2014

The impact of herbal saponins on gut microflora in animal models

Lei Chen
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The Impact of Herbal Saponins on Gut Microflora in Animal Models

CHEN Lei

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Principal Supervisor: Prof. HSIAO Wen Luan Wendy

Hong Kong Baptist University

May 2014
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

Signature: CHENG

Date: May 2014
ABSTRACT

Human gut harbors 100 trillion microbial organisms that is intrinsically linked to individual’s health and diseases, including cancer. Food fiber and phytochemicals such as polyphenols are considered as prebiotic-like dietary modifiers. They can influence the gut microbial communities, and in turn to modulate disease outcome and drug responses of the host. Saponins belong to a family of phytochemicals commonly found in many medicinal and edible plants. Herbal saponins have raised keen interest among scientists for their health-promoting effects, but have not been investigated for their potential as prebiotics. *Gynostemma pentaphyllum* (Gp) is riched in triterpenoid saponins and has been consumed in China and other part of the world as an herbal tea and as a folk medicine. In our lab, we have demonstrated that Gp possesses strong anticancer and anti-inflammatory effects. Whether Gp possesses prebiotic property and whether gut microbiota plays any part of the anticancer effect of Gp are the questions addressed in the present study. Thus, we hypothesized that Gp saponins (GpS) might modulate the gut microbiota, which in turn enhance its anticancer activities. In the study, the gut microbiome analysis were carried out using two main techniques, namely the enterobacterial repetitive intergenic consensus (ERIC-PCR) and 16S pyrosequencing approaches. Both xenograft nude mice and *Apc*<sup>min/+</sup> mice were employed as the animal models to investigate the interaction between the herbal saponins and the gut microbiota in the host.

Athymic nude mice have been employed for tumorigenic research for decades, however, the relationships between the gut microbiome and host's response to the grafted tumors and drug treatments are unexplored. For the first part of the thesis, we investigated the relationship between the gut microbiota and grafted tumor in the nude mice under the treatment of Gp saponins. Partial least squared discriminant analysis (PLS-DA) of ERIC-PCR data showed that the microbiota profile of xenograft nude mice departed from that of the nonxenograft mice. However, prolonged treatment of GpS seems to realign the fecal microbiota with the pretreatment control. Pyrosequencing data reiterated the differences in fecal microbiome between the nonxenograft and xenograft animals. GpS treatment had a much stronger impact on the phylotypes of the xenograft than the nonxenograft mice. In addition, GpS treatment markedly induced the relative abundance of *Clostridium cocleatum* and *Bacteroides acidifaciens*, for which the beneficial effects on the host have been well documented.
Apc<sup>Min/+</sup> colorectal cancer mouse model was further employed for the investigation of the association of the gut microbiota and cancer occurred inside the gut, which was a more direct site to interact with the gut microbiota. In the Apc<sup>Min/+</sup> mouse model, we found distinct difference of fecal microbiome between the Apc<sup>Min/+</sup> and the wild-type littermates. GpS treatment significantly reduced the number of intestinal polyps. GpS also increased the ratio of Bacteroidetes/Firmicutes and reduced the sulfate- and sulfur-reducing bacteria lineage and potential opportunistic pathogens, which might cause certain deleterious effects to the host. The impact of GpS on the gut mucosal environment was also examined. We found GpS treatment improved the gut barrier function by increasing the numbers of Paneth cells, goblet cells, up-regulating the expression of E-cadherin and down-regulating the expression of N-cadherin in the intestine. In addition, GpS treatment down-regulated the protein expression of beta-catenin and p-STAT3. Furthermore, higher levels of anti-inflammatory and tissue repair-related cytokines as well as Arginase I, but lower level of iNOS expression were found in GpS-treated Apc<sup>Min/+</sup> mice, indicating increased anti-inflammatory macrophage phenotype M2 (associated with tissue repair) and reduced proinflammatory phenotype M1. Furthermore, in addition to GpS, other herbal saponins also showed prebiotic-like effects in C57BL/6 mice.

In summary, this study provides first hand evidence for the impact of herbal saponins on the gut microbial ecosystem and new insight into mechanisms responsible, at least in part, for the activities of GpS. We demonstrate that tumor growth induce intestinal dysbiosis. GpS treatment can inhibit tumor progression and concurrently alter the microbiome by increasing symbionts and/or decreasing pathobionts, which may contribute to its chemopreventive effect against tumorigenesis. Herbal saponins showing prebiotic-like effects may be used for improving the health of the host by manipulation of the gut microbiota.
ACKNOWLEDGEMENTS

It was really a nice journey, also a great adventure pursuing my Doctor of Philosophy study. I extremely enjoyed exploring the unknown world with my strong curiosity. In addition to gaining invaluable experiences, knowledge and joyfulness, I learned how to deal with tough situations, fallen expectations and setbacks, thus making me more mature. During this journey, I gradually felt that science is art. As Richard Dawkins says, "You need a combination of that and genuine inspiration, which is akin to poetry; it's akin to great art; it's the inventive, creative leap which great scientists have." This journey would not be possible without the continued support from my supervisor, colleagues, friends and family.

I would like to express my sincere gratitude to my principal supervisor Prof. Wendy Hsiao for providing me the opportunity to join her team and start my PhD study. I appreciate her great foresight for choosing such an interesting topic, which becomes more and more important and popular. I am grateful for her continuous guidance throughout my whole study and the preparation of this thesis. Thanks for her full support for allowing me to test all hypotheses for which we were interested in. I am also thankful to her encouragement when I encountered the difficulties. Spark of inspiration always came after pleasant discussions, and her insightful advices empowered me the power to discover more beautiful scenery. Thanks for continuously developing my potential and research interests. Thanks for giving me so many chances to share my research in overseas conferences, which had broadened my horizon, refreshed my knowledge and strengthened my communication skill. Altogether, I feel very fortunate to meet Prof. Hsiao and be her student in this wonderful journey.

I am very grateful to Dr. William Tai for his generous support and his good laboratory management. Thanks for his kind help on reagent ordering and animal supplying. I would like to thank all the present and past members of Prof. Hsiao’s lab for creating such an excellent environment for learning and working. Special thanks to Mr. Huang Chen-hu for his kind assistance in animal experiments. I am also appreciated the technical support from our enthusiastic technicians, Mr. Michael Wong, Ms. Hilda Cheung, Ms. Nickie Chan, Mr. Alan Ho, Ms. Irene Koo, and Ms. Sally Lee, and the help from Ms. Lisa Song in literature retrieval. I would like to express my sincere thanks to Ms. Patty Lam and Mr. Lo Kam Fai for their help in my thesis submission.
and oral examination. I am extremely thankful to our collaborators Prof. Frederick CC Leung in The University of Hong Kong and his student Manreet for their help in pyrosequencing. In addition, I particularly want to thank all the colleagues who give me a hand during my study. I thank Mr. Chan Chi Leung, Ms. Ma Xiao-qing, and Ms. Liang Xu for their help in metabolomics data analysis. I thank Dr. Qin Hong-yen for her kind help in discussion of histological techniques. I thank Dr. Alexander Leung for his helpful suggestions in microbiological experiments. Also, I would like to thank Dr. Tang Jing and Dr. Tu Xing in Model Animal Research Center of Nanjing University for their help in data collection of the comprehensive laboratory animal monitoring system.

Furthermore, I would like to give heartfelt thanks to my friends, our PhD ladies club, Dr. Qin Hong-yen, Dr. Zhu Lin, Dr. Wu Meng-hua, Dr. Chen Xiao-yu, Ms. Pook Supawadee Parhira, Ms. Liang Xu, Ms. Xiao Ting-ting, Ms. Cao Hui-hui, Ms. Cheng Zhen, Ms. Zhang Zui, and Ms. Chen Lei-lei. As the old saying goes, “Fixed barrack, floating soldiers.” Although some of them have already left BU, the wonderful memories are timeless. Thanks for the numerous moments of joy, excitement and disappointment we shared together. I would particularly like to thank Ms. Xiao Ting-ting and Ms. Cao Hui-hui for their daily companionship and being my eternal sunshine. Long live our friendship. Finally, I would like to express my countless and sincere thanks to my family and my boyfriend for their unconditioned love and enormous support. Thanks for their understanding, toleration, and encouragement all the time. Thanks for giving me strength to overcome the moments of stress and frustrations. Thanks for always being by my side.
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LIST OF ABBREVIATIONS

5-FU 5-fluorouracil
AOM azoxymethane
APC adenomatous polyposis coli
APRIL a proliferation-inducing ligand
ASD autism spectrum disorder
BAFF B cell-activating factor
BLAST Basic Local Alignment Search Tool
Cc correlation coefficient
CD Crohn's disease
CLAs conjugated linoleic acids
CLAMS comprehensive laboratory animal monitoring system
CMC carboxymethyl cellulose
CPT-11 irinotecan
CRC colorectal cancer
Cs similarity coefficient
DCs dendritic cells
DSS Dextran sodium sulfate
emPCR emulsion-PCR
ERIC enterobacterial repetitive intergenic consensus
ESI electrospray ionization
ETBF enterotoxigenic Bacteroides fragilis
FAO Food and Agriculture Organization
FAP familial adenomatous polyposis
FOS fructo-oligosaccharides
FOXP3 forkhead box P3
GF germ-free
GOS galacto-oligosaccharides
Gp Gynostemma pentaphyllum
GpS Gynostemma pentaphyllum saponins
GS Ginseng saponins
H&E hematoxylin and eosin
H' index Shannon-Wiener diversity index
IBD inflammatory bowel disease
IgA immunoglobulin A
IHC Immunohistochemistry
IL-4 Interleukin-4
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LDA</td>
<td>linear discriminant analysis</td>
</tr>
<tr>
<td>LEfSe</td>
<td>linear discriminant analysis effect size</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>M cell</td>
<td>microfold cell</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
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<td>NKT</td>
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<td>NOD</td>
<td>nucleotide-binding oligomerization-like receptor</td>
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<td>NORA</td>
<td>New-onset untreated RA</td>
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<td>OTUs</td>
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<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
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<td>sTNFRI</td>
<td>soluble tumor necrosis factor receptor I</td>
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<td>TCM</td>
<td>Traditional Chinese Medicine</td>
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TGFβ  transforming growth factor-β
Th1  T helper type 1
Th2  T helper type 2
Th17 T helper type 17
TLC thin-layer chromatography
TLRs Toll-like receptors
TLR4 Toll-like receptor 4
TNBS Trinitrobenzene sulfonic acid
Treg cell regulatory T cell
TRUC $T{\text{-}}bet^{-/-} \times RAG2^{-/-}$ ulcerative colitis
UC ulcerative colitis
UHPLC ultra high-performance liquid chromatography
UPLC ultra performance liquid chromatography
WHO World Health Organization
WT wild-type
CHAPTER 1

INTRODUCTION
1.1 Overview of commensal gut microflora

Microflora (also known as microorganism or microbiota) can easily be found in parts of the human body, such as the mouth, skin, colon, esophagus, stomach, and vagina. The human gut, which has a mucosal surface >200 m², is a nutrient-rich environment and contains 100 trillion bacteria. The human gut microbiome has been estimated to harbor millions of genes, specifically, at least 100-fold more genes than the complete human genome contains (Backhed et al., 2005, Gill et al., 2006). Humans can be regarded as “superorganisms” because of their close symbiotic relationships with the microbiota (Li et al., 2008). Approximately 10% of the metabolites circulating in hosts are attributed to the gut microflora (Wikoff et al., 2009). The gut microflora plays a crucial role in health and disease (Neish, 2009, Nicholson et al., 2005). Physicians understood the importance of these microbes as early as 400 B.C., when Hippocrates wrote, “death sits in the bowels” and “bad digestion is the root of all evil” (Hawrelak and Myers, 2004). Asher Mullard (2008) drafted a news feature in Nature indicating the proliferation of human microbiome projects and the global efforts to elucidate this large microbiome. The Human Microbiome Project of the National Institutes of Health (NIH) of United States, launched in 2008, is a 5-year project with a total budget of US$115 million. The mission of this project is to “generate resources to enable
comprehensive characterization of the human microbiota and analyze its role in human health and disease” (NIH). In 2010, the NIH awarded US$42 million as additional funding to expand the scope of 8 demonstration projects designed to study the association between the human microbiome and human diseases. This new field provides a powerful lens for exploring the microbial world and might revolutionize knowledge on the entire living world. In recent years, interest in research on the field of gut microflora has increased considerably (Fig. 1.1).

![Figure 1.1](image-url) Number of annual publications related to gut microflora in the previous two decades. Data were obtained by searching Pubmed and by using the following terms: intestinal microbiota, gut microbiota, intestinal flora, gut flora, intestinal microflora, gut microflora, intestinal microbe, gut microbe, intestinal microorganism, and gut microorganism (http://www.ncbi.nlm.nih.gov/pubmed/).

Several factors, such as the mode of fetus delivery (Domínguez-Bello et al., 2010, Palmer et al., 2007), host genotype (Benson et al., 2010, Spor et al., 2011), dietary patterns (Walker et al., 2011, Wu et al., 2011a), antibiotics (Jernberg et al., 2007,
Willing et al., 2011), probiotics (Rauch and Lynch, 2012), age (Biagi et al., 2010, Tiihonen et al., 2010), and stress (Konturek et al., 2011), have been shown to affect the gut microflora. Nevertheless, the gut microbial communities of most people can be divided into 3 statistically robust clusters, known as enterotypes (Arumugam et al., 2011). Furthermore, when germ-free (GF) wild-type (WT) mice are colonized with a microbiota harvested from diseased mice, the WT mice exhibit symptoms similar to those exhibited by the diseased mice, indicating that the gut microbiota has a transmissible trait (Couturier-Maillard et al., 2013, Garrett et al., 2009, Turnbaugh et al., 2006, Vijay-Kumar et al., 2010). In addition, recent findings have indicated that the microbiota from a healthy individual can be transplanted into a diseased recipient to restore gut functionality and reestablish a certain state of intestinal flora (Mattila et al., 2012).

Most current drug development is focused on identifying a novel candidate against a specific target, such as a receptor or an enzyme. However, gut microbial ecosystem has long been oversighted. Jeremy Nicholson et al. (2005) reviewed and assessed the importance of the gut microbiota in drug metabolism, efficacy, and toxicity. Previous studies have proposed the gut microbiota to be a potential therapeutic strategy and a rich resource for drug development (Cani and Delzenne,
2011, Jia et al., 2008, Lemon et al., 2012, Shanahan, 2010). The gut microbiome was involved in a broad, drug-like phase II metabolic response to the host, generating numerous sulfated, glycine-conjugated, and glucuronide adducts in the serum (Wikoff et al., 2009). In addition, the enzymes secreted by the gut microbiota contribute to the potential for the metabolism or biotransformation of xenobiotics. At least 30 drugs are known to be transformed by these bacterial enzymes (Sousa et al., 2008). Recent advancement in metagenomics and metabolomics has contributed to the fast development of the field of mammalian commensal microbiota. It is believed that the understanding of the host microbes will open up a new territory for drug discovery and therapy, and even personalized health care.

1.2 The gut microbial ecosystem

Colonization of the human gut by environmental microbes begins immediately after birth and becomes increasingly complex and diverse with age. The human gastrointestinal tract is estimated to comprise 500 to 1000 species of bacteria (Eckburg et al., 2005, Steinhoff, 2005). More than 50 bacterial phyla exist in human microbial communities; Firmicutes and Bacteroidetes are the predominant intestinal phyla (Ley et al., 2008b). Most of the gut microbiota is composed of strict anaerobes (97%), and only 3% consists of aerobic bacteria (facultative
anaerobes). The most common anaerobic genera are *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Lactobacillus*, *Eubacterium*, and *Fusobacterium*, and common aerobes are Gram-negative enteric bacteria (*Escherichia coli* and *Salmonella* spp.) and the Gram-positive cocci (*Enterococcus*, *Staphylococcus*, and *Streptococcus*) (Noverr and Huffnagle, 2004). Temporal and spatial characterizations of microbial composition, as well as the functions of gut microflora, are provided in the following subsections.

### 1.2.1 Temporal development of the microbial composition

The microbiota is linked to humans throughout their entire lives. Microorganisms colonize the gastrointestinal tract of infants at the moment of birth (Mandar and Mikelsaar, 1996). The acquisition and structure of the initial microbiota in newborns differ according to the mode through which the newborns are delivered. Vaginally delivered infants acquire microbial communities similar to those present in the mother’s vagina, predominantly consisting of *Lactobacillus*, *Prevotella*, or *Sneathia* spp., whereas the microbiota of infants delivered using a C-section closely resembles that of the mother’s skin surface, predominantly consisting of *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. (Dominguez-Bello *et al.*, 2010). Thus, humans inherit the gut microflora from the
mother, just as they inherited half of the mother’s genes. Compared with vaginally delivered infants, infants delivered using C-sections are more susceptible to allergies and asthma (Bager et al., 2008, Negele et al., 2004). The bacteria acquired from a mother’s vagina may prevent harmful pathogens from colonizing in newborns.

During the first year of life, the composition of the gut microflora is relatively simple and varies widely among individuals as well as over time (Mackie et al., 1999, Mandar and Mikelsaar, 1996). At 1 year of age, infants begin developing a microbial profile that is highly similar to that observed in the adult gastrointestinal tract (Palmer et al., 2007). At 2.5 years of age, infants’ microbial profile completely resembles that observed in the adult gastrointestinal tract (Koenig et al., 2011). In adulthood, the intestinal microbiota is considered to be stable until old age. The change in microbial composition in elderly adults was investigated by the ELDERMET consortium. Compared with young adults, elderly adults exhibited an apparent alteration in the proportions of Bacteroides spp. and the Clostridium group (Claesson et al., 2011).
1.2.2 Spatial characterization of the microbial composition

The distribution of the gut microflora varies along the gastrointestinal tract; the number of bacteria is low in the stomach and duodenum ($10^1$ to $10^3$ bacteria per gram), increases in the jejunum and ileum ($10^4$ to $10^7$ bacteria per gram), and peaks in the colon ($10^{11}$ to $10^{12}$ bacteria per gram) (O'Hara and Shanahan, 2006). In addition, the microbial composition differs among these sites. For example, bacilli of the Firmicutes and Actinobacteria are prevalent in the small intestine, whereas Bacteroidetes and the Lachnospiraceae family of the Firmicutes are predominant in the colon (Frank et al., 2007). In addition to the longitudinal variation, the microbial composition exhibits latitudinal variation. The microbiota differs among the structural layers of the gut wall, such as the epithelial surface, the mucus layer, and the intestinal lumen (Swidsinski et al., 2005).

1.2.3 Functions of the gut microflora

The gastrointestinal tract is colonized by a vast community of symbionts and commensals. The interactions between intestinal microbiota and the host can be summarized as a continuum ranging from symbiosis, to commensalism, to pathogenicity (Hooper and Gordon, 2001). “Symbiosis” generally refers to a relationship from which at least one partner benefits without harming the other.
“Commensalism” generally refers to a relationship between partners that coexist without detriment or obvious benefit. “Pathogenicity” refers to a pathogenic relationship that causes damage to the host. Gut microbes are essential contributors to numerous processes for maintaining host health, including metabolic functions, immune regulation, epithelial development, and protection against pathogens.

1.2.3.1 Metabolic function

The gut microflora plays crucial roles in digesting dietary compounds, harvesting energy, and supplying nutrients. The microbial metabolism is responsible for nutrient conversion from dietary substances. Gut microbes are involved in fermenting nondigestible dietary fibers and anaerobically metabolizing peptides and proteins, thus enabling energy from food ingested but not digested by the host to be harvested (Jia et al., 2008). Recent evidence has suggested that the gut microbiota can affect the efficiency of energy harvesting (Ley et al., 2006, Turnbaugh et al., 2006). In addition, gut microbes participate in synthesizing vitamins, such as B12 and K, deconjugating and dehydroxylating bile acids, and degrading oxalate-based complexes (Montalto et al., 2009). Furthermore, the gut microbiota can influence the production of tryptophan-derived metabolites such as
ndoxylo sulfate (uremic toxins) and indole-3-propionic acid (antioxidant).

1.2.3.2 Immune regulation

Recent studies have revealed how the microbiota or microbiota-derived products affect the innate and adaptive immune system, thus protecting the host and enabling intestinal homeostasis to be maintained. The intestinal microbiota profoundly influences the postnatal development of the immune system. For example, the intestinal microbiota affects the transient expansion of germinal center reactions between B and T cells in the Peyer’s patches, increases the levels of serum immunoglobulin, and produces immunoglobulin A (IgA) from B cells (Tlaskalova-Hogenova et al., 2004). The components that are released from commensal microbiota, such as lipopolysaccharides, peptidoglycans, bacterial CpG-DNA motifs, and heat-shock proteins, are responsible for various immunomodulatory effects (Tlaskalova-Hogenova et al., 2004). In the regulation of mucosal immune responses, intestinal microflora recognizes molecules through multiple, such as signal transduction mediated by Toll-like receptors (TLRs), nucleotide-binding oligomerization-like receptors (NODs), formylated peptide receptors, IL-1β-converting enzyme protease activator factor, and C-type lectin receptors (Krisztina et al., 2011).
1.2.3.3 Intestinal epithelial development

The first indication that the gut microflora played a role in mammalian intestinal epithelial development was provided in a study showing that the renewal of gut epithelial cells in GF mice was slower than that of their colonized counterparts (Pull et al., 2005). Other studies have determined that the metabolic products of the gut microbiota, such as short-chain fatty acids (SCFAs), provide essential nutrients for the proliferation, angiogenesis, and restitution of intestinal epithelial cells (Hooper, 2004, Neish, 2009, Sharma et al., 2010). In addition, microbes induce the production of intestinal mucus, promote the development of intestinal microvilli (Nicholson et al., 2005), and modulate the intestinal glycoconjugate repertoire (Hooper, 2004).

1.2.3.4 Protection against pathogens and other functions

The gut microbiota provides the host with a physical and immunologic barrier against incoming pathogens in a “competitive-exclusion” manner by occupying attachment sites, blocking invasion, exhausting nutrient sources, and stimulating the host to produce antimicrobial compounds (Sekirov et al., 2010). GF animals have yielded numerous valuable clues on the functions of microbiota. For example, compared with their colonized counterparts, GF animals exhibit a
smaller heart and liver (Wostmann et al., 1982), but higher locomotor activity (Backhed et al., 2007). Increasing in-depth research has provided a more extensive understanding of the comprehensive functions of microflora.

1.3 Interaction of intestinal mucosal cells with microbiota

The intestinal mucosa is exposed to various dietary components as well as numerous and complex microbiota and is the main interface where the interaction between host and gut microflora occurs. The host-microbe interaction plays a vital role in health and disease. Numerous chronic diseases have been linked to disturbances of the dysregulation of mucosal immunity or intestinal mucosal barrier function (Tlaskalova-Hogenova et al., 2004). In the following subsections, we focused on 2 primary aspects: (1) the interaction between the gut microflora and immune homeostasis, and (2) the interaction between the gut microflora and the intestinal mucosal barrier.

1.3.1 Anatomical illustration of the gut barrier and distribution of the various types of immune cells and their function

The intestinal wall is composed of 4 layers: the mucosa, which is exposed to the gut contents; the submucosa, which is located beneath the mucosa; and 2 outer
layers, namely the muscularis externa and serosa (Fig. 1.2). The intestinal epithelium is a vital part of the mucosa. Terminally differentiated epithelial cells are divided into 4 main types (Fig. 1.3). The predominant cell type is the enterocytes or absorptive cells, and the other 3 types are secretory cells, namely goblet cells, Paneth cells, and enteroendocrine cells (Crosnier et al., 2006). The enterocytes are the major structural components of the intestinal barrier. They contain digestive enzymes and participate in gap junction and absorption. In the intestinal epithelium, the constant renewal of enterocytes is crucial for maintaining dynamic homeostasis. Goblet cells generate mucus, and Paneth cells secrete antimicrobial substances (lysozyme and cryptdins or defensins), and enteroendocrine cells are required for producing various gut hormones (peptides and catecholamines) as well as neuroendocrine molecules (Crosnier et al., 2006, Tlaskalova-Hogenova et al., 2011). Underlying the epithelium is the lamina propria and the muscularis mucosa. The lamina propria contains myofibroblasts, blood vessels, nerves, and various immune cells, such as dendritic cells, IgA+ plasma cells, Th1, Th2, Th17, and Treg cells. Figure 1.4 illustrates the modulatory effects of the immune cells on immune responses in the gut environment. The interaction between the gut microbiota and immune cells (e.g., Th17/Treg balance and Th1/Th2 balance) is discussed as follows.
**Figure 1.2** Anatomical illustration of the intestinal wall.

