, 31]. During the first year of life, the microbiota species increases and composition trends towards a distinct adult-like microbial profile with unique temporal patterns to its host [32]. By around 2.5 years of age, the composition, diversity and functional capabilities of the infant microbiota resemble those of adult microbiota [25, 26]. In adulthood, the composition of the gut microbiota is relatively stable, but it is still subject to perturbation by life events [33]. By the age of 65 and older, the microbial community shifts, with increased abundance of *Bacteroidetes* and *Clostridium* cluster IV, comparing with younger subjects where cluster XIV is more prevalent [34]. In contrast, a separate study suggested that the microbiota of a young cohort and an elderly population (70 years) were relatively comparable, whilst the diversity of the microbiota from a cohort of centenarians was significantly reduced [35]. In the elderly population, a significant relationship has been identified between diversity and living arrangements, such as community dwelling or long-term residential care [36].

2.1.3 Composition and Structure of Gut Microbiota

Compiled data from different studies identified 2172 species isolated from human
beings, indicating the large diversity of microorganisms. Among them, more than 500 species are identified in the colon [20]. O'Hara et al. reported that the density increase following the GI track. It is estimated that less than $10^4$ cells per gram lumen content in the upper GI tract while about $10^{12}$ cell in the lower GI tract [37, 38]. Cohabited bacteria at different site of GI track differs at not only the number but also the species. A study analyzed the composition of bacteria in small intestine and colon and concluded that the majority bacteria phyla in small intestine are *Firmicutes* and *Actinobacteria* while the *Fimicutes* were enriched at colon [39]. The situation is similar in mice. In mice, the small-intestine microbial community is largely dominated by *Lactobacillaceae* [40]. In contrast, *Prevotellaceae, Lachnospiraceae* and *Rikenellaceae* have been shown to dominate in colon [40, 41].

The colon is constituted by three different parts, epithelial surface, mucosa and feces/lumen, in which the types of flourishing bacteria are different. Swidsinski et al. indicated that *Bacteroides, Streptococcus, Bifidobacterium* only present in the feces [38, 42]. the abundance of *Bacteroidetes* appears to be higher in faecal/luminal samples than in the mucosa [20, 43]. In contrast, *Firmicutes*, specifically *Clostridium* cluster XIVa, is enriched in the mucus layer compared with the lumen [43]. Even in the same colon mucosa, the gut microbiome differs at both composition and diversity at difference mucosa regions [20, 44]. Interestingly, recent experiments in mice colonized with a diverse specific pathogen-free microbiota showed that the outer mucus of the large intestine forms a unique microbial niche and that bacterial species present in the mucus show differential proliferation and resource utilization properties compared with that in
intestinal lumen [45].

2.1.4 Role of Gut microbiota in Health

Owing to its large genomic content and metabolic complement, the gut microbiota provides a range of health beneficial properties to the host. Some of the most important roles of these microbes are maintaining the integrity of intestinal barrier function, providing nutrients such as vitamins or protecting against pathogens. In addition, the interaction between commensal microbiota and the mucosal immune system is crucial for host immune system.

Colonic bacteria release different types of carbohydrate-active enzymes, which endow them with the capacity to ferment carbohydrates generating metabolites such as SCFAs [46]. Three predominant SCFAs, propionate, butyrate and acetate, are typically found in a proportion of 1:1:3 in the GI tract [11]. These SCFAs are rapidly absorbed by epithelial cells in the GI tract where they are involved in the regulation of cellular processes such as gene expression, chemotaxis, differentiation, proliferation and apoptosis [47]. In human gut, acetate is produced by most gut anaerobes, propionate is mainly produced by Bacteroidetes, whereas the production of butyrate is dominated by Firmcutes [48, 49]. Propionate is primarily absorbed by the liver, whilst acetate is released into peripheral tissues [50]. Propionate is known for its beneficial effects in humans acting on β-cell function [51] and attenuating reward-based eating behavior via striatal pathways [52]. Butyrate has strong anti-inflammatory and anticancer potential [49, 51] and is an important energy source for colonocytes [47]. Butyrate can inhibit bacterial translocation and improve gut barrier function by promoting tight-junction assembly and mucin synthesis [49]. SCFAs also play a role in hepatic lipid and glucose
homeostasis via complementary mechanisms. In the liver, propionate can activate gluconeogenesis, whilst acetate and butyrate are lipogenic [49]. SCFAs also contribute to immune system and inflammatory response regulation [49]. Except SCFAs, microbial metabolites have an impact role on intestinal barrier functions, epithelium proliferation and the immune system also [53].

The GI microbiota is also crucial to the de novo synthesis of essential vitamins which the host is incapable of producing [54]. Lactic acid bacteria are key organisms in the production of vitamin B12, which cannot be synthesized by either animals, plants or fungi [54, 55]. Bifidobacteria are main producers of folate, a vital vitamin involved in host DNA synthesizing and repairing [56]. In addition, gut microbiota in human have also been shown to synthesize in humans, include vitamin K, riboflavin, biotin, nicotinic acid, panthotenic acid, pyridoxine and thiamine [57]. Colonic microbiota can also metabolize bile acids that are not reabsorbed for biotransformation to secondary bile acids [58]. The alteration of the co-metabolism of bile acids, branched fatty acids, choline, vitamins (i.e. niacin), purines and phenolic compounds has been associated with the progress of metabolic diseases such as obesity and type 2 diabetes [59].

There are many lines of evidence in support of a role for the gut microbiota in affecting epithelial homeostasis [60]. Germ-free mice exhibit impaired epithelial cell turnover which is reversed upon colonization with microbiota [61]. It has been demonstrated also that bacteria could help to promote cell renewal and wound healing, such as Lactobacilli rhamnosus GG [62]. Furthermore, several species have been reported in enhancing epithelial integrity, for example, in the case of Akkermansia muciniphila [63] and Lactobacillus plantarum [64]. Except the effect in modulating
epithelial properties, bacteria are implicated in modulating mucus properties and turnover. Germ-free mice have an extremely thin adherent colonic mucus layer, but when exposed to bacterial products (peptidoglycan or LPS), the thickness of the adherent mucus layer can be restored to levels observed in conventionally reared mice [65]. *B. thetaiotaomicron* and *F. prausnitzii* are associated with the co-ordination of mucus production [66]. *R. gnavus* E1, *Lactobacillus casei* DN-114 001 and *B. thetaiotaomicron* can remodel mucin glycosylation, for example, by regulating glycosyltransferase expression [67-69]. It is proposed that these functions mediate the ability of other commensals or pathogens to colonize, potentially providing some commensal species a competitive advantage for some commensal species in the gut [67].

The GI microbiota is also important for the development of both the intestinal mucosal and systemic immune system as demonstrated by the deficiency in several immune cell types and lymphoid structures exhibited by germ-free animals. A major immune deficiency exhibited by germ-free animals is the lack of expansion of CD4+ T-cell populations. This deficiency can be completely restored by treating Germ-free mice with polysaccharide A from the capsule of species *Bacteroides fragilis* [70]. This process is mainly mediated via the pattern recognition receptors (PRRs) of epithelial cells, such as Toll-like or Nod-like receptors, which are able to recognize the molecular effectors that are produced by intestinal microbes. These effectors mediate processes that can ameliorate certain inflammatory gut disorders, discriminate between beneficial and pathogenic bacteria or increase the number of immune cells or PRRs [71]. Segmented filamentous bacteria (SFB) is a class of anaerobic and clostridia-related spore-forming commensals present in the mammalian GI tract, and can actively interact with the
immune system [72]. Specifically, SFB are closely associated with the epithelial lining of the mammalian GI tract membrane, stimulating the secretion of serum amyloid A1 by epithelial cells [53]. Colonized SFB may also direct postnatal maturation of the gut mucosal lymphoid tissue, trigger a potent and broad IgA response, stimulate the T-cell compartment and upregulate intestinal innate defense mediators, indicating the immune-stimulation ability of SFB [73]. Akkermanisa muciniphila has been reported to be associated with protection effect against several inflammatory diseases [5, 74-77], suggesting that this species possesses anti-inflammatory capacity [78]. Individuals suffering Crohn’s disease has unbalanced microbiota in mucosa characterized by reduced diversity of core microbiota and lower abundance of F. prausnitzii [79]. Therefore, F. prausnitzii may be monitored to serve as the biomarker to help gut disease diagnostics [80]. Recently, an anti-inflammatory protein released by F. prausnitzii was shown to inhibit the NF-κB pathway in intestinal epithelial cells and prevent colitis in an animal model [81].

The physical presence of gut microbiota also influences pathogen colonization by competing for attachment sites or nutrient sources, and by producing antimicrobial substances [82]. Antibiotics have a profound impact on the microbiota that alter the microbiota composition and reduce diversity in the gut and lead to the proliferation of pathogenic populations [83]. Dietary fiber deficiency, together with a fiber-deprived mucus-eroding bacteria, promotes greater epithelial access and lethal colitis by the mucosal pathogen Citrobacter rodentium in mice [84]. The gut microbiota, via its structural components and secretions, also stimulates the host to produce various antimicrobial compounds. These include AMPs such as cathelicidins and C-type lectins
by the host Paneth cells via a PRR-mediated mechanism [85]. The other mechanism by which the gut microbiota can limit pathogen overgrowth is by inducing mucosal SIgA [86]. SIgA's induction directed against gut commensal bacteria occurs via M-cell-mediated sampling mechanism [87]. SIgA's are then anchored in the outer layer of colonic mucus through combined interactions with mucins and gut bacteria, thus providing immune protection against pathogens whilst maintaining a mutually beneficial relationship with commensals [88].

2.1.5 Gut Microbiota and Inflammatory Bowel Disease (IBD)

In United States and Europe, there are total 3.6 million people suffering from IBD and the number is keeping increasing. There are two major clinical phenotypes of IBD: ulcerative colitis disease (UC) and Crohn’s disease (CD), referring to the aberrant inflammation in small intestine and colon. The importance of microbiota with IBD was evidenced by researches that colitis is significantly attenuated or absent in germ-free animals and those treated with antibiotics [89-91]. Specifically, Bacteroides, Enterobacteriaceae, Porphyromonas, Akkermansia muciniphila, and Clostridium ramosum are enriched in several animal models of IBD and are associated with higher levels of inflammation [91]. In humans, there is an overall trend toward decreased microbiome richness, higher abundance of Bacteroides and Enterobacteriaceae, and lower abundance of Firmicutes in people with IBD compared with healthy people [92, 93]. Studies note a reduction of certain commensal microbes in IBD, particularly SCFAs producers. These include Faecalibacterium prausnitzii (F. prausnitzii), Leuconostocaceae, Odoribacter splanchnius, Phascolarctobacterium, and Roseburia [94]. F prausnitzii and Roseburia hominis both are butyrate producer and their abundance
are inversely correlate with disease degree of UC [95]. Furthermore, reduction of \textit{F. prausnitzii} has been linked with postoperative recurrence of Crohn’s disease, and its administration ameliorates gut inflammation in mouse [96]. \textit{Phascolarctobacterium} produces propionate in the presence of \textit{Paraprevotella}, which stimulate the production of \textit{T}_{\text{regs}} [97]. At the same time, several pathogens are enhanced in IBD, including \textit{Escherichia coli, Shigella, Rhodococcus, Stenotrophomonas maltophilia, Prevotellaceae, Clostridium difficile, Klebsiella pneumoniae, Proteus mirabilis, and Helicobacter hepaticus}[9]. This unbalanced shift in favor of pathogens colonization is thought to lead to perturbations in the immune function of lamina propria cells, ultimately resulting in inflammation and progression to disease [10].

\textbf{2.1.6 Diet Shaping the gut microbiota}

Disruption to the balance of cohabited gut microbiota has been associated with host metabolic disorders, such as obesity, diabetes, coronary heart disease [6, 98], and inflammatory bowel disease [99], and it is also associated with neurodevelopment and cognitive processes as well [100, 101]. However, human microbiota is sensitive to both multiple host intrinsic and extrinsic factors. A newly published research indicated that human microbiome composition is dominated by environmental factors rather than by host genetics [12]. Extrinsic factors, especially diet, may be crucial in shaping the gut microbiota. Levels of \textit{Prevotella} were enriched in children from a rural African village who consume high-fiber diet [102], whilst in children and adults from Malawi and Venezuela with plant-derived polysaccharide dominated foods. By contrast, the microbiota of those in USA has greater \textit{Bacteroides} [103]. \textit{Bacteroides} is associated with a long-term diet enrich in animal protein, several amino acids and saturated fats, and
Preoptella is correlated with carbohydrates and simple sugars [104]. Besides, small molecules can help to restore the microbiota. For example, the fecal microbiota is significantly altered and the gut physiology is markedly improved after 4 weeks’ treatment of non-digestible fructans in high-fat induced obsess mice [105]. The administration of small molecular photochemical quercetin restored the mice gut microbiota from dysbiosis induced by high-fat sucrose diet in mice [106]. Therefore, dietary interventions can be a viable strategy to restore or enhance gut microbiota function depending on the desired outcome.

2.2 Bioactive Components from Orange Peel

2.2.1 Limonin

Limonin is a bitter white crystalline substance, widely found in citrus fruits such as orange and lemon seeds. It belongs to a group of bioactive triterpenoid aglycone derivatives named limonoids [107]. Limonin has been reported to possess various beneficial health effects including anti-carcinogenic, anti-inflammation, antibacterial, antiviral [13-15, 108]. Accordingly, they have been recognized as one of the most important components of medical foods [109]. Limonin has low bioavailability due to the large molecular size and lipophilic property[110]. Thus, it is believed that limonin may evade digestion and absorption by the host during transit through GI tract. The unabsorbed limonin may reach the colon intact and interact with the gut microbial community.

2.2.2 Nobiletin

Dietary nobiletin (5,6,7,8,3’,4’-hexamethoxyflavone), the major PMFs mostly found in citrus fruits, has been demonstrated to be a potential anticancer [18, 111-113],
anti-inflammatory [114, 115], neuroprotective [116] and anti-obesity and insulin tolerance agent [117]. Previously, we demonstrated that nobiletin inhibited colon cancer in azoxymethane (AOM)-induced colon carcinogenesis in rats [113] and AOM/dextran sulfate sodium (DSS)-induced colitis-associated colon carcinogenesis in mice[18]. Three demethylated metabolites of nobiletin were reported, and the total amount of three metabolites was 20-fold higher than its parent compound, and two out of the three metabolites were more efficient in cancer inhibition than its parent compound-nobiletin[113]. Herein, the anti-cancer and anti-inflammatory effects of nobiletin were induced by not only nobiletin itself but also its metabolites produced by the gut microbiota [18, 114]. The biotransformation of nobiletin by gut microbiota improved its bioactivities. However, the influence of nobiletin on gut microbiota has been poorly understood, and insights into this could reveal details on the initiation and development of several diseases [118-120].

2.3 The effect of gut microbiota on xenobiotic metabolism

Dietary xenobiotics can modulate gut microbiota composition and functions[121-124]. Conversely, the gut microbiota can metabolize numerous xenobiotic compounds found in our diet, including macronutrients, micronutrients and phytochemicals[125, 126]. Xenobiotics can be metabolized into active, inactive, or toxic metabolites. Before entering systemic circulation, gut microbiota is a significant component of first-pass metabolism. The gut microbiota may metabolize compounds prior to absorption, after efflux from the intestinal epithelium or following biliary excretion from the liver.

It was identified that members of the Gordonibacter genus in the Actinobacteria
phylum in colon can metabolize ellagitannins [127, 128]. Ellagic acid released by ellagitannins hydrolysis can be metabolized into several structurally related urolithins can at very high in colon and blood[129]. Plant-derived two phytoestrogen, isoflavones and lignans, are metabolized by a diverse range of gut bacteria, such as members of the Actinobacteria, Bacteroidetes and Firmicutes phyla[130-132]. For instance, daidzin, is a glycosidic isoflavone in soy products, which can be metabolized to equol by several species of bacteria residing in the gut (for example, Enterococcus faecium, Lactobacillus mucosae, Bifidobacterium sp. and Eggerthella sp.) by glycosidic cleavage and reduction of an α,β-unsaturated ketone[133]. Several gut bacteria involved in the metabolism of ligans (such as pinoresinol and secoisolariciresinol) includes E. faecalis, E. lenta, Blautia producta, Eubacterium limosum, Clostridium scindens and Lactonifactor longoviformis, and produced bioactive metabolites enterodiol and enterolactone[134]. Conversely, microbial biotransformation may exacerbate the effect of harmful compounds that are derived from the diet. The glucuronidated compounds produced in liver by phase II metabolism are excreted into the small intestinal lumen through bile and flow into the colon, where microbial β-glucuronidases could theoretically release the conjugate group, reactivating the toxic compound and thereby augmenting its genotoxicity[135]. For example, the activity of microbial β-glucuronidases may contribute to associations between the risk of CRC and the intake of heterocyclic amines, which are compounds formed during the charring of meat[135]. On the other sides, bacteria β-glucuronidases could activate the bioactivity of xenobiotic or its metabolites [18]. Though a lot attention has turn to the metabolic property of gut microbiota, limited information is available so far.
2.4 Bioactivities of bacteria secretions

In rodents, probiotics can benefit host by secreting surface molecules or secret bioactive factors, which might help to stimulate host immune system. SCFAs produced by gut bacteria is most well-known bacteria secretion, whose host benefits has been well studied[47, 49, 51]. While biofactors released by gut microbiota is not limited to SCFAs, there’re researches indicates that gut bacteria can produce other bioactive components. *Helicobacter pylori* infection can be inhibited by *Bacillus subtilis* 3 fermentation supernatant, in which the effective components were identified as at least two antibiotics. One was amicoumacin A with anti-inflammation capacity, and the other was MICs[136]. *Bifidobacterium infantis* secretion improved human epithelia cells (T84) barrier function by increasing the expression of tight junction protein *in vitro*, and the oral treatment of bacteria secretion ameliorate gut inflammation, increased colon barrier function in IL-10−/− mice model [137]. Conditioned medium of *Bifidobacterium longum sup infantis* attenuated IL-6 production in response to IL-1β through TLR-4 in cell H4- a human fetal small intestine epithelial cell [19]. Gastric cancer cell growth was suppressed by ferrichrome, derived from *Lactobacillus casei* ATCC334[138]. Studies centered at bioactivities of the bacteria secretion can contribute the mechanism study of probiotic beneficial effects.

2.5 Oral delivery system for probiotics

Encapsulation of probiotics in colloidal delivery systems can improve the shelf life and viability of probiotics during storage and in the GIT [139-141]. Encapsulation of *Lactobacillus casei* 01 in alginate-starch beads improved cell viability during freeze-drying and storage [142]. Encapsulation of *B. longum* in alginate microgels coated with chitosan improved their survival during passage through a simulated GIT [139]. However, there are few studies showing that colloidal delivery systems can successfully
preserve the viability of probiotics after 2 hours’ exposure to gastric conditions [139, 143]. The main reason for the poor gastric stability of probiotics encapsulated in microgels is that hydrogen ions (H\(^+\)) can easily diffuse through the hydrogel network and inactivate the encapsulated bacteria. Previously, we prepared alginate microgels loaded with an antacid agent, Mg(OH)\(_2\), that could neutralize hydrogen ions when they entered the microgels and thereby maintain a neutral internal pH even when they were dispersed in acidic gastric fluids [144]. These antacid microgels may therefore be particularly useful for increasing the cell viability of probiotics for oral delivery.
CHAPTER 3

THE GASTROINTESTINAL FATE OF LIMONIN AND ITS EFFECT ON MICE

GUT MICROBIOTA

3.1 Introduction

Human’s GI tract is colonized by environmental microorganisms rapidly after birth [145]. After several years, the GI track microbial community becomes stable, and the bacteria number reaches $10^{13}$ to $10^{14}$, close to total human body cell count [146]. The interaction between gut microbiota and host is associated with host metabolic disorders, such as obesity, diabetes, coronary heart disease [6, 98], and inflammatory bowel disease [99], and it is also implicated in neurodevelopment and cognitive processes as well [100, 101]. Emerging evidence has suggested that the composition of the gut microbiota is associated with several external elements, including host genome, diet, lifestyle, and the use of xenobiotics (prebiotics or antibiotics) [147-149]. Of these factors, dietary interventions can be a viable strategy to restore or enhance gut microbiota function depending on the desired outcome. In a particularly illustrative example, the fecal microbiota of high-fat diet induced obese mice was markedly shifted and the gut physiology was significantly improved after the mice were treated with the non-digestible fructans for 4 weeks [105]. The administration of small molecular photochemical quercetin restored the mice gut microbiota from dysbiosis induced by high-fat sucrose diet [106].
Limonin is a bitter white crystalline substance, widely found in citrus fruits such as orange and lemon seeds. It belongs to a group of bioactive triterpenoid aglycone derivatives named limonoids [107]. Limonin has been reported to possess various beneficial health effects including anti-carcinogenic, anti-inflammation, antibacterial, antiviral [13-15, 108]. Accordingly, it has been recognized as one of the most important components of medical foods [109]. Limonin has low bioavailability due to the large molecular size and lipophilic property [110]. Thus, it is believed that limonin may evade digestion and absorption by the host during transit through GI tract. The unabsorbed limonin may reach the colon intact and interact with the gut microbial community. However, research regarding the interaction between limonin and gut microbiota is limited. In this study, we examined the effect of limonin on gut microbiota in unchallenged CD-1 mice. We hypothesized that limonin would evade metabolism on the upper GI tract and persist to the colon, altering the gut microbiota.

3.2 Materials and methods

3.2.1 Animal and diet

20 male wide-type CD-1 mice (6-8 week of age) from Charles River Laboratories (Wilmington, MA, US) were transported to the specific pathogen free (SPF) facility. Mice were housed in an air-conditioned room (temperature 23±2 °C, 50±10% humidity, 12-hour light-dark cycle) with free access to water and basal diet. After 1-week acclimation, 20 male mice were randomly assigned to limonin treatment group (10 mice/group, 5 mice/cage) and control group. The control group was fed with AIN93G diet, while the limonin treatment group was fed with the AIN-93G diet containing 0.05% (w/w) limonin. Fecal pellets from metabolic cage were collected and stored at -80 °C.
After 9 weeks, mice were sacrificed with CO$_2$ asphyxiation after overnight fasting. Stool from distal colon were collected for colonic fecal microbiome analysis. This animal study was based on protocol approved by the University of Massachusetts, Amherst Institutional Animal Care and Use Committee (#2014-0079).

3.2.2 Sample preparation and liquid chromatography-mass spectrometry (LC-MS) conditions

Limonin in all mice organs and the whole GIT was extracted based on the methods by Liang et al. [150]. The obtained samples were re-dissolved in 50% acetonitrile for LC-MS analysis (Model 2020, Shimadzu, Kyoto, Japan) on a Zorbax SB-Aq C18 column (150 mm × 4.6 mm, 5 µm, Agilent Technologies, USA) at a flow rate of 0.8 mL/min. The linear gradient elution condition was: started with 80% mobile phase A (5% ACN/water, v/v)/20% mobile phase B (100% ACN) (v/v) for 5 min, then processed to 80% B/20% A over 30 min, and held at 80% B for 5 min. The elution was monitored on the m/z 469.

3.2.3 Cecal short chain fatty acids (SCFAs) analysis

Cecum contents were homogenized with 6-fold volume of pure water, and supernatants were obtained by centrifugation (12,000 rpm, 10 min, 4 °C), and then filtered through a 0.22 µm membrane. A chromatographic system composed of a 6890 N GC (Agilent Technologies Inc., Palo Alto, CA, USA) connected with an ion flame detector and a mass spectrometry 5973N detector (Agilent) was used for quantification and identification of cecum content SCFAs as described previously [151].
3.2.4 Microbial DNA extraction

Total fecal DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instruction with the addition of the bead-beating step to increase yield. Extracted DNA quantity was measured with a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, US) and quality was verified with the gel electrophoresis.

3.2.5 Terminal restriction fragment length polymorphism (T-RFLP) analysis

The T-RFLP analysis was conducted as described previously [152]. Briefly, the universal primers designed to anneal at specific sequence in bacteria 16S rRNA genes, FAM 8F (5’-AGAGTTTGATCCTGGCTCAG-3’) and HEX 1492R (5’-GGTTACCTTGTTACGACTT-3’) were labeled at the 5’ end with phosphoramidite dye (Life Technology, MA, USA). PCR products were amplified with 2.0 U KAPA HiFi PCR master mix (KAPA Biosystem, Wilmington, MA, US). Each PCR product (4 µL) was visualized after electrophoresis on 1.0% agarose TAE gel after ethidium bromide staining. The remaining PCR products were subjected to a PCR purification column.

The purified PCR products were aliquoted and digested separately with 15 U of MspI, HaeI and HaeIII (Roche Diagnostics) in a total volume of 12 µL as recommended by the manufacturer for 5 h at 37 ºC. The resulting fragments of different sizes were electrophoresed in 4% (wt./ vol.) of polyacrylamide gel (Acrylamide/Bis, 19:1; Bio-Rad) with 1.25 µM Buffer (Bio-Rad) at a constant voltage of 120 V for 4 h. In general, T-RF size corresponds to specific bacteria groups.
3.2.6 Microbial phylogenetic profiling by sequencing of the 16S rRNA gene amplicon

PCR was performed to amplify the V3-4 regions of the 16S rRNA gene, which incorporates targeted primers and the Illumina overhang adaptor. The primer set was developed by Illumina (16S Amplicon PCR Forward Primer = 5'TCGTCGGCAGCCTCAGATGTGTATAAGAGACAG)

GACAGCCTACGGGNGGCWGCAG) and (16S Amplicon PCR Reverse Primer = 5'GTCTCGTGGGCTCGAGATGTGTATAAGAGACAG)

Yasir et al., 2015). PCRs were performed in a 96 well format on a Veriti thermal cycler (Life technology, Carlsbad, CA, US) with 2x KAPA HiFi Hotstart ReadyMix (KAPA Biosystem, Wilmington, MA, US). After purification on AMPure XP beads (Beckman Coulter, Danvers MA, US), a limited cycle PCR was performed using the Nextera XT Index Kit (Illumina, San Diego, CA, US) to attach dual indices and Illumina sequencing adapters, followed by an additional purification on AMPure XP bead. The quantity of the purified PCR products was measured by Qubit dsDNA BR Assay kit (Life technology, Carlsbad, CA, US) and the amplicon quality was estimated by ScreenTape Assay on Tape Station 2200 (Agilent Technologies, Santa Clara, CA, US). After quantification and qualification, samples were pooled in equimolar amount and pair-end 2×300 bp sequencing was performed on Illumina MiSeq platform (Illumina, San Diego, CA, US).

3.2.7 Microplate growth assay

*Lactobacillus plantarum* ATCC BAA-793 (*L. plantarum* 793) and *Bifidobacterium longum* subsp. *longum* ATCC 15707 (*B. longum* 15707) were got from
the American Type Culture Collection (ATCC). The two strains were verified by Dr. Sela’s lab [153]. In brief, two trains confirmed by *Bifidobacterium*-specific phosphoketolase assay [154] and through microscope, and *B. longum* subspecies was differentiated with PCR-based *B. longum/B. infantis* ratio analysis [155]. The two strains were propagated in de Man-Rogosa-Sharpé (MRS; Oxoid, Hampshire, England) medium supplemented with 0.05% (wt/vol) L-cysteine (Sigma-Aldrich, St. Louis, MO) [156] at 37 °C under anaerobic conditions (Coy Laboratory Products, Grass Lake, MI).

Growth phenotypes were monitored in a 96-well microplate. For each studied strain, 2 μL of overnight culture was inoculated to 200 μL MR S medium with or without different concentration of limonin (10 μM or 100 μM). The growth assay was conducted anaerobically at 37 °C for 48 h by assessing optical density at 600 nm (OD$_{600}$) using an automated PowerWave HT microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Each strain was evaluated in biological triplicate with three technical replicates. Bacterial growth kinetics were calculated with Wolfram Mathematica 10.3 Student Edition with the equation $\Delta$OD($t$) = $\Delta$OD$_{\text{asym}}${$1/[1+\exp(ktc-t)] - 1/[1+\exp(ktc)]$}, where growth parameter $\Delta$OD$_{\text{asym}}$ is the growth level at stationary phase, $k$ represents the maximum growth rate, and $tc$ is the inflection point’s location indicating the time needed to reach the highest growth rate [157].

3.2.8 Statistical analysis

The sequencing data was processed by QIIME software pipeline v1.9.1[158]. In general, the high quality (quality value>25) sequence data was demultiplexed. Sequences were then clustered into operational taxonomic unites (OTUs) using open reference OTU
picking was performed against Greengenes bacterial 16S rRNA database (13_8 release) [159] with 97% similarity threshold.

\[ \alpha \] -diversity (diversity within sample community species richness) was determined with ten iterations at a maximal sequence depth where all samples could be included. \[ \beta \] -diversity (between sample communities dissimilarity) was calculated using weighted and unweighted UniFrac distances [160]. To investigate the effect of limonin treatment on relative abundance of taxa, Student’s t-test and linear discriminant analysis effective size (LEfSe) analysis were performed.

Galaxy Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to explore the predicted functional metagenome shifts between communities. According to the requirement form PICRUSt algorithm, operational taxonomic unites (OTUs) were aligned to the Greengenes 16S rRNA database using a closed reference picking protocol [161]. Statistical analysis was used to compare functional shifts between groups in the STAMP software [162]. For all analyses, statistical significance was declared if \( p < 0.05 \).