**Figure 1.3** Distribution of epithelial cell types in the small intestine.  
(Crosnier et al., 2006)

(a) A villus with one of the crypts that contribute to renewal of its epithelium. Arrows indicate the upwards flow of cells out of the crypts. Stem cells lie near the crypt base. Above the stem cells are transit-amplifying cells; and above these, in the neck of the crypt and on the villus, lie post-mitotic differentiated cells. (b) There are four classes of terminally differentiated cells. Absorptive cells have a brush border (a dense array of microvilli) on their apical surface. The other three classes are all secretory: goblet cells secrete mucus; enteroendocrine cells are smaller and secrete various gut hormones (peptides and catecholamines); and Paneth cells secrete antibacterial proteins (lysozyme and cryptdins or defensins). Cited from Crosnier et al., 2006.
Figure 1.4 Modulation of adaptive immune responses by the gut microbiota.
(Cerf-Bensussan and Gaboriau-Routhiau, 2010)

(a) Intestinal adaptive immune responses can be initiated in Peyer’s patches or in mesenteric lymph nodes (MLNs). Activated T and B cells subsequently leave these lymphoid tissues and home to the intestinal lamina propria via the bloodstream. Bacteria are mainly sampled by Peyer’s patch dendritic cells (DCs) after transcytosis across the specialized epithelium that overlays these lymphoid organs. (b) Microbiota-derived products activate Toll-like receptors (TLRs) that are expressed by intestinal epithelial cells, which leads to the production of B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL); these cytokines promote both T cell-dependent and T cell-independent IgA class-switching responses in the intestine. (c) CD103^+ DCs are ‘conditioned’ by epithelial cell-derived factors, such as thymic stromal lymphopoietin (TSLP), transforming growth factor-β (TGFβ) and retinoic acid, to acquire a tolerogenic phenotype; these DCs can promote the induction of forkhead box P3 (FOXP3)^+ regulatory T (Treg) cells. Bacteria-derived products can further promote the induction of Treg cells. T helper 17 (Th17) cell differentiation can be promoted by bacteria-derived ATP or by serum amyloid A protein (SAA). Segmented filamentous bacteria (SFB) can drive the expansion of mucosal Th1 cell populations. M cell, microfold cell. Cited from Cerf-Bensussan and Gaboriau-Routhiau, 2010.
1.3.2 The gut microflora and immune homeostasis

Increasing evidence has indicated that the gut microbiota is associated with balancing effects in immune homeostasis. In contrast to the composition of the human genome, that of the gut microflora can likely be altered because of the plasticity of the microbiome. Host homeostasis contributes to shaping the composition of the gut microflora, and a balanced microflora composition enables symbiosis and facilitates the maintenance of immune homeostasis. Dysbiosis may cause dysregulation of the immune system (Fig. 1.5) (Maslowski and Mackay, 2010). Alterations in the composition of the microflora might disturb the partnership between the microflora and the human immune system. An altered immune response may underlie various human disorders (Maslowski and Mackay, 2010, Round and Mazmanian, 2009).
The balance between Th17 and Treg cells is crucial for ensuring intestinal immune homeostasis. Commensal microbiota has been suggested to modulate the Th17/Treg balance. Segmented filamentous bacteria (SFB) were identified as the first members of the mouse commensal microbiota that can modulate murine intestinal homeostasis. SFB were determined to induce lamina propria Th17 cells and influence mucosal immune responses (Gaboriau-Routhiau et al., 2009, Ivanov et al., 2009, Ivanov et al., 2008). In addition, among the indigenous commensal bacteria, Clostridium clusters, particularly clusters IV and XIVa, were determined to be outstanding inducers for promoting the accumulation of Treg cells in the
colonic lamina propria (Atarashi et al., 2011). Furthermore, DNA derived from commensal bacteria was proposed to modulate the Th17/Treg balance by limiting Treg cell conversion (Hall et al., 2008). Furthermore, commensal-microbiota-produced ATP was reported to promote Th17 cell differentiation in the lamina propria (Atarashi et al., 2008).

Mazmanian et al. (2005 & 2008) provided a suitable example of how a human symbiont-derived factor can affect a mucosal immune system. Bacteroides fragilis was determined to protect animals from experimental colitis in several models. Furthermore, the B. fragilis polysaccharide was the first identified member of “symbiosis factors” and was reported to mediate a beneficial relationship between the gut microbiota and mammalian hosts, providing benefits such as the establishment of an optimal Th1/Th2 balance and alleviation of intestinal inflammation.

1.3.3 The gut microflora and intestinal mucosal barrier

The intestinal mucosal barrier is a vital contributor to host defense and is composed of the intestinal epithelium, underlying lamina propria, and muscularis mucosa. Although numerous bacteria are pathogenic and can disrupt the intestinal
barrier (Ashida et al., 2011), most bacteria are harmless or beneficial, constituting a natural barrier that plays a fundamental role in fortifying mucosal barrier function (Tlaskalova-Hogenova et al., 2011).

1.3.3.1 The gut microflora maintains epithelial cell turnover

Epithelial cell turnover as well as cell death and shedding are pivotal for health preservation, and diseases can occur when the balance shifts toward either excessive or insufficient apoptosis or proliferation (Siggers and Hackam, 2011). Commensal bacteria have been implicated in modulating intestinal epithelial cell turnover and controlling cell apoptosis, proliferation, and differentiation. Compared with conventionally raised animals, GF animals exhibited reduced epithelial apoptosis and crypt cell proliferation (Yu et al., 2012b) and abnormally long villi (Danielsen et al., 2007) in previous studies. Additionally, short chain fatty acids generated by bacteria are essential energy sources for the colonic epithelium and can regulate the growth and differentiation of epithelial cells (O'Keefe, 2008).

1.3.3.2 The gut microflora regulates intestinal permeability

Intestinal epithelial cells are arranged in a single layer. The intercellular space
between adjacent epithelial cells is sealed by dynamic junctional complexes, such as tight junctions (Groschwitz and Hogan, 2009, Ulluwishewa et al., 2011). Disturbances in a tight junction can induce increased permeability and defective intestinal barrier function, which are observed in several diseases (Forster, 2008). Signals from commensal bacteria or probiotics (e.g., Lactobacillus plantarum and Bifidobacterium infantis) have been suggested to regulate tight junctions and maintain epithelial integrity (Ivanov and Littman, 2011, Ulluwishewa et al., 2011). Conversely, the colonization of bacterial pathogens (e.g., Salmonella Typhimurium, Vibrio parahaemolyticus, and Citrobacter rodentium) can disrupt tight junctions and impair intestinal epithelial integrity (Ashida et al., 2011).

### 1.3.3.3 The gut microflora influences the mucus layer

The intestinal epithelium is covered by a protective mucus layer, constituting the first line of immune defense. Mucin degradation caused by bacteria can disturb the homeostasis of a mucosal barrier and is often recognized as the initial stage of some intestinal diseases (Derrien et al., 2010). Mucin supplementation can stimulate the growth of sulfate-reducing bacteria, particularly Desulfovibrio spp., and subsequently generate toxic substances, such as sulfide for the intestinal epithelium (Attene-Ramos et al., 2010, Gibson et al., 1988). Conversely,
mucin-degrading bacteria can occasionally exert positive effects. *Akkermansia muciniphila*, a human intestinal mucin-degrading bacterium, has been observed to increase the intestinal levels of endocannabinoids and thus alleviate metabolic disorders (Everard *et al.*, 2013). Furthermore, adhesion to mucins has been regarded as a possible mechanism through which probiotics can persist in the gastrointestinal tract and stimulate the host immune system (Kankainen *et al.*, 2009).

### 1.3.3.4 Antimicrobial substances secreted by the gut microflora

Paneth cells secrete substantial quantities of α-defensins, which are predominant antimicrobial peptides in the human intestine (Bevins and Salzman, 2011). Paneth cell dysfunction may affect the secretion of antimicrobials, predisposing the host to intestinal inflammation or other diseases. Low levels of the α-defensin production have been associated with susceptibility to Crohn’s disease (Menendez and Brett Finlay, 2007). Further evidence has suggested that Paneth cells and their antimicrobial substances play a crucial role in host defense against enteric pathogens. For example, matrilysin-deficient mice (α-defensin deficiency) are more susceptible to enteric *Salmonella typhimurium* infection (Wilson *et al.*, 1999), and HD5-transgenic mice (expressing a human intestinal defensin) are
more resistant to such infection than WT mice (Salzman et al., 2003). In addition, these antimicrobial substances can affect the microbial composition of a host. In contrast to WT mice, Bacteroidetes were overrepresented and Firmicutes were underrepresented in DEFA5 tg (+/+ ) mice (expressing a human α-defensin gene), whereas Mmp7−/− mice (α-defensin deficiency) exhibited a significant decrease in the relative abundance of Bacteroidetes but an increase in Firmicutes in one study (Salzman et al., 2010).

1.3.3.5 The gut microflora promotes secretory immunoglobulin A generation

The secretory form of IgA, known as sIgA, is the primary immunoglobulin in mucus secretions and has been determined to protect mucosal colonization or invasion by pathogens as well as limit the growth of pathogens, respond to microbial antigens, and neutralize toxins and enzymes (Fagarasan and Honjo, 2003, Woof and Kerr, 2006). Commensal bacteria can stimulate IgA, contributing to the protection of mucosal barrier and host-microbe interactions. Reduced gut secretory IgA has been observed in GF mice (Round and Mazmanian, 2009), indicating that the effect of the gut microbiota on sIgA is nonnegligable. Colonizing GF mice with a gut commensal microbiota can lead to a bacteria-specific IgA response (Shroff et al., 1995). Furthermore, specific bacteria,
such as *Bacteroides acidifaciens*, have been observed to promote IgA production (Yanagibashi *et al.*, 2012).

1.4 The gut microflora and diseases

Accumulating evidence has indicated that the gut microflora plays a vital role in the development of obesity, diabetes, inflammatory bowel disease, allergic diseases, rheumatoid arthritis, autism, and cancer. We discuss nonpathogenic bacteria, or commensal microbiota. However, whether alterations in the gut microbiota are the cause or consequence of most of the aforementioned diseases remains unclear. Some diseases might affect the composition of the gut microbiota. Conversely, perturbations in the microbiota might cause aforementioned diseases or at least contribute to pathological development.

1.4.1 The gut microflora and metabolic disorders

The gut microbiota has been linked to several metabolic disorders, such as obesity and diabetes. In one study, compared with lean mice (*ob/+* or *++*), *ob/ob* mice exhibited a 50% reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes (Ley *et al.*, 2005). Similarly, in contrast to lean people, obese people exhibited decreased numbers of Bacteroidetes in another study. However, a
low calorie diet caused obese people to lose weight and the proportion of Bacteroidetes to increase (Ley et al., 2006). The colonization of GF WT mice with a microbiota harvested from ob/ob mice caused a marked increase in body fat (Turnbaugh et al., 2006). Gut microbiota were suggested to be involved in obesity by affecting energy harvesting and fat storage, as well as inducing chronic inflammation (Cani and Delzenne, 2009).

A previous study reported that high-fat feeding increased the lipopolysaccharide (LPS) concentration in plasma; this process was considered “metabolic endotoxemia” (Cani et al., 2008). A similar finding was observed in ob/ob and db/db mice (Brun et al., 2007). In animals fed a high-fat diet, the number of Gram-negative Bacteroides-like mouse intestinal bacteria substantially decreased. The number of bacteria in the Eubacterium rectale-Clostridium coccoides group and Bifidobacterium spp. also significantly decreased (Cani et al., 2007a). Multiple-correlation analyses revealed that metabolic endotoxemia was significantly and negatively associated with Bifidobacterium spp. (Cani et al., 2007b), which have been shown to contribute to reducing intestinal endotoxin levels and improving mucosal barrier function (Griffiths et al., 2004, Wang et al., 2006). Evidence from prebiotic intervention supported this finding. Oligofructose
is a well-known prebiotic that can stimulate the growth of \textit{Bifidobacterium} spp. The metabolic endotoxemia induced by a high-fat diet can be abolished by dietary supplementation with oligofructose (Tuohy \textit{et al.}, 2005). Prebiotic treatment has been suggested to increase the number of \textit{Bifidobacterium} spp., reduce plasma LPS levels, normalize low-grade inflammation, and improve metabolic disorders. In addition, several studies have indicated that the modulation of gut microbiota by prebiotics can regulate the gut peptides, which are crucial modulators of food intake and energy expenditure (Chaudhri \textit{et al.}, 2008, Druce \textit{et al.}, 2004). Moreover, a recent study indicated that \textit{Akkermansia muciniphila}, a mucin-degrading bacterium, can alleviate high-fat-diet-induced metabolic disorders (Everard \textit{et al.}, 2013).

1.4.2 The gut microflora and inflammatory bowel disease

The gut microbiota is involved in inflammatory bowel disease (IBD). A decrease in the number of protective commensal bacteria (symbionts) and an increase in the number of potential pathogens (pathobionts), known as dysbiosis, were linked to IBD (Chow \textit{et al.}, 2010). The alleviation of intestinal inflammation was observed in mice pretreated with antibiotics (Videla \textit{et al.}, 1994). Abnormal microbial composition has been characterized by the depletion of 2 phyla, Firmicutes and
Bacteriodetes, accompanying concomitant increases in Proteobacteria and Actinobacteria in IBD patients (Frank et al., 2007). The major types of IBD are Crohn’s disease (CD) and ulcerative colitis (UC). In one study, fecal stream diversion from a segment of an inflamed small bowel attenuated intestinal inflammation in patients with CD. In other studies, the restoration of the fecal stream to a segment of a surgically resected bowel (histologically normal) triggered intestinal inflammation, implicating that the microbiota function as a major “driver” in IBD (D’Haens et al., 1998, Rutgeerts et al., 1991). The temporal stability and diversity of the gut microbial composition in patients diagnosed with CD were significantly lower than those in healthy control subjects (Scanlan et al., 2006). Dysbiosis in CD patients is characterized by increased adherent and invasive E. coli and decreased clostridial groups IV and XIVa (Sartor, 2010).

A reduction in beneficial bacteria, such as Lactobacillus and Bifidobacterium spp., has been linked to UC (Cummings et al., 2003). Similarly, decreased Faecalibacterium and Roseburia have been observed in ileal CD. These genera are known as principal sources of butyrate-producing bacteria, which exert protective effects in the intestine (Sartor, 2010). Faecalibacterium prausnitzii, an antiinflammatory commensal bacterium, was observed to ameliorate dysbiosis and
mediate protective effects in CD patients (Sokol et al., 2008). A previous study suggested using the ratio of *F. prausnitzii* to *E. coli* to evaluate dysbiosis in IBD patients by microbial analyses of specimens (Chassaing and Darfeuille-Michaud, 2011). Probiotics have been suggested to prevent the attachment of pathogens, maintain the intestinal epithelial barrier function, and activate adaptive immunity in IBD patients (Gareau et al., 2010).

1.4.3 The gut microflora and allergic diseases

Allergies (e.g., asthma, allergic rhinitis, atopic dermatitis, and food allergies) are chronic inflammatory diseases caused by deregulated immune responses against innocuous antigens. Epidemiological and clinical studies have linked the gut microbiota to an increased incidence of allergic diseases (Hormannsperger et al., 2012). Environmental antigens are essential to the development of mature and healthy gut microbiota. Immature microbiota can influence the proper maturation of the immune system and development of immunological tolerance, increasing the incidence of allergic hypersensitivity (Noverr and Huffnagle, 2005).

Recent findings have indicated that Caesarean delivery, breastfeeding, probiotics, antibiotics, and perinatal stress affect the development of asthma because of the
modification of the infant gut microbiota (Azad and Kozyrskyj, 2011). In one study, children who developed allergies were colonized by few *Lactobacillus* spp. (*L. rhamnosus, L. casei*, and *L. paracasei*), *Bifidobacterium adolescentis*, and *Clostridium difficile* during the first 2 months of infancy (Sjogren *et al.*, 2009). In addition, infants with a high risk of atopy exhibited increased Enterobacteriaceae, Clostridia, *Bacteroides*, and *Staphylococcus* (Festi *et al.*, 2011). A previous study reported that allergic infants, aged 2 years, exhibited a higher *Bacteroides/Bifidobacterium* ratio than nonallergic infants did (Suzuki *et al.*, 2008). Probiotics can potentially be used for treating allergic diseases by modulating early microbial colonization, enhancing gut-specific IgA responses, or alleviating local and systemic allergic inflammation (Isolauri *et al.*, 2012). In addition, the effects of probiotics on the prevention and/or treatment of allergic diseases have been assessed in several human clinical studies (Toh *et al.*, 2012). Colonizing GF mice with the gut microbiota from healthy infants, primarily composed of *Bifidobacterium* and *Bacteroides*, produced a protective effect on allergic sensitization and food allergies (Rodriguez *et al.*, 2012).

1.4.4 The gut microflora and rheumatoid arthritis

The development of rheumatoid arthritis (RA) has been linked to both genetic and
environmental factors (Edwards, 2008). Numerous animal and human studies have indicated that the intestinal microbiota participates in the etiopathogenesis of RA. Several reports have suggested that the gut microflora exerts protective effects in arthritic diseases. For example, compared with conventional rats, GF rats developed severe joint inflammation with 100% incidence in an adjuvant-induced arthritis model, whereas specific-pathogen-free rats exhibited an intermediate susceptibility (Kohashi et al., 1979). Similarly, in a streptococcal cell wall-induced rat arthritis model, F344 rats were resistant to chronic joint inflammation, but GF rats were susceptible to arthritic disease (van den Broek et al., 1992). In addition, deleterious effects of the gut microbiota have been reported in some studies. When K/BxN mice were raised in a GF environment, the development of arthritis was attenuated. Monocolonization with segmented filamentous bacteria aggravated the disease in GF K/BxN mice (Wu et al., 2010a). A similar finding was reported for Il1rn−/− mice monocolonized by Lactobacillus bifidus (Abdollahi-Roodsaz et al., 2008). Certain intestinal microbes seem to be required for triggering forms of autoimmune arthritis, such as RA, in some genetically susceptible animals.

In one study, patients diagnosed with early RA exhibited decreased Bifidobacteria and bacteria of the Bacteroides-Porphyromonas-Prevotella group, Bacteroides
fragilis subgroup, and Eubacterium rectale-Clostridium coccoides group, compared with fibromyalgia patients (Vaahtovuo et al., 2008). Recently, Prevotella copri was determined to play a potential role in the pathogenesis of RA by using a pyrosequencing approach. New-onset untreated RA (NORA) patients exhibited a higher abundance of Prevotella copri than did healthy individuals. The presence of P. copri corresponded to a reduction in Bacteroides and a loss of beneficial microbes in NORA patients (Scher et al., 2013). Furthermore, Liu et al. (2013b) summarized the various effects (inhibitory or promoting effects) of Lactobacillus spp. on RA, and indicated that more Lactobacillus was observed in the feces of RA patients than in healthy controls. In a randomized clinical trial, Lactobacillus casei 01 supplementation was determined to improve the disease activity and inflammatory status of patients diagnosed with RA (Vaghef-Mehrabany et al., 2013).

1.4.5 The gut microflora and autism

Recent studies have suggested that the gut microbiota is associated with autism spectrum disorders (ASDs), such as autism (Midtvedt, 2012, Stilling et al., 2013). A high percentage of people diagnosed with autism have a history of extensive antibiotic use (Bolte, 1998). Clostridium species, including C. bolteae, and clusters
I and XI of *Clostridium* spp. have been reported to be more abundant in ASD patients than in healthy controls (Song et al., 2004). *C. histolyticum*, known as a toxin producer, was elevated in ASD children compared with unrelated healthy children, but not compared with healthy siblings (Parracho et al., 2005). Furthermore, increased *Sutterella* spp. and *Ruminococcus torques* (Wang et al., 2013) as well as decreased Bifidobacteria (Adams et al., 2011, Wang et al., 2011) and *Akkermansia muciniphila* (Wang et al., 2011) have been observed in ASD children compared with healthy controls. A pyrosequencing report indicated that autistic children exhibited a high proportion of Bacteroidetes and a low level of Firmicutes, as well as high levels of *Desulfovibrio* species and *Bacteroides vulgatus* (Finegold et al., 2010). Another recent report provided additional detailed information. *Caloramator*, *Sarcina*, and *Clostridium* were overrepresented, whereas *Faecalibacterium* and *Ruminococcus* were underrepresented in children with autism compared with healthy controls (De Angelis et al., 2013).

Modulation of the gut microbiota has been suggested as a treatment for ASD. Short-term antibiotic treatment can temporarily improve the behavioral symptoms in some people diagnosed with ASD. Probiotics have been suggested to play a potential role in ameliorating childhood ASDs (Critchfield et al., 2011). In
addition, human commensal *Bacteroides fragilis* has also been reported to correct gut permeability, alter microbial composition, and modulate levels of several metabolites, contributing to amelioration of the features of ASD (Hsiao *et al.*, 2013).

### 1.4.6 The gut microflora and cancer

The microbial ecosystem is a complex community. Some bacteria have been implicated in the pathogenesis of cancer. Conversely, some bacteria, such as *Lactobacillus acidophilus* and *Bifidobacterium longum*, have been observed to exert inhibitory effects on cancer development (McIntosh *et al.*, 1999, Rowland *et al.*, 1996). It is well known that gut bacteria play an active role converting dietary procarcinogens into carcinogens through various enzymatic activity (Arimochi *et al.*, 2006, Bingham *et al.*, 1996, Chung *et al.*, 1992, Davis and Milner, 2009, Mirvish *et al.*, 2002, Schut and Snyderwine, 1999, Xu *et al.*, 2007). In addition, the gut microflora produces harmful substances, thus contributing to the pathogenesis of cancer. For example, *Bacteroides fragilis* and *Clostridium perfringens* have been linked to increased colon cancer risk because they produce α-toxins and enterotoxins (Arimochi *et al.*, 2006, Toprak *et al.*, 2006). Furthermore, commensal bacteria, such as segmented filamentous bacteria and *Enterococcus faecalis*, have
been reported to alter the epithelial redox environment by producing hydrogen sulfide or oxygen radicals (Huycke and Gaskins, 2004). These oxidants may be regarded as a crucial cause of chromosomal instability that gives rise to cancer.

Nevertheless, the gut microflora can influence cancer risk by generating protective metabolites from dietary components. Short chain fatty acids (SCFAs) produced by the microbial fermentation of dietary fibers are used as nutrients by intestinal epithelial cells (Sharma et al., 2010). Among these SCFAs, butyrate has been observed to exhibit anticancer activity (Berni Canani et al., 2012, Fung et al., 2012). In addition, bacteria are responsible for forming conjugated linoleic acids (CLAs), which possess antiinflammatory and cancer-preventive properties (Ip et al., 2002, Kelley et al., 2007, Yu et al., 2002). Davis and Milner (2009) reviewed other bacterial metabolites exhibiting cancer-preventive properties, including equol from isoflavones, enterodiol and enterolactone from lignans, and urolithins from ellagic acid. Moreover, the gut microflora has bioremediation-like functions, such as detoxifying ingested carcinogens, which might lower the susceptibility of the host to cancer (Turnbaugh et al., 2007).

Recently, the relationship between the gut microbiota and cancer has been
described in cancer development and treatment. Commensal bacteria have been proven to shape the anticancer immune response (Viaud et al., 2013) and mediate the anticancer effect by regulating myeloid-derived cell functions in the tumor microenvironment (Iida et al., 2013). Conversely, tumor-associated gut microbiome alterations (dysbiotic community) have been determined to exacerbate colon tumorigenesis in GF mice treated with azoxymethane/dextran sodium sulfate (Zackular et al., 2013), for reviews, please see Bultman (2013), Hullar et al. (2014), and Schwabe and Jobin (2013). Our study indicated a potential association between the gut microflora and the anticancer effect of herbal medicine; the work is discussed in the following chapters. We will elaborate more the relationship between the gut microbiota and colorectal cancer in Section 1.5 for the reason that CRC is the model cancer in this thesis work.

1.4.7 The gut microflora and other diseases

In addition to the aforementioned diseases, the gut microflora has been associated with other diseases. A reduced microbial diversity was observed in familial mediterranean fever (Khachatryan et al., 2008). Systemic inflammatory response syndrome was associated with a reduction in obligate anaerobes (Bacteroidaceae, Bifidobacterium, and Veillonella spp.) as well as a decrease in Lactobacillus and
an increase in *Staphylococcus* and *Pseudomonas* (Shimizu et al., 2006). The suppression of antibiotic-sensitive bacteria and overgrowth of antibiotic-resistant bacteria have been observed in antibiotic-associated diarrhea (Johnston et al., 2007, Rohde et al., 2009). In addition, liver disease was linked to the intestinal microbiota (Henao-Mejia et al., 2013).

### 1.5 The gut microflora and colorectal cancer (CRC)

Colorectal cancer (CRC) is the third most commonly diagnosed cancer, with more than one million new cases per year worldwide. Moreover, 600,000 deaths per year are attributed to CRC, and the mortality rate is approximately 33% in developed areas (Cunningham et al., 2010, Jemal et al., 2011). Among all types of cancer, CRC might be most closely linked to the gut microbiota. The bacterial density in the large intestine is much higher than that in the small intestine (approximately $10^{12}$ vs. $10^2$ cells/ml). An approximately 12-fold higher risk of cancer is associated with the large intestine compared with the small intestine (Sobhani et al., 2013). Thus, the colon has been described as a “bioreactor,” containing the highest amount of bacteria. Segregation of gut microbiota between normal and CRC patients was observed (Wang et al., 2012). Increased pH, and decreased SCFAs, including acetic acid, propionic acid, and butyric acid, were
also found in CRC patients (Ohigashi et al., 2013). The role of gut microbiota in cancer development will be reviewed in the following sections.