### 3.3 Results

#### 3.3.1 Limonin has no effect on mice general properties

There was no difference in mouse initial body weights (results not shown), and after an 8-week intervention period, there was no observed difference in mouse final body weights (Control=39.08±1.83 g, Limonin=40.32±3.89 g, \( p=0.62 \)) (Table 3.1). Additionally, no differences were found for the liver or spleen weight (\( p=0.61 \) and \( p=0.74 \)), indicating that 500 ppm limonin diet has no toxicity to mice.
Table 3.1 General property of mice fed with limonin

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight/g</th>
<th>Liver/mg</th>
<th>Spleen/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39.08±1.83</td>
<td>1922.58±162.20</td>
<td>138.52±17.77</td>
</tr>
<tr>
<td>Limonin</td>
<td>40.32±3.89</td>
<td>2032.70±92.36</td>
<td>89.70±7.01</td>
</tr>
<tr>
<td><em>p</em> value</td>
<td>0.62</td>
<td>0.61</td>
<td>0.74</td>
</tr>
</tbody>
</table>

3.3.2 Determination of limonin distribution in mice

To explore the effect of limonin on gut microbiota, it was critical to ensure whether limonin could evade GIT metabolism and reach the colon to interact with gut microorganisms. Herein, GIT tissues were subjected to limonin extraction and the extracts were detected using LC-MS. As shown in Figure 3.1A, the concentration of limonin increased following transit through the small intestine (SI) in the digesta, and the concentration of stomach digesta was close to the SI-4. Mice cecum and colon were the organs to further concentrate the limonin, and the limonin in colon digesta was as high as 523.14±95.67 nmol/g tissue. However, limonin distribution in mucosa along the GIT was different from the digesta (Figure 3.1B). Cecum mucosa had the highest limonin concentration (15.02±3.80 nmol/g tissue), which may be due to the long villi and transition time. When compared to the high concentration of limonin in colon digesta, limonin amount in the colon mucosa was low (3.82±1.17 nmol/g tissue), which was only 0.7% of the amount in colon digesta. However, the highest concentration of limonin in organs was 2.76±0.85 nmol/g in spleen, which was near 50% of the average concentration (5.43 nmol/g) in GIT mucosa and 1.4% of the average concentration (191.57 nmol/g) in GIT digesta (Figure 3.1C). Limonin concentration in the liver and plasma was all below 0.5 nmol/g tissue. Taken the tissue weight into account, the absorbed limonin was no more than 1% of the total administrated limonin (data not
show). Therefore, we concluded that most of the limonin was unabsorbed, evaded the GIT metabolism and accumulates in the distal colon digesta, in which lives the highest density of bacteria ($10^{11}$/mL digesta) [146].

Figure 3.1 Limonin distribution in mice. (A) Limonin distribution in the digesta along the gastrointestinal track (GIT) (nmoL/g digesta); (B) Limonin distribution in the mucosa along the GIT (nmoL/g mucosa); (C) Limonin distribution in mice organs (nmoL/g).

3.3.3 SCFAs production in cecum

SCFAs are the end products of bacteria fermentation in cecum and colon. As a measure of colonic microbial activity, fecal SCFAs concentrations were analyzed. Limonin supplementation increased the production of 6 SCFAs (acetate, propionate, isobutyric, butyric, isovaleric, and valeric). However, no significant differences were
observed. (Figure 3.2). Since limonin itself couldn’t be the substrate of SCFAs production, the insignificant changes may be due to the change of gut microbiota composition. In consistent with most researches, acetate was the predominant SCFA in the cecum [163, 164].

![Figure 3.2 Cecum short chain fatty acids (SCFAs). Data was shown as mean ±SD (n=5), Student’s t-test was used to perform the statistical analysis, and no significant difference was observed between control and limonin treatment groups.](image)

**3.3.4 Mice fecal microbial community structure changed with limonin treatment**

At week 9, mice were sacrificed and distal colon feces were picked for T-RFLP analysis to reduce the environmental contamination as much as possible. The microbial community from the limonin group was significantly separated from the control group (Figure 3.3), indicating that 9 weeks limonin treatment altered the fecal microbial community profile.
Figure 3.3 T-RFLP analysis of gut microbiota structure change with limonin diet intervention (at week 9).

3.3.5 Variation of fecal microbial community structure

To investigate the details about the microbial community shifts, colon fecal samples were subjected to the microbial 16S rRNA gene sequencing on Illumina MiSeq platform. A total of 953581 16S rRNA-based counts were obtained, with a mean of 95358.1 counts (range =56470-151193)/sample. The data set was rarified to 56470 sequences for analysis of diversity and species richness.

α-diversity including phylogenetic diversity whole tree matrix (PD_whole tree), Observed OTUs richness, Chao1 and Shannon index were estimated using a linear mixed model. Compared to the control, fecal microbiota species richness was increased by limonin treatment significantly [observed species at the 97% level (OTUs):
3415.80±306.51 compared with 2305.60 ± 622.43, (p < 0.01); Chao 1 index: 7005.83 ± 578.54 compared with 5303.89 ±1375.58 (p < 0.03); and PD_whole tree (phylogenic
branch distance): 101.06 ±8.76 compared with 81.31 ± 20.92 (P < 0.09)]. Even when considering the evenness, the diversity of limonin treatment group was extremely increased [Shannon index: 6.98 ± 0.26 comparing with 5.36 ± 0.39 (p<0.01)] (Table 3.2).

In addition, principal coordinates analysis (PCoA) of weighted and unweighted UniFrac distances performed on the 97% OTU abundance matrix revealed a distinct separation (p < 0.05) on the gut microbial community structures (β-diversity) between limonin treatment and control groups (Figure 3.4A and 3.4B, respectively). ANOSIM with 999 permutations was used to test the significant differences between the two groups based on unweighted and weighted UniFrac [165]. As expected, limonin treatment significantly shift the gut microbial community away from the control group (p=0.01 for unweighted and p=0.003 for weighted), indicating that limonin

<table>
<thead>
<tr>
<th></th>
<th>PD_whole tree</th>
<th>Observed OTUs</th>
<th>Chao1</th>
<th>Shannon index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>p value</td>
<td>Value</td>
<td>p value</td>
</tr>
<tr>
<td>Control</td>
<td>81.31±</td>
<td>0.09</td>
<td>2305.60±</td>
<td>0.01</td>
</tr>
<tr>
<td>Limonin</td>
<td>101.06±</td>
<td></td>
<td>3415.80±</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.4 Principal coordinate analysis (PCoA) of unweighted (A) and weighted (B) UniFrac distance of fecal microbial communities performed on the 97% OTU abundance matrix revealed a distinct separation (p<0.05) between limonin treatment group and control group. Each dot represents a sample from each mouse (n=5) fed diets.

3.3.6 Taxomic shifts by limonin treatment

Greengenes classifier assigned usable raw reads to 9 phyla, 18 families, and 81 genera. The most abundant phyla included Firmicutes (49.10% of sequences), Bacteroidetes (48.40% of sequences), Actinobacteria (1.24% of sequences), Proteobacteria (0.354% of the sequences), TM7 (0.14% of sequences), Verrucomicrobia
(0.02% of sequences), *Deferribacteres* (0.02% of sequences), and *Tenericutes* (0.01% of sequences). Compared with the control group, phyla *Proteobacteria* and *Bacteroidetes* were significantly enriched (from 0.04±0.01% to 0.354±0.14% p=0.0, and from 19.07±4.45% to 48.04±5.98%, p=0.03), while the phylum *Actinobacteria* was suppressed significantly by limonin supplement (from 13.82±0.18% to 1.24±0.10%, p=0.03) and *Firmicutes* decreased by 25% (from 65.39±2.90 to 49.10±6.09%, p=0.09).

To further explore the variation of microbial community composition between the two groups, LEfSe test was performed to investigate the significant changes in relative abundance (only average relative abundance >0. 01% included) of bacterial taxa at genus level across samples. Our LEfSe analysis revealed that, 18 known genera (*f* _S24-7*, *Bacteroides*, *Ruminococcus*, *Oscillossira*, *Dtreptococcus*, *Dorea*, and so on) were significantly enriched and five genera (*Peptostreptococcaceae*, *Turicibacter*, *Allobaculum*, *Bifidobacterium* and *Lactobacillus*) were significantly decreased by limonin treatment (LDA > 2.0, p < 0.05) (Figure 3.5). Our data demonstrate that limonin treatment may dramatically impact microbial taxonomy when compared with the control. Genus *Oscillossira* was increased from 0.33% to 2.95%, about 9-fold, which has been associated with leanness in humans [166] and decreased incidence of inflammatory bowel disease [167]. Unexpectedly, the genus *Lactobacillus* and *Bifidobacterium* were both decreased significantly (43.99% to 13.17%, p=0.00, and 13.3% to 0.04%, p= 0.01).
Figure 3.5 Gut microbiota genera represented between control and limonin treated groups identified by linear discriminant analysis coupled with effect size (LEFSe) (LDA>2, P<0.05). Red box: suppressed by limonin treatment, green box: enriched by limonin treatment.

3.3.7 Variation of predicted functional metagenomes induced by limonin supplement

Galax PICRUSt was used as a predictive exploratory tool. 18 level 2 KEGG pathways were represented in the data set with significantly abundances shifts between samples (Figure 3.6). Despite the accuracy of predictions being lower in other mammals than for humans (mean NSTI = 0.03 ± 0.02), it can still provide useful information about
functional predictions for mammalian microbiomes [161]. For level 2, we found that 9 KEGG pathways (Cell motility, Amino Acid Metabolism, Metabolism of Cofactors and Vitamins, Signal Transduction, Lipid Metabolism, Biosynthesis of other Secondary Metabolism, Transport and Catabolism, Immune System, and Neurodegenerative Diseases) were significantly increased by limonin treatment, and 9 KEGG pathways (Immune System Disease, Infection Disease, Signaling Molecules and Interaction, Enzyme Families, Xenobiotic Biodegradation and Metabolism, Nucleotide Metabolism, Translation, Cell Growth and Death, and Replication and Repair) were significantly suppressed by limonin treatment (LDA > 2, p < 0.05) (Figure 3.6A). Particularly, staphylococcus aureus infection belonging to infectious disease was markedly downregulated by limonin treatment (LDA > 2, p < 0.05) (Figure 3.6B). In summary, limonin treatment changed the profile of gut microbiota, which in turn could influence gut microbiota functions and the health of the host.
Figure 3.6 Microbial functional pathways significantly shifted with limonin treatment using predictive metagenomics. (A) Functional pathway changes based on LEfSE analysis. (B) The relative abundance of bacteria functional pathways associated with Staphylococcus aureus infection. PICRUSt was used to predict the microbial metagenomes from bacteria MiSeq data.
3.4 Discussion

Limonin, a triterpene derivate from citrus fruits, has been recognized to have a wide range of bioactive activities including a beneficial effect on colon health [13, 15, 108]. For example, it previously has been reported to inhibit the proliferation of human colon adenocarcinoma (SW480) cells through mitochondria mediated intrinsic apoptosis [108], and suppressed AOM-induced colon cancer in male rats [13]. In addition, researchers found that limonin could be metabolized by some specific bacteria (Acinetobacter sp.) from soil [168]. Thus, Limonin might interact with gut bacteria. However, limited information has been reported about the effect of limonin on gut microbiota. Therefore, we studied the effect of limonin on mice gut microbiota.

Oral ingested xenobiotics have diverse bioavailability, depending on each compound’s properties. Oral bioavailability in human studies revealed that the bioavailability of xenobiotic with molecular weight (MW) >400 was less than 20% [169], while limonin has a MW of 470.52, indicating that limonin’s in situ bioavailability should be below 20%. As expected, our results showed that a large fraction of the orally administrated limonin was unabsorbed and persisted to the colon, interplaying between diet and the gut microenvironment.

Our results indicated that the gut microbial community was shifted substantially during the treatment time as the PCA figure showed distinct separation of fecal microbial communities between control and limonin treatment groups (Figure 3.3A). It was probably caused by the exposure to limonin that provided a selective advantage to certain members of the community. At week 9, the distal colon fecal microbiota was subjected to 16S rRNA gene analysis, revealing that the gut microbial community (α-diversity and β-
diversity) was strongly shifted by the limonin treatment. One important aspect of the diversity is species richness (the number of species present in certain microbiota ecosystem), which was significantly increased by limonin treatment. Communities has higher species richness are more resistant to invasion as they are more efficient in competing for limited resources [170]. Besides, higher species richness could improve the stability of the host gut microbiota ecosystem [171]. On the opposite, obesity and diet with high fat and sugar has been linked with decreased diversity comparing with low-fat and plant based diet [172, 173].

Besides, the composition of the colon fecal microbiota was altered in response to dietary treatment. At the phylum level, the relative abundance of Proteobacteria in mice’s gut was significantly higher in limonin treatment group (p<0.05). The alteration in relative abundance of Proeroebacteria may results in a change in host energy accumulation [174-176]. While, in our study, we didn’t see significant difference in weight change during the experimental period. On the other hand, the phylum Actinobacteria decreased dramatically (p=0.03) The relative abundance of Actinobacteria would have different effects on host health depending on age and health status. Previously , it shows that children suffering with autism had lower Actinobacteria in their gut microbiota [177], while people with inflammatory bowel disease had higher level of Actinobacteria [178]. The relative abundance of Bacteroidetes and Firmicutes are typically reported to be associated with obesity where decreased F/B ratio is highly related to the gut microbiota dysbiosis [4] and western high fat diet [179].The inconsistent results may result from the sharing number of bacterial genera or species that were altered, and their yet unknown health effects.
The relative abundance of 23 genera were modified significantly by limonin. Among them 18 genera were increased, while only 5 genera decreased in abundance. Unexpectedly, the relative abundance of genus *Bifidobacterium* and *Lacobacillus*, which are widely regarded as beneficial bacteria, are significantly decreased by limonin [180, 181]. To confirm the inhibition effect of limonin on the growth of *Lactobacillus* and *Bifidobacterium*, the growth curve of two strain from the two genera was studied in vitro. Interestingly, limonin significantly increased the maximum bacteria optical density, reduced the time to reach the highest growth rate for both of two bacteria at the concentration of 10 µM and 100 µM, much higher than the concentration in the mice colon (Figure 3.7, Figure 3.8, and Table 3.3 and 3.4).

Figure 3.7 The growth curve of *B. longum subsp longum* 15707 with limonin treatment at different concentrations.
Table 3.3 The growth parameters of *B. Longum subsp Longum* 15707 with limonin treatment at different concentrations

<table>
<thead>
<tr>
<th></th>
<th>tc</th>
<th>k</th>
<th>yasymp</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.93±0.39a</td>
<td>0.50±0.02</td>
<td>1.32±0.01a</td>
<td>1.00</td>
</tr>
<tr>
<td>Limonin 100 μM</td>
<td>13.09±0.09b</td>
<td>0.47±0.00</td>
<td>1.29±0.01b</td>
<td>1.00</td>
</tr>
<tr>
<td>Limonin 10 μM</td>
<td>12.66±0.26b</td>
<td>0.51±0.01</td>
<td>1.34±0.00a</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Figure 3.8 The growth curve of *Lactobacillus. plantarum* 793 with limonin treatment at different concentrations.

Table 3.4 The growth parameters of *Lactobacillus plantarum* 793 with limonin treatment at different concentrations.

<table>
<thead>
<tr>
<th></th>
<th>tc</th>
<th>k</th>
<th>yasymp</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.20±0.28</td>
<td>0.59±0.01</td>
<td>1.60±0.01</td>
<td>0.999</td>
</tr>
<tr>
<td>Limonin 100 μM</td>
<td>6.22±0.28</td>
<td>0.59±0.01</td>
<td>1.60±0.02</td>
<td>0.999</td>
</tr>
<tr>
<td>Limonin 10 μM</td>
<td>6.22±0.28</td>
<td>0.58±0.02</td>
<td>1.61±0.01</td>
<td>0.999</td>
</tr>
</tbody>
</table>
Herein, the significantly reduced abundance of *Bifidobacterium* and *Lactobacillus* might due to the increased growth of the other bacteria, or reduced excretion of these bacteria into the colon digesta. The increased diversity of gut microbiota community should be associated with the increased growth of some non-dominant bacteria. In addition, the reduced excretion of *Lactobacillus* and *Bifidobacterium* would be potentially associated with the increased colonization in the mucosa, which could interact with host immune response [182, 183]. Thus, the effect of limonin on *Bifidobacterium* and *Lactobacillus* colonization on mucosa would be determined in future study.

Three out five genera in Phyla *Bacteroidetes* were significant increased, including *Bacteroides*, f__Rikenellaceae; other and f__S24-7. Certain commensal *Bacteroides* species could induce IBD in dnKO mice with or without antibiotic pretreatment, and innate and adapted immune responses were activated in a host-genotype-specific fashion [184]. The increased abundance of f__S24-7 could contribute to the plant carbohydrate fermentation [185]. In phylum *Firmicutes*, 23 genera were increased more or less, and the most significant ones were o__Clostridiales;f__;g__;f__;Lachnospiraceae;Other, [Ruminococcus], Oscillospira and Ruminococcus. The genus Oscillospira was negative correlated with body mass index (BMI) and inflammatory disease [166, 186]. The genus Ruminococcus was increased by 9-fold, which might enhance the gut microbiota ability in degrading and utilizing fiber and carbohydrate in diet [187]. No significant changes were observed for the genera in Phylum *Proteobacteria*, TM7, Tenericutes and *Verrucomicrobia*.

Knowing the composition changes alone was not enough to know how the limonin treatment could influence the host gut ecosystem, and we need to know its
function shifts. Our PICRUSt analysis revealed that predicted genes from the metagenome related to cellular processes and human disease were significantly increased in limonin-treated samples. Xenobiotics Biodegradation and Metabolism were reduced, whereas limonene and pinene degradation was increased, which was consistent with the increased environmental adaption. Bacterial toxin behavior was reduced. Besides, the immune system behavior was predicted to be enhanced, and the primary immunodeficiency was reduced. Infectious disease signals were decreased, with the most significant changes being associated with *Staphylococcus aureus* infection, which was decreased to 22% (p=0.001) (Figure 3.6B). These results indicated that the changes measured in the gut microbial community with limonin treatment may be beneficial for our immune system and our human health.

### 3.5 Conclusion

In summary, this study investigated the influence of 9-week oral admission of limonin on the mice gut microbiota. No significant changes in physical characteristics (bodyweight, spleen, heart, liver and kidney) were observed in mice. Our study confirmed that limonin could evade metabolism through GI track and persist to the colon. The gut microbiota community was modified significantly, and the microbiota function was also altered in response to dietary intervention.
CHAPTER 4

ANTI-INFLAMMATORY EFFECT OF NOBILETIN WAS ASSOCIATED WITH
ALTERED GUT MICROBIOTA

4.1 Introduction

Inflammatory bowel diseases (IBD) is a kind of heterogeneous chronic and relapsing inflammatory disorder, which will cause epithelial dysfunction or increasing mucosal permeability of gastro-intestinal track. In the past decade, IBD has emerged as a public health challenge worldwide [188]. Currently survey reported that the prevalence of IBD in North America, and many countries in Europe exceeded 0.3% [189]. Multiple research proved that the pathogenesis of IBD is correlated with dysregulated gut microbiota [190-192]. The importance of microbiota with IBD was evidenced by researches that that colitis is significantly attenuated or absent in germ-free animals and those treated with antibiotics [89-91]. And comparing with healthy individuals, the gut microbiota composition and structure in IBD patients is significantly disrupted, with an increase in certain opportunistic pathogens and a decrease in beneficial bacteria [192]. This dysbiotic shift in favor of pathobionts is thought to contribute to perturbations in the immune function of lamina propria cells, ultimately resulting in inflammation and progression to disease [10].
However, Human microbiota is sensitive to both multiple host intrinsic and extrinsic factors. A newly published research indicated that human microbiome composition is dominated by environmental factors rather than by host genetics [12]. Cultural factors, especially diet, may be crucial in shaping the gut microbiota. The ratio of two of the main genera of gut bacteria, *Prevotella* and *Bacteroides*, correlates well with the overall pattern of diversity across healthy adults (see discussion of entero-types later) [104, 193]. Levels of *Prevotella* were enriched in children from a rural African village who had a high-fiber diet [102], and in children and adults from Malawi and Venezuela whose diet was dominated by plant-derived polysaccharide foods such as maize and cassava. By contrast, the microbiota of those in the United States had more *Bacteroides* [103]. For healthy adults in the United States, differences in long-term diet between individuals also was correlated with these bacteria. *Bacteroides* was associated with a long-term diet rich in animal protein, several amino acids and saturated fats, and *Prevotella* was associated with carbohydrates and simple sugars [104]. The relative importance of changes in the microbiota as a result of diet, compared with other factors that affect health, remains a subject of active investigation. Besides, small molecules can help to restore the microbiota also. In a particularly illustrative example, the fecal microbiota of high-fat diet induced obese mice was markedly shifted and the gut physiology was significantly improved after the mice were treated with the non-digestible fructans for 4 weeks [105]. The administration of small molecular photochemical quercetin restored the mice gut microbiota from dysbiosis induced by high-fat sucrose diet [106]. Therefore, dietary interventions can be a feasible strategy to restore or enhance gut microbiota function depending on the desired outcome.
Dietary nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), the major PMFs mostly found in citrus fruits, has been demonstrated to be a potential anticancer [18, 111-113], anti-inflammatory [114, 115], neuroprotective [116] and anti-obesity and insulin tolerance agent [117]. Previously, we demonstrated that nobiletin inhibited colon cancer in azoxymethane (AOM)-induced colon carcinogenesis in rats [113] and AOM/dextran sulfate sodium (DSS)-induced colitis-associated colon carcinogenesis in mice[18]. Three demethylated metabolites of nobiletin were reported, and the total amount of three metabolites was 20-fold higher than its parent compound, and two out of the three metabolites were more efficient in cancer inhibition than its parent compound-nobiletin[113]. Herein, the anti-cancer and anti-inflammatory effects of nobiletin were induced by not only nobiletin itself but also its metabolites produced by the gut microbiota [18, 114]. The biotransformation of nobiletin by gut microbiota improved its bioactivities [18]. However, it’s still unclear, whether gut microbiota involved in the bioactivities of nobiletin except biotransformation or not.

Herein, the aim of this study was to investigate the effect of nobiletin on mice gut microbiota and the relationship between gut microbiota and inflammatory response in mice to provide key information to lay fundamental information for health benefits of dietary bioactive compounds.

4.2 Materials and methods

4.2.1 Animals, diets and experimental procedure

The protocol for the animal experiment was approved by Institutional Animal Care and Use Committee of University of Massachusetts Amherst (#2014-0079). 30 male
wild-type CD-1 mice (6-8 weeks old) were obtained from Charles River Laboratories (Wilmington, MA, USA) and kept in a temperature-controlled animal room of 22 ± 2 °C and 50 ± 10% humidity in a 12-h light-dark cycle with free access to water and a basal diet. The mice were randomly divided into 3 groups (n=10/group) to receive either standard chow (AIN-93G diet) or nobiletin diet. Nobiletin diet is made of AIN93G diet mixed with 500 ppm nobiletin powder. After one-week acclimation with chow diet, the three groups were distributed as follows: the control group, which was fed with standard chow diet and regular water; the DSS group, which received chow diet and 1.5 % DSS water (wt/v, dextran sulfate sodium salt, average molecular weight 36,000–50,000) (International Lab, Chicago, IL, USA); the DSS+Nobiletin group, which received the nobiletin diet and 1.5% DSS drinking water (wt/v). All experimental groups were provided with respective drinking water ad libitum for 4 days followed by 1 week of regular water for recovery, and this cycle was repeated for times. The weight of the mice was recorded every other day during the whole experiment. At the end of the fourth cycles of DSS water treatment, all mice were sacrificed by CO₂ asphyxiation. The entire colons were removed and rinsed with PBS, the weight and the length of the colon then were measured and recorded. After measurement, the whole colon was opened longitudinally and cut into two part longitudinally then kept separately for further analysis. One part of the colon was stored at -80 °C for ELISA analysis while the other part was first fixed in 4% buffered formalin (pH 7.4) for 24 h for further histopathological and immunohistochemically analysis. The fecal pellets were collected from the colon and stored at -80°C for further microbiota analysis. Both the liver and the
spleen were dissected from the body and weighted for evaluation of disease progression of ulcerative colitis.

4.2.2 Disease activity index (DAI) and histological evaluation of colon

Disease activity index were determined basing on the scoring for weight loss, stool consistency and rectal bleeding, which were recorded every other day [194]. For histological evaluation, the fixed colon segments were first dehydrated by using ethanol and isopropanol, then were embedded in paraffin. The specimens were then sectioned into slides, which get stained by hematoxylin and eosin (H&E). Histological grading was evaluated based on the inflammatory criteria as described by Park et al. [195].

4.2.3 Enzyme-linked immunosorbent assay (ELISA)

Intestinal mucosa was collected and bead homogenized in lysis buffer (0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1mM benzethonium, 1% protease inhibitor cocktail (Boston Bioproducts, Ashland, MA, USA)). Sample supernatants were collect after centrifugation (10,000 × g, 30 min, 4 °C), and protein concentration in the supernatant was detected with BCA method [196]. The supernatant samples were loaded in sandwich enzyme-linked immunosorbent assay (ELISA) kits (Meso Scale Diagnostics, LLC, Rockville, Maryland, USA) to measure the concentrations of 10 inflammatory cytokines.

4.2.4 16S rRNA gene sequencing

Total genomic DNA in mice feces was extracted with QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s protocol with the addition of mild bead-beating step to increase yield.
Extracted DNA quantity was measured with a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, US) and quality was verified with gel electrophoresis and imaging. To characterize the microbial profile of each sample, the hypervariable regions V3-4 of the genes encoding the 16S rRNA were sequenced from the extracted DNA. PCR was performed to amplify the V3-4 region, which incorporates targeted primers and the Illumina overhang adaptor. The PCRs were performed in a 96 well format on a Veriti thermal cycler (Life technology, Carlsbad, CA, US) with 2x KAPA HiFi Hotstart ReadyMix (KAPA Biosystem, Wilmington, MA, US). After purification with AMPure XP beads (Beckman Coulter, Danvers MA, US), a limited cycle PCR was performed with the Nextera XT Index Kit (Illumina, San Diego, CA, US) to attach dual indices and Illumina sequencing adapters, followed by an additional purification with AMPure XP bead. The quantity of the purified PCR products was measured by Qubit dsDNA BR Assay kit (Life technology, Carlsbad, CA, US) and the amplicon quality was estimated by ScreenTape Assay on Tape Station 2200 (Agilent Technologies, Santa Clara, CA, US). After quantification and qualification, samples were pooled in equimolar amount and pair-end 2×300 bp sequencing was performed on Illumina MiSeq platform (Illumina, San Diego, CA, US).

4.2.5 Statistic analysis

The sequencing data was processed by QIIME software pipeline v1.9.1[158]. In general, the high quality (quality value>25) sequence data was demultiplexed. Sequences were then clustered into operational taxonomic unites (OTUs) using open reference OTU picking against Greengenes bacterial 16S rRNA database (13_8 release) [159] with 97% similarity threshold.
α-diversity (diversity within sample community species richness) was determined with ten iterations at a maximal sequence depth where all samples could be included. β-diversity (between sample communities dissimilarity) was calculated using weighted and unweighted UniFrac distances [160].

Galaxy’s Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to explore the predicted functional metagenome shifts between communities. According to the requirement form PICRUSt algorithm, operational taxonomic unites (OTUs) were aligned to the Greengenes 16S rRNA database using a closed reference picking protocol [161]. Functional profiling and comparing was performed with STAMP software [162]. Unpaired Students’ t-test was used to calculate the statistical difference between the two groups. The results were considered statistically significant at \( p < 0.05 \). Pearson correlation was examined in SPSS Statistics, and cluster and heatmap was created in R.

4.3 Results

4.3.1 General observations

In this study, DSS was used to induce the colonic inflammation in mice and anti-inflammatory effect by nobiletin was evaluated. At day 32, DSS induced severe illness in mice which evidenced by a dramatically higher DAI scores (including weight loss, diarrhea and evident rectal bleeding) (Table 4.1). Dietary nobiletin intervention significantly decreased the DAI scores compared with DSS group. It is generally accepted that colon length is negatively correlated with the severity of colitis in mice. As shown in Table 4.1, a significant shortening of colon length was observed in mice treated
with DSS compared with control mice (p<0.05), while nobiletin treatment had reversed
the change. The mean liver weight in DSS group was much lower than Control group,
while DSS+Nobiletin group has significantly greater liver weight than DSS group
(p<0.05) (Table 4.1). However, there were no pathological alterations suggesting toxicity
of test compounds in the liver, kidneys, lung, and heart of mice (data not shown).

Table 4.1 Disease activity index (DAI), spleen weight colon length and histological
score of each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>DSS</th>
<th>DSS+N1</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAI</td>
<td>0c</td>
<td>2.26±0.20a</td>
<td>1.56±0.17b</td>
</tr>
<tr>
<td>Colon length/mm</td>
<td>84.95±2.52a</td>
<td>69.57±2.98b</td>
<td>76.02±6.27ab</td>
</tr>
<tr>
<td>Liver weight/g</td>
<td>2392.37±181.66a</td>
<td>1808.42±59.48c</td>
<td>2197.81±90.22b</td>
</tr>
<tr>
<td>Histological score</td>
<td>0c</td>
<td>6.00±0.58a</td>
<td>2.67±0.88b</td>
</tr>
</tbody>
</table>

4.3.2 Effects of dietary nobiletin on the histological changes and expression of
inflammatory cytokines

The potential of dietary nobiletin on histological damages in the colons were
examined following the histological grading system as described [195]. Colonic
inflammation is characterized by severe lesions in the colon mucosa, alteration of
epithelial structure, increase in neutrophil population and lymphocyte infiltration into the
mucosal and submucosal layers, and loss of normal crypts. The colon specimens from the
negative control group showed normal features of typical glands, abundant goblet cells in
epithelium. In contrast, the colonic specimens obtained from the DSS group showed
serious erosive lesions with extensive loss of glands, cellular infiltration of immune cells
into the submucosa and lamina propria and crypt shortening, resulting in a high
histological score (Table 4.1). However, in the DSS+Nobiletin group, dietary nobiletin
remarkably reduced the total histological score compared with the DSS group. The colonic specimens had no erosion with just mild inflammatory cell infiltration. In both control group and control-cranberry group, no aberrant tissue was observed (Figure 4.1A, Table 5.1).