1.5.1 The critical role of commensal microbiota in CRC development

The impact of commensal microbiota on CRC development has been investigated in several animal models by using GF mice and microbiota transplantation experiments. \(Apc^{Min/+}\) mice carrying a point mutation in the Apc gene develop multiple neoplasias in their intestinal tracts several weeks after birth (Mai et al., 2007). In one study, germ-free (GF) \(Apc^{Min/+}\) mice exhibited an obvious reduction in intestinal tumor load compared with specific-pathogen-free \(Apc^{Min/+}\) mice (Li et al., 2012). GF azoxymethane (AOM)-treated \(Il-10^{-/-}\) mice (a colitis-associated CRC model) exhibited normal colon histology and exhibited no intestinal inflammation or tumors (Uronis et al., 2009). The ability to transfer host characteristics through microbiota transplantation implies that microbes play an active rather than passive role in health and disease. Garrett et al. (2007) determined that the microbiota from colitis-prone TRUC (\(T-bet^{-/-}\times RAG2^{-/-}\) ulcerative colitis) mice have a transmissible trait. Merely cohousing adult WT mice with TRUC mice induced aggressive colitis in WT mice. \(Nod2\)-deficient mice exhibited a high risk of colitis and colitis-associated carcinogenesis.
Microbiota transplantation with the dysbiotic fecal microbes from *Nod2*-deficient mice can increase the disease risk of WT mice (Couturier-Maillard *et al.*, 2013).

### 1.5.2 Bacterial toxins and carcinogenesis

Bacterial toxins are crucial in carcinogenesis, and their role has been extensively studied. Intestinal inflammation caused by altering the gut microbial composition and inducing the expansion of microbes through genotoxic actions has been suggested to promote CRC (Elinav *et al.*, 2013). Arthur *et al.* (2012) observed a relationship among the gut microbiota, host immune system, and genotoxic events in an inflammation-induced CRC model. Monocolonization with the commensal *E. coli* NC101 can promote invasive carcinomas in AOM-treated *Il10*−/− mice. The polyketide synthase (pkS) genotoxic island of *E. coli* NC101 has been indentified as a molecular trigger. This finding has also been confirmed for humans. IBD and CRC patients harbor a high abundance of mucosa-associated pkS+E. coli.

In addition, enterotoxigenic *Bacteroides fragilis* (ETBF) is more prevalent in CRC patients than in control patients (Toprak *et al.*, 2006). ETBF, a human colonic commensal bacterium, can cause an inflammatory colitis and strongly promote colon tumorigenesis in Min^Apc^{716+/-} mice (Wu *et al.*, 2009).
However, Mazmanian et al. (2008) demonstrated that the prominent human symbiont *Bacteroides fragilis* can protect *Helicobacter hepaticus*-induced experimental colitis in animals, and that a single microbial molecule, polysaccharide A, is required for this beneficial effect.

1.5.3 Microbial sensors mediate host-microbe interactions in intestinal tumorigenesis

The host microbial sensors, representing by a group of pattern recognition receptors (PRRs), can recognize specific conserved microbial molecules, such as components of bacterial cell walls or nucleic acids. TLRs are the most extensively characterized PRRs. They act as innate immune signal sensors and participate in host defense (Takeda and Akira, 2005). Functional polymorphisms in the human TLR2 and TLR4 genes were linked to the risk for CRC (Pimentel-Nunes et al., 2012). TLR4 signaling plays a critical role in colon carcinogenesis. One study reported that TLR4-deficient mice were protected against the development of colitis-associated CRC (Fukata et al., 2007). Nevertheless, TLR4-mediated signal transduction of the host microbiota is more complex than originally thought. Constitutively active TLR4 signalling in the intestinal epithelium was reported to cause a dramatic reduction in tumor load in *Apc^{Min+}* mice (Li et al., 2013).
Most TLRs, including TLR2 and TLR4, perform signal transduction through the adaptor protein myeloid differentiation factor 88 (MyD88). The TLR/MyD88 pathway is essential for the microbiota-induced development of CRC. Il10$^{-/-}$ Myd88$^{-/-}$ mice treated with AOM exhibited no signs of tumor development in a previous study (Uronis et al., 2009). In addition, Myd88 deficiency can attenuate the development of spontaneous intestinal tumorigenesis, reducing polyp numbers and size in Apc$^{Min/+}$ MyD88$^{-/-}$ mice (Rakoff-Nahoum and Medzhitov, 2007). In contrast to the TLR family, the NOD-like receptor family exerts a protective role in CRC development. Nod1 deficiency causes the augmented development of both AOM and dextran sodium sulfate (DSS)-induced colitis-associated and spontaneous CRC (Chen et al., 2008). All of these findings indicate the complex interaction between the host and the microbiota.

1.5.4 Microbial components regulate immune homeostasis in colorectal cancer

Microbial components may participate in tumor development. In Apc$^{Min/+}$ mice, the defective barrier function contributes to the translocation of microbial lipopolysaccharides and activation of tumor-elicited inflammation, causing IL-23/IL-17-mediated (Th17 signature) tumor growth (Grivennikov et al., 2012).
This study provides a suitable example for explaining the integrative mode of CRC pathogenesis, indicating the relationship among the genetic mutation, the mucosal barrier, the microbiota, and the immune system. Another clinical report suggested that Th1 and Th17 clusters play a critical role in the survival of CRC patients. Patients exhibiting highly expressed Th17 were associated with a poor prognosis, whereas patients with highly expressed Th1 exhibited prolonged disease-free survival (Tosolini et al., 2011). In addition to T helper cells, Tregs play a crucial role in maintaining immune homeostasis. Spore-forming bacteria, Clostridium clusters IV and XIVa, have been reported to promote the accumulation of Tregs (Atarashi et al., 2011). These 2 groups are crucial butyrate-producing microbes (Van den Abbeele et al., 2013) that have been determined to be significantly reduced in the gut microbiota of CRC patients (Wang et al., 2012).

1.5.5 Advancement of the gut microbiome toward the understanding of colorectal cancer

The advancement of next-generation sequencing sheds light on the field of the gut microbiome. Although recent studies have not identified a consensus or core group of microbes linked to CRC, structural segregation of gut microbial
communities has been observed in CRC patients and healthy individuals. For example, *Bacteroides vulgatus* and *Bacteroides uniformis* were abundant in the fecal microbiota of healthy subjects, whereas *Bacteroides fragilis* was enriched in that of CRC patients. Compared with healthy volunteers, CRC patients harbored a relatively high abundance of *Enterococcus*, *Escherichia/Shigella*, *Klebsiella*, *Streptococcus*, and *Peptostreptococcus*, but exhibited an obvious reduction of butyrate-producing bacteria, such as *Roseburia* (Wang et al., 2012). In other reports, microbial dysbiosis was observed in CRC patients (Chen et al., 2013, Sobhani et al., 2011). In addition, the adherent bacterial communities of mucosal biopsy samples were compared. Only 5 taxa were more prevalent in subjects without adenomas (controls), whereas 87 taxa were relatively abundant in subjects diagnosed with adenomas (Sanapareddy et al., 2012). One group determined that the microbial structures of cancerous tissue are similar to those of noncancerous tissue, but the intestinal lumen microbiota and the mucosa-adherent microbiota differed between CRC patients and healthy people (Chen et al., 2012).

Striking differences were observed in the microbiome of CRC tissue and the adjacent nonmalignant mucosa. A decrease in Firmicutes and an increase in Bacteroidetes as well as commensals exhibiting probiotic features were observed
in the tumor tissue compared with the adjacent mucosa (Marchesi et al., 2011). The author attributed these findings to the recruitment of the tumor microenvironment and suggested that a CRC-associated niche recruits tumor-foraging commensal-like microbes. These probiotic microbes have an apparent competitive advantage and might replace the pathogenic bacteria potentially involved in CRC development.

Unlike other microbes, the expansion of *Fusobacterium* in CRC patients has been independently reported in several studies (Chen et al., 2012, Marchesi et al., 2011, Wang et al., 2012). Further studies indicated that a significantly higher abundance of *Fusobacterium* has been observed in colorectal adenomas (Kostic et al., 2012, McCoy et al., 2013). *Fusobacterium nucleatum* bacterial strain has been identified as an overrepresented microbe in colorectal carcinoma by using RNA-seq followed by host sequence subtraction (Castellarin et al., 2012). However, the alteration of microbiome in CRC patients might be a consequence of disease development or tumor environmental changes. The factors that contribute to the overrepresentation of certain microbes remain undetermined. Thus, the roles of *Fusobacterium* and other bacteria in the pathogenesis of CRC require further investigation.
1.5.6 Driver and passenger microbes in the development of colorectal cancer

According to previous relevant studies, linking CRC carcinogenesis to a single bacterium or phylogenetic core changes in the microbiome remains difficult. Tjalsma et al. (2012) hypothesized that CRC is initiated by particular bacterial drivers and promoted by certain bacterial passengers. In this review, bacterial drivers are defined as the indigenous intestinal microbes that can induce epithelial DNA damage, such as Enterococcus faecalis, the producer of extracellular superoxide and hydrogen peroxide, Escherichia coli NC101, the producer of genotoxin colibactin (Cuevas-Ramos et al., 2010), and enterotoxigenic Bacteroides fragilis, the producer of B. fragilis toxin, fragilysin (Goodwin et al., 2011). In addition, bacterial passengers are defined as opportunistic bacteria that hold a competitive advantage in the tumor microenvironment. Tjalsma et al. (2012) speculated that Streptococcus gallolyticus and Fusobacterium function as candidate bacterial passengers. Streptococcus bovis has long been associated with CRC. Streptococcus bovis accounts for 2.5% to 15% of the intestinal microbiota in healthy people, but an increased proportion can be detected in 56% of CRC patients (Abdulamir et al., 2011). A recent meta-analysis indicated that patients presenting with Streptococcus gallolyticus (previously known as S. bovis biotype I)
infection exhibited a markedly increased risk of CRC development (Boleij et al., 2011).

1.5.7 Interaction between the gut microbiota and chemotherapeutic medicines

Increasing evidence has suggested that chemotherapy can disrupt the homeostasis of intestinal microbial ecosystem. For example, in a rat model exhibiting irinotecan-induced diarrhea, the intestinal microbiota was altered, with increases in *E. coli*, *Staphylococcus* spp. and *Clostridium* spp., and decreases in certain beneficial bacteria, such as *Lactobacillus* spp. and *Bifidobacterium* spp., as well as *Bacteroides* spp. (Stringer et al., 2008). Another study determined that tumor progression can increase the abundance of *Enterobacteriaceae* and *Clostridium* clusters I and XI. Moreover, irinotecan (CPT-11) chemotherapy can increase cecal *Enterobacteriaceae* and *Clostridium* cluster XI, particularly after dose-intensive therapy (Lin et al., 2012).

Conversely, other studies have suggested that intestinal microbes are involved in chemotherapy-induced toxicity. In 1993, 18 acute deaths in Japanese patients diagnosed with cancer and herpes zoster were due to the interactions of a new oral
antiviral drug, called sorivudine (SRV), with one of the oral 5-fluorouracil (5-FU) prodrugs. The enhanced toxicity of 5-FU was attributed to a key enzyme inactivation, which was associated with a SRV metabolite generated by the gut microflora (Okuda et al., 1998). Similarly, the common colon cancer chemotherapeutic CPT-11 has a dose-limiting side effect of severe diarrhea. This effect is caused by the reactivation of the drug in the gut, which is mediated by the bacterial β-glucuronidases (Takasuna et al., 1996). Wallace et al. (2010) screened an inhibitor that was highly effective against the activity of this undesirable enzyme and produced no disturbance in the microbial ecosystem. When CPT-11 was combined with this inhibitor, a high dosage was allowed, and the mice were protected from the CPT-11-induced side effect. Furthermore, the intestinal microflora contributes to the delayed diarrhea caused by the anticancer agent irinotecan (Brandi et al., 2006). In addition, commensal intestinal bacteria may play a prominent role in developing the severity of chemotherapy-induced mucositis in cancer patients (van Vliet et al., 2010).

1.6 Functional foods and colorectal cancer

“A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional
effects, in a way that is relevant to either improved stage of health and well-being and/or reduction of risk of disease” (Diplock et al., 1999). The intestinal microbiota can be modulated by varying the diet or consuming functional foods, including probiotics, prebiotics, synbiotics, and phytochemicals. These agents can affect the microbial composition, gut epithelial barrier, and the interactions between microbes and the immune system, contributing to CRC prevention.

### 1.6.1 Probiotics

Probiotics are “live microorganism which when administered in adequate amounts confers a health benefit to the host” (Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation) (FAO/WHO, 2002). Probiotics should be able to survive in the gastrointestinal tract. *Lactobacillus* and *Bifidobacterium* contain the most commonly used probiotic microorganisms. Members of *Bacillus* and *Propionibacterium* are also used as probiotic microorganisms, as well as some gram-negative bacteria and yeasts (e.g., *Saccharomyces*) (Gerritsen et al., 2011).

Probiotics play a vital role in microbe-microbe interaction and can prevent the adhesion of pathogens. For example, *Lactobacillus* and *Bifidobacterium* have been
shown to share carbohydrate-binding specificities with some enteropathogens, contributing to their abilities to inhibit, displace, and compete with pathogens (Fujiwara et al., 2001). Probiotics can secrete antimicrobial substances and compounds. Lactic acid bacteria can produce antimicrobial metabolites, including antimicrobial compounds (e.g., organic acids) and proteins (e.g., bacteriocins) (Chen and Hoover, 2003, Niku-Paavola et al., 1999). Furthermore, some probiotics exhibit resistance against microbial pathogens by activating the immune system (Cross, 2002). In addition to microbe-microbe interaction, probiotics are involved in microbe-gut epithelium interaction and microbe-immune system interaction (Collado et al., 2009). Probiotics can improve intestinal cell junctions (Luyer et al., 2005, Montalto et al., 2004) and stimulate mucin secretion (Mack et al., 2003). Probiotics have been reported to induce the production of secretory IgA and other nutrients, such as SCFAs (Viljanen et al., 2005).

Accumulating evidence has suggested that probiotics exert protective effects on CRC in cellular models, animal models, and clinical trails. Probiotic strains of the following species have been extensively studied: Lactobacillus rhamnosus GG, Lactobacillus acidophilus, Lactococcus lactis, Lactobacillus casei, Lactobacillus plantarum, Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium
infantis, and Bifidobacterium breve. VSL#3 is a commercial probiotic mixture composed of 4 strains of Lactobacillus (Lactobacillus casei, L. plantarum, L. bulgaricus, and L. acidophilus), 3 strains of Bifidobacterium (Bifidobacterium longum, B. breve, and B. infantis), and a Streptococcus salivarius subspecies, thermophilus. Some Gram-negative bacteria, such as Escherichia coli Nissle 1917, and yeasts (e.g., Saccharomyces boulardii) have been reported to benefit the host (Gareau et al., 2010, Zhu et al., 2011). Biological activities are exerted by components of probiotics, such as cell-bound exopolysaccharides isolated from Lactobacillus acidophilus 606 (Kim et al., 2010), polysaccharide fractions extracted from Bifidobacterium bifidum BGN4 (You et al., 2004), supernatants and lipopolysaccharides isolated from Escherichia coli Nissle 1917 (Stetinova et al., 2010), and a soluble protein p40 isolated from Lactobacillus rhamnosus GG (Yan et al., 2011, Yan et al., 2013, Yan and Polk, 2012). Some genetically modified probiotic strains exhibit an inhibitory effect on the development of CRC (Lightfoot et al., 2013).

The possible protective mechanisms of probiotics against CRC development have been extensively documented (Uccello et al., 2012): (1) competition with pathogenic microbiota; (2) regulation of cell proliferation and apoptosis; (3)
regulation of the mucosal inflammatory response; (4) generation of SCFAs and CLAs; (5) modulation of the host immune system; (6) regulation of the activity of bacterial enzymes such as β-glucuronidase, nitroreductase, and azoreductase; and (7) inactivation of carcinogens such as heterocyclic aromatic amines and N-nitroso compounds.

1.6.2 Prebiotic

A prebiotic is “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health” (Roberfroid, 2007). Prebiotics are nondigestible food ingredients that can stimulate the growth of carbohydrate-using bacteria, including Bifidobacteria and lactic acid bacteria. The organic acids released by these beneficial bacteria can inhibit enteropathogens by generating an antimicrobial environment (Vulevic et al., 2004). The common prebiotics are inulins, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), lactulose, pyrodextrins, soya-oligosaccharides, xylo-oligosaccharides, and isomalto-oligosaccharides. These prebiotics are ingredients in numerous high-fiber foods (DuPont and DuPont, 2011, Tuohy et al., 2005). Rycroft et al. (2001) compared the fermentation properties of commercial prebiotic oligosaccharides.
They determined that FOS enhanced the *Lactobacillus* population, whereas xylo-oligosaccharides and lactulose exhibited a substantial increase in Bifidobacteria. GOS substantially reduced the population of Clostridia. In addition, lactulose and GOS exhibited strong abilities for generating SCFAs.

Increasing evidence has suggested that prebiotics are conducive to CRC prevention. Prebiotics can affect fecal bulking, reduce colonic pH, produce SCFAs, regulate the activity of exogenous carcinogens, and modulate gene expression and immune response (Lim *et al*., 2005). However, several reports have indicated that prebiotics exert no effect on CRC (Burn *et al*., 2008, Limburg *et al*., 2011, van Gorkom *et al*., 2002)

### 1.6.3 Synbiotics

Synbiotics are a combination of probiotics and prebiotics that exhibit synergistic effects. Synbiotics have been suggested to be more effective than probiotics or prebiotics used alone in CRC prevention (Zhu *et al*., 2011). For example, carbohydrate resistant starch (RS) can be used as a substrate for *Bifidobacterium lactis*. Synbiotic intervention of *Bifidobacterium lactis* and RS exhibited protective effects against CRC development that were not observed in rats treated with
Bifidobacterium lactis only (Le Leu et al., 2010).

1.6.4 Phytochemicals

Biologically active plant-based compounds, also known as phytochemicals, are regarded as effective chemopreventive agents that can be applied in treating CRC (Chung et al., 2013, Rafter, 2002). Daily intake of fruit and vegetables (e.g., berries (Brown et al., 2012), dry beans (Hughes et al., 1997), apples (Gerhauser, 2008), and onions (Tache et al., 2007)) is associated with a reduced risk of CRC because of their various phytochemicals. Phenolics, flavonoids, and phytoestrogens have attracted much attention because of their beneficial effects (e.g., anticarcinogenic, antioxidant, antiinflammatory, and cardioprotective effects) (Laparra and Sanz, 2010). Polyphenols derived from tea or red wine can influence colon carcinogenesis, reduce oxidative damage, and induce an increase in the gut colonization of some probiotic strains, such as Lactobacillus and Bifidobacterium spp. (Dolara et al., 2005). Isoflavones are considered beneficial to reducing the risk of cancer, including colon cancer (Branca and Lorenzetti, 2005). Furthermore, some dietary herbal supplements, such as herbal flavonoids (e.g., quercetin, curcumin, rutin, and silymarin) or herb mixtures (e.g., ginseng) exhibit potentially beneficial effects on colon cancer prevention (Volate et al., 2005). In addition, a
case-control study revealed that the intake of dietary flavonoids, particularly quercetin, may be linked to a reduced risk for colon cancer (Kyle et al., 2010).

In addition, functional foods can enhance the antitumor efficacy and reduce the side effects of CRC chemotherapy. Dietary phytochemicals (e.g., anthocyanin-rich tart cherry extract) are suggested to be combined with anticancer drugs at a suboptimal dosage to reduce the potential adverse side effects and enhance the protective effect against colon cancer (Bobe et al., 2006). Celecoxib combined with a butyrate-producing fiber, such as FOS, exhibited a protective effect in the development of aberrant crypt foci (Buecher et al., 2003). Other nutrients, such as glutamine and n-3 fatty acids, can reduce the CPT-11-induced severe diarrhea and enhance the efficacy of CPT-11 (Xue et al., 2007).

1.7 Methodology for microbiome study

Recent advances in the study of microbial ecosystem, including genomic and metabolic profiling, have made great strides in the characterization of microbial communities. PCR-based molecular methods have overcome the limitations of traditional culture-based techniques (Juste et al., 2008). In addition, advances in pyrosequencing have provided a powerful tool for studying the microbiome. In
this study, enterobacterial repetitive intergenic consensus (ERIC) sequences-based PCR and pyrosequencing of the 16S rRNA gene were used to characterize the fecal microbiome of the experimental mice.

1.7.1 ERIC-PCR

ERIC sequences, also known as intergenic repetitive units, are highly conserved noncoding sequences (127-bp imperfect palindromes), and dispersed throughout various bacterial genomes (Wilson and Sharp, 2006). ERIC sequences were first reported in *Escherichia coli*, *Salmonella typhimurium*, and other members of *Enterobacteriaceae*, as well as *Vibrio cholerae* (Hulton *et al.*, 1991, Sharples and Lloyd, 1990). Based on the conserved central core, two opposing primers were designed by Versalovic *et al.* (1991) (See Fig. 1.6). ERIC sequences empowered a rapid identification and genetic analysis of various microorganisms.

![ERIC sequence and the targeted primers](image)

Adapted and modified (Wilson and Sharp, 2006)
1.7.1.1 Discrimination of various bacteria

ERIC-PCR has been used as a molecular tool for distinguishing different bacterial species within the same genus, such as the species among *Yersinia* (Souza *et al.*, 2010), *Streptococcus* (Okada *et al.*, 2011), *Lactobacillus* (Ju *et al.*, 2009, Saito *et al.*, 2011), and *Mycobacterium* (Englund, 2003). In addition to species-specific profiles, ERIC-PCR is able to generate strain-specific profiles for *Bacteroides fragilis* (Moraes *et al.*, 2000), *Streptococcus pyogenes* (Matsumoto *et al.*, 2001), and *Actinobacillus seminis* (Appuhamy *et al.*, 1998). Furthermore, ERIC-PCR has been applied as a rapid identification method for serotyping of *Salmonella* (Bennasar *et al.*, 2000, Van Lith and Aarts, 1994) and epidemiological typing of *Stenotrophomonas maltophilia* (Marty, 1997). Moreover, ERIC-PCR can be used as a robust, reliable and rapid molecular typing method for the discrimination of different genera of *Proteobacteria* (Lee *et al.*, 2012).

1.7.1.2 Molecular typing of pathogens

ERIC-PCR has been applied as a useful tool for molecular typing of pathogens, including human opportunistic pathogens such as *Pseudomonas aeruginosa* (Wolska and Szweda, 2008, 2009), *Vibrio cholerae* (Balaji *et al.*, 2013), and *Enterobacter cloacae* (Glatz *et al.*, 2007, 2011), animal pathogens such as
*Paenibacillus larvae* (Antunez et al., 2007, Di Pinto et al., 2011, Rusenova et al., 2013), *Pasteurella multocida* (Biswas et al., 2004, Chrzastek et al., 2012, Leotta et al., 2006, Loubinoux et al., 1999, Sellyei et al., 2008), and *Haemophilus parasuis* (Jablonski et al., 2011, Macedo et al., 2011, Oliveira et al., 2003, Olvera et al., 2007), and phytopathogens such as *Ralstonia solanacearum* (Ivey et al., 2007, Jaunet and Wang, 1999, Norman et al., 2009) and *Pseudomonas syringae* (Little et al., 1998, Oguiza et al., 2004, Scortichini et al., 2005, Sesma et al., 2000).

### 1.7.1.3 Food processing and environmental monitoring

ERIC-PCR has been used in the detection of the bacterial contamination during food processing, such as *Listeria monocytogenes* (Chen et al., 2010), *Escherichia coli*, and potential enteric pathogens (Warriner et al., 2002). In addition, the functional bacteria, such as acetic acid bacteria, can be determined by ERIC-PCR during vinegar production (Fernandez-Perez et al., 2010, Wu et al., 2010b) and wine fermentation (Gonzalez et al., 2005, Gonzalez et al., 2004). Furthermore, ERIC-PCR has been shown to identify and characterize the microbiota isolated from environment, such as bacteria isolated from endosulfan contaminated soil (Kumar et al., 2008).
1.7.1.4 Characterization of the gut microbial ecosystem

In recent years, ERIC-PCR has been applied to monitor and characterize the structure and dynamic change of intestinal microbiota (Cheng et al., 2011, Li et al., 2007, Pang et al., 2007, Yang et al., 2011). Microbial communities can be regarded as a mixture of microbial genomes. Different populations of the gut microbiota exhibit different numbers and locations of ERIC sequences, thus generating different banding patterns (Di Giovanni et al., 1999). The ERIC-PCR bands represent the predominant microbial members, and the intensity of these bands reflects the abundance of these bacterial populations. ERIC-PCR is a very rapid and reliable molecular method to obtain highly reproducible and unique banding patterns for microbial communities (Wei et al., 2004). In this study, ERIC-PCR was used to analyze the fecal microflora profiles of the experimental mice.