Figure 4.1B showed the concentration of three significantly changed inflammatory cytokines (IFN-γ, IL-10 and KC/GRO) in mice serum. Among the three groups, DSS group has the highest concentration of anti-inflammatory cytokine IL-10, while control group has the lowest level (p<0.05); nobiletin treatment increased the level of inflammatory cytokines IFN-γ and KC/GRO comparing with DSS group. As shown in Figure 4.1 C and D, the expression of eight immune cytokines in colon mucosa were different from that in serum. For all the eight cytokines (IFN-γ, GM-CSF, IL-2, IL-10, TNF-α KC/GRO, IL-1β, and IL-6), DSS group had greater expression than both control and DSS+Nobiletin group (p<0.05), indicating that nobiletin suppressed the inflammatory response in colon mucosa.
Figure 4.1: Dietary nobiletin intervention reduced colon inflammation in mice. (A) HE staining of mice colon mucosa. (B) The expression level of immune cytokines in mice serum. (C and D) The expression level of inflammatory cytokines in mice colon mucosa.

4.3.3 Impact of nobiletin on mice gut microbiota

4.3.3.1 Microbiome diversity

An average of 173219.8 counts/sample was passed the filters applied through the QIIME, and were clustered into 1406 identified OTUs (data not shown). All samples were rarefied to 67143 sequences to run the α- and β-diversity analysis, the lowest number of reads obtained from all sample datasets. Shannon index and Simpson value are indicators for evenness of the bacteria community in each group. Nobiletin interruption didn’t influence the evenness of microbiota community, and was much lower than the control group (Figure 4.2), indicating that nobiletin treatment was too weak to increase diversity of gut microbiota community.
Figure 4.2: Evenness of gut microbial community within the three mice groups

The UniFrac Principal Component Analysis (PCoA) based on the relative abundance of OTUs from each sample provided the information about the phylogenetic relationship among the fecal bacterial microbiota in all study groups. The unweighted PCoA figure we observed that DSS treatment separated the microbiota structural of the microbial community from the Control group, while nobiletin intervention attenuated the alteration as DSS+Nobiletin group was much closer to the Control group (Figure 4.3). ANOSIM with 999 permutations was used to calculate the significant separation the three groups with distance matrices of unweighted UniFrac. As expected, Bacteria community of DSS group separated from both Control and DSS+Nobiletin groups extremely (p<0.01). Moreover, microbiome community of DSS+Nobiletin group was significantly different from the Control group (p<0.05). These suggested that the nobiletin diet could alleviate the gut microbiota disruption by DSS treatment.
Figure 4.3: Principal coordinate analysis (PCoA) of unweighted UniFrac distance of fecal microbial communities performed on the 97% OTU abundance matrix revealed a distinct separation (p<0.05) among three groups (red dot indicated samples from Control group, blue dot indicated samples from DSS group, and yellow dot meant the sample from DSS+Nobiletin group). Each dot represents a sample from each mouse (n=5) fed diets.

In this study, significant variations of bacteria composition were observed among the three groups. *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Verrucomicrobia* and *Actinobacteria* were five major phyla in mice colon fecal microbiota (Figure 4.4), which was consistent with other mice gut microbiota research [197]. Linear discriminant analysis (LDA) effect size (LEfSe) was applied to determine differentially relative abundant bacterial phyla among the three groups (Figure 4.4). As observe din the figure, DSS group has great higher abundance for phyla TM-7 and *Proteobacteria*, while
nobiletin intervention promoted several genera in phyla *Firmicutes* and *Verrucomicrobiota*. At the genus level, DSS+Nobiletin group has greater abundance of *Turicibacter, Dehalobacterium, Dorea, Lachnospiraceae, Akkermansia* (p<0.05). DSS group was much higher abundance for 6 genera (p<0.05), such as *Parabacteroides, Ruminococcus, rc4-4, F16*, and *Bilophia*. For the commonly recognized probiotics, Bifidobacterium and Lactobacillus, Control group has much higher abundance. These results indicated that nobiletin diet could reverse the changes induced by DSS, while there’re some differences between Control and DSS+Nobiletin groups.

Figure 4.4: LEfSe identified the most differentially abundant taxons among the three groups. Taxonomic cladogram obtained from LEfSe analysis of 16S sequences.
(relative abundance $\geq 0.1\%$). (Red) Control-enriched taxa; (Green) taxa enriched in DSS group; (Blue) taxa enriched in Nobiletin treated colitic mice. The size of each dot is proportional to its effect size.

### 4.3.5 Predicted microbiome functions

Given the modification of microbiota profile following the exposure to dietary nobiletin in colitic mice, Galaxy’s PICRUSt was used as to explore the gut microbiota functions and the metagenomic functional pathways were predicted based on KEGG database [161]. DSS and DSS+Nobiletin groups were far separated for the microbiome community structure, while the functions were much closer as shown in the PCA figure, and both were separated from the Control group (Figure 4.5 A). The expression of 12 functional pathways were significantly modified (Figure 4.5 B). It’s obvious that the significance mainly caused by DSS, while the effect of nobiletin on the gene expression was weak. Therefore, changes of gene expression and bacteria composition might not be consistent.
Figure 4.5: Predicted gut microbiome functions among the three groups based on KEGG database. (A) PCA figure of gut microbiota functions among the three group. (B) The gene copy numbers of samples within the same sample group were pooled. Ratio of each functional gene to references was made and the significant enrichment and deplete was shown in the heatmap at the second level of KEGG pathway (p<0.05).

4.3.6 Correlation analysis

4.3.6.1 Correlation between bacteria at genus and immune cytokines

As DSS induced colitis was colonic inflammation associated with mucosa, Pearson correlation as run to investigate the possible relationship between bacteria abundance at gene level and the expression level of inflammatory cytokines in mice colon mucosa. As shown in the heatmap, genus Dorea, whose had greater abundance in DSS group, was extremely positive correlated with the expression of cytokines GM-CSF, IFN-γ, IL-10, IL-1β (p<0.01) (Fig. 4.6). The four cytokines were expressed at higher level in DSS group, followed by DSS+Nobiletin and Control group had the lowest expression. Take genus Lactobacillus as an example for the strong negative correlation,
the expression of IL-10 was negatively associated with the abundance of *Lactobacillus*. The level of IL-10 was lowest in Control group, which had the highest abundance of *Lactobacillus* in mice feces. Therefore, these results might contribute to the development of quick diagnosis for colonic inflammation without damage.

![Figure 4.6: Heatmap showing Pearson correlation coefficients between immune cytokines and bacteria abundance at genus level. (Red correspond to significant positive correlation value, and blue indicate markedly inversed correlation value).](image)

**4.3.6.2 Correlation between bacteria relative abundance at genus level**

Pearson correlation was run to monitor the relationship among the bacteria relative abundance at genus level, and the r value with strong correlation was listed in the heatmap. In our study, the abundance of *Akkermansia* was negatively associated with the
abundance of *Bifidobacterium, Adlercreutzia, Lactobacillus*, while positively correlated with *Dorea, Dehalobacterium, Bilophila* (p<0.05) (Figure 4.7). It was indicated in the correlation heatmap that the bacteria changes were not alone. Bacteria might change in group format, and the group member was determined by different factors.

**Figure 4.7:** Pairwise genetic correlation matrix of the linear distances. Genetic correlation was calculated from >15 million common genetic variants. Traits that have high positive genetic correlations with each other are shown in blue. Traits that have high negative genetic correlations with each other are shown in red.
4.4 Discussion

Previously, our lab found that dietary nobiletin exerted anti-inflammation with cell culture model and confirmed the effect with mice ear inflammation model [17]. In mice colon, nobiletin was metabolized by colonic bacteria, and some of the metabolites were even more effective than the parent compound [198]. However, the anti-inflammatory effect of nobiletin with IBD associated disease was limited and the role gut microbiota play was rare to date. As previously characterized [199, 200], murine chemical models of colitis have many similarities to human IBD, are characterized by cytokine dysregulation, have consistent colitis with a defined onset, and have become useful tools to study the innate and adaptive arms of the intestinal immune response in IBD. The mucosal immune system is the central effector of intestinal inflammation and injury, with cytokines playing a central role in modulating inflammation [190]. Therefore, DSS induced chronic colitic model was used to explored the anti-inflammatory effect of nobiletin in mice, especially focused on colonic microbiome and systemic and mucosal immune response.

It was reported that murine DSS-induced chronic colitis had distinct cytokine pattern [201]. The serum cytokine profile was a Th2-biased (IL-4 and IL-10) and B-cell differentiation (IL-6 and IFN-γ) induction synergistically inflammatory profile [202], which showed similar patterns of changes observed in local tissue (mucosa) [201]. In our study, IL-10 and IFN-γ was upregulated in the circulation system by DSS treatment in both serum and colonic mucosa. Besides, more cytokine changes were observed in local site, like GM-CSF, IL-6 and IL-12. The difference might due to host difference. TNF-α plays an important role on activation of various inflammatory cells and modulation of
epithelial cell permeability [195]. IL-12p70 is linked with autoimmunity. It stimulated the production of IFN-γ and TNF-α, and the significant increase of TNF-α might be contributed by IL-12p70 in our research. Strikingly, bladder cancer, bacteria invasion at epithelial cells and RIG-I-like receptor signaling pathway expression level was strongly positively correlated with the level of IL-12p70 and TNF-α, indicating nobiletin treatment might reduce its incidence. GM-CSF formation could be induced by pro-inflammatory cytokines, such as TNF-α and IL-1β, and it has been demonstrated in several different cell types, for example fibroblasts and endothelial cells [203, 204]. These results suggested the key role nobiletin played in modulating immune response, indicating that nobiletin could attenuate the progress of chronic disease.

Emerging evidence points that interrupted microbiota was associated with onset and progress of IBD [192, 205]. In addition to examining nobiletin intervention on the disease recovery, we also accessed the impact of nobiletin on gut microbial community composition and diversity. In this study, 500 ppm nobiletin diet trended toward recovery from DSS-induced dysbiosis to closer to the unchallenged microbial community based on UniFrac distance. At the meantime, the microbial composition responded dramatically to DSS administration and nobiletin supplement.

Compared with Control and DSS group, microbiota of DSS+Nobiletin group exhibited greater abundance of in phylum Verrucomicrobial, containing one specie Akkermanisa Muciniphila (A. muciniphila) so far. It is a key organism at the mucosal interface between lumen and epithelium cells due to its specific mucin degrading property. After DSS treatment the abundance of A. muciniphila was markedly enriched, which is consistent with others research [206-208]. However, in most human studies,
reduced levels or depletion of *A. muciniphila* is observed in IBD mucosa and in fecal samples [78]. Additional research indicates that the abundance of *A. muciniphila* positively correlated with mucin level in cecum [209]. A striking observation was that the Control group had the highest abundance of order *Lactobacillales* and phylum *Actinobacteria*, which contains the general regarded probiotic *Lactobacillus* and *Bifidobacterium*. These microbial results indicated that nobiletin had the capacity to modify the bacteria composition and provide protective effect for the gut health, though the details about how microbiota contributed is still unknown. As such, further investigations into the mechanism how microbiota works to improve gut health may be necessary.

Collectively, oral administration of 500 ppm nobiletin treatment benefited mice gut health through the modulation of microbiota and immune response. Nobiletin treatment modulated certain immune cytokines in colon mucosa and serum. Meanwhile, microbiota should be involved in the performance of nobiletin. This study, to our knowledge, is the first study to study the possible role of the anti-inflammation effect of nobiletin in DSS-induced colitis.

These findings enlarged the knowledge of gut microbiota in mice micro-ecosystem facing phytochemicals in diet. Our study provides the first line of evidence that dietary administration of nobiletin could alter gut microbiota and their functions, which warrants further investigation to determine its implication in human health.
CHAPTER 5
IDENTIFY AND CHARACTERIZE BACTERIA WITH DECONJUGAION CAPACITY FROM HUMAN FECES

5.1 Introduction

It’s documented that gut microbiota dysbiosis is associated disease, like inflammatory bowel disease [210, 211], while diet is crucial for the dynamics of gut microbiome. A xanthone isolated from mangosteen fruit-α-mangostin, exacerbates mice colitis and promoted gut microbiota dysbiosis [212]. Inversely, cranberry enriched diet ameliorated the mice colitis and modulated gut microbiota [213]. Our lab has long outstanding interest in the interaction between dietary phytochemicals and gut microbiota. We have observed the effect of nobiletin intervention on inflammation and fecal microbiota composition in mice. Interestingly, nobiletin residue was at readily low abundant compared with its metabolites in mice colon. In colon mucosa, total amount of nobiletin metabolites (named M1, M2, M3) is about 30-fold of nobiletin. The three metabolites (M1, M2, M3) have even stronger anti-inflammatory effect [18], indicating that the metabolites contribute to the anti-inflammatory effect of nobiletin. Whilst, more than 80% of nobiletin metabolites were in conjugated form in the small intestine, suggesting that certain bacteria in the colon deconjugated the conjugates formed by Phase II metabolism in liver [18]. Gut microbiota contributed a lot to xenobiotic metabolism, such as Clostridium sporogenes metabolites amino acids into nine circulating
metabolites[214], Clostridium and Eubacterium genera are commonly involved in the metabolism of some polyphenols [215], and strain belonging to species Blautia could hydrolyze the aryl methyl ether group from polymethoxyflavones [216]. Therefore, we hypothesize that there’re certain strains could deconjugate the conjugates in the colon or feces.

In addition to the xenobiotic metabolizing capacity of gut microbiota, probiotics can benefit host health with their secretion. Bifidobacterium infantis secretion improved human epithelia cells (T84) barrier function by increasing the expression of tight junction protein in vitro, and the oral treatment of bacteria secretion ameliorate gut inflammation, increased colon barrier function in IL-10−/− mice model [137]. Conditioned medium of Bifidobacterium longum sup infantis attenuated IL-6 production in response to IL-1β through TLR-4 in cell H4- a human fetal small intestine epithelial cell [19].

In this study, we will isolate and characterize the bacteria from human feces with nobiletin metabolites deconjugation capacity. The bioactivities of the isolated bacteria secretion will be studied also, including anti-inflammation and anti-cancer capacity.