1.7.2 Pyrosequencing of 16S rRNA gene

Next-generation sequencing technology, such as Roche/454 pyrosequencing (Fig. 1.7), is revolutionizing microbial ecology studies (Siqueira et al., 2012). Pyrosequencing, with a resolution of 10-100 fold higher than clone library approach, has provided an insight into the comprehensive microbial communities (Tamaki et al., 2011). Unlike Sanger-based 16S rRNA sequencing method,
pyrosequencing allows hundreds of communities to be analyzed simultaneously by using error-correcting barcoded primers (Hamady et al., 2008). Bacterial 16S rRNA gene contains nine hypervariable regions (V1-V9), and pyrosequencing primers are designed to target these hypervariable regions and enable the unprecedented characterization of the complex microbial communities (Chakravorty et al., 2007). The fusion primers contain four components, including adaptor sequence, key sequence, multiplex identifier (barcode sequence), and template specific sequence (Fig. 1.8). Briefly, the adaptor sequence is required for the emulsion-PCR (emPCR) and 454 pyrosequencing. The key sequence TCAG is used as a quality control to validate the reads. Multiplex identifier (MID) is used like a DNA barcode to identify amplicons or samples. In this study, pyrosequencing was performed by using 563F and 1064R primers that are designed to target the V4-V6 regions of 16S rRNA gene.
Figure 1.7 The 454 pyrosequencing approach.  
(Siqueira et al., 2012)

Figure 1.8 Components of fusion primers for amplicon sequencing.
1.7.3 Main method of data analysis

1.7.3.1 ERIC-PCR data analysis

Partial least squares discriminant analysis (PLS-DA) model is commonly used to process metabolomics data. In this study, we established a PLS-DA-based method for analyzing the ERIC-PCR data, thus visualizing the dynamic changes in the microflora composition. Briefly, ERIC-PCR was used to profile the gut microbiome by using fecal genomic DNA and a pair of ERIC specific primers. Agarose gel electrophoresis was used to separate the DNA fragments of PCR product. Gel pictures were photographed using a Gel Doc™ XR+ System and digitized with an Image Lab 3.0 system (Bio-Rad). Based on the distance and the intensity of each DNA band, PLS-DA was applied to analyze the ERIC-PCR banding patterns by using SIMCA-P 12.0 tool (Fig. 1.9).

![Flowchart of ERIC-PCR data analysis](image)

**Figure 1.9** Flowchart of ERIC-PCR data analysis.

1.7.3.2 Pyrosequencing data analysis

Recent advances in bioinformatics analysis have provided powerful tools for analyzing the massive pyrosequencing data. In this study, Quantitative Insights
Into Microbial Ecology (QIIME) and Linear discriminant analysis effect size (LEfSe) were used as the main methods.

1.7.3.2.1 QIIME software
QIIME (available at http://qiime.sourceforge.net/) is an open-source software and widely used to process data of high-throughput 16S rRNA sequencing. The differences of the microbial community can be visualized using the robust platform QIIME by network analysis, OTU heatmap, and histograms of within- or between-sample diversity (Fig. 1.10) (Caporaso et al., 2010).
Figure 1.10 Overview of the QIIME analysis pipeline.
(Caporaso et al., 2010)
1.7.3.2.2 LEfSe method

LEfSe method (available at http://huttenhower.sph.harvard.edu/lefse/) (Segata et al., 2011) is used to support the comparison of high-dimensional data. This method couples statistical significance with biological consistency and effect size estimation, and is designed for metagenomic biomarker discovery. LEfSe identifies differentially abundant features (e.g., taxa, genes, and functions) that are most likely to characterize consistent differences between compared classes (Fig. 1.11). Linear discriminant analysis (LDA) is used to estimate the effect size of these differentially abundant features. The following scheme illustrates how the algorithm works (Fig. 1.12)

![Figure 1.11 Overview of the LEfSe analysis pipeline.](image)

(Segata et al., 2011)
1.8 Why focus on Saponins?

An immunological equilibrium between the symbionts and pathobionts exists in the gut microbial ecosystem (Candela et al., 2011). Traditional Chinese Medicine (TCM) is believed to modulate homeostasis by balancing Yin and Yang. It is possible that TCM can regulate the balance of the gut microbial system, thus achieving homeostasis and exerting therapeutic effects on the host (Fig. 1.13). Recent research on the association of the gut microflora and Chinese herbal medicine has focused mainly on two aspects. On one hand, the gut microflora plays a crucial role in transforming pro-drugs into active drugs. These microbes can metabolize the nonabsorbable, nonbioavailable, and nonbioactive herbal components to new compounds with therapeutic activities (Akao et al., 2000, Hasegawa and Uchiyama, 1998, He et al., 2007, Kim et al., 1998, Leng-Peschlow,
1986, Park et al., 2005, Puupponen-Pimia et al., 2004, Shibata, 2000, Yagi et al., 1997). On the other hand, Chinese herbal medicine can promote the growth of beneficial microbes, inhibit harmful microorganisms, and modulate the imbalanced gut ecosystem (Jeon et al., 2006, Kato et al., 2007, Kong et al., 2006, Peng et al., 2009, Zhang et al., 2003).

![Diagram of microbial ecosystem balance]

**Figure 1.13** The balance of microbial ecosystem. Adapted and modified (Maslowski and Mackay, 2010, Round and Mazmanian, 2009)

After reviewing the literature, it seems only a small amount of research has been done on the association of the gut microflora and Chinese herbal medicine, and no systematic investigations have been carried out. With that in mind, it becomes even more critical to explore the impact of gut microflora on host metabolism and response to TCM treatment. This unexplored area of research is important for the development of evidence-based TCM, and the issue has not yet been clearly addressed.
Saponins are commonly found in a large number of natural sources and particularly abundant in many different plant species. Saponin components generally lower the surface tension of water, and cause soap-like foaming when shaken in aqueous solutions, hence the name “saponin”. In this project, we try to understand the association between the gut microflora and Chinese herbal medicine. It is possible that TCM with a longer residence time in the intestinal tract may have a great chance to affect the gut microbial ecosystem. Saponins have the following traits underlying poor membrane permeability, including relative high molecular mass (>500 Da), high hydrogen-bonding capacity (>12) and high molecular flexibility (>10) (Yu et al., 2012a). These characteristics result in poor intestinal absorption. The nonabsorbable saponins are too difficult to be absorbed through the intestinal wall, thus enabling the interaction with the gut microflora. Saponins can be hydrolysed by the intestinal flora. After absorption, the deglycosylated metabolites (aglycones) undergo phase I and/or II metabolism.

1.8.1 Chemical characteristics of saponins

Saponins are a group of amphiphilic glycosides containing one or more sugar chains bound to a nonpolar triterpene (Fig. 1.14A) or steroid aglycone (Fig. 1.14B) skeleton. Some other classes of compounds have the same biosynthetic precursor
as saponins, such as the steroid alkaloid saponins, also named glycosteroidealalkaloids (Harinantenaina et al., 2002). The glycosteroidealalkaloids contain steroids with a nitrogen atom integrated into a ring or in a substituent. Sometimes they are not considered as saponins because of the characteristic of the aglycone skeleton (Vincken et al., 2007). The number as well as the length of the saccharide chains attached to the aglycone core (sapogenin) can vary. Both linear and branched chain saccharides are present in saponins. The carbohydrate portion is composed of one or more sugar moieties, including D-glucose, D-galactose, L-rhamnose, L-arabinose, D-xylose, D-glucuronic acid, and D-galacturonic acid. The length of the saccharide chains can vary from 1 to 11, most containing with 2-5 sugar molecules. D-glucose and D-galactose are the most frequent sugars of the attached chains (Hostettmann and Marston, 1995).

![Figure 1.14 Common skeletons of saponins.](image)

(A) Triterpenoid saponins (B) Steroid saponins

1.8.2 Distribution of saponins

The aglycone (glycoside-free) portion of the saponins is called sapogenin. Based
on the type of sapogenins, the saponins can be divided into two major classes, triterpenoid saponins and steroid saponins. Triterpenoid saponins are mainly found in the plants of Umbelliferae, Araliaceae, Leguminosae, Campanulaceae, Polygalaceae, and Ranunculaceae, while steroid saponins are found in Liliaceae, Dioscoreaceae, Scrophulariaceae, and Amaryllidaceae. Famous saponin-containing TCM includes *Panax ginseng*, *Glycyrrhiza glabra*, *Radix Bupleuri*, *Platycodon grandiflorus*, *Gynostemma pentaphyllum*, *Radix Astragali*, *Radix Notoginseng*, *Dioscorea opposita*, and *Ophiopogon japonicus*.

1.8.3 Pharmacological activities of saponins

Saponins are believed to be responsible for the pharmacological activities of most Chinese herbal medicine. Saponins exhibit many different biological and pharmacological effects, including antitumor, immunomodulatory, antiinflammatory, cardiovascular, hepatoprotective, cholesterol-lowering, and antiviral activities (Table 1.5).
Table 1.1 Pharmacological Activities of Saponin-containing Chinese Herbal Medicines.  
(Francis et al., 2002, Lacaille-Dubois and Wagner, 1996, Sparg et al., 2004)

<table>
<thead>
<tr>
<th>Pharmacological Activities</th>
<th>Saponins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antitumor activity</td>
<td>Ginsenosides, gypenosides, dioscins, avicins, astragalosides, saikosaponins, soyasaponins, ruscogenin diglycoside, furcreastatin, chloromaloside A, Hederacolchiside A1, Avicins D, Dragonins A, B and C, kinmoonosides A, B and C, etc.</td>
</tr>
<tr>
<td>Immunomodulatory activity</td>
<td>Ginsenosides, gypenosides, saikosaponins, Quillaja saponins, etc.</td>
</tr>
<tr>
<td>Antiinflammatory activity</td>
<td>Esculentoside A, saikosaponins, ginsenosides, escins, glycyrrhizins, platycodins, etc.</td>
</tr>
<tr>
<td>Cardiovascular activity</td>
<td>Notoginsenosides, astragalosides, diosgenins, etc.</td>
</tr>
<tr>
<td>Hepatoprotective activity</td>
<td>Ginsenosides, gypenosides, araliosides, glycyrrhizins, saikosaponins, lonicera saponins, etc.</td>
</tr>
<tr>
<td>Cholesterol-lowering activity</td>
<td>Soybean saponins, gypenosides, etc.</td>
</tr>
<tr>
<td>Antiviral activity</td>
<td>Saikosaponins, glycyrrhizins, ginsenoside Rb, soybean saponins, Arganine C, maesasaponins, Escin saponins, etc.</td>
</tr>
</tbody>
</table>

1.9 Why focus on Triterpenoid saponins?

Triterpenoid compounds play a very important role in a plant’s defense. They protect the plants through either constitutive or induced defensive responses against insects, pathogens and other environmental damage or stress (Franceschi et al., 2005, Keeling and Bohlmann, 2006). The poor intestinal absorption characteristic of saponins gives a higher chance of interaction with the gut microflora. In addition, the defensive characteristic of triterpenoid compounds may protect the gut microflora ecosystem from dysbiosis.
1.9.1 Chemical characteristics of triterpenoid saponins

Triterpenes, terpenes consisting of six isoprene units (molecular formula C$_{30}$H$_{48}$), are a large group of compounds arranged in a four or five ring configuration. Through the cytosolic mevalonate pathway, triterpenes are assembled from C5 isoprene units to form a C30 compound (Yadav et al., 2010). Triterpenoid saponins are triterpene units with various sugar molecules attached by glycoside or ester bonds. Based on the chemical structure, pentacyclic triterpenoid saponins can be divided into three major types; oleanane, ursane, and lupine. Tetracyclic triterpenoid saponins can be classified into two main types; lanostanes and dammaranes.

1.9.2 Distribution of triterpenoid saponins

Triterpenoids are secondary metabolites of isopentenyl pyrophosphate oligomers in various species. More than 20,000 triterpenoids have been estimated to be found in nature (Liby et al., 2007). Triterpenoids are widely distributed and can be found in fungi, ferns, monocotyledonous and dicotyledonous plants, animals, and marine life. They are particularly abundant in the dicotyledonous plants (Dinda et al., 2010). There are two categories of triterpenes; free triterpenoids and triterpenoid saponins. The free triterpenoids are mainly found in Compositae, Leguminosae,
and Euphorbiaceae, while triterpenoid saponins are predominantly present in Leguminosae, Araliaceae, Campanulaceae, Polygalaceae, and Cucurbitaceae.

1.9.3 Pharmacological activities of triterpenoid saponins

Triterpenoid saponins have shown various pharmacological activities, including anticancer, antiinflammatory, immunomodulatory, antibacterial, and antiviral effects. The most attractive properties of triterpenoid saponins are the anticancer and antiinflammatory effects (Setzer and Setzer, 2003, Yadav et al., 2010). Among more than 100 families of saponin-containing plants, at least 150 kinds of natural saponins exhibit significant anticancer activity. Among these saponins, dammaranes, oleananes, lupanes, cycloartanes, and steroids show a strong inhibitory effect on multiple types of cancers (Man et al., 2010). Dammaranes, oleananes, and lupanes belong to the triterpenoid saponins.

In addition, many triterpenoid compounds derived from botanical sources are of vital importance in reducing inflammation. Yadav, et al. (2010) reviewed the role of triterpenoids in targeting inflammatory pathways for prevention and treatment of cancer. Many of the triterpenoids exhibit great potential for targeting NF-κB, leading to its downregulation. Mounting evidence has revealed that chronic
inflammation mediates many chronic diseases, including cancer (Lu et al., 2006, Shacter and Weitzman, 2002). In addition, disturbances of the intestinal microflora may be also involved in the development of chronic diseases because of the triangular relationship between the gut microbial system, the immune system and the inflammatory responses. The fascinating effects of triterpenoid saponins on cancer and inflammation give us insight in the study of the association between their possible impact on the gut microbial ecosystem and their anticancer activity on the host.

1.10 Why focus on *Gynostemma pentaphyllum* saponins?

*Gynostemma pentaphyllum* (Gp), also called Jiaogulan, is a popular herb in China and some Asian countries. It has a history of about 500 years, and is recorded in Jiuhuangbencao (*Herbs for Famine*) and Bencaogangmu (*Compendium of Materia Medica*). The wide range of medicinal properties Gp possesses has earned its reputation as the “immortality herb”. Gp saponins are the major compounds in *Gynostemma pentaphyllum*, and are responsible for its pharmacological activities.
1.10.1 Chemical characteristics of Gp saponins

The major active components of Gp are the dammarane-type triterpenoid saponins, also known as gypenosides or gynosaponins. A general chemical structure of the dammarane-type gypenosides is illustrated in Fig. 1.15. The main sugars on C-3 (β) and C-20, mostly pyranoses, are β-D-glucose, β-D-xylose, α-L-arabinose, and α-L-rhamnose. Characteristic functional groups on C-19 include hydroxyls, methyls, aldehydes, alcohols, and least commonly ketones. A hydroxyl group is also found at C-2 (α) and C-12 (β). New ocotillone-type saponins containing an epoxy ring on C-17 have also been isolated (Razmovski-Naumovski et al., 2005).

More than 100 different gypenosides exist in Gp, and some are very similar to the ginsenosides. Gypenosides are structurally identical to known ginsenosides, such as Rd, Rb1, Rb3, F2, Rc, Rg3, and malonylginsenosides Rb1 and Rd, and make up around 25% of the total gynosaponins in Gp. Gypenosides are the first examples of ginseng saponins found outside of the Araliaceae family (Cui et al., 1999).
Figure 1.15 Dammarane skeleton of Gp saponins with typical chains.
(Razmovski-Naumovski et al., 2005)

1.10.2 Pharmacological activity of Gp saponins

Gp saponins possess important pharmacological and biological functions and reported clinical effects. Studies have shown that gypenosides exhibit protective effects on the cardiovascular system (Circosta et al., 2005, Tanner et al., 1999). In addition, Gp saponins show extensive modulating effects on hyperlipidemia and hyperglycemia (Megalli et al., 2006), and exert therapeutic effects in the central nervous system (Qi et al., 2000, Zhang et al., 2002), immune functions, and cancer (Hou et al., 1991). Furthermore, they exhibit inhibitory effects on platelet aggregation (Wu et al., 1990), and show regulatory effects on arachidonic acid metabolism (Satoh et al., 1981). Moreover, Gp saponins possess hepatoprotective activity (Lin et al., 2000), antisenescent and antioxidant activities (Li and Lau, 1993, Ma and Yang, 1999), and protective effect against chemotherapy-induced
toxicity (Arichi et al., 1985). In brief, the adaptogenic properties of Gp saponins may be highly beneficial to general health, strengthening the immune and digestive system, improving brain function, maintaining healthy metabolic function, increasing stamina and preventing the progress of aging.

1.10.3 Anticancer effects of Gp saponins

In previous studies, we demonstrated that treatment with total saponins of *Gynostemma pentaphyllum* (GpS) displays anticancer effects. The anticancer activities of Gp saponins have also been documented in many studies. Gp suppressed the carcinomatous conversions of leukoplakia (Zhou et al., 2000), and inhibited the development of oral mucosal premalignancies in golden hamster cheek pouches (Zhou et al., 1996b). In addition, Gp exerted a preventive effect on esophageal cancer in rats (Wang et al., 1995). Furthermore, Gp saponins have been reported to prevent mutagenesis caused by cyclophosphamide in mice, and promote DNA repair (Qian, 2001, Wang and Bai, 1994). Chiu et al. (1999) found that Gp saponins may act as noncompetitive inhibitor of *N*-acetyltransferase. In addition, Gp saponins inhibited the growth of human lung tumor cells, and induced apoptosis in human hepatoma cell lines in a dose-dependent manner (Chen et al., 1999). Gp saponins exhibit significant anticancer activities, however,
no systematic study has been published on the impact of ingested saponins on the composition of the gut microflora.

1.11 Rationals and aims of the project

1.11.1 Rationals

The impact of gut microflora on the bioavailability and bioactivity of herbal saponins is profound. Metabolic activation of ginseng saponins, ginsenosides by the intestinal bacteria have been investigated extensively (Bae et al., 2004, Hasegawa et al., 1997, Hasegawa et al., 1996, Lee et al., 2009, Shin et al., 2003). For example, the gut microflora can convert ginsenoside Rb1 to 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol by β-D glucosidases. The new metabolite exhibits significant antitumor and antiallergic activities (Hasegawa and Uchiyama, 1998). It is well reported that metabolism by intestinal microflora may play an important role in pharmacological effects of herbal saponins (Bae et al., 2004, Bae et al., 2005, Lee et al., 2010, Lee et al., 2005, Yim et al., 2004). Several pharmacokinetic studies have been done on other important saponins, including licorice saponins, dioscorea saponins, astragalosides, and saikosaponins (Yu et al., 2012a). Never the less, studies have been confined to the metabolites of the drugs, no investigation of the impact of ingested herbal saponins on the gut
microflora and the metabolic profiles of the host. Thus, in this current project, we postulate that herbal saponins may change the composition of the gut microbes, which in turn alter the host-microbe interaction. As the fundamental first step toward the understanding of the role of gut microflora in the efficacy of Chinese herbal medicines in treating diseases, in particular for cancer, we propose to investigate the profiles of the host-microbe changes under the influence of herbal saponins by using genomic and metabolomics approaches. Our ultimate goal is to be able to lend what we learn and extend the study to human subjects, and be able to classify drug responses of individuals in the population and provide guidance for optimized or personalized drug management in treating diseases, particularly cancers.

1.11.2 Aims

The current study aimed to reveal the potential association between the anticancer effect of Gp saponins and the gut microflora. The objectives are in five main areas:

1. Define the changes in the gut microbiome associated with the anticancer effect of Gp saponins in subcutaneous xenograft tumor model;

2. Define the changes in the gut microbiome and gut mucosal environment
associated with the anticancer effect of Gp saponins in colorectal cancer model;

3. Compare the effect on Gp on gut microflora with three other herbal saponins from *Radix notoginseng*, *Radix ginseng* and red ginseng (steamed ginseng).

In this study, to achieve the above objectives, ERIC-PCR and pyrosequencing of the 16S rRNA gene were conducted as the main methodologies to characterize the fecal microbiome of the experimental mice. Bioinformatics analysis on microbial profiles was performed using tools such as PLS-DA, QIIME and LEfSe. The histological staining was used to investigate the gut mucosal environment.
CHAPTER 2

MATERIALS AND METHODS
2.1 Preparation of herbal saponins

The total saponins (GpS), extracted from the aerial parts of *Gynostemma pentaphyllum*, was purchased from the Hauduo Natural Products (Guangzhou, China). The GpS contains about 85 to 88% of triterpenoid saponins determined by silica gel thin-layer chromatography (TLC) in our laboratory. The ginsenoside Rb1 was used as a titration standard. Authentication and chemical profiling were monitored for qualitative control (Wu et al., 2011b). Each batch of GpS was first generated an ultra performance liquid chromatography (UPLC) profile, and then compared to the UPLC profile established with 10 pure saponins isolated from the GpS in our laboratory (Fig. 2.1). Total saponins of *Radix Notoginseng* (NGS), *Radix ginseng* (GS) and red ginseng (RGS) were purchased from Hongjiu Biotech Company Ltd., China. The standardization of these three herbal saponins will be described in Chapter 7.
1. Gynosaponin TN1
2. Gypenoside LXXVII
3. Gypenoside XLVEd
4. Gypenoside LVII
5. Ginsenoside Rd
6. Ginsenoside Rb3
7. Gypenoside XLVI
8. Gypenoside XLIII
9. Gypenoside LVI
10. Gypenoside XLII

Figure 2.1 Quality control of GpS. The GpS contains about 85 to 88% of triterpenoid saponins determined by silica gel thin-layer chromatography (TLC). The ginsenoside Rb1 was used as a titration standard. Each batch of GpS was first generated a UPLC profile, and then compared to the UPLC profile established with 10 pure saponins isolated from the GpS for quality control.

2.2 Animals and treatments

Animal welfare and experimental procedures were performed strictly in accordance with the care and use of laboratory animals. All procedures were approved by the University Ethics Review Committee for animal research.

2.2.1 Nude mice (BALB/c-nu/nu)

The athymic nude mice (BALB/c-nu/nu) were purchased from Chinese University
of Hong Kong and maintained in IVC cages, on a 12-h light/dark cycle and with free access to food and water. Xenograft was done by injecting $10^6$ R6/GFP-Ras transformed cells into the flank of each 7-8 weeks old mice. The Rat6/GFP-Ras cell line is a transformed clonal cell line established from a transformed focus derived from R6 rat fibroblast cultures transfected by a GFP-tagged ras oncogene vector in our laboratory (Hsiao et al., 2004). GpS was dissolved in 0.5% carboxymethyl cellulose (CMC) at 50 mg/ml. Single dose of GpS at 750 mg/kg or solvent control was given daily by gavages, started the second day after the implant of GFP-Ras cells.

### 2.2.2 Apc<sup>Min/+</sup> mice (C57BL/6J-Apc<sup>Min/+</sup>)

Heterozygous male Apc<sup>Min/+</sup> (C57BL/6J-Apc<sup>Min/+</sup>) and female wild type C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). An in-house breeding colony has been maintained by breeding C57BL/6J-Apc<sup>Min/+</sup> male to wild type female C57BL/6J mice. The Apc<sup>Min/+</sup> genotype of offspring is confirmed by polymerase chain reaction analysis. Mice were housed in a 12-h light/dark cycle facility with free access to food (LabDiet® 5053) and water. The body weight, collected fecal weight, diet and water consumption of mice were monitored every week. GpS was dissolved in 0.5% CMC at 50 mg/ml. Single dose
of GpS at 500 mg/kg or solvent control was given daily by gavages, started at 6 weeks of age for the mice and was carried out for 8 weeks.

2.2.3 C57BL/6 mice

The C57BL/6 mice (8 weeks old) were purchased from Chinese University of Hong Kong, on a 12-h light/dark cycle and with free access to food and water. Total saponins of Gynostemma pentaphyllum (GpS), Radix Notoginseng (NGS), Radix ginseng (GS) and red ginseng (RGS) were dissolved in milli-Q H₂O at 50 mg/ml respectively and then filtered (0.2 μm). Single dose of these four different saponins at 500mg/kg or milli-Q H₂O control was given to different treatment groups of mice daily by gavages, started the second day after the first collection of feces.

2.3 Fecal samples collection

All the fecal samples were collected from mice in the morning (8:00-10:00 a.m.). For nude mice, fecal samples were collected at day 0 (before xenograft), and 5 days and 10 days after GpS treatment. For the baseline study of microbiota in Apo<sup>Min/+</sup> mice, fecal samples were collected every week, started at 6 weeks of age for the mice, and continued until 30 weeks of age. For the treatment study in Apo<sup>Min/+</sup> mice, fecal samples were collected before treatment and every week after
treatment. For C57BL/6 mice, fecal samples were collected at day 0 (before treatment), and 5 days, 10 days and 15 days after treatment. All fecal samples were immediately stored at -20°C and kept for later DNA extraction.

### 2.4 Bacterial genomic DNA extraction from fecal samples

Total genomic DNA was isolated from fecal samples as described with slight modification (Kong et al., 2006, McCracken et al., 2001). 0.1g of fecal samples were vortexed in 4 ml sterile PBS (pH7.4) for 5 minutes, then centrifuged at 40×g for 8 minutes to collect the upper phase containing the bacteria. After repeating this procedure once, the supernatant was centrifuged at 2000×g for 8 minutes. The supernatant was discarded and the bacterial pellets were then washed twice with PBS. The bacterial pellets were used for DNA extraction as described. QIAamp DNA Stool Mini Kit (QIAGEN Inc., Valencia, CA) was used to extract the fecal genomic DNA from Apc<sup>Min/+</sup> mice and their WT littermates according to the manufacturer’s instruction and kept for later pyrosequencing. The DNA concentration was determined by NanoDrop 1000 spectrophotometry.
2.5 Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

2.5.1 ERIC-PCR system

ERIC sequences are noncoding, highly conserved intergenic repeated sequences that reside in the genome of various bacterial species in addition to enterobacteria as it was first discovered (Delihas, 2007, Wang et al., 2009, Wilson and Sharp, 2006). ERIC-PCR was used to profile the gut microbiome using fecal genomic DNA as the template and a pair of ERIC specific primer sequences: ERIC 1R (5’-ATGTAAGCTCCTGGGGATTCAC-3’) and ERIC 2 (5’-AAGTAAGTGACTGGGGTGAGCG-3’) (Versalovic et al., 1991). The PCR reaction was optimized and determined with orthogonal array design. A 25 μl reaction mixture containing 5 μl 5×PCR reaction buffer, 250 μM dNTP, 2 mM Mg2+, 0.4 μM primers, 1.5 unit Hotstart Taq polymerase, and 50 ng fecal genomic DNA. PCR was performed under the following conditions: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturing at 95°C for 50 seconds, annealing at 49°C for 30 seconds, 46°C for 30 seconds, and extension at 72°C for 3 minutes; and then a final extension at 72°C for 9 minutes (Peng et al., 2009). 10 μl of each PCR product was loaded into a 2% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide and run for 40 minutes at 100 V. A DNA ladder (0.1-10.0 kb) was used as DNA marker (NEB, N3200). Agarose gels were photographed using a Gel
2.5.2 Data analysis of ERIC-PCR fingerprints

Partial least squares discriminant analysis (PLS-DA) was performed to visualize the dynamic changes of microflora composition before and after treatment. Based on the distance and the intensity of each DNA bands, the banding patterns of ERIC-PCR products separated on the gel were digitized by Image Lab 3.0 system (Bio-Rad) and performed PLS-DA analysis using SIMCA-P 12.0 tool (Umetrics, Umea, Sweden). The correlation coefficient (Cc) was used to assess the correlation between two samples using the CORREL function in Microsoft Office Excel 2003. Sorenson’s pairwise similarity coefficient (Cs) was used to perform a paired comparison on the microflora profiles before and after treatment. Two identical profiles create a Cs value of 100%, whereas two completely different profiles (no common bands) result in a Cs value of 0%. Cs (\%) = (2 \times j)/(a+b) \times 100\%, where a is the number of total bands in the ERIC-PCR pattern for one sample, b is the number for the other, and j is the number of the common bands shared by the two samples (Peng et al., 2009, Scanlan et al., 2006). Shannon-Wiener diversity index, also called H’ index, refers to the community richness, was used to describe the microflora distribution of PCR bands in our study, although each ERIC-PCR band
does not have to stand for one individual bacterial species. $H' = \sum - (P_i \times \ln P_i)$, where $P_i$ refers to the relative abundance of each band in the lane of the fingerprint (lane %) (McCracken et al., 2001, Peng et al., 2009).

2.6 Identification of major bacterial genera using 16S rRNA gene sequences for PCR detection

Specific primer sequences for 16S rRNA were employed for PCR reaction to detect major bacteria genera. Primers specific to 16S rRNA of all eubacteria were used as an endogenous control to normalize gene intensity data between different samples. All primer sets used are listed in Table 1. Each PCR mixture (25 μl) contained 5 μl 5×PCR reaction buffer, 200 μM dNTP, 2.5 mM Mg²⁺, 0.4 μM primers, 1 unit Taq polymerase, and 50 ng fecal genomic DNA. The optimal annealing temperature for each primer set was determined by using a gradient PCR program (Applied Biosystems Veriti™ Thermal Cycler). The amplification conditions were one cycle at 95°C for 5 minutes followed by the indicated cycles (see Table 1) at 95°C for 30 seconds, the indicated annealing temperature (see Table 1) for 1 minute, 72°C for 1 minute, final extension at 72°C for 8 minutes and then cooling to 4°C. PCR products were examined for expected bands on 1% or 2% (according to the size of PCR product) agarose gel containing 0.5 μg/ml
ethidium bromide by running 10 μl of the PCR product. The size of the PCR fragments was determined using a 1 kb DNA ladder. The agarose gels were photographed using a Gel Doc™ XR+ System and digitized by Image Lab 3.0 system (Bio-Rad).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Product Size (bp)</th>
<th>Annealing Temp (°C)</th>
<th>Cycles</th>
<th>Reference</th>
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</thead>
<tbody>
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<td>62</td>
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<tr>
<td></td>
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<td></td>
<td></td>
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<tr>
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<tr>
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</table>

2.7 16S rRNA pyrosequencing of fecal DNA samples

2.7.1 16S rRNA pyrosequencing system

PCR was performed for each sample in a final reaction volume of 25 ul comprising 0.1-2 μl of extracted DNA, 300 nM of each primer (563F and 1064R of 16S rRNA gene), 2.5 μl of 10x Expand High Fidelity buffer (Roche), 200 μM PCR Grade Nucleotide Mix, and 2.6 units of Expand High Fidelity Enzyme mix (Roche) with
the reaction volume adjusted using milli-Q H$_2$O. The forward primer of each reaction had a unique 11-nt barcode to enable demultiplexing of reads post-sequencing. The PCR conditions for the fecal genomic DNA from nude mice were conducted with an initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 15s, 58°C for 20s, and 72°C for 1 min. Finally, an elongation reaction for 7 min at 72°C was performed followed by cooling at 4°C until collection. To obtain a better amplification efficiency of the fecal genomic DNA (100 ng template) from $Apc^{Min/+}$ mice and their WT littermates, the PCR conditions were modified by adding 0.1 μg/μl BSA in PCR reaction system and extending the denaturation and annealing time (95°C for 5 min followed by 35 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 1 min). Amplicon sizes were confirmed on 1.5% agarose gel and purified with PureLink Quick Gel Extraction Kit (Life Technologies). Amplicon libraries were quantified with Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) using FLUOstar OPTIMA F fluorometer (BMG Labtech GmbH, Offenburg, Germany) and visually assessed using the FlashGel System (Lonza Group Ltd., Basel, Switzerland). Emulsion-PCR and pyrosequencing using titanium chemistry on the GS Junior System (454 Life Sciences Corp., Branford, CT, USA) was carried out as detailed by the manufacturer.
2.7.2 Denoising and analysis of pyrosequencing data

Pyrosequencing data were processed and analyzed using the Quantitative Insights Into Microbial Ecology software (QIIME version 1.5.0) (Caporaso et al., 2010), available at http://qiime.sourceforge.net/. Denoising of raw sequences was performed to reduce the amount of erroneous operational taxonomic units (OTUs). Sequences were removed if they were <200 or >1000 nucleotides, with quality score below 25, contained primer mismatches or uncorrectable barcodes, or had a homopolymer run or ambiguous bases in excess of 6. The denoised sequences were assigned to OTUs with a 97% identity threshold, and the most abundant sequence from each OTU was selected as a representative sequence showing up in that OTU. Taxonomy was assigned to OTUs by using the Basic Local Alignment Search Tool (BLAST) for each representative sequence. For tree-based analyses, PyNAST was used to align these representative sequences of each OTU, and FastTree algorithm was used to build a phylogenetic tree (Price et al., 2009). The differences in overall community composition between compared samples were determined using the unweighted UniFrac metric. A matrix of pairwise distances between communities was constructed and used to generate Principal Coordinates Analysis (PCoA) plots. Linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011) was used to evaluate the key phylotypes responsible
for the observed differences between microbial communities. OTU network was generated by QIIME and visualized with Cytoscape. Shannon-Wiener diversity index (H') was used to evaluate the diversity of microbial communities. Venn diagram was used to figure out the unique and shared taxa between microbial communities.

### 2.8 Gut samples collection and polyp counting

At the end of the experiment, $Apc^{Min/+}$ mice were sacrificed and the intestinal tract was removed. Small intestine and colon were divided at cecal junction. 2 cm of small intestine and colon were cut from the adjacent cecum, rinsed with phosphate buffered saline (PBS) and then fixed in 10% formalin for later histological sections. The remaining part of colon and 8 cm of distal small intestine were used for mucosal scrapings. Other part of the intestinal tract was opened longitudinally and rinsed with PBS and then fixed in 10% formalin. After 24-36 hours, the intestine was rinsed with PBS and then stained with methyl blue. The number and sizes of polyps in the intestine were determined with a dissecting microscope.
2.9 Mucosal protein extraction

The protein of mucosal scraping samples from small intestine of colon were extracted by homogenization, and followed by sonication in Raybiotech cell lysis buffer with the following protease inhibitors: 0.3 μM aproptinin, 2 μM E64, 10 μM leupeptin, 1μM pepstatin, 5 μM PMSF, 5 μM Na₃VO₄, and 2 μM NaF. After a 30 min lysation on ice, the mucosal lysates were centrifuged at 20800×g for 15min at 4°C, and the supernatant containing the protein sample was collected. Protein concentration was determined by DC Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer’s instruction.

2.10 Cytokine array

Mucosal lysates from five selected mice per group were pooled together and applied to a mouse cytokine array (RayBiotech, Inc.). Each cytokine was represented in duplicate on the membrane. Two independent experiments were performed to evaluate the expression level of various cytokines. The intensity of signal was quantified by densitometry (ImageJ, NIH). The positive control was used to normalize the results from different membranes being compared.
2.11 Western blot

Protein lysates were mixed with 5× loading buffer (250 mM Tris-HCl, pH 6.8, 30 % glycerol, 10 % SDS, 0.02 % bromophenol blue, 5% β-mercaptoethanol) and boiled for 10 min. Equal amount (30 μg) of individual protein samples were resolved by 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane (Amersham Biosciences, USA). The membranes were blocked with 3 % bovine serum albumin in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1 % Tween-20). Immunodetection was performed using specific antibodies against beta-catenin (1:2000, sc-7963), c-Myc (1:500, sc-789), TLR4 (1:1000, sc-293072) and GAPDH (1:1000, sc-20357) purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and p-Stat3 (1:1000, cell signaling #9138), Stat3 (1:1000, cell signaling #9132), p-Src (1:1000, cell signaling #6943) and Src (1:1000, cell signaling #2123) purchased from Cell Signaling Technology (Beverly, MA). The membranes were then incubated with peroxidase-coupled secondary antibodies. ECL Detection Reagents (Amersham Biosciences, USA) were used to detect the signal of protein bands according to the manufacturer’s instruction.
2.12 Histology and Immunohistochemistry

5 μm thick paraffin sections were used for hematoxylin and eosin (H&E) staining, Alcian blue-staining, and immunohistochemical staining using standard procedures. Immunohistochemistry was performed using antibodies against Lysozyme (1:200, A0099, Dako Cytomation, Glostrup, Denmark), E-Cadherin (1:200, #3195S, Cell Signaling Technology, Beverly, MA), N-Cadherin (1:100, 610920, BD Biosciences, San Jose, CA), Stat3 (1:200, #9139, Cell Signaling Technology, Beverly, MA), beta-catenin (1:50, sc-7963, Santa Cruz Biotechnology, Santa Cruz, CA), IL-4 (1:100, PAB16160, Abnova Corporation, Taipei, Taiwan), iNOS (1:200, ab129372, Abcam Ltd., Cambridge, UK), Arginase I (1:100, 610708, BD Biosciences, San Jose, CA), and LSAB+System-HRP kit (K0679, DAKO, Carpinteria, CA). All procedures were carried out according to the manufacturer’s instructions. The slides were mounted and viewed on a Nikon Eclipse 80i microscope. Images were photographed with a SPOT RT3 CCD camera and SPOT Advanced software (Diagnostic Instruments, Sterling Heights, MI, USA).
2.13 Metabolomic analysis of fecal samples

Metabolomic studies on the fecal samples collected from mice treated with different herbal saponins were performed by using ultra high-performance liquid chromatography (UHPLC) coupled with quadrupole time-of-flight (Q-TOF) mass spectrometry. The Mass Profiler Professional (MPP) B.02.00 software was used to analyze the metabolomics data.

2.13.1 Sample preparation

The metabolites of fecal samples were extracted with methanol. The volume of 100% methanol in the extraction was 250 μl per 0.1 g of feces. Fecal samples were homogenized in methanol, followed by vortexing and incubating for 15 min at room temperature, and then centrifuged at maximum speed (~20000g) for 15 min. The supernatant was transferred and filtered (0.22 um Hydrophilic PVDF, Millipore). The metabolite extracts were frozen at -20 °C until analysis.

2.13.2 UHPLC analysis

The chromatography was performed on Agilent 1290 Infinity UHPLC equipped with G4220A binary pump, G4226A automatic sample injector and G4212A Diode Array Detector (Agilent Technologies, Santa Clara, CA, USA). The
separation was conducted with an ACQUITY UPLC BEH C8 column, 2.1 x 100 mm i.d., 1.7 μm (Waters Corp., Milford, MA, USA). A mobile phase consisted of 0.1% acetic acid and 5 mM ammonium acetate in milli-Q water (A) and acetonitrile (B) was used for separation. The system was programmed with the following gradients: 0-0.25 min, 10% B; 0.25-5 min, 10-75% B; 5-22 min, 75-99% B; 22-27 min, 99% B. The flow rate was kept constant at 0.4 ml/min at 45 °C for a total run time of 30 min. The volume of sample injection was 8 μl.

2.13.3 Q-TOF mass spectrometry

An Agilent 6540 Ultra High Definition Accurate-Mass Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) was coupled to the UHPLC system described above via an electrospray ionization (ESI) ion source with Jet-Stream technology for the comprehensive LC/MS analysis of fecal samples. The ESI-MS spectra were acquired in the positive and negative ion modes. Ultra-high-purity nitrogen was used as collision gas in product ion scanning experiments. The capillary voltage was set at 4.5 kV. The drying gas and sheath gas were delivered at flow rate of 8 l/min, and temperatures were 300 °C and 350 °C, respectively. The pressure of nebulizer gas was 35 psi. The
fragmentor voltage is 135 V. The mass analyzer was scanning from 80 to 1700 (m/z). Data were collected at a spectral acquisition rate of 2 Hz.

### 2.13.4 Metabolomics data analysis

MassHunter Qualitative Analysis was used to create the Molecular feature extraction (MFE) method for the metabolomics data. MassHunter DA Reprocessor was then used to automate MFE on all of the samples in a single batch processing. The molecular features for each sample data file were exported as a CEF file and imported into Mass Profiler Professional (MPP) software. Principal Component Analysis (PCA) was used to find differences between samples and weigh relative contributions of compounds to the separation of the groups by MPP. A series of differential metabolites were obtained, and heatmap was generated based on statistical analysis (ANOVA, p<0.05) by MPP.

### 2.14 Statistical analysis

The data are presented as mean ± SEM, and statistical comparisons were performed using one-way ANOVA followed by Student’s t-test at $P < 0.001$ (***) , $P < 0.01$ (**) or $P < 0.05$ (*).
CHAPTER 3

OPTIMIZATION OF

EXPERIMENTAL CONDITIONS
3.1 Introduction

The microflora is linked to human beings throughout the whole life. Many factors have been shown to affect the gut microflora, such as the mode of fetus delivery, geographic origin, host genotype, diet, antibiotics, probiotics, age or even stress (Backhed et al., 2012). The gut microflora plays a crucial role in health and disease (Neish, 2009, Nicholson et al., 2005). In this study, we planned to investigate the impact of herbal saponins on the gut microbiota. As the first step, we tried to optimize the experimental conditions and establish a consistent platform to carry out our study.

Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences are highly conserved 127-bp imperfect palindromes that are dispersed throughout various bacterial genomes (Wilson and Sharp, 2006). ERIC-PCR has been widely used to provide a snapshot of gut microbial communities (Cheng et al., 2011, Li et al., 2007, Pang et al., 2007, Yang et al., 2011). It is a very effective method to generate highly reproducible profiles for microbial ecosystem (Wei et al., 2004). In this study, fecal samples were collected and applied to display the dynamic changes of microbial communities by ERIC-PCR. In this section, we tried to
obtain the optimum experimental conditions for fecal sample collection and ERIC-PCR system.

3.2 Results

3.2.1 Optimization of experimental conditions for fecal sample collection

In order to find the appropriate conditions for fecal sample collection, the following two factors were taken into consideration: biological rhythms (feeding clock and fecal excretion clock) and the amount of collected fecal samples. C57BL/6 mice were used in the following experiments.

3.2.1.1 Food intake in mice

The comprehensive laboratory animal monitoring system (CLAMS; Columbus Instruments, Ohio, USA) was used to measure the food intake in mice on a 12-h light/dark cycle. Mice were allowed to acclimatize to the kept environment 3 days prior to the start of the data collection. The CLAMS system enables real-time continuous monitoring of food intake. Data was collected in a 30 minute intervals. Recoding started at 20:00, last for 24 hours. Data showed that mice were more likely to eat more at night (from 20:00 to 8:00) than during the day (from 8:00 to 20:00) (Fig. 3.1).
Figure 3.1 Measurement of mice food intake. I. Food intake records of mice for a whole day. Food intake was monitored automatically by CLAMS (n=4). Each mouse was placed in an individual metabolic cage. Data collection started at 20:00, recordings made at every 30 minute intervals and continued for 24 hours. II. Food intake during night and day time in mice. Data are presented as mean ± SEM (** P < 0.01).

3.2.1.2 Timing for fecal sample collection

To determine whether the best timing of fecal collection for microflora analysis, we collected the fecal samples every three days in the morning hours (8:00-10:00) and in the afternoon hours (16:00-18:00) and repeated 3 times. The fecal samples were weighed and used for microbial DNA extraction. The fecal DNA was analyzed using ERIC-PCR to compare the microflora profiles of fecal samples. Collection time periods of two and three hours were also tested. It appeared that the amount of feces collected in the morning was much more than in the afternoon (P<0.001) (Fig. 3.2-I). In order to determine the most suitable time in the morning, we further investigated the different time periods. When comparing collection times, two hours (8:00-10:00) obtained more fecal samples than three hours.
(10:00-13:00) (P<0.05) (Fig. 3.3). There were no significant differences in the ERIC-PCR patterns between the fecal samples collected in the morning and in the afternoon (Fig. 3.2-II).

**Figure 3.2** Comparison of fecal samples collected in the morning and in the afternoon hours. I. Comparison of fecal amounts collected from each mouse (n=5, collected 3 times). Data are presented as mean ± SEM (*** P < 0.001). II. Comparison of the microflora profiling of fecal samples. ERIC-PCR was used to assess the differences in the microflora profiling. Mice are coded 1 to 5.

**Figure 3.3** Comparison of fecal samples collected from different time periods. The collection interval of fecal samples was once every three days. Collection was carried out three times (n=5). Two hours of collection was from 8:00 to 10:00; and three days later, three hours of collection was from 10:00 to 13:00. The collected fecal samples were weighed and data are presented as mean ± SEM (* P < 0.05).
3.2.1.3 Comparison of fresh and frozen fecal samples

To test whether the bacterial pellets isolated from the fecal samples could be kept frozen (-20°C), the fresh and frozen samples were subjected to ERIC-PCR. The results indicated there were no significant differences in the ERIC-PCR patterns between the fresh and frozen samples (Fig. 3.4).

Figure 3.4 Comparison of the ERIC-PCR profiles of the fresh and frozen fecal samples. Fecal samples were obtained from three individual mice.
3.2.2 Optimization of experimental conditions for ERIC-PCR

To obtain the best PCR results, we employed orthogonal design to optimize the concentrations of four factors; dNTP, Mg$^{2+}$, primers and HotStart Taq polymerase.

3.2.2.1 Orthogonal design

Orthogonal design is used to test the comparative effectiveness of multifactor interventions and seek the optimal combination. The four common factors affecting ERIC-PCR were considered at 4 levels (Table 3.1). The orthogonal array in Table 3.2 was generated by $4^4$ factorial design in SPSS 11.5. The amplification conditions were as follows: 50 ng genomic DNA, 5 μl 5×PCR reaction buffer and other corresponding components in Table 3.2. PCR H$_2$O was added up to 25 μl. All the experiments were performed under the following PCR condition: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturing at 95°C for 50 seconds, annealing at 49°C for 30 seconds, 46°C for 30 seconds, and extension at 72°C for 3 minutes; and then a final extension at 72°C for 9 minutes (Peng et al., 2009).
#### Table 3.1 Different Levels and Factors Used in $4^4$ Factorial Design.

<table>
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<tr>
<th>Factors</th>
<th>Levels</th>
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<td>dNTP ($\mu$M)</td>
<td>100</td>
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<tr>
<td>Mg$^{2+}$ (mM)</td>
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<tr>
<td>Primers ($\mu$M)</td>
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<td>HotStart Taq polymerase (U)</td>
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#### Table 3.2 Orthogonal Array for the Optimization of ERIC-PCR.

<table>
<thead>
<tr>
<th>Components</th>
<th>dNTP ($\mu$M)</th>
<th>Mg$^{2+}$ (mM)</th>
<th>primers ($\mu$M)</th>
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#### 3.2.2.2 The Main Effect analysis of parameters for ERIC-PCR reaction

To investigate the effect of the four factors in the PCR system on the DNA fingerprint patterns, we defined scores according to the amplification of 3 samples. Based on the number of amplified bands (assuming the more the better), intensity and clarity (with smear or not), scores were determined for the best (16 points)
and the worst (1 point) combinations. The main effect of each factor in the ERIC-PCR reaction system was statistically analyzed by SPSS 11.5. It suggested that variations of these four factors affected the fingerprints significantly (Table 3.3). The order of the effects was primers (F=54.143, P=0.000), HotStart Taq polymerase (F=10.590, P=0.000), Mg$^{2+}$ (F=6.099, P=0.002) and dNTP (F=5.324, P=0.004).

**Table 3.3** Tests of Between-Subjects Effects.

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A = dNTP; B = Mg$^{2+}$; C = primers and D = HotStart Taq polymerase

**3.2.2.3 Optimized experimental conditions for ERIC-PCR reaction**

ERIC-PCR amplification generated multiple DNA fragments, and components 9, 13 and 15 were selected as the top 3 by calculating the average scores of each combination (Fig. 3.5-II). For each factor, the scores of individual levels were also calculated (Fig. 3.5-III), and the new component was combined with the highest ones (250 μM dNTP, 1.5 mM Mg$^{2+}$, 0.6 μM primers and 2unit Hotstart Taq polymerase). In order to find the best performance, the top 3 and the new
component were further compared by testing more samples. Component 15 proved to be the best (Fig. 3.6-II), and the concentrations of the four factors in component 15 also showed the highest scores (Fig. 3.6-III). We also optimized the PCR cycle numbers and genomic DNA amount, as well as the percentage of DNA agarose gel (data not shown). We found that 35 cycles, 50 ng genomic DNA template and 2% agarose gel were appropriate for DNA profiling analysis.