5.2 Material and Methods

5.2.1 Bacteria isolation

Fresh fecal sample from a healthy male donor (27-32 years old) was collected and homogenized with sterilized peptone water (1:10). Fecal bacteria were collect after centrifugation (200 g ×5 min, 4 ℃) to remove food residues. Then serial diluted fecal microbiota streaked on Gifu anaerobic agar (GAM, Nissui Pharmaceutical Co., Tokyo, Japan) and cultured in an anaerobic cabinet (Whitley A35 anaerobic workstation, Don
Whitley Scientific) under an atmosphere of 5% CO\(_2\), 10% H\(_2\), and 85% N\(_2\) at 37 °C for 48 h. Single colonies were incubated with small intestine content (10 mg/mL) collected from mice fed with dietary nobiletin. After 48 h fermentation, the supernatant and bacteria were collected for future analysis.

5.2.2 Determine the biotransformation potential of isolated colonies

200 \(\mu\)L fermentation supernatant was extracted with double volume of ethyl acetate for three times. Pooled ethyl acetate fractions were dried under speed vacuum condition and re-dissolved in 50% acetonitrile for HPLC analysis (Model 2000, Agilent Technologies, USA) on an Amino acid C18 column (250 mm \(\times\) 4.6 mm, 5 \(\mu\)m, Agilent Technologies, USA) at a flow rate of 0.8 mL/min. The linear gradient elution condition was: started with 75% mobile phase A (5% ACN/water, v/v)/25% mobile phase B (100% ACN) (v/v) for 5 min, then processed to 100% B over 20 min, and held at 100% B for 5 min. The elution was monitored at UV length 326 nm [217]. Nobiletin (Quality Phytochemicals LLC, Edison, NJ, USA) and its free form metabolites (M1, M2, and M3, synthesized within our lab with purity \(\geq 98\%\)), were used as external standards for nobiletin and its metabolites identification and quantification.

5.2.3 16S rRNA gene sequencing and phylogenetic analysis

Strain Isolate G7 was isolated from the fecal sample, which showed deconjugation potential. 16S rRNA gene sequencing was applied for bacteria identification. Partial 16S rRNA gene was amplified by PCR with primers 27F (5’-AGAGTTTGATCMTGCTCAG-3’) and 1492R (5’-GGYTACCCTGTTACGACTT-3’) [216], and sequencing was done by the sequencing service at GENWIZ (NJ, USA). The
16S rRNA sequence was analyzed with the BLAST tool in National Center for Biotechnology Information (NCBI) to identify bacterial species based on sequence similarity[218]. Phylogenetic trees were constructed with neighbor-joining method with MEGA 7 program [219].

5.2.4 Isolate G7 secretion collection

Stored bacteria Isolate G7 was subcultured on MRS agar (Difco Laboratories, MI, USA) containing 0.5 g/L L- cysteine (Sigma- Aldrich, St. Louis, MO, USA) in anaerobic condition at 37 °C for 2 days. Single colony was inoculated in fresh MRS broth overnight and the OD_{600} nm was adjusted to 0.5. Then, the adjusted Isolated G7 inoculate was inoculated with MRS broth (1: 100, v:v) supplemented with small intestine content from mice fed with nobiletin (final concentration 10 mg/mL), and incubated at 37 °C in anaerobic condition for 48 h. Fermentation samples were collected every 12 h and filtered through 0.22 um filter to obtain sterile isolate G7 secretion. Samples collected at different time point were stored at -20 °C for future treatment.

5.2.5 Anti-inflammation assay with RAW 264.7 cells

RAW 264.7 cells purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) were cultured in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% heat-inactivated foetal calf serum (FBS) (Medistech, Herndon, VA, USA), 100 units/mL of penicillin and 0.1 mg/mL of streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C with 5% CO2 and 95% air. Cells were seeded into 96-well plate (1×10^6 cells/mL). After 24 h attachment, cells were stimulated by incubation in a medium containing100 ng/mL lipopolysaccharides (LPS) and Isolate G7
secretion (7.5%) collected at different time were added together with LPS. Cells were treated with 7.5% MRS as the vehicle control and cells treated with 7.5% MRS without the presence of LPS as the blank control.

Nitric oxide production by RAW 264.7 cells was determined as nitrite concentration in the culture medium according to the Griess reaction [220]. The culture medium of the control and treated cells was collected and mixed with an equal volume of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture was read at 550 nm with microplate reader (Dynatech MR-7000, Dynatech Laboratories, Chantilly, VA). The Nitric oxide production was normalized by MTT results.

The MTT assay is based on the reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), by actively growing cells. After cells being treated for 24 h, cell culture medium was replaced with 100 μL MTT solution (1.0 mg/mL RPMI) and cells were incubated for 2 h. Thereafter, dumping the culture supernatant, 100 μL DMSO was added into each well and the plate was gently agitated to dissolve crystal MTT. Absorbance was measured at 540 nm, with microplate reader (Dynatech MR-7000, Dynatech Laboratories, Chantilly, VA). DMSO was used as a negative control. Normalized nitric oxide production = OD_{550} nm/OD_{540} nm. For cell cytotoxicity, active cells were detected by MTT assay. Cell viability (%)= (OD of treatment group/OD of control group) × 100.

5.2.6 Analysis of cell viability, cell cycle and cell apoptosis

Human colon cancer cell HCT 116 (ATCC, Manassas, VA, USA) were cultured
in RPMI-1640 media supplemented with 5% heat-inactivated FBS and antibodies (100 units/mL penicillin and 0.1 mg/mL streptomycin) at 37 °C with 5% CO₂ and 95% air. Assays for cell viability, cell cycle and apoptosis were conducted as we previously described [221-223]. In brief, cell HCT116 were seeded in 96-well plates (2 ×10⁴ cells/mL). After 24 h, cells were treated with 7.5% isolate G7 secretion for 24 h, and the cell viability was quantified by MTT assay. HCT116 were seeded in 6-well plates for cell cycle and apoptosis analysis (5×10⁴ cells/mL). After 24 h incubation for cell attachment, cells were treated with 7.5% isolate G7 secretion. After 24 h treatment, floating cells in medium were harvested and combined with attached cells that were detached by brief trypsinization. Then cell pellets were washed with 1 mL of ice-cold PBS and subject to cell cycle and apoptosis analysis by flow cytometry as we described previously [221-223]. 7.5% MRS treatment was used as control.

5.2.7 Immunoblotting

Whole cell lysate was prepared as previously described [223, 224]. RAW 264.7 cells were seeded in 10-cm culture dishes. After 24 h of incubation, RAW 264.7 cells were treated with 1.0 µg/mL LPS alone or with 15% isolate G7 secretion. HCT116 cells were treated with 7.5% isolate G7 secretion only. The same percentage of MRS was used as control. After 24 treatment, the cells were harvested and extracted with RIPA lysis buffer (Boston Bioproducts, Ashland, MA, USA) containing 1% protease inhibitor cocktail (Boston Bioproducts). The homogenates were centrifuged at 12,000×g for 20 min at 4 °C. Protein concentrations were determined with the BCA method (Pierce, Rockford, IL, USA). The cell lysates were further subjected to Western blotting analysis as previously described [223, 224]. Antibodies for iNOS, COX-2, Bcl-2, cleaved-PARP,
cyclin D1, cyclin E, CDK4, CDK6 and p21 were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibody for β-actin was purchased from Sigma-Aldrich.

5.2.8 qRT-PCR

qRT-PCR was performed as previously described [225]. Total RNA was extracted from HCT116 cells with Trizol Reagent (Ambion) according to the manufacturer’s instructions. Total RNA (2 μg) was converted to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer’s instructions. The qRT-PCR was carried out with Applied Biosystems ViiA 7(TM) Real Time PCR System (Life Technology) with Maxima SYBR-green Master Mix (Thermo Fisher Scientific). The sequences of human colon cell specific primer (Thermo Fisher Scientific) were listed in Table 5.1. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal control. The copy number of each transcript was calculated with respect to the Gapdh copy number, using the $2^{-\Delta\Delta CT}$ method [226].

Table 5.1 Sequence of primers used in qRT-PCR for Wnt signaling

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>AGGTCGGGTGTAACCGGATTTG</td>
<td>TGTAGACCATGTAGTTGAGGTCA</td>
</tr>
<tr>
<td>c-MYC</td>
<td>TGAAGTCACAGTTGAGGGG</td>
<td>AGAGCTCCTCGAGCTGTTC</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>AGAGGCAGGAGGAAACAAAC</td>
<td>GGCAGGTGAAATGAAC</td>
</tr>
<tr>
<td>AXIN2</td>
<td>TGCACTCTCTGTTGAGCTG</td>
<td>ACTGACCGACGATCCATGT</td>
</tr>
<tr>
<td>CDC-2</td>
<td>CGCCCTTTTCTCTTTCTTCTT</td>
<td>ATCGGGTAGCCCGTAGACTT</td>
</tr>
</tbody>
</table>
5.2.9 Statistic analysis

Data were presented as means ± standard deviation (S.D.) for the indicated number of independently performed experiments. All statistical analyses were performed using SPSS. Unpaired student's t-test was used to assess the mean difference between two groups. A p-value<0.05 was considered statistically significant.

5.3 Results and Discussion

5.3.1 Strain Isolate G7 isolation

Humans have co-developed with trillions of microorganisms living in and on human body, whose genomes extend our metabolic capacity beyond those encoded by human genome. Xenobiotic metabolism is one of the major influence of gut microbiota on host, which will affect the bioactivity, bioavailability and toxicity of xenobiotics. Deconjugation is one of the most common reaction type, which contributed to the anti-inflammation effect of nobiletin in mice. As we mentioned before, free form of metabolites of M1, M2 and M3 has way stronger anti-inflammation and anti-cancer effect than nobiletin [113, 114]. Here, we wanted to isolate bacteria with deconjugation capacity.

From the diluted fecal samples, 46 single colonies were isolated and incubated with small intestine content from mice fed with nobiletin to determine the deconjugation activity. After 48 h fermentation, supernatants extracted by ethyl acetate were loaded into HPLC. For nobiletin metabolite conjugates incubated with Isolate G7, peaks for free form nobiletin metabolites M1, M2 and M3 appeared (Figure 5.1), indicating that Isolate G7
could secret enzyme to release glucose or sulfate group from the conjugates formed by Phase II metabolism. Then, Isolate G7 was successfully subcultured on GAM plates anaerobically without losing deconjugation activity.

![Chromatograph](image)

Figure 5.1 Chromatograph for small intestine content from mice fed with nobiletin fermented with the presence or absence of Isolate G7.

### 5.3.2 Strain Isolate G7 identification

To identify the bacteria Isolate G7, its partial 16S rRNA gene sequence was obtained by Sanger sequencing. After blasting in NCBI, gene sequence was most closely related with *Bifidobacterium Pseudocatenulatum* DSM20438 and IPLA36007, showing 98% similarity. Other strains in the family *Bifidobacteriaceae* and genus *Bifidobacterium*, particularly *Bifidobacterium catenulatum* DSM16992, *Bifidobacterium adolescentis Reuter* 1963, *Bifidobacterium angulatum* DSM20098, *Bifidobacterium dentium* DSM20436, *Bifidobacterium breve* DSM20213 and *Bifidobacterium bifidum*
ATCC29521, also showed high sequence similarity (> 90% similarity) to the isolated bacterium (Figure 5.2). Therefore, the isolated G7-metabolizing anaerobic bacterium was identified as *Bifidobacterium pseudocatenulatum* G7. *Bifidobacterium pseudocatenulatum* IPLA 36007, isolated as from human feces, exhibited the capability of releasing aglycones from soy isoflavone glycosides[227, 228]. There’re some other species showed the deconjugation capacity, like *Bifidobacterium animalis* [229], *Bifidobacterium breve*[230], *Bifidobacterium angulatum*, *Bacteroides thetaiotaomicron*, and *Eubacterium siraeum*[231].

![Phylogenetic tree of Isolate G7 with strains belong to the same family.](image)

Figure 5.2 Phylogenetic tree of Isolate G7 with strains belong to the same family.

### 5.3.3 Anti-inflammatory effect of Isolate G7 secretion

In addition to xenobiotic metabolizing capacity, species *Bifidobacterium pseudocatenulatum* is generally regarded as probiotic. Several researches indicated that oral administration of *Bifidobacterium pseudocatenulatum* could benefit host health [232-235]. However, research on how probiotic benefit host health is still overlooked. Colon is
the niche where most bacteria grow and colonize. The biofactors released during bacteria growth might improve host health.

Macrophages is a major component of the mononuclear phagocyte system, like blood monocytes, and tissue macrophages. They play a critical role in the initiation, maintenance, and resolution of inflammation [236]. Therefore, we tested the anti-inflammation effect on macrophages, which is the common immune cells. From the Figure 5.3a, it’s obvious that Isolate G7 secretion collected at different time points showed marginal effect on cell viability (>93% cell survived), indicating that Isolate G7 secretion has no toxic effect on macrophages. Cell inflammation induced by LPS was significantly inhibited by bacteria secretion, as shown by the reduced production of nitric oxide. The anti-inflammation effect showed a fermentation time dependent response, indicating that most effective components was secreted during the whole 48 h fermentation time. At the time 48 h, the nitric oxide produced was as low as about 6.5%, which was 92.7% at time 0 h.

To make clear the pathway involved in the anti-inflammation effect, we checked key protein expression. Pro-inflammatory enzyme iNOS catalyzes the oxidative deamination of L-arginine to generate NO, which is a critical inflammatory mediator. Overproduction of NO can further cause the structural modification of DNA and mutagenesis [237]. Since iNOS and its downstream products play key roles in the occurrence of chronic inflammatory diseases including colon cancer, iNOS and related signaling molecules have become critical targets for anti-inflammation and anticancer remedy [238]. Enzyme COX-2 is a rate-limiting step for Prostaglandin E2 synthesis, which is produced by macrophages and contributes to vasodilation, pain and fever [239].
Comparing with the negative control group (without LPS and Isolate G7 secretion treatment), LPS treatment stimulated the production of proteins iNOS and COX-2 with a dark bold band. While the expression of protein iNOS and COX-2 were almost depleted when treated with Isolate G7 secretion (48 h), with iNOS downregulated to 0.001 and COX-2 was decreased to 0.14 of the positive control group (Figure 5.3b). Both enzyme iNOS and COX-2 are downstream key proteins in NF-κB pathway, suggesting that isolate G7 secretion could interrupt NF-κB pathway in macrophages. Therefore, isolate G7 could inhibit LPS-induced inflammation on macrophages by blocking the NF-κB pathway.