**Figure 3.5** Optimization of mouse fecal microflora ERIC-PCR by orthogonal array design. I. Representative ERIC-PCR fingerprints based on the orthogonal array design. Each component (for parameters see Table 3.2) obtained a score according to the ranking principles. II. The average scores of each component in orthogonal array design (n=3). Arrows indicate the top 3 scoring systems. III. Effects of different concentrations of (a) dNTP, (b) Mg$^{2+}$, (c) primers and (d) Hotstart Taq polymerase on ERIC-PCR amplification, based on the orthogonal array design and scoring systems. Arrows indicate the concentration which obtained the highest score.
Figure 3.6 Confirmation of ERIC-PCR optimization results in orthogonal array design. I. Representative ERIC-PCR fingerprints of the top 3 and the new component. The new component was a combination of the four individual concentrations with the highest scores. Each component (for parameters see Table 3.2) obtained a new score according to the ranking principles. II. The average scores of each component in further optimization of the ERIC-PCR system (n=3). Arrow indicates the component that obtained the highest score. III. Effects of different concentrations of (a) dNTP, (b) Mg²⁺, (c) primers and (d) Hotstart Taq polymerase on ERIC-PCR amplification, based on the further optimization and scoring systems. Arrows indicate the concentration which obtained the highest score.
3.3 Discussion

A reproducible and comprehensive analysis is very important for establishing the experimental platform of microbial profile study. Through the assessments of various factors, we concluded the most optimal conditions are as following: 1). Two hours of fecal collection between 8:00-10:00 a.m. obtained sufficient fecal amount and was matched to the biological clock; 2). Frozen samples can be used to carry out our study; 3). 250 μM dNTP, 2 mM Mg$^{2+}$, 0.4 μM primers, 1.5 unit Hotstart Taq polymerase, 50 ng genomic DNA template, 35 cycles and 2% agarose gel were suitable for the ERIC-PCR profiling.
CHAPTER 4

TUMOR GRAFTING INDUCES CHANGES OF GUT MICROBIOTA IN ATHYMIC NUDE MICE IN THE PRESENCE AND ABSENCE OF GP SAPONINS
4.1 Introduction

Accumulated evidence shows that the efficacy of drug treatments varies greatly from individual to individual, possibly influenced by the genetic polymorphism of the individuals. It is also well recognized that the environmental factors, such as nutritional status, age, health status, and gut bacterial distribution, influence the drug metabolism of an individual. Trillions of microbes live in the gut of a higher organism in symbiotic relationship. Normal gut microbes make significant contributions to the overall health of their host including protection against potentially harmful microorganisms and stimulation of the immune system. Recent findings have revealed that gut microbes play an even greater role in modulating human metabolic phenotypes and individuals’ drug responses. On one hand, the host’s dietary and drug uptake can alter the gut microbial composition (Ley et al., 2005, Ley et al., 2006, Nicholson et al., 2005). Conversely, microbes can influence the bioavailability and bioactivity of ingested products, including functional foods and herbal medicines (Akao et al., 2000, Wang et al., 2005) as well as the host’s metabolic phenotypes and syndrome (Vijay-Kumar et al., 2010). Strikingly, accumulating evidence shows that the gut microflora can influence diseases incurred in organs or tissues distant from the gut, such as obesity (Ley et al., 2005, Ley et al., 2006), diabetes (Diamant et al., 2011, Musso et al., 2011),
autism (Critchfield et al., 2011, Wang and Kasper, 2013), rheumatoid arthritis (Scher and Abramson, 2011), allergy (Hormannsperger et al., 2012, Russell and Finlay, 2012), and chronic liver disease (Almeida et al., 2006).

Recent studies have presented strong evidence showing that intestinal microbiota modulates the efficacy of chemotherapy. For example, it was found that beta-glucuronidase in intestinal microflora contributes to the delayed diarrhea caused by the antitumor camptothecin derivative irinotecan hydrochloride (Stringer et al., 2008, Takasuna et al., 1996). Later study showed that mice tolerated higher dosage of CPT-11 when the drug was administrated together with a beta-glucuronidase inhibitor (Wallace et al., 2010). Commensal intestinal microbiota might also play a prominent role in the development of the severity of chemotherapy-induced mucositis in cancer patients (Stringer, 2013, van Vliet et al., 2010). Studying in animal demonstrated that intestinal microbiota modulates the antitumor efficacy of the cyclophosphamide, in part, through the induction of “pathogenic” Th17 cells (Viaud et al., 2013). Along this line, recent report showed that disruption of commensal microbiota impairs the response of subcutaneous tumors to CpG-oligonucleotide immunotherapy and platinum chemotherapy (Iida et al., 2013). In addition to the impact on chemotherapy, the gut microflora has
been suggested to mediate the effect of diet or dietary compounds in cancer prevention and cancer risk. For instance, resveratrol-supplemented diet significantly reduced the colonic tumors in rats through the reduction of bacterial glucuronidase (Sengottuvelan et al., 2006). Combination of beneficial microbes and dietary compounds has suggested a preventive role in colonic polyps in both animal and human studies (Ishikawa et al., 2005, Pierre et al., 1997, Rao et al., 1998). Overall, the dynamic interplay between the gut microbiota and the ingested drugs or dietary compounds impacts on cancer risk and treatment.

Athymic nude mice have been a major animal model for cancer research. Their impaired immune system facilitates tumor grafting avoiding the risk of graft rejection by the animal’s immune system. Early study on the gastrointestinal microecology showed that the loss of T-cell function does not drastically alter the cultivated gut microflora from the Balb/c athymic (nu/nu) nude mice compared to the heterozygous (nu/+). However, the impact of the grafted tumors on the gut microbiota in nude mice and how gut microbiome would influence the host’s response to the grafted tumors and drug treatments are unexplored.
Saponins are found in many herbal and edible plants. *Gynostemma pentaphyllum* (Gp) has been consumed as an herbal tea, and is also well documented as a folk medicine dating from the sixteenth century in the Chinese *Compendium of Materia Medica*, where it was recommended for the treatment of various diseases, including cancer. The main active components in Gp are triterpenoid saponins (Razmovski-Naumovski *et al.*, 2005). In this study, we first demonstrated that GpS exert anticancer effects on tumor-bearing nude mice. We compared the gut microbial composition of nonxenograft and tumor-bearing xenograft nude mice and investigated how GpS treatment could shape the composition of gut microflora in healthy and tumor-bearing nude mice.

4.2 Results

4.2.1 A significant alteration in the gut microbiota of the xenograft nude mice

To investigate whether tumor implanted at the flank of the mice would affect microbiota in the gut, the fecal microflora profiles of xenograft and nonxenograft mice were analyzed using ERIC-PCR method. The banding patterns of ERIC-PCR (Fig. 4.1-I) were then digitized using the Image Lab 3.0 system (Bio-Rad) and analyzed using PLS-DA analysis (Fig. 4.1-II). Although
inter-animal variation in microbial profile appeared at Day 0, clear difference
between the samples from Day 10 normal and tumor-bearing mice obtained in the
PLS-DA plot (Fig. 4.1-III) and in the correlation coefficient plot (Fig. 4.1-IV).
These findings suggest that xenograft tumor in mice caused alteration of
microflora composition compared to the nonxenograft mice.
Figure 4.1 Comparison of fecal microflora between nonxenograft and xenograft nude mice. **I.** Representative ERIC-PCR DNA fingerprints of the fecal microflora of individual nonxenograft and xenograft nude mice. Fecal samples were collected before xenograft (Day 0), and 5 & 10 d upon saline or tumor cells injection. **II.** Digitization of ERIC-PCR DNA fingerprints. Gel images were digitized by Image Lab 3.0 system (Bio-Rad). The peak area corresponded to the intensity of ERIC-PCR band shown in the gel image. **III.** PLS-DA plot of ERIC-PCR data from fecal microflora of nonxenograft and xenograft nude mice at Day 10. Black square: nonxenograft nude mice; Red dot: xenograft nude mice. **IV.** Correlation coefficients of fecal microflora of nonxenograft and xenograft nude mice. All data were normalized by self-control at day 0 and presented as mean ± SEM (* P < 0.05, ** P < 0.01 versus nonxenograft group); n=6.
4.2.2 GpS inhibited tumor growth in nude mice

To test the effect of GpS on tumor growth, a single daily dose of GpS (750mg/kg) or the solvent control (0.5% CMC) by gavages was initiated on the second day after the implant of the transformed R6/GFP-ras cells. The tumor volume and tumor weight of the GpS-treated group were 60% and 50% lower than the untreated group (Fig. 4.2-I & II). No weight loss was observed in the experimental animals (Fig. 4.2-III).

Figure 4.2 Effect of GpS on tumor growth in nude mice. I. Tumor volume. II. Tumor weight. III. Body weight. All data are presented as mean ± SEM (* P < 0.05, ** P < 0.01 versus control); n=6.
4.2.3 GpS treatment induced unique alteration of microbiota in xenograft, but not in the nonxenograft mice

Fecal samples were collected from four experimental groups: nonxenograft and xenograft animals with and without GpS treatment at Days 0, 5, and 10 (Fig. 4.3-Ia & IIa). The PLS-DA plots, based on the ERIC-PCR banding patterns of the fecal DNA samples, showed no clear difference between samples collected in the presence or absence of GpS in nonxenograft mice (Fig. 4.3-Ib & c). In the xenograft mice, by contrast, clustering of samples collected from different time points were observed (Fig. 4.3-IIb & c). Intriguingly, in the xenograft group treated with GpS, the cluster of Day 10 samples seemed to be merged with the cluster of Day 0 samples (Fig. 4.3-IIc). This alteration was not observed in the untreated xenograft mice.
Figure 4.3 ERIC-PCR analysis of the effect of GpS on fecal microbial composition of nonxenograft mice (I) and xenograft mice (II). (a) Treatment scheme, (b) PLS-DA plots of nontreatment group, and (c) PLS-DA plots of GpS treatment group.
4.2.4 16S pyrosequencing further revealed marked differences between the nonxenograft and xenograft nude mice in microbial communities

We performed 16S rRNA pyrosequencing on the DNA obtained from fecal samples on Day 10 time point. Three fecal samples from each of the four experimental groups (as indicated in Fig. 4.3) were analyzed. A total of 147,128 reads passing quality control were produced in this study, with an average of 12,261 sequences per sample. A total of 399 distinct operational taxonomic units (OTUs) were determined after denoising by using QIIME (Caporaso et al., 2010). OTU is defined as a terminal node in phylogenetic analysis. OTUs in QIIME are clusters of sequences, which are used to represent the taxonomic relatedness. All of the sequences from all of the samples will be clustered into OTUs based on their sequence similarity.

We performed OTU network analysis on the denoised OTUs to generate an overall picture of the clustering of the test samples. Tumor progression is the likely cause of the differences in fecal microflora composition between nonxenograft and xenograft mice. Similar results were observed in the OTU network analysis (Fig. 4.4-Ia). Nonxenograft and xenograft mice had 112 and 91 unique OTUs respectively and shared 110 OTUs (Fig. 4.4-Ib). Xenograft mice
exhibited reduced microbial diversity compared with nonxenograft mice based on the calculated Shannon-Wiener diversity index (Fig. 4.4-Ic).

The 16S pyrosequencing data were analyzed using LEfSe (Segata et al., 2011) to identify the key phylotypes responsible for the differences in fecal microflora composition between the nonxenograft and xenograft mice. As shown in Fig. 4.4-IIa, the taxonomic distribution of fecal microbiota between the two groups varied significantly at all taxonomic levels. At the phylum level, the most differentially abundant bacterial taxon in the feces of nonxenograft mice was TM7, whereas Bacteroidetes was overrepresented in xenograft mice. Compared with the nonxenograft mice, xenograft mice had relatively high levels of Deltaproteobacteria but low levels of Gammaproteobacteria, both of which are Gram-negative proteobacteria. Mollicutes, in the phylum Tenericutes, were also underrepresented in xenograft mice. The histogram of the linear discriminant analysis (LDA) scores (Fig. 4.4-IIb) and the relative abundance scores (Fig. 4.4-IIc) further illustrated a clear difference between the nonxenograft and xenograft mice in the composition of biologically clades. Several clades in the phylum Firmicutes, such as Catabacteriaceae, Peptococcaceae, and Coprococcus, were particularly abundant in nonxenograft but not in xenograft mice (Fig.
4.4-IIc).
**Figure 4.4** Pyrosequencing analysis of fecal microbiota of nonxenograft and xenograft nude mice. **I.** Comparisons of overall OTU networks (a), the number of shared and unique OTUs (b), and the diversity (c) of fecal microbiota between the nonxenograft (red) and xenograft (green) mice. The analysis is based on the percentage of classified OTUs of each sample. Shannon-Wiener diversity index (H') was used to calculate the diversity of microbial communities. H' = ∑ - (Pi * ln Pi). **II.** Comparison of fecal microbial structures between nonxenograft and xenograft mice. (a) Taxonomic representations of fecal microbiome of nonxenograft and xenograft nude mice. The differentially abundant taxa are presented with designated colors using LEfSe method. The taxa from nonxenograft and xenograft mice are colored in red and green, respectively. The taxa with nonsignificant changes between the nonxenograft and xenograft mice are colored in yellow. Each small circle’s diameter represents the taxon abundance. (b) Histogram of the LDA scores of fecal 16S rRNA sequences of nonxenograft (red color) and xenograft (green color) mice. LDA scores characterized the magnitude of differential abundance in the microbial taxa between compared samples. (c) The relative abundance (%) of differentially abundant families and genera. Nonxenograft nude mice, n=3; Xenograft nude mice, n=3. Data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, nonxenograft versus xenograft group).
4.2.5 GpS treatment altered the taxonomic composition of fecal microbiome

The 16S pyrosequencing data showed that GpS treatment influenced the relative abundance of dominant microflora taxa at both phylum (Fig. 4.5-I) and genus levels (Fig. 4.6). Upon GpS treatment, Tenericutes, Proteobacteria, Bacteroidetes and Actinobacteria were elevated, while TM7, Firmicutes and Deferribacteres were decreased. The range of changes was more prominent in the tumor-bearing nude mice than the mice free of tumor xenograft (Fig. 4.5-I). To identify which taxa were unique to different treatment groups, we compared the bacterial families by using the Venn diagram. As shown in Fig. 4.5-II, there were seven and two unique bacterial families found in the nonxenograft and xenograft mice respectively, whereas 19 bacterial families appeared in both groups. Comparing nonxenograft mice with and without GpS treatment, 22 bacterial families were shared, whereas the control and GpS-treated groups each had 4 unique bacterial families. By contrast, in xenograft mice, 20 bacterial families were found in both the control and GpS-treated mice. Only one unique bacterial family was found in the control whereas 6 unique families were found in the GpS-treated xenograft mice. These unique bacterial families with a mean relative abundance > 0.01% are listed in Fig. 4.5-II.
Figure 4.5 Effect of GpS on taxonomic composition of fecal microbiome. I. Relative abundance of the main phyla of gut microbial communities. 16S pyrosequencing was performed on fecal DNA samples from nonxenograft and xenograft nude mice with or without GpS treatment (10 d). Triplicate animals were employed for each treatment group. Data are presented as mean ± SEM. II. Unique fecal bacterial families identified from different treatment groups. Venn diagram showing the number of unique and shared bacterial families of nonxenograft and xenograft nude mice, with or without GpS treatment. Yellow: nonxenograft nude mice; Green: xenograft nude mice; Blue: nonxenograft nude mice with 10 d GpS treatment; Red: Xenograft nude mice with 10 d GpS treatment. The table indicates the unique families to each treatment group.
Figure 4.6 Taxonomic composition of microbial communities at the genus level in nude mice with or without GpS treatment. A bar chart of relative abundance of bacterial genera in the individual mice of different treatment groups.
4.2.6 Identification of key phylotypes in fecal microbiome associated with different treatment groups

The phylogenic compositions of fecal samples from each experimental group were analyzed using LEfSe tool. The taxonomic data are displayed as cladograms in Fig. 4.7. In the nonxenograft mice, Clostridia and Mollicutes were found to be particularly differentially abundant classes (Fig. 4.7-Ia). Within Clostridia, families such as Catabacteriaceae, Peptococcaceae, and Ruminococcaceae, and genera such as Clostridium, Coprococcus, and Oscillospira, were all found to be more abundant in the untreated nonxenograft mice than in the GpS-treated mice. Within the class Mollicutes in the phylum of Tenericutes, lineages in including Anaeroplasmatales, Anaeroplasmataceae, and Anaeroplasma were the prevalent clades in the nonxenograft group without GpS treatment. Anaerotruncus, a genus in Clostridia, was the only differentially abundant taxon detected in the treated mice (Fig. 4.7-I).

In the xenograft mice, three dominant phyla showed differential responses to GpS treatment: Firmicutes, Proteobacteria, and Tenericutes. Within these phyla, Clostridia, Betaproteobacteria, and Erysipelotrichi were the predominant classes, respectively. The control and GpS-treated mice exhibited different numbers of
Clostridia (95.84% vs. 49.08%), Betaproteobacteria (0.81% vs. 8.01%) and Erysipelotrichi (1.65% vs. 39.58%) (Fig. 4.7-IIa). Differences in bacterial community structure between the control and GpS-treated mice were also observed. In the GpS-treated xenograft mice, two lineages were notably high in abundance compared with the controls: the Proteobacteria-Betaproteobacteria-Burkholderiales-Alcaligenaceae lineage and the Tenericutes-Erysipelotrichi-Erysipelotrichales-Erysipelotrichaceae-Clostridium lineage (Fig. 4.7-IIa). At the family level of these two particular lineages, GpS-treated xenograft mice showed higher levels of Alcaligenaceae (8.01% vs. 0.81%) and Erysipelotrichaceae (39.58% vs. 1.65%) compared with the control. At the genus level, Clostridium presented the greatest difference in abundance between the two groups (Fig. 4.7-IIc). Clostridium constituted less than 0.5% of total bacteria in controls, but was more prevalent in GpS-treated mice (mean 38.48%).
Figure 4.7 Effects of GpS on fecal microbiome in nonxenograft (I) and xenograft (II) nude mice. (a) Taxonomic representations of fecal microbiome. (b) Histogram of the LDA scores for differentially abundant clades. (c) The relative abundance of differentially abundant families and genera. Red: nontreatment control; Green: GpS treatment group (10 d). Data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, versus control group).
4.2.7 Two bacterial species were markedly enhanced upon GpS treatment

The consensus lineage map of OTUs generated by QIIME enabled bacterial identification at the genus level, and in some cases, at the species level, where OTUs related to *Clostridium cocleatum* and *Bacteroides acidifaciens* were identified. In both xenograft and nonxenograft mice, these species increased in number following GpS treatment. GpS-treated nonxenograft mice showed 28 times higher levels of *C. cocleatum*, compared to the control mice. In the Gps-treated xenograft mice, *C. cocleatum* elevated 80 times more than those found in the control group. The relative abundance of *B. acidifaciens* increased five-fold compared with the untreated controls (Fig. 4.8). The substantial increase of *C. cocleatum* appeared to be a driver of fecal bacterial community structures in GpS-treated tumor-bearing mice.
Figure 4.8 Differentially abundant bacterial species responding to GpS treatment. 

I. OTU heatmap based on the 16S rRNA reads identified bacterial species. The figures displayed in the OTU heatmap are the raw OTU counts of individual mouse sample. II. The relative abundance of (a) *Clostridium cocleatum* and (b) *Bacteroides acidifaciens* in nonxenograft and xenograft nude mice with or without GpS treatment. Data are presented as mean ± SEM (n =3 per group).
4.3 Discussion

Athymic nude mice have been the most used animal model for tumorigenic study. However, the impact of the grafted tumors on the gut microbiota in nude mice and how gut microflora would influence the host’s response to the grafted tumors and drug treatments are unexplored. This study presents an investigation on how gut microflora in nude mice is influenced by the xenografted tumor and by the medicinal saponins. ERIC-PCR and 16S pyrosequencing data demonstrated dynamic responses of intestinal microflora communities in nude mice to tumor grafting in the presence or absence of GpS-treatment. Early report on the gastrointestinal microecology of Balb/c nude mice suggested that the lack of a thymus did not significantly affect the normal microbial flora of the mouse (Brown and Balish, 1978). Studies also cited the presence of activated macrophages upon stimulations with exogenous factors, indicating that there might be intracellular components linking the tumor cells to the host flora (Rao et al., 1977). In fact, gut microflora is mobile. Alterations in anatomical location, in addition to the composition, of intestinal commensal bacteria are associated with numerous chronic diseases (Ley et al., 2006, Ott and Schreiber, 2006). Significant alteration of intestinal microbial composition in mice bearing subcutaneous tumor upon feeding with anticancer agent cyclophosphamide were also reported in a
very recent study (Viaud et al., 2013), which is consistent with our observation. A
decline in microflora diversity was observed in tumor-bearing mice, which may
have resulted from the impact of cancer. Other diseases have also been shown to
reduce microflora diversity, such as inflammatory bowel disease (Ott and
Schreiber, 2006) and obesity (Turnbaugh et al., 2009). Our results may indicate
the characteristic features of cancer-induced dysbiotic microflora composition.
Tumor progression might lead to dysregulation of the immune system, accounting
for the alteration in microflora.

ERIC-PCR has been used to detect bacterial species in the Enterobacteriaceae and
Vibrionaceae families, including well-known organisms such as Escherichia coli,
Salmonella enterica, Yersinia pestis, and Vibrio cholera. ERIC sequences have
also been found in the genome of various bacterial species (Delihas, 2007, Wang
et al., 2009, Wilson and Sharp, 2006). We observed that tumor growth rapidly
altered the composition of gut microbiota. Intriguingly, as the graduate shrinkage
of the tumor in responding to GpS treatment, the microbiota seems to behave
closer to the microbiota of the animals before implantation of the tumor cells. This
phenomenon was not observed in nonxenograft mice received the same GpS
treatment. Such alterations in microbiota composition demonstrate a dynamic
interaction of the gut microbes, the tumor-bearing host and the ingested phytosaponins. Whether this change was the result of tumor regression or it was the result of GpS treatment requires further investigation.

Fecal microflora communities were assessed using 16S pyrosequencing to obtain a comprehensive profile. At the phylum level, Tenericutes, Proteobacteria, and Bacteroidetes were more abundant in GpS-treated mice than in the controls, whereas Firmicutes showed the opposite pattern, particularly in tumor-bearing nude mice. A shift in the ratio between Firmicutes and Bacteroidetes has been reported in many other studies, and such a shift has been linked to numerous diseases, such as obesity (Ley et al., 2005, Ley et al., 2006). The pyrosequencing data indicated that the Bacteroidetes/Firmicutes ratio showed an increased trend after 10 d of GpS treatment in xenograft mice. GpS treatment also increased the relative abundance of Proteobacteria, the major group of Gram-negative bacteria in the gut. The lipopolysaccharide (LPS) in the outer layer of the Gram-negative bacteria is known to stimulate the immune system and has been recognized as a treatment for cancer (Goto et al., 1996). The increased numbers of Proteobacteria in GpS-treated mice might enhance the secretion of LPS, thus activating an immune response against tumors growth, directly or indirectly.
The pyrosequencing analysis identified few species of bacteria responding to GpS treatment. *C. cocleatum* and *B. acidifaciens*, which have several well-documented beneficial effects, markedly increased in both nonxenograft and xenograft mice treated with GpS. A previous study indicated that a strain of *C. cocleatum* protects against the colonization of the gut by the pathogenic *Clostridium difficile*, revealing multiple glucosidase activities that might degrade the oligosaccharide chains of mucin in the digestive tract (Boureau et al., 1993). *C. cocleatum* has been shown to have significantly decreased in irritable bowel syndrome patients (Kassinen et al., 2007, Malinen et al., 2010). *C. cocleatum* also plays a role in the conversion of diglucoside, and has a deglycosylation function (Clavel et al., 2007). *Clostridium* bacteria are a major component of mammalian gut microflora and are responsible for promoting antiinflammatory immune responses. We speculated that *C. cocleatum*, which increased considerably in GpS-treated xenograft mice, was responsible for most of the differences between the GpS-treated xenograft mice and the controls. In addition to the mentioned well-documented beneficial effects, *C. cocleatum* might play a role similar to symbionts by taking part in the metabolism of Gp saponins through glucosidase activities.

*B. acidifaciens* was first isolated from the cecum of mice (Miyamoto and Itoh,
B. acidifaciens and its close relative, B. uniformis, were found to be associated with the degradation of the isoflavone in human feces (Renouf and Hendrich, 2011). A recent study demonstrated that B. acidifaciens promoted IgA production (Yanagibashi et al., 2012). It is reasonable to assume that the beneficial effects of C. cocleatum and B. acidifaciens might support the anticancer effects of GpS. Changes in gut microflora observed in GpS-treated xenograft mice were more apparent than in GpS-treated nonxenograft mice; that is, the pathological condition of the xenograft mice enhanced the effects of GpS treatment. The reason for this might be because GpS treatment reversed the dysbiosis of the microbiota, increasing beneficial bacteria, which in turn exerted an inhibitory effect on tumor growth.

In summary, our results indicated that tumors grafted in the flank of the animal can significantly alter the composition of the gut microbiota, indicating an active interaction of flora-tumor bearing host. We also demonstrated that GpS alters the gut microbiota, in particular, by boosting the level of beneficial bacteria and contributing to the restoration of eubiosis from dysbiosis. Findings also showed that a much wider changes in phylogenic composition to GpS treatment in the tumor-bearing than the healthy nude mice. At this point, the mechanism that
accounts for these observations is unclear. Whether this alteration of gut microflora induced by GpS treatment is linked to the anticancer effect of GpS treatment warrants further investigation. Nevertheless, this study provides a fundamental importance in term of understanding of the role of gut microbiota in the tumorigenic study in the nude mice, which have been an area largely overlooked.
CHAPTER 5

BASELINE STUDY OF MICROFLORA

IN THE WILD TYPE AND $APC^{MIN/+}$ MICE
5.1 Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer deaths in the world. The colon has been described as a “bioreactor”, containing the greatest amounts of bacteria. Among all types of cancers, CRC might be the most closely linked to the gut microbiota. The bacterial density in the large intestine ($\sim 10^{12}$ cells per ml) is much greater than that in the small intestine ($10^2$ cells per ml). In addition, an almost 12-fold higher risk of cancer is associated with the large intestine in contrast to the small intestine (Sobhani et al., 2013). Recent evidence has further demonstrated the crucial role of commensal microbiota in the development of CRC. In order to extend our study on the responses of commensal microflora to cancer and anti-cancer herbal saponins, we adapted colorectal $Apc^{Min/+}$ mice for the thesis project. Mutations in the adenomatous polyposis coli (APC) gene are involved in sporadic CRC tumorigenesis. $Apc^{Min/+}$ mice, carrying a point mutation in the $APC$ gene, develop spontaneous multiple neoplasias in their intestinal tracts within several weeks of birth and is they are thus regarded as one of the models for colorectal tumorigenesis (Mai et al., 2007). In order to establish a reproducible protocol for the microflora study in this mouse model, we characterized the physiological conditions and the gut microbiota profile of the $Apc^{Min/+}$ mice and their C57BL/6J littermates.
5.2 Results

5.2.1 Tumorigenesis is accompanied with changes in body weight and fecal amount in $Apc^{Min/+}$ mice at the late-disease stage

In order to investigate the microbial baseline of $Apc^{Min/+}$ mice, fecal samples of the WT and $Apc^{Min/+}$ mice were collected every week. Normally, the intestinal polyps can be found in $Apc^{Min/+}$ mice at 8 weeks of age. We supposed that the microbial composition of $Apc^{Min/+}$ mice at 6 weeks of age is relatively close to the normal status. Thus, the baseline study was started at 6 weeks and ended at 30 weeks of age for the WT and $Apc^{Min/+}$ mice as shown in Fig. 5.1-I. The diet and water consumptions, body weight, and the amount of feces were monitored during the entire period. Fecal amount in the WT was much more consistent than that in the $Apc^{Min/+}$ mice (Fig. 5.1-IIc). Decreased body weight and fecal amount were observed in the $Apc^{Min/+}$ mice at the late-disease stage (Fig. 5.1-IIc&d). However, the dietary and water intake were increased in the $Apc^{Min/+}$ mice at the late stage (Fig. 5.1-IIa&b). All these findings suggested that the changes in food intake, water consumption, body weight, and fecal amount might be linked to the disease progression in the $Apc^{Min/+}$ mice.
5.2.2 The predominant size of polyps was 2-4 mm in $Apc^{Min/+}$ mice at the late-disease stage

To evaluate the tumorigenesis in the WT and $Apc^{Min/+}$ mice, all animals were sacrificed in the end of 30 weeks. The guts were fixed with 10% formalin and stained with methyl blue, and then the number and size of polyps were scored. Polyps were categorized as < 1 mm, ≥1-2 mm, ≥ 2-3 mm, and ≥ 3-4 mm in size, and measured under a dissecting microscope. Our observation showed that WT exhibited regular alignment of intestinal villi and free of polyps, while the
Apc\textsuperscript{Min/+} mice exhibited a disrupted alignment of intestinal villi and replaced by different sizes of polyps (Fig. 5.2-I). At this late-disease stage, the dominant size of polyps was \( \geq 2-3 \) mm and followed by \( \geq 3-4 \) mm (Fig. 5.2-II).

![Image of intestinal polyps in both WT and Apc\textsuperscript{Min/+} mice at 30 weeks of age. I. Methyl blue staining of intestinal polyps in Apc\textsuperscript{Min/+} mice. Arrows indicate the polyps. (a) WT littermates (n=3); (b) Apc\textsuperscript{Min/+} mice (n=3). II. Size distribution of polyps in Apc\textsuperscript{Min/+} mice. Polyps along the intestinal tract were counted and measured under a dissecting microscope. All data are presented as mean ± SEM (n=3).]

### Figure 5.2

Intestinal polyps in both WT and Apc\textsuperscript{Min/+} mice at 30 weeks of age. I. Methyl blue staining of intestinal polyps in Apc\textsuperscript{Min/+} mice. Arrows indicate the polyps. (a) WT littermates (n=3); (b) Apc\textsuperscript{Min/+} mice (n=3). II. Size distribution of polyps in Apc\textsuperscript{Min/+} mice. Polyps along the intestinal tract were counted and measured under a dissecting microscope. All data are presented as mean ± SEM (n=3).

#### 5.2.3 Apc\textsuperscript{Min/+} mice developed blood stools and intestinal ischemia

The blood feces and anemia are the common symptoms in both CRC patients and Apc\textsuperscript{Min/+} mice model. In the baseline study, we found that fecal extracts of the Apc\textsuperscript{Min/+} mice showed darker color than their WT littermates. This phenomenon
was observed in the $Apc^{Min/+}$ mice at 17 weeks of age and getting more apparent as the time progressed. (Fig. 5.3-Ia). At 30 weeks of age, when we sacrificed the mice and removed the intestinal tract, the dark colored cecum was found in the $Apc^{Min/+}$ mice (Fig. 5.3-Ib). In addition, the intestinal tracts were pale in color in the $Apc^{Min/+}$ mice compared to the WT (Fig. 5.3-II).

<table>
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<th></th>
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</tr>
<tr>
<td></td>
<td>$Apc^{Min/+}$ (30 week)</td>
<td><img src="image5" alt="Intestinal tracts" /></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5.3** The fecal suspensions and intestinal tracts collected from $Apc^{Min/+}$ mice and WT littermates. I. (a) Fecal extracts; (b) Representative comparison of the cecum collected from the WT and $Apc^{Min/+}$ mice at 30 weeks of age. Arrows indicate the dark colored cecum in $Apc^{Min/+}$ mice. II. Intestinal tracts of the WT and $Apc^{Min/+}$ mice at 30 weeks of age. WT: n=3; $Apc^{Min/+}$ mice: n=3.
5.2.4 ERIC-PCR revealed the different microbial communities between 

\( Apc^{Min/+} \) mice and WT littermates

We further characterized the microbial profiles from 6 weeks to 30 weeks of age for 25 consecutive weeks. The fecal samples were prepared for genomic DNA and subjected for ERIC-PCR. Relatively consistent DNA banding patterns were observed with WT, but not with \( Apc^{Min/+} \) mice (Fig. 5.4-I). In the subsequent PLS-DA plots, the fecal microbiota of WT and \( Apc^{Min/+} \) clustered together at the early stage (6-8 weeks of age), but the two groups of mice distinctively drifted apart at the late stage (Fig. 5.4-II). Furthermore, in the PLS-DA plot for \( Apc^{Min/+} \) alone, it seemed that the alterations of microbial composition could be divided into five distinct groups (Fig. 5.4-III), which might be associated with the disease progression. We then further identified the discriminative ERIC-PCR fragments between \( Apc^{Min/+} \) mice and their WT littermates. As shown in Fig. 5.5, eight fragments were identified as the main contributors to the distinct ERIC-PCR profiles between \( Apc^{Min/+} \) mice and WT littermates. Compared with the WT, \( Apc^{Min/+} \) mice exhibited higher abundance in the 1000 bp, 600 bp, 250 bp and 400 bp fragments, but lower abundance in the 720 bp, 370 bp, 300 bp and 140 bp fragments.
Figure 5.4 Comparison of fecal microbial compositions between the WT and $Apc^{Min/+}$ mice. **I.** Representative ERIC-PCR fingerprints of the fecal microbial composition of (a) WT littermates and (b) $Apc^{Min/+}$ mice. **II-III.** PLS-DA score plots of ERIC-PCR data. **II.** $Apc^{Min/+}$ mice versus WT. Open symbols: WT; Solid symbols: $Apc^{Min/+}$ mice. **III.** $Apc^{Min/+}$ mice at different disease stages. Different symbol shapes represented for different disease stages. Gel pictures were digitized by Image Lab 3.0 system (Bio-Rad). Based on the distance and the intensity of each DNA band, SIMCA-P 12.0 tool was applied to obtain the PLS-DA score plots. WT: n=3; $Apc^{Min/+}$ mice: n=3.
Figure 5.5 The discriminative ERIC-PCR fragments between the WT and Apc<sup>Min/+</sup> mice. I. Summary plots of up-regulated and down-regulated fragments in Apc<sup>Min/+</sup> mice. ▲: each identified DNA bands of PCR reactions; Red: up-regulated fragments; blue: down-regulated fragments. II. The intensity of the discriminative fragments. (a) ~1000 bp fragment; (b) ~600 bp fragment; (c) ~520 bp fragment; (d) ~400 bp fragment; (e) ~720 bp fragment; (f) ~370 bp fragment; (g) ~300 bp fragment; (h) ~140 bp fragment. All data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001 versus WT littermates); n=3/group.
5.2.5 The major bacteria genera showed an age and disease dependent manner in \( Apc^{Min/+} \) mice

We investigated the major bacterial genera in the feces of \( Apc^{Min/+} \) mice and their WT littermates by 16S rRNA PCR. There are two dominant bacterial phyla in the gut ecosystem, Gram-positive *Firmicutes* (most notably *Clostridium* spp., *Enterococcus* spp. and *Lactobacillus* spp.) and Gram-negative *Bacteroidetes* (*Bacteroides* spp.) (Cerf-Bensussan and Gaboriau-Routhiau, 2010, Dethlefsen et al., 2007, Ley et al., 2008a, Mahowald et al., 2009). Using PCR primer sets of the 16S rRNA specific for these bacteria genera as well as *Bifidobacterium* spp., we found *Bacteroides* and *Bifidobacterium* showed an age dependent manner in both \( Apc^{Min/+} \) mice and their WT littermates. However, the differences between the WT and \( Apc^{Min/+} \) mice fluctuated during 6 to 30 weeks of age (Fig. 5.6-I). By comparing these major bacterial genera in cecal contents at 30 weeks of age, the \( Apc^{Min/+} \) mice exhibited a higher level of *Bifidobacterium* and *Clostridium* than the WT (Fig. 5.6-II).
Figure 5.6 Comparison of the major bacteria genera in fecal samples (I) and cecal contents (II) from the WT and Apc\textsuperscript{Min/+} mice. Fecal samples were collected every week and cecal contents were obtained in the end of 30 weeks. All these samples were analyzed by PCR with 16S rRNA specific primers of Bacteroides, Bifidobacterium, Clostridium, Enterococcus, and Lactobacillus. Based on the intensity of the amplified bands, the normalized values were calculated as the ratio of the specific genus to the total bacteria. The expression of 16S rRNA of the specific genus was presented by the fold change of the self-control at 6 weeks of age in each sample. All data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001 versus samples of 6 weeks old; # P < 0.05, ## P < 0.01, ### P < 0.001 Apc\textsuperscript{Min/+} mice versus WT); n=3/group.
5.3 Discussion

In this study, we characterized the gut microbiota profiles during the course of tumor development in $Apc^{Min/+}$ mice, using the WT littermates as the normal control. Baseline study of microbiota in $Apc^{Min/+}$ mice and their WT littermates provided the basic information for the subsequent study. By comparing the ERIC-PCR fingerprints, different microbial communities can be found in $Apc^{Min/+}$ mice in contrast to their WT littermates. In the WT, the microbial community seemed to gradually mature and reached to steady stage after 4 weeks. Unlike WT littermates, the microbial composition of $Apc^{Min/+}$ was seemingly affected by the onset and progression of the colonic tumors. We also found that tumorigenesis was accompanied with changes in body weight and fecal amount in $Apc^{Min/+}$ mice in the late-disease stage, as well as the appearance of blood stools and intestinal ischemia. The color of fecal extracts can somehow be used as an indicator of disease progression in the later on study.
CHAPTER 6

THE IMPACT OF GP SAPONINS ON GUT MICROBIOTA IN $APC^{MIN/+}$ MICE
6.1 Introduction

The mammalian intestine is harbored by roughly 100 trillion commensal bacteria, by which the intestinal health are maintained (Backhed et al., 2005, Sekirov et al., 2010). However, alteration of the gut microbiota homeostasis or “dysbiosis” contributes to the pathogenesis of various chronic diseases, such as obesity, autism, asthma, rheumatoid arthritis, inflammatory bowel disease and cancer, in particular to the colorectal cancer (CRC). According to the reports, the distribution of microflora along the gastrointestinal tract varies greatly, with low numbers of bacteria in the stomach and duodenum, increasing in the jejunum and ileum and peaking in the colon (O'Hara and Shanahan, 2006). Furthermore, microbiota in the colon also shows spatial organization of morphologically distinct species (Nava et al., 2011).

CRC can be triggered by both genetic and environmental factors (see reviews Fearon, 2011, Hall and Crowe, 2011, Lin, 2009). Increasing evidence has implicated that commensal microbiota may also have a role in the development of CRC. For example, certain indigenous intestinal microbes such as Enterococcus faecalis, Escherichia coli NC101 (Cuevas-Ramos et al., 2010), and enterotoxigenic Bacteroides fragilis (Goodwin et al., 2011), have been reported to
induce epithelial DNA damage and trigger intestinal tumorigenesis. In addition, germ-free (GF) azoxymethane (AOM)-treated Il-10−/− mice were devoid of intestinal inflammation and tumors (Uronis et al., 2009). Similarly, GF ApcMin+/− mice also showed reduced intestinal polyps compared to the specific pathogen-free ApcMin+/− mice (Li et al., 2012). In colitis-associated CRC models, colonization of dysbiotic microbes from TRUC (T-bet−/−; RAG2−/− ulcerative colitis) mice (Garrett et al., 2007) or Nod2-deficient mice was able to induce an enhanced disease incidence in wild-type (WT) mice (Couturier-Maillard et al., 2013). Furthermore, pyrosequencing of 16S rRNA gene has revealed the microbiome related with colorectal cancer, and structural segregation of gut microbiota between CRC patients and healthy volunteers (Chen et al., 2013, Sanapareddy et al., 2012, Sobhani et al., 2011, Wang et al., 2012).

In the gut of mammalian hosts, microbiota is found to engage a dynamic interaction with the host immune cells residing at the surface of the intestinal tract where the commensal bacteria colonize. The host-microbes are maintained in a symbiotic stage. Such interaction may lead to an optimal proinflammatory cytokine release by the host immune cells in order to regulate the resident microbiota, and block the foreign pathological bacteria. Such delicate and dynamic homeostasis can be
deregulated by various factors in the gut microenvironment and results in prolonged inflammation in the gut. This persistent inflammation may account for the link between the gut microbiota and various chronic inflammatory diseases, including colonic carcinogenesis. For example, segmented filamentous bacteria (SFB) were determined to induce lamina propria Th17 cells, influence mucosal immune responses, and modulate intestinal homeostasis (Gaboriau-Routhiau et al., 2009, Ivanov et al., 2009, Ivanov et al., 2008). Bacteroides fragilis was reported to protect animals from experimental colitis in several models. The B. fragilis polysaccharide was the first identified member of “symbiosis factors”, and was reported to mediate a beneficial relationship between the gut microbiota and mammalian hosts by driving the differentiation of IL-10-secreting Treg cells (Round and Mazmanian, 2010), suppressing proinflammatory IL-17 and correcting and Th1/Th2 imbalance (Mazmanian et al., 2005, Mazmanian et al., 2008), thus alleviating the intestinal inflammation. In addition, administration of probiotic cocktail VSL#3 was able to increase the IL-10 production and the percentage of TGFβ-expressing T cells in colitic mice (Di Giacinto et al., 2005). Furthermore, clinical report suggested that patients exhibiting highly expressed Th17 were associated with a poor prognosis, whereas patients with highly expressed Th1 exhibited prolonged disease-free survival (Tosolini et al., 2011).
In our previous study, we have demonstrated that the treatment with total saponins of *Gynostemma pentaphyllum* (GpS) exerts anti-cancer effect in *Apc<sup>Min/+</sup>* mice (Tai *et al.*, unpublished data). The different microbial communities have also been found between *Apc<sup>Min/+</sup>* mice and their WT littermates by the baseline study of microbiota as we showed in Chapter 5. Here, the question we like to address is whether pretreatment of GpS would have preventive effect on the polyp development and can alter the gut microflora in *Apc<sup>Min/+</sup>* mice, and may have implication for cancer prevention. In this study, we show the dynamic alteration of the profiling of fecal microbiota and the host immune responses under the interventions of GpS, by investigating the phylotypes of the gut microbiota, the cytokine profiles, and the immune cells resided on the surface of the intestinal barrier.

### 6.2 Results

#### 6.2.1 GpS treatment reduced the spontaneous intestinal polyps in *Apc<sup>Min/+</sup>* mice

To investigate the effect of GpS on tumorigenesis and the gut microbial profiles in *Apc<sup>Min/+</sup>* mice, single dose of GpS at 500mg/kg or solvent control (0.5% carboxymethyl cellulose) was given daily by gavages, starting 6 weeks of age of the
mice and was carried out for 8 weeks. Fecal samples were collected before treatment and every week after treatment to measure the dynamic changes of gut microbiota over time. The treatment scheme is illustrated in Fig. 6.1-I. Throughout the experimental period, none of the treated animals showed weight loss and abnormal food or water intake (Fig. 6.1-II). Fig. 6.1-III showed that administration of GpS significantly reduced the number of polyps by 40.68% (P < 0.05) when compared with the control mice. In our baseline study of microbiota in \( Apc^{Min/+} \) mice, we found that the polyp formation in \( Apc^{Min/+} \) mice was often accompanied with bloody feces and darker color of the fecal extracts compared with their WT littermates. Interestingly, the fecal extracts again appeared in darker color in the untreated, but not in the treated \( Apc^{Min/+} \) mice, (Fig. 6.1-IV).
Figure 6.1 Effect of GpS on the intestinal polyp formation in the $Apc^{Min/+}$ mice. I. Schematic diagram of experimental design. II. The records of (a) body weight, (b) diet consumption and (c) water consumption in the WT and $Apc^{Min/+}$ mice with or without GpS treatment. These parameters were recorded every week (from 6 to 14 weeks of age, n=6). III. Effect of GpS on size distribution of polyps in the $Apc^{Min/+}$ mice. All data are presented as mean ± SEM (* $P < 0.05$ versus control); n=6/group. IV. Fecal extracts of the WT and $Apc^{Min/+}$ mice with or without 8 weeks of GpS treatment.

6.2.2 ERIC-PCR revealed structural segregation of fecal microbial communities of the treated and untreated mice

The comparative study of microbial profiles between GpS-treated and untreated mice was conducted for 8 consecutive weeks, starting from 6 weeks to 14 weeks of age using ERIC-PCR analysis of the fecal samples. The cecal contents were also collected at the end point of experiment and subjected for ERIC-PCR (Fig. 6.2-I).
The resulting digitized data of ERIC-PCR fingerprints was analyzed by partial least squares discriminant analysis (PLS-DA). Results showed a clear segregation of the microbial communities between the controls and GpS treated mice. This phenomenon existed in both the WT (Fig. 6.2-IIa) and $Apc^{Min/+}$ mice (Fig. 6.2-IIb). However, the microbial communities of GpS treated $Apc^{Min/+}$ mice from different time points seems to form a tighter cluster than the control group. The PLS-DA also identified the discriminative ERIC-PCR fragments between untreated and treated mice. As shown in Fig. 6.3, nine and six PCR fragments were distinctively different between the controls and GpS treated individuals found in WT and $Apc^{Min/+}$ mice, respectively. In the WT mice, GpS treated mice, compared to the controls, showed increased intensity in the 1600 bp, 1200 bp, 370 bp and 300 bp bands, but decreased intensity in the 2500 bp, 1700 bp, 570 bp, 140 bp and 110 bp bands. On the other hand, in the $Apc^{Min/+}$ mice, GpS treated mice, compared to the untreated controls, showed higher intensity in the 1200 bp and 300 bp DNA fragments, but lower intensity in the 2500 bp, 1700 bp, 570 bp and 140 bp DNA fragments. Among all of these discriminative ERIC-PCR fragments, the alteration of the 300 bp DNA fragment showed the most remarkable association as the result of GpS feeding with the consistent change in both GpS treated $Apc^{Min/+}$ mice and WT littermates when compared to the control mice.
Figure 6.2 Comparison of microbial compositions between the controls and GpS-treated WT and $Apc^{Min/+}$ mice. I. Representative ERIC-PCR fingerprints of the microbial composition. II. PLS-DA score plots of ERIC-PCR data. (a) WT mice: control vs. GpS treatment. (b) $Apc^{Min/+}$ mice: control vs. GpS treatment. Open symbols: control mice; Solid symbols: GpS-treated mice (n=6/group). Gel pictures were digitized by Image Lab 3.0 system (Bio-Rad). Based on the distance and the intensity of each DNA bands, SIMCA-P 12.0 tool was applied to obtain the PLS-DA score plots.
Figure 6.3 The discriminative ERIC-PCR fragments between the controls and GpS-treated WT and Apc\textsuperscript{Min/+} mice. I. Summary plots of up-regulated and down-regulated fragments in GpS-treated mice. ▲: each identified DNA bands of PCR reactions; Red: up-regulated fragments; blue: down-regulated fragments. (a) WT mice; (b) Apc\textsuperscript{Min/+} mice. II. The intensity of the discriminative fragments. (a) ~1600 bp fragment; (b) ~1200 bp fragment; (c) ~370 bp fragment; (d) ~300 bp fragment; (e) ~2500 bp fragment; (f) ~1700 bp fragment; (g) ~570 bp fragment; (h) ~140 bp fragment; (i) ~110 bp fragment. All data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001; Black asterisk: Apc\textsuperscript{Min/+} vs. WT; Red asterisk: WT GpS vs. WT control; Green asterisk: Apc\textsuperscript{Min/+} GpS vs. Apc\textsuperscript{Min/+} control); n=6/group.
6.2.3 GpS treatment reduced the intra-group variations among the fecal microflora of the treated animals

Comparing the PCR fingerprints of the fecal microbiota from each treatment group, the individual mice fed GpS for 8 weeks displayed similar patterns within each experimental group. This phenomenon existed in both \( Apc^{Min/+} \) mice and their WT littermates (Fig. 6.4-I), but not observed in the non-treatment groups. Indeed, the correlation coefficient of the microflora composition was higher in the treated than in the control groups (Fig. 6.4-II). It seems that there are variations of gut microflora among individual mice, however, GpS treatment seems to be able to synchronize the microflora profiles of the treated animals. This finding was more prominent in \( Apc^{Min/+} \) mice.
6.2.4 Identification of bacterial genera sensitive to GpS treatment

The ratio of Bacteroidetes and Firmicutes has been linked to the metabolic disease. In our previous study in nude mice, it seems that the Bacteroidetes/Firmicutes ratio has been associated with cancer. *Bacteroides* spp. is a major genus under phylum Bacteroidetes. Here, we detected the presence of *Bacteroides* spp., as well as two known beneficial bacteria genera including *Bifidobacterium* spp. and *Lactobacillus* spp. in the cecal contents of all mice in the experiments. Using PCR
primers of the 16S rRNA specific for these three bacteria genera, we found GpS treatment could significantly increase the levels of *Bacteroides* spp., *Bifidobacterium* spp. and *Lactobacillus* spp. in the WT mice. In addition, GpS-treated *Apc<sup>Min/+</sup>* mice exhibited a higher abundance of *Bifidobacterium* spp. than the control mice. Furthermore, a dramatic increase of *Bacteroides* spp. was observed in the *Apc<sup>Min/+</sup>* mice compared with their WT littermates (Fig. 6.5).