![Graph](image)

Figure 5.3 Anti-inflammation effect of isolate G7 secretion on LPS stimulated inflammation on RAW 264.7. (a) Cytotoxicity and nitric oxide production of Isolate G7 secretion treatment obtained at different fermentation time. (b) The expression of enzymes iNOS and COX-2 with or without isolate G7 secretion treatment.
5.3.4 Anti-cancer effect of Isolate G7 secretion

The connection between inflammation and tumorigenesis is well-established and in the last decade has received a great deal of supporting evidence from genetic, pharmacological, and epidemiological data. Inflammatory bowel disease is an important risk factor for the development of colon cancer[240]. Here, we studied the anti-cancer effect on human colon cancer cell HCT116. After cells treated with bacteria secretion for 24 h, cell viability was reduced dramatically, specifically for sample collected at 24 h fermentation time, which can be lower than 40% (Figure 5.4), suggesting that isolate G7 secretion could inhibit cancer cell proliferation effectively.

Figure 5.4 Cell viability of HCT116 after treated with Isolate G7 secretion obtained at different fermentation time.
Dysfunction in the physiological pathways of programmed cell death may promote proliferation of malignant cells, and correction of such defects may selectively induce apoptosis in cancer cells. Cell apoptosis is an effective way to lead cancer cell death. Isolate G7 secretion treatment markedly increased both early and late apoptosis for cell HCT116 (p<0.01), and the total apoptotic cells with treatment was about 3-fold of the control group (Figure 5.5a). To confirm this finding, we checked the key protein expression related with cell apoptosis. Protein Bcl-2 is an anti-apoptotic protein[241], whose expression was downregulated to 66% comparing with control group (Figure 5.5b). Protein Cleaved-PARP is named damaged protein sensor, and hyperactivation of the PARP pathway may be exploited to selectively kill cancer cells. During apoptosis, the presence of the 89-kDa PARP cleavage fragment is considered a marker of cells that have undergone apoptosis[242, 243]. In Figure 5.5b, it’s obvious that protein Cleaved PARP expression was upregulated by 42% with isolate G7 secretion treatment. Here, we could point out that the isolate G7 secretion treatment could induce human colon cancer cell HCT116 apoptosis.
Figure 5.5 (a) Effects of Isolate G7 secretion obtained at 48h fermentation time on cell apoptosis. (b) Effects of Isolate G7 secretion obtained at 48h fermentation time on cell apoptosis-related signaling proteins.

Except cell apoptosis, cell cycle progression plays essential roles in cancer. In normal cells, cell cycle is strictly regulated by a series of signaling pathways. However, in cancer, this regulatory process is often malfunctioned due to genetic mutations [244]. Thus, induction of cell cycle arrest is an effective strategy to control cancer. Therefore, we tested the cell population at different cell cycle stages after treated with Isolate G7 secretion. Cell cycle is divided into three phases, G0/G1 phase, S phase and G2/M phase. With Isolate G7 secretion treatment, cell population was arrested at G2/M phase significantly (p<0.05) (Figure 5.6a). The effect of Isolate G7 on the expression of several cell cycle related proteins was monitored also. Cyclins and cyclin-dependent kinases (CDKs) are two important classes of proteins that regulate the cell cycle progression. Misregulated cyclins and CDKs may stimulate abnormal cell proliferation and genomic and chromosomal instability. The transition of cell cycle from G1 to S phase is mainly
driven by cyclin D-CDK4/6 and cyclin E-CDK2 complexes. CDK inhibitors, such as p21 can arrest cell growth by holding the cell cycle at the G1/S transition point [221]. In figure 5.6b, protein cyclin D1, cyclin E, CDK4, CDK6 and P21 were all misregulated by isolate G7 secretion, suggesting that isolate G7 secretion could modulate cell cycle and contribute to the lower active cell number.

![Graph showing cell population/% for cyclin D1, cyclin E, CDK4, CDK6, P21, and β-actin in HCT116 cells with control and Isolate G7 secretion.](image)

**Figure 5.6** (a) Effects of Isolate G7 secretion obtained at 48h fermentation time on cell cycle. (b) Effects of Isolate G7 secretion obtained at 48h fermentation time on cell cycle-related proteins in human colon cancer cell HCT116.

Overactivation of Wnt signaling is a hallmark of colorectal cancer. Wnt pathway is a key regulator of both the early and the later, more invasive, stages of CRC development[245]. In CRC, aberrant activation of the Wnt signaling pathway is a central oncogenic driver in 90% of patients, mostly resulting from mutations in the adenomatous polyposis coli gene[246]. CDC-2, Cyclin D, c-MYC and AXIN2 are all genes involved in
Wnt pathway. All these genes were remarkably downregulated when treated with isolate G7 secretion (Figure 5.7). The deactivation capacity of Wnt signaling pathway proved the anticancer effect of isolate G7 secretion. Simply, we could conclude that isolate G7 secretion has strong anti-inflammation effect by promoting cell apoptosis, arrest cell cycle and deactivate Wnt pathway.

![Graphs showing gene expression changes](image)

Figure 5.7 Effects of Isolate G7 secretion obtained at 48h on the gene expression associated with Wnt signaling pathway in cell HCT116.

### 5.4 Conclusions

After oral administration of xenobiotics, it will interact with gut microbiota in vivo. Xenobiotic could be metabolized by specific bacteria and influence its bioactivities.
Besides, xenobiotic could modify the gut microbiota, which may influence host health. For the strong anti-inflammation effect of nobiletin, we found that bacteria Isolate G7 in mice colon could release the free form of M1, M2 and M3 which contributed to the anti-inflammation effect. Besides, the Isolate G7 is comes from species Bifidobacterium pseudocatenulatum, which is a probiotic. Isolate G7 secretion has strong anti-inflammation effect by blocking the NF-κB pathway. What’s more, Isolate G7 secretion exerted distinguished anti-cancer potent by promoting cancer cell apoptosis, arrest cell cycle at G2/M phase, and downregulate the Wnt signaling pathway. Therefore, we can infer that the interaction between nobiletin and gut microbiota contributed to the anti-inflammation effect of nobiletin. Probiotic isolate G7 has the potential to be a functional food to benefit human gut health.
CHAPTER 6

ENCAPSULATION OF BIFIDOBACTERIUM PSEUDOCATENULATUM G7 IN GASTROPROTECTIE MICORGEELS: IMPROVEMENT OF CELL VIABILITY UNDER SIMULATED GASTROINTESTINAL CONDITIONS

6.1 Introduction

The human gastrointestinal tract (GIT) typically contains hundreds to thousands of different bacterial species, forming a complicated microbial ecosystem [2]. The genus Bifidobacterium is one of the most common bacteria in the feces (3.2 %) and cecum (5.2 %) of humans [247-249]. Bifidobacterium species are regarded as probiotics due to their potential health benefits and have been generally recognized as safe (GRAS) by the US government [250]. There are more than 50 species belonging to the genus Bifidobacterium, with Bifidobacterium pseudocatenulatum (B. pseudocatenulatum) being one of the most dominant species.

As a probiotic, B. pseudocatenulatum has been studied for its efficacy in the prevention and treatment of a broad spectrum of animal and/or human gastrointestinal disorders. It has been reported to attenuate abnormal vascular function in obese mice [8], to partially restore neuroendocrine function in obese mice [251], and to reduce inflammation in mice with cirrhosis [252]. However, probiotics have to reach their site of action at a sufficiently high level before they can exhibit their potential health benefits. Unfortunately, environmental challenges encountered in the human GIT, such as gastric
acids, bile salts, and digestive enzymes, have limited the application of probiotics in vivo [253].

Encapsulation of probiotics in colloidal delivery systems can improve the shelf life and viability of probiotics during storage and in the GIT [139-141]. Encapsulation of Lactobacillus casei 01 in alginate-starch beads improved cell viability during freeze-drying and storage [142]. Encapsulation of B. longum in alginate microgels coated with chitosan improved their survival during passage through a simulated GIT [139]. However, there are few studies showing that colloidal delivery systems can successfully preserve the viability of probiotics after 2 hours’ exposure to gastric conditions [139, 143]. The main reason for the poor gastric stability of probiotics encapsulated in microgels is that hydrogen ions (H⁺) can easily diffuse through the hydrogel network and inactivate the encapsulated bacteria. Previously, we prepared alginate microgels loaded with an antacid agent, Mg(OH)₂, that could neutralize hydrogen ions when they entered the microgels and thereby maintain a neutral internal pH even when they were dispersed in acidic gastric fluids [144]. These antacid microgels may therefore be particularly useful for increasing the cell viability of probiotics for oral delivery.

B. pseudocatenulatum G7 (BPG7) has been isolated from healthy human feces in our laboratory and shown to have strong anti-inflammatory and anti-cancer activities (data not shown). The availability of an effective oral delivery system for this potent probiotic that would protect it throughout the GIT would be highly advantageous for commercial applications [254]. In this study, we encapsulated BPG7 in alginate microgels loaded with two different kinds of antacids, either Mg(OH)₂ or CaCO₃. Both Mg(OH)₂ and CaCO₃ are widely used food-grade antacids, which are insoluble at neutral
and basic pH but dissolve at acidic pH thereby releasing hydrogen ions (OH\(^-\)). We hypothesized that these antacid microgels would maintain an internal neutral pH under acidic gastric conditions so as to enhance the viability of the probiotic in the gastrointestinal tract. The results obtained from the current study should provide valuable information for optimizing the performance of probiotic-loaded delivery systems for applications in functional foods and beverages.

6.2 Material and methods

6.2.1 Bacteria propagation and general growth conditions

The probiotic (BPG7) was stored at -80 °C in deMan, Rogosa, Sharpe (MRS) broth (Difco Laboratories, Sparks, MD) containing 50% glycerol (Sigma, St Louis, MO, USA). After growing on a MRS agar plate at 37 °C for 48 h, a single colony was selected and propagated in fresh 40 mL MRS for 24 h, anaerobically. Anaerobic conditions were maintained using an anaerobic chamber with an airlock (82% N\(_2\), 10% CO\(_2\) and 7% H\(_2\); Whitley A35 Anaerobic Workstation, Microbiology International, Frederick, MD).

6.2.2 Probiotic Microencapsulation

Cells were harvested by centrifugation at 4000 g for 10 min at 4 °C and then washed twice with sterile peptone water. The washed cells were re-suspended in 20.0 mL sterile deionized water. BPG7-loaded microgels were prepared by mixing the bacteria with 2% sodium alginate solution in the absence of presence of an antacid agent (either Mg(OH)\(_2\) or CaCO\(_3\)) (1:1, v/v). As shown in Figure 1, the polymeric matrix was agitated to uniformly distribute the bacterial cells throughout the mixture. The mixture was left to stand for 5 min to allow any dissolved air to leave the solution prior to microgel
preparation using an automated encapsulation device (Büchi B-390 Encapsulator, Flawil, Switzerland). An injection nozzle with a diameter of 200 µm was used and standard operating conditions were utilized: vibration frequency = 800 Hz, electrode potential = 800 V, and driving pressure = 500 mbar. The microgels were collected in 60 mL of 10% calcium chloride solution (Sigma, St Louis, MO, USA). The microgels were then vacuum-filtered and rinsed with sterile deionized water (200 mL).

6.2.3 Surface potential characterization of encapsulated calcium alginate microgels

The surface potential (φ-potential) of the microgels was determined using an electrophoretic light scattering device (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). For each sample, filtered microgels (~0.1 g) were re-suspended in appropriate pH-adjusted solutions prior to measurements to avoid multiple scattering effects. Specifically, phosphate buffer (5 mM, pH 7.0) was used for the dilution of initial and small intestine samples, while acidified distilled water (pH 2.5) was used for dilution of the stomach samples.

6.2.4 Particle size characterization of encapsulated calcium alginate microgels

The particle size distribution of all the samples was measured using a static light scattering instrument (Mastersizer S, Malvern Instruments, Worcestershire, UK). Again, initial and small intestine samples were diluted in phosphate buffer (5 mM, pH 7.0) while stomach samples were diluted in acidified distilled water (pH 2.5) to avoid multiple scattering effects. The particle sizes are reported as the surface-weighted mean diameter (d_{43}).
6.2.5 Cold-stage scanning electronic microscopy (cryo-SEM)

Microgels were fixed by immersion in 2.5% (v/v) glutaraldehyde in 0.5 M sodium cacodylate-hydrochloric acid buffer (pH 7.2) for 1 h at room temperature. The fixed samples were then washed three times in deionized water. The alginate microgels were then placed on an aluminum SEM stud and a second stub was placed on top to create a cap on top of the alginate microgels. This sandwich was then plunge frozen in liquid nitrogen. Once frozen, the aluminum stubs were quickly pulled apart, which fractured the microgels. The two aluminum stubs were then placed in a vacuum lyophilizer and completely dried overnight. The next morning the fractured and dried alginate microgels were mounted to carbon tape on a SEM stub and sputter coated with 6 nm of Au/Pd (80/20). The mounted and coated specimens were examined and digitally imaged at various magnifications using a scanning electron microscope (FEI Quanta 200 MKII FESEM, FEI Company, Hillsboro, Oregon, USA).

6.2.6 Confocal scanning laser microscopy (CLSM)

Cell viability and distribution were characterized using confocal scanning laser microscopy (CLSM). The probiotic was labelled with fluorescent molecular probes using a LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, MA, USA). Dye SYTO 9 is a green-fluorescent nucleic acid stain for both live and dead cells, whereas propidium iodide (PI) is a red-fluorescent nucleic acid stain that only interacts with cells that have damaged membranes, leading to a reduction in the SYTO 9 fluorescence signal. Therefore, live bacteria fluoresce green, while dead bacteria fluoresce red. Equal volumes of the SYTO 9 and PI dyes were mixed together. Then, 3 µL of this dye mixture was added to 1 mL microgel solution and incubated in the dark for
15 min at room temperature prior to visualization with CLSM [255]. Images were obtained with a 20-objective lens (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, USA). For the SYTO 9 dye, an excitation wavelength of 488 nm was used and an emission wavelength of 590 nm/50 nm. For the PI dye, an excitation wavelength of 488 nm was used and an emission wavelength of 650 nm/LP. The confocal fluorescent images were analyzed using image analysis software (NIS-Elements, Nikon, Melville, NY, USA).

6.2.7 Enumeration of viable cells

To determine viable cell counts of encapsulated bacteria, alginate microgels (0.10 g) were suspended in 9.9 mL of 10% sodium citrate dihydrate solution (pH 8.2; Fisher, Fairlawn, NJ, USA) followed by mixing for 10 min at room temperature to dissociate them. After serial dilutions of the dissociated microgels (10⁻²–10⁻⁵), the number of surviving cells was determined by plate culture on MRS agar in duplicate and anaerobically incubated at 37 °C for 48 h. All the samples were counted initially, after the simulated stomach phase, and after the simulated stomach and small intestinal phase.

6.2.8 Simulated gastrointestinal fate of microgels

Samples with the same concentrations of free or encapsulated BPG7 cells and the same total volumes were prepared by diluting the original samples with buffer solution (5 mM PBS, pH 7). The samples were then passed through an in vitro GIT model that simulated the stomach and small intestine phases. Specifically, simulated gastric fluids (SGF) was prepared by adding sodium chloride (2 g) and 6 M hydrochloric acid (7 mL) into one-liter of distilled water and then filter sterilized. The simulated intestinal fluids (SIF) was prepared by dissolving calcium chloride (0.25 M) and sodium chloride (3.75 M) in phosphate buffer (5 mM PBS, pH 7). A bile salts solution was prepared by
dissolving 1.5 g porcine bile extract in 28 mL phosphate buffer (5 mM PBS, pH 7), and 3.5 mL of the bile salts solution was added to each intestinal phase. All the simulated stock solutions were subjected to autoclaving before the experiments.

Free and encapsulated BPG7 cells were sequentially added to SGF (45 mL, pH adjusted to 2.5) for 2 h, and then incubated with SIF (45 mL, pH adjusted to 7.0) for 2 h. All the incubation experiments were carried out in an anaerobic chamber set at 37 °C. Dilutions \((10^{0}-10^{-5})\) of the released cells were plated on MRS agar for samples collected initially, after 2 h exposure to a simulated gastric phase, or after 4 h exposure to simulated gastric and then intestinal phases. Then MRS plates were incubated anaerobically for 48 h at 37 °C for viable cell counting.

6.2.9 Statistical analysis

Experiments on the physicochemical characteristics of the microgels were repeated in duplicate. Experiments on viable cells counts were repeated at least six times independently. All values are expressed as mean ± standard deviation (SD) unless stated otherwise. The difference among samples were analyzed by one-way ANOVA with significance level of \(p < 0.05\).

6.3 Results and Discussion

6.3.1 Particle size analysis

Alginate is an anionic polysaccharide that has been widely used for the microencapsulation of probiotics because of its ability to form microgels using a simple injection process. Two different antacid agents, either Mg(OH)\(_2\) or CaCO\(_3\), were co-encapsulated with the probiotics inside the alginate microgels to enhance their viability
during gastrointestinal transit (Figure 6.1). A series of controls was also studied: alginate microgels containing no antacids or probiotics; Mg(OH)$_2$-loaded microgels with no probiotics; and, CaCO$_3$-loaded microgels with no probiotics. Changes in the particle size of the microgels were then determined as they passed through various stages of the simulated GIT. Incubation under oral conditions was not simulated in the current study because the short duration and neutral pH conditions in the human mouth would not have been expected to impact cell viability.

Figure 6.1 Schematic diagram of delivery systems fabrication containing probiotic loaded beads with or without buffer agent encapsulation.

The general shape of the particle size distribution of all the microgels remained fairly similar throughout the entire GIT (Figure 6.2), suggesting that they retained their overall integrity when exposed to upper gastrointestinal conditions. There was a slight decrease in the mean particle diameter ($d_{43}$) for all the samples after exposure to the simulated stomach phase, which has been previously attributed to a reduction in the electrostatic repulsion between the anionic alginate chains when the carboxyl groups
become partially protonated under acidic conditions. Interestingly, the CaCO$_3$-loaded microgels were initially larger (665 µm) than the Mg(OH)$_2$-loaded ones (540 µm). This may have been because the initial alginate-CaCO$_3$ solution had a higher viscosity than the alginate-Mg(OH)$_2$ solution before injection (data not shown). This may have been because some of the solid CaCO$_3$ particles partially dissolved and released a few calcium ions (Ca$^{2+}$) that cross-linked the alginate and increased the solution viscosity.

Figure 6. 2 Particle size distribution of different delivery systems after exposure to successive GIT stage: (a) Control beads (b) CaCO$_3$-loaded beads and (c) Mg(OH)$_2$-loaded beads.

6.3.2 Surface potential analysis

The electrical characteristics of the microgels were also determined during the simulated GIT process. All the microgels initially had a strong negative charge of around -25 mV (Figure 6.3), which can be attributed to the presence of anionic carboxylic acid groups (–COO$^-$) from the alginate molecules being present at the hydrogel surfaces. An appreciable reduction in the magnitude of the potential occurred for all the microgels after exposure to the simulated stomach phase. This effect is due to the fact that the simulated gastric fluids have a low pH and high ionic strength, which reduced the
ionization of the anionic charged groups and caused electrostatic screening effects. This result is consistent with the observed shrinkage of the microgels under simulated stomach conditions, as mentioned in the previous section. After exposure to the small intestine phase, all the microgels again had relatively high negative charges. This was due to the deprotonation of the carboxyl groups on the alginate molecules under simulated intestine conditions (pH 7), as well as the presence of anionic species such as bile salts. Overall, all the antacid-loaded microgels had fairly similar charge characteristics throughout the simulated GIT, indicating that encapsulation of the antacids did not change their surface potentials.

![Graph showing electrical properties of particles in different delivery systems](image)

Figure 6. 3 Electrical properties of the particles in different delivery systems measured after exposure to different GIT stages.
6.3.3 Cryo-SEM for hydrogel beads with presence or absence of antacid agent

Cryo-SEM was used to probe the internal structure of fractured alginate microgels in the absence or presence of antacid agents (Figure 6.4). Cryo-SEM allows detailed observation of microgels in the frozen hydrated state. Compared with conventional SEM, potential artifacts like shrinkage produced by dehydration and fixation are avoided using cryo-SEM [256]. To study their inner structure, alginate microgels were cracked by cryo-fracturing and then the cracked surfaces were visualized by SEM. The probiotics were clearly observed at the surfaces of the antacid-free microgels (Figure 6.4a). Both probiotics and irregular shaped particles (presumably antacid crystals) were observed in the probiotic-loaded microgels containing Mg(OH)$_2$, which were indicative of the presence of antacid crystals (Figure 6.4b). Probiotics were visible in clusters at the surfaces of the probiotic-loaded microgels containing CaCO$_3$, which appeared to have a highly variegated surface morphology (Figure 6.4c).

In the absence of probiotics, the surfaces of the antacid-free microgels appeared smoother than in the two antacid-loaded microgels (Figure 6.4d-f). The irregular surfaces of the antacid-loaded microgels was probably because of the presence of antacid crystals embedded inside them. The SEM images suggested that the size of the Mg(OH)$_2$ crystals were considerably smaller than those of the CaCO$_3$ crystals. In addition, the structure of the hydrogel network in the microgels appeared to be different when they contained different antacids. This effect might have been caused by the presence of different ions (magnesium versus calcium) that altered the cross-linking between the alginate chains in the hydrogel network [257].
Figure 6. 4 Cold-stage scanning electron microscopy (cryo-SEM) of calcium alginate microcapsules. (a) Fractured alginate micorgels with bacteria. (b) Fractured microcapsules with bacteria with the presence of antacid agent-Mg(OH)$_2$. (c) Fractured microcapsule with bacteria with the presence of antacid agent-CaCO$_3$. (d) Fractured structure of calcium alginate microgels. (e) Fractured structure of alginate micorgels filled with antacid agent-Mg(OH)$_2$ without bacteria. (f) Fractured structure of alginate microgels loaded with antacid agent- CaCO$_3$ without bacteria. Magnification is indicated individually on data bar at the bottom of micrograph.

6.3.4 Viability of encapsulated BPG7 under simulated digestion

Previous studies have shown that probiotics tend to be inactivated during their passage through the upper GIT and therefore do not reach the colon intact [258]. Therefore, we examined the impact of microgel encapsulation on cell viability when exposed to simulated GIT conditions. BPG7 cells encapsulated in alginate microgels in
the absence or presence of antacids (Mg(OH)$_2$ or CaCO$_3$) were subjected to simulated stomach or stomach/small intestine conditions [259]. The level of viable cells that survived was measured before and after exposing them to simulated GIT conditions (Figure 6.5 and 6.6). A live/dead fluorescent stain method was used to visualize probiotic viability, where viable bacteria with intact cell membranes fluoresce green and dead bacteria with damaged cell membranes fluoresce red. The confocal microscopy images showed that all the free bacteria lost their viability after 2 h incubation in the gastric fluids (Figure 6.5). Similarly, none of the cells inside the antacid-free alginate microgels survived after exposure to the simulated stomach phase, indicating that hydrogen ions (H$^+$) from the gastric fluids rapidly diffused into the microgels and deactivated the probiotic bacteria (Figure 6.5 and 6.6). Conversely, many of the cells remained viable in the microgels containing Mg(OH)$_2$ or CaCO$_3$ after exposure to the stomach phase (Figure 6.5). After 2 h gastric digestion, there was a 1.5 log$_{10}$ CFU reduction in the number of viable bacteria in the microgels containing Mg(OH)$_2$ and a 1.0 log$_{10}$ CFU reduction for the microgels containing CaCO$_3$ (Figure 6.6). This result highlighted the ability of the antacids to protect the probiotics against acidic gastric conditions. Our previous study showed that the pH inside Mg(OH)$_2$-loaded alginate microgels remained close to neutral when they were incubated in acidic gastric fluids for 2 h [144]. This suggests that the level of insoluble Mg(OH)$_2$ inside the microgels was sufficient to neutralize the gastric fluids throughout the entire incubation time. This phenomenon was probably because the Mg(OH)$_2$ particles only dissolved slowly in the gastric fluids [144]. The microgels containing CaCO$_3$ gave even better gastric protection than the ones containing Mg(OH)$_2$ (Figure 6.6). After exposure to the intestinal phase, no viable cells were detected for the
microgels containing Mg(OH)$_2$, which corresponded to a greater than 5.43 log$_{10}$ CFU loss in cell viability. On the other hand, the microgels containing CaCO$_3$, only suffered a 2.5 log$_{10}$ CFU reduction. These results suggest that CaCO$_3$ also had a better protective effect on cell viability than Mg(OH)$_2$ in the small intestinal phase.

The origin of the different protective effects of Mg(OH)$_2$ and CaCO$_3$ is still unclear. There are several possible reasons that might attribute for this observation. Firstly, the slowly released calcium ions might interact with bile salts or intestinal enzymes and thereby reduce cell injury. The bile salts in the small intestine are known to have a strong antimicrobial activity due to their ability to disrupt the structure of the cell membrane and trigger DNA damage [260]. Secondly, the cryo-SEM images suggested that CaCO$_3$ crystals had a larger particle size than Mg(OH)$_2$ crystals, which may have reduced their dissolution rate or sterically hindered the ability of hydrogen ions to diffuse through the hydrogels [261]. Third, the released calcium ions may have cross-linked the alginate molecules in the hydrogel thereby reducing the pore size and inhibiting diffusion. Fourth, the size of the microgels containing CaCO$_3$ was larger than the ones containing Mg(OH)$_2$, which would have also slowed down diffusion processes [262]. Clearly, further work is required to work out the detailed physicochemical mechanisms underlying the difference between the two types of antacids.
Figure 6.5 Fluorescence confocal microscopy images of viable bacteria in different systems after exposure to successive GIT stage (stained with LIVE/DEAD BacLight Bacterial Viability Kit). Red indicates live cells, while green indicates cell with damaged membrane.
6.4 Conclusion

In order to colonize the colon, probiotics must survive the harsh conditions they experience during passage through the upper gastrointestinal tract. In particular, they must resist deactivation by the deleterious actions of acids and bile salts within the gut. In this study, we showed that antacid-loaded microgels could improve probiotic survival under both stomach and small intestine conditions. It was postulated that the antacids helped maintain neutral pH conditions within the interior of the microgels as they passed through the highly acidic gastric fluids, thereby protecting the probiotics trapped inside them. CaCO₃ proved to be a more effective antacid than Mg(OH)₂ at protecting the probiotics for reasons that are currently unknown. Overall, the microgels developed in
this work may be useful for encapsulating, protecting, and delivering sensitive probiotics in functional foods, supplements, and pharmaceuticals. Future work will focused on determining the mucoadhesion properties of the encapsulated probiotics within the intestinal mucosa.
CHAPTER 7

CONCLUDING REMARKS

Emerging evidence have support the opinion that gut microbiota dysbiosis is associated with the onset and progress of several chronic diseases, such as inflammatory bowel disease, diabetes, and obesity. Intrinsic and environmental factors can influence gut microbiota composition and diversity. Dietary intervention has been recognized as a powerful method for microbiota modification, even more effective than gene. However, the interaction between dietary component and gut microbiota is bidirectional, indicating that gut microbiota can work on administrated xenobiotics. Gut microbiota metabolism on xenobiotic will activate, deactivate or toxify the parent compound. There’re trillions of bacteria co-evolved with human and residing in human body, while only some species or strains has the metabolizing capacity. Most of the metabolizing capacity of bacteria remains unknown. Besides, genus Bifidobacterium is regarded as probiotic, however, research focuses on bacteria secretion bioactivities is kind of overlooked. To perform the host health benefits, probiotic has to reach the site with sufficient amount. However, it’s hard for bifidoabcteria to pass through the digestive system successfully. Because both high acid condition in stomach and bile salts in small intestine are detrimental for cells. Therefore, it’s necessary to develop an effective oral delivery system to help probiotic reach colon with abundant cells.

In this dissertation, the impact of dietary citrus components was demonstrated in unchallenged mice and colitic mice model. Our results demonstrated that oral intake of limonin and nobiletin modified mice fecal microbiota. In colitic mice, gut microbiota
dysbiosis was partially reversed by nobiletin intervention. Mice inflammation induced by DSS was inhibited by nobiletin treatment, which was proved by reduced disease activity index, lower histological score and inflammatory cytokines in colon mucosa. The strong anti-inflammation of nobiletin was partially contributed by nobiletin metabolites, which was produced by gut microbiota. Gut microbiota in colon deconjugated the nobiletin metabolite conjugates. Therefore, we isolated and identified the bacteria (Isolate G7) with deconjugation capacity, who belongs to species *Bifidobacterium pseudocatenulatum*. Except the deconjugation potential of Isolate G7, its secretion has strong anti-inflammation and anti-cancer capacity. Isolate G7 interrupted the NF-κB pathway in cell RAW 264.7. Isolate G7 secretion performed its anti-cancer ability on human colon cancer cell HCT116 by promoting cell apoptosis, arresting cell cycle and downregulating gene expression of wnt signaling. Together, our results suggested that dietary components and gut microbiota could interact with each other, and gut microbiota and dietary component worked together to inhibited mice inflammation, providing a scientific basis for using dietary nobiletin as a nutraceutical to promote gut health.

Accumulating studies have suggested that probiotic encapsulation can help to increase cell viability after gastrointestinal digestion. To increase the efficiency of the delivery system, antacid agents were used as gastroprotective agent. Our results indicated that both cells and antacid agents can be encapsulated into alginate micorgels. The micorgels keep intact during the gastrointestinal digestion. Most importantly, the system could protect cells from acidic condition in simulated gastric juice and antacid agent-CaCO₃ could help to protect from bile salts in the simulated small intestine fluid also.

Overall, the microgels developed in this work may be useful for encapsulating,
protecting, and delivering sensitive probiotics in functional foods, supplements, and pharmaceuticals. The dissertation present here provides important knowledge concerning the interaction between gut microbiota and dietary components and the beneficial effect of probiotics and developed an effective delivery system for sensitive probiotic or proteins.
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