![Figure 6.5](image)

**Figure 6.5** Comparison of the cecal bacteria genera. 16S rRNA PCR was used to detect the bacteria genera in the cecal contents of the controls and 8 weeks of GpS-treated WT and *Apc<sup>Min/+</sup>* mice. I. *Bacteroides*; II. *Bifidobacterium*; III. *Lactobacillus*. All data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001, GpS vs. control group); n=6/group.
6.2.5 Identification of the key changes in the fecal microbiome between the 

$Apc^{Min/+}$ and the WT mice using 16S pyrosequencing

16S rRNA pyrosequencing was performed on the fecal DNA obtained from the WT and $Apc^{Min/+}$ mice, with or without 8 weeks of GpS treatment described in the experiment showed in Figure 6.1-I. Five fecal samples per group and a total of 20 samples were subjected for sequencing. A total of 591,640 reads that passed quality control were produced in this study, with an average of 29,582 sequences per sample. We then analyzed the 16S pyrosequencing data using the combination of QIIME (Caporaso et al., 2010) and LEfSe methods (Segata et al., 2011). LEfSe tool was used to identify the key phylotypes responsible for the differences in fecal microbial communities between $Apc^{Min/+}$ mice and their WT littermates. As shown in Fig. 6.6-I, the fecal microbiome between the WT and $Apc^{Min/+}$ mice varied significantly at all taxonomic levels. At the phylum level, compared with the WT, $Apc^{Min/+}$ mice showed a significant decrease in the relative abundance of Verrucomicrobia and Actinobacteria, but an increase in Tenericutes. Three lineages within these two phyla were also identified as the differentially abundant clades (from phylum to genus level) between the WT and $Apc^{Min/+}$ mice. For instance, WT mice harbored a fecal microbiota relatively enriched in *Bifidobacterium* and *Akkermansia*, whereas $Apc^{Min/+}$ mice showed a relatively
higher abundance in *Anaeroplasma*. These genera were the main contributors to the differentially abundant lineages in the WT and *Apc^{Min/+}* mice. In addition, within phylum Proteobacteria, *Apc^{Min/+}* mice were overrepresented by Gammaproteobacteria and underrepresented by Epsilonproteobacteria. The histogram of the linear discriminant analysis (LDA) score (Fig. 6.6-II) and the relative abundance of identified bacterial genera (Fig. 6.6-III) exhibit the statistically and biologically differential clades appeared in *Apc^{Min/+}* mice and their WT littermates.
Figure 6.6 Comparison of the fecal microbial structures between the WT and Apc<sup>Min/+</sup> mice. I. Taxonomic representations of fecal microbiome. The differentially abundant taxa are presented with different colors using LEfSe method. The taxa from the WT and Apc<sup>Min/+</sup> mice are colored in green and red, respectively. The taxa with non-significant changes between the WT and Apc<sup>Min/+</sup> mice are colored in yellow. Each circle’s diameter represents the taxon abundance. II. Histogram of the LDA scores of fecal 16S rRNA sequences of the WT (green color) and Apc<sup>Min/+</sup> (red color) mice. LDA scores characterized the magnitude of differential abundance in the microbial taxa between compared samples. III. The relative abundance of differentially abundant genera. Data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001, WT vs. Apc<sup>Min/+</sup> mice); n=5/group.
6.2.6 16S Pyrosequencing revealed a reduced microbial diversity in GpS-treated mice

Our data so far has demonstrated that the gut microbiome of $Apc^{Min/+}$ mice is significantly different from that of the wild-type. Here, we presented our pyrosequencing findings on the influence of GpS on fecal microbiome. 645 distinct operational taxonomic units (OTUs) (filter by counts per OTU = 5) were determined after denoising using QIIME method. By comparing the number of OTUs in $Apc^{Min/+}$ mice and their WT littermate, we found only 309 OTUs were shared. 98 and 134 unique OTUs can be found in WT and $Apc^{Min/+}$ mice, respectively (Fig. 6.7-Ia). The sequencing results showed that 235 shared OTUs and 172 unique OTUs from the control and 102 unique OUTs from the treated were obtained from the WT. For the $Apc^{Min/+}$ mice, the control and treated mice shared 273 OTUs, whereas 170 and 109 unique OTUs existed in the controls and treated-mice, respectively (Fig. 6.7-Ib&c). Collectively, GpS reduced the overall microbial diversity based on the calculated Shannon-Wiener diversity index (Fig. 6.7-II).
Comparison of overall OTUs between the WT and $Apc_{Min/+}$ mice. I. Numbers of shared and unique OTUs. (a) WT vs. $Apc_{Min/+}$ mice; (b) WT mice: Control vs. GpS; (c) $Apc_{Min/+}$ mice: Control vs. GpS. II. The diversity of fecal microflora in the WT and $Apc_{Min/+}$ mice with or without GpS treatment. Based on the percentage of classified OTUs in each sample, Shannon-Wiener diversity index ($H'$) was used to calculate the diversity of microbial communities. $H' = \sum - (P_i \times \ln P_i)$. Data are presented as mean ± SEM (** $P < 0.01$, GpS vs. Control); n=5/group.

6.2.7 GpS treatment significantly altered the fecal microbiome of the WT and $Apc_{Min/+}$ mice

In addition to the reduced microbiota diversity, the relative abundance of dominant phylum in the fecal microbiota also altered upon GpS treatment (Fig. 6.8-Ia). In the WT mice, GpS treatment markedly reduced the abundance of Firmicutes (from 39.42% down to 21.58%). In the meantime, it substantially increased the relative abundance of Proteobacteria (from 44.95 to 62.24%). In $Apc_{Min/+}$ mice, compared with the controls, mice treated with GpS exhibited relatively lower abundance of Tenericutes (from 6.10 down to 1.08%). In addition, in contrast to the untreated mice, the increased Bacteroidetes/Firmicutes ratio can
also be observed in both GpS-treated $Apc^{Min/+}$ and the WT (Fig. 6.8-Ib). Firmicutes, Actinobacteria and TM7 are the major Gram-positive bacteria. Other phyla such as Proteobacteria, Verrucomicrobia, Tenericutes, Bacteroidetes and Cyanobacteria are Gram-negative bacteria. It seems GpS treatment can somehow decrease the ratio of Gram-positive to Gram-negative bacteria. As is known, Proteobacteria is a main Gram-negative bacterial phylum. In this study, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria have been detected in the experimental samples, whereas Betaproteobacteria was the most abundant class under phylum Proteobacteria. The Deltaproteobacteria is one of the major phylogenetic lineages of sulfate- and sulfur-reducing bacteria (Kersters et al., 2006). Compared with the controls, GpS-treated $Apc^{Min/+}$ mice showed a significant reduction in the relative abundance of Deltaproteobacteria but a substantial increase in Epsilonproteobacteria (Fig. 6.8-Ic). Furthermore, the sequencing data also revealed that GpS altered the microbial communities at genus level (Fig. 6.8-II). Principal coordinates analysis (PCoA) plots showed a clear separation among the fecal microbiome of different experiment groups (Fig. 6.8-III).
**Changes in relative abundance of the main phyla of microbial communities in the feces**

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<th>Apc&lt;sup&gt;Min/+&lt;/sup&gt;</th>
<th>Percent change</th>
<th>Ctrl (%)</th>
<th>GpS (%)</th>
<th>Percent change</th>
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<td>Actinobacteria</td>
<td>0.139 ± 0.019</td>
<td>0.414 ± 0.287</td>
<td>↑ 197.84%</td>
<td>0.056 ± 0.012 **</td>
<td>0.073 ± 0.017</td>
<td>↑ 30.36%</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>0.73 ± 0.12</td>
<td>1.02 ± 0.18</td>
<td>↑ 39.73%</td>
<td>0.53 ± 0.10</td>
<td>1.08 ± 0.27</td>
<td>↑ 103.77%</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>0.015 ± 0.007</td>
<td>0.024 ± 0.005</td>
<td>↑ 60.00%</td>
<td>0.021 ± 0.008</td>
<td>0.02 ± 0.008</td>
<td>↓ 4.76%</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>39.42 ± 4.17</td>
<td>21.58 ± 3.36</td>
<td>↓ 45.26% **</td>
<td>40.16 ± 1.36</td>
<td>37.08 ± 8.20</td>
<td>↓ 7.76%</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>44.96 ± 2.98</td>
<td>62.24 ± 3.44</td>
<td>↑ 38.46% **</td>
<td>50.50 ± 1.63</td>
<td>58.95 ± 8.68</td>
<td>↑ 16.73%</td>
</tr>
<tr>
<td>TM7</td>
<td>0.005 ± 0.005</td>
<td>0.001 ± 0.001</td>
<td>↓ 80.00%</td>
<td>0.001 ± 0.001</td>
<td>1.08 ± 0.21</td>
<td>↓ 82.30% **</td>
</tr>
<tr>
<td>Tenericutes</td>
<td>1.86 ± 0.20</td>
<td>2.29 ± 0.80</td>
<td>↑ 23.12%</td>
<td>6.10 ± 0.99 #</td>
<td>1.08 ± 0.21</td>
<td>↓ 82.30% **</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>12.89 ± 2.62</td>
<td>12.43 ± 4.95</td>
<td>↓ 3.57%</td>
<td>2.63 ± 1.04 **</td>
<td>1.71 ± 0.65</td>
<td>↓ 34.98%</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM (n=5). **p<0.01, GpS versus Control; #p<0.05, #p<0.01, Apc<sup>Min/+</sup> versus WT.
Figure 6.8 Taxonomic composition of the microbial communities in the WT and Apc\textsuperscript{Min/+} mice with or without GpS treatment. I. The comparison at the phylum level. (a) Changes in the relative abundance of the main phyla of microbial communities in the feces. Pyrosequencing was performed on the fecal samples from the WT and Apc\textsuperscript{Min/+} mice with or without 8 weeks of GpS treatment (n=5/group). Beta diversity was calculated by QIIME software. (b) The ratio of Bacteroidetes to Firmicutes. (c) The distribution of the classes under the phylum Proteobacteria. Data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, GpS vs. Control). II. A bar chart of relative abundance of bacterial genera in the individual mice of different treatment groups. III. Principal coordinate analysis (PCoA) of all samples from different treatment groups.
**Phylotyping of the fecal microbiome in the WT mice treated with GpS:**

We then further investigated the key phylotypes responsible for the differences of fecal microbiome by LefSe tool. In the untreated WT mice, the most differentially abundant bacterial phylum in the feces was Firmicutes and the lineages of Mollicutes under Tenericutes phylum (Fig. 6.9-I). In the Firmicutes phylum, several families of Clostridia, such as Clostridiaceae, Catabacteriaceae, Lachnospiraceae, Peptococcaceae, and an unclassified Clostridiales were all observed enriched in the control mice relative to the GpS-treated mice. In the GpS-treated WT, Bacilli and Proteobacteria were overrepresented. The LDA scores showed in Fig. 6.9-II illustrated the magnitude of differential abundance. We then further calculated the relative abundance of the identified genera for the elucidation of the differential microbial phylogenetic structures between GpS-treated and untreated WT mice (Fig. 6.9-III).
Figure 6.9 Effect of GpS on the fecal microbiome in the wild-type mice. I. Taxonomic representations of the fecal microbiome of the WT mice with or without GpS treatment. II. Histogram of the LDA scores of the differentially abundant clades. Red: samples from the controls; Green: samples from the WT mice with 8 weeks of GpS treatment. III. The differentially abundant genera found in the control and GpS-treated WT mice. Data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001, GpS vs. control group); n=5/group.
Phylotyping of the fecal microbiome in the $Apc^{Min/+}$ mice treated with GpS:

Within this experimental group, four lineages were identified as the main contributors to the differences in the fecal microbiome structure between untreated and GpS-treated $Apc^{Min/+}$ mice. Three lineages, including Deltaproteobacteria-Desulfovibrionales-Desulfovibrionaceae-LE30, Tenericutes-Mollicutes-RF39- Unclassified RF39, and Tenericutes-Mollicutes-Anaeroplasmatales-Anaeroplasmataceae-Anaeroplasma, were overrepresented in the untreated $Apc^{Min/+}$ mice, whereas Epsilonproteobacteria-Campylobacterales-Helicobacteraceae-Helicobacter lineage was relatively enriched in the GpS-treated $Apc^{Min/+}$ mice (Fig. 1.10-I). It was noteworthy that genus LE30, affiliated with the sulfate- and sulfur-reducing bacterial family Desulfovibrionacea was identified with a very high LDA score (Fig. 6.10-II), reflecting marked abundance in $Apc^{Min/+}$ control mice. Interestingly, LE30 was depleted in GpS-treated $Apc^{Min/+}$ mice. Likewise, Anaeroplasma and Eubacterium were also absent from the GpS-treated individuals. Conversely, Ruminococcus, Coprobacillus and Escherichia were unique to GpS-treated $Apc^{Min/+}$ mice. All these unique genera showed statistically significant difference in the relative abundance between GpS-treated and untreated $Apc^{Min/+}$ mice (Fig. 6.10-III).
Figure 6.10 Effect of GpS on the fecal microbiome in the Apc<sup>Min/+</sup> mice. I. Taxonomic representations of the fecal microbiome of the Apc<sup>Min/+</sup> mice with or without GpS treatment. II. Histogram of the LDA scores of the differentially abundant taxa. Red: samples from controls; Green: samples from the Apc<sup>Min/+</sup> mice with 8 weeks of GpS treatment. III. The differentially abundant genera found in the control and GpS-treated Apc<sup>Min/+</sup> mice. Data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001, GpS vs. control group); n=5/group.
6.2.8 GpS treatment enhanced the beneficial bacterial species and suppressed the harmful species

Based on the consensus lineage map of OTUs generated by QIIME software, we identified few altered bacterial species upon GpS treatment (Table. 6.1). Among the identified bacterial species, compared with the WT, Apc\textsuperscript{Min/+} mice showed a significant increase in the relative abundance of \textit{Bacteroides uniformis} but a substantial decrease in \textit{Bifidobacterium pseudolongum}. However, GpS treatment caused a certain extent restore of these two species. Additionally, \textit{Lactobacillus intestinalis} was unique to the GpS-treated WT mice. Compared with the Apc\textsuperscript{Min/+} control mice, \textit{Allobaculum spID4}, \textit{Clostridium cocleatum} and \textit{Streptococcus thermophilus} significantly increased upon GpS feeding. \textit{Allobaculum} is one of the butyrate producers that can be promoted by Fe repletion (Dostal \textit{et al.}, 2012). \textit{Allobaculum spID4} is an identified species under genus \textit{Allobaculum}, which showed more than a 2-fold increase in both the GpS-treated WT and Apc\textsuperscript{Min/+} mice compared with the controls. In contrast to the untreated controls, \textit{Clostridium cocleatum} increased more than 17 fold in the GpS-treated Apc\textsuperscript{Min/+} mice. In addition, \textit{Streptococcus thermophilus} and \textit{Parabacteroides distasonis} were only detected in the GpS-treated Apc\textsuperscript{Min/+} mice. \textit{Bacteroides acidifaciens} has been demonstrated to promote IgA production (Yanagibashi \textit{et al.}, 2012) and exists as
an important host protein forager (Berry et al., 2013). The relative abundance of *Bacteroides acidifaciens* increased by 212.00% compared with the untreated controls. It seemed that GpS treatment increased the levels of several bacterial species showing various beneficial effects to the host. In addition to the reduction in sulfate- and sulfur-reducing bacteria, GpS treatment showed a decreased trend in certain potential opportunistic pathogen. For instance, *Acinetobacter lwaffii* has been reported to cause infections as well as a bacteremia associated with acute gastroenteritis (Regalado et al., 2009). In this study, *Acinetobacter lwaffii* was only observed in *Apc*\(^{Min/+}\) mice and exhibited a 93.10% decrease upon GpS treatment.

**Table 6.1** The effects of GpS on the relative abundance of the bacterial species found in fecal microbiota of the mice

<table>
<thead>
<tr>
<th>Species</th>
<th>Ctrl (%)</th>
<th>GpS (%)</th>
<th>Percent change</th>
<th>Ctrl (%)</th>
<th>GpS (%)</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter lwaffii</em></td>
<td>0.0561 ± 0.0093</td>
<td>0.1668 ± 0.0732</td>
<td>232.98%</td>
<td>0.0087 ± 0.0087</td>
<td>0.0006 ± 0.0006</td>
<td>93.10%</td>
</tr>
<tr>
<td><em>Allobaculum sp</em></td>
<td>0.0690 ± 0.0329</td>
<td>0.1193 ± 0.0547</td>
<td>37.92%</td>
<td>0.0040 ± 0.0117</td>
<td>0.1246 ± 0.049</td>
<td>212.00%</td>
</tr>
<tr>
<td><em>Bacteroides uniformis</em></td>
<td>0.0015 ± 0.0009</td>
<td>0.0147 ± 0.0691</td>
<td>860.00%</td>
<td>0.0267 ± 0.0057</td>
<td>0.0160 ± 0.0057</td>
<td>70.30%</td>
</tr>
<tr>
<td><em>Bifidobacterium pseudolongum</em></td>
<td>0.1229 ± 0.0190</td>
<td>0.3972 ± 0.2824</td>
<td>224.47%</td>
<td>0.0268 ± 0.0066</td>
<td>0.0604 ± 0.0156</td>
<td>63.13%</td>
</tr>
<tr>
<td><em>Clostridium cocleatum</em></td>
<td>0.0379 ± 0.0220</td>
<td>0.0929 ± 0.0497</td>
<td>147.07%</td>
<td>0.0044 ± 0.0017</td>
<td>0.0807 ± 0.0162</td>
<td>1734.09%</td>
</tr>
<tr>
<td><em>Lactobacillus intestinalis</em></td>
<td>-</td>
<td>0.0172 ± 0.0066</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Parabacteroides distasonis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0081 ± 0.0040</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM (n=5). *p<0.05, **p<0.01, GpS versus Control, #p<0.01, Apc*\(^{Min/+}\) versus WT. 
\(a\): (GpS-Ctrl)/Ctrl×100%
6.2.9  GpS treatment improved the intestinal barrier function in the \textit{Apc}^{\textit{Min}+/+} mice

To investigate the effect of GpS on the gut barrier function, we first examined the general intestinal morphology by H&E staining. There were no obvious differences between the control and GpS treatment groups (Fig. 6.11-I). Paneth cells are a principal source of antimicrobial substances in the small intestine. Dysfunction of Paneth cell may predispose to intestinal inflammation (Bevins and Salzman, 2011). Immunohistochemistry (IHC) staining for lysozyme, which is used as the marker for the presence of Paneth cells, demonstrated a reduction of Paneth cells in the \textit{Apc}^{\textit{Min}+/+} mice compared with their WT littermates (Fig. 6.11-II). Goblet cells take responsibility for generating mucus, which constitutes the first line of immune defense, and degradation of mucin can disturb the homeostasis of mucosal barrier, and is often recognized as an initial stage of some intestinal diseases (Derrien \textit{et al.}, 2010). To check the status of goblet cells in the gut barrier of the mice, we stained the gut section with Alcian blue that is used to detect the mucin-containing goblet cells. The result indicated a decrease of goblet cells in the \textit{Apc}^{\textit{Min}+/+} mice than the WT mice, particularly in the colonic region (Fig. 6.11-III). However, GpS treatment reversed the reduction of Paneth cells and goblet cells in the \textit{Apc}^{\textit{Min}+/+} mice. We also examined expression of E-cadherin and N-cadherin in the small
intestines as impaired expression of E-cadherin has been linked to a defective gut barrier function, which is reflected in many intestinal diseases (Schneider et al., 2010). Switching from E-cadherin to N-cadherin is found to be associated with CRC progression (Halbleib and Nelson, 2006). In contrast to the WT mice, an obvious decrease in E-cadherin and high level of N-cadherin were observed in the small intestines of the ApcMin/+ mice. Intriguingly, GpS treatment effectively reversed the trend, for which the level of E-cadherin was up-regulated and N-cadherin was significantly down-regulated. GpS treatment also brought down the level of N-cadherin in the gut of the WT animals (Fig. 6.12).
Figure 6.11 Effect of GpS on the intestinal structure (I), Paneth cells (II) and goblet cells (III). Hematoxylin and eosin (H&E) staining was used to visualize the formalin-fixed sections of small intestine. IHC staining of lysozyme was applied to...
identify the Paneth cells in the small intestine, and the dark brown indicates the presence of Paneth cells. Alcian blue staining was used to identify the goblet cells, and the blue staining indicates the presence of the goblet cells. Tissues of $Apc^{Min/+}$ mice and their WT littermates were collected after 8 weeks of treatment.

**Figure 6.12** IHC staining of E-cadherin and N-cadherin in the small intestine with or without GpS treatment. Small intestine and colon tissues of the WT and $Apc^{Min/+}$ mice were collected after 8 weeks of treatment. Positive expression is indicated by the brown color staining. Nuclear is stained and appeared in blue color that was done by hematoxylin staining.

6.2.10 GpS down-regulated the protein expression of beta-catenin and the phosphorylation of STAT3 in the intestinal mucosa

We investigated the impact of GpS on the expressions of beta-catenin, c-Myc, Toll-like receptor 4 (TLR4), Signal transducer and activator of transcription 3 (STAT3) phosphorylation and Src phosphorylation in the small intestinal and colonic mucosa. Three individual animal samples were assayed using western blot analysis. It seemed that GpS down-regulated the expression of beta-catenin in
both the WT and *Apc<sup>Min/+</sup>* mice. The expression of c-Myc is much lower in the small intestine than the colon. However, there were no obvious effects of GpS on the expressions of c-Myc and TLR4 (Fig. 6.13-I). STAT3 is a critical node for numerous oncogenic signaling pathways. It mediates tumor-induced immunosuppression and is constitutively activated in tumor cells as well as immune cells (Yu *et al*., 2007). Blocking STAT3 may become a promising target for cancer immunotherapy (Lee *et al*., 2011, Yu *et al*., 2009). STAT3 can be activated by oncogenic proteins such as Src (Aggarwal *et al*., 2009, Yu *et al*., 2007). Here, we found GpS treatment decreased the phosphorylation of both STAT3 and Src, in particular in the colonic mucosa (Fig. 6.13-II). IHC staining further revealed the down-regulated effect of GpS on the expression of beta-catenin (Fig. 6.14-I). As shown in Fig. 6.14-II, nuclear STAT3 was observed in the small intestine of the *Apc<sup>Min/+</sup>* mice, but hardly observed in the nuclear fraction upon GpS treatment, which was consistent with the down-regulated effect of GpS on the phosphorylation of STAT3 that is required for nuclear translocation of the protein.
Figure 6.13 Effects of GpS on protein expressions of beta-catenin, c-Myc, TLR4 (I), p-STAT3 and p-Src (II). Mucosa from the small intestine and colon of the WT and $Apc^{Min/+}$ mice were collected after 8 weeks of treatment with or without GpS. Mucosal protein lysates were analyzed by western blotting using the indicated antibody. GAPDH was used as loading control. Each lane indicates the sample obtained from individual mouse (3 mice per experimental group).
Figure 6.14 IHC staining of beta-catenin (I) and STAT3 (II) in the small intestine and colon with or without GpS treatment. Small intestine and colon tissues of the WT and Apc^{Min/+} mice were collected after 8 weeks of treatment. Positive expression is indicated by the brown color staining. Nuclear is stained and appeared in blue color that was done by hematoxylin staining. Arrows indicate the STAT3 nuclear translocation.
6.2.11 GpS modulated the mucosal cytokine profiles

Cytokines are molecular messengers that participate in the cell communication and cell response in immune system (Fowler et al., 2011), and are recognized as immunomodulating agents (Fantini and Pallone, 2008). Cytokines have been suggested to play a crucial role in regulating immune response between the mucosal barrier and the commensal microbiota (Owens and Simmons, 2013, Tlaskalova-Hogenova et al., 2011). To investigate the effect of GpS treatment on cytokine profiles, RayBiotech mouse cytokine array containing 22 main cytokines (Fig. 6.15-I) was used to detect the cytokines in the intestinal mucosal protein from both the control and GpS-treated $Apc^{Min/+}$ mice and their WT littermates. The expression of cytokines were quantified by densitometric analysis and normalized to the positive control. Distinct cytokine profiles were obtained in the $Apc^{Min/+}$ mice compared with the WT. The cytokine profiles also differed in the mice with or without GpS treatment (Fig. 6.15-II). In particular, the levels of Interleukin-4 (IL-4), monocyte chemoattractant protein-1 (MCP-1) and MCP-5 were significantly increased, whereas soluble tumor necrosis factor receptor I (sTNFRI) was significantly decreased in the intestinal mucosa from GpS-treated $Apc^{Min/+}$ mice compared with the controls. However, the regulatory effect of GpS treatment was not apparent in the WT littermates (Fig. 6.15-III). IHC staining of
IL-4 further confirmed the finding in the cytokine array (Fig. 6.16). Macrophages play an essential role in the mucosal environment and can be polarized by IL-4. Therefore, we further investigated the effect of GpS on two different subset phenotypes, a proinflammatory macrophage phenotype M1 and an antiinflammatory phenotype M2. The latter is known to be associated with tissue repair (Novak and Koh, 2013). iNOS and Arginase I are the common markers for M1 and M2 macrophages, respectively (Liu et al., 2013, Sica and Mantovani, 2012), which were used for IHC staining. In contrast to the WT mice, the $Apc^{Min/+}$ mice exhibited relatively higher expression of iNOS and lower expression of Arginase I. Meanwhile, GpS-treated $Apc^{Min/+}$ mice demonstrated increased Arginase I and decreased iNOS expression compared with the controls (Fig. 6.17). The intestinal mucosa is an essential protective barrier in the immune system where the multiple interactions occur between the gut microbiota and the host. These results indicated that in addition to the regulation of microbial ecosystem, GpS could modulate the mucosal cytokines, which might affect the host-microbe interactions and contribute to its chemopreventive efficacy.
Figure 6.15 Effect of GpS on the mucosal cytokine profiles. Mucosal lysates from five selected mice per group were pooled together, and analyzed for various cytokines by cytokine array kit. I. The location of detected cytokines in the membrane. II. Representative cytokine array blots of mucosal protein from the control and GpS-treated Apc\textsuperscript{Min/+} mice and WT littermates. III. Comparison of cytokine expression between the control and GpS-treated mice by densitometric analysis. (a) Apc\textsuperscript{Min/+} mice; (b) WT mice. Data were normalized to the positive control and shown as fold changes relative to the controls. Results were representative of two independent experiments with duplicate in each membrane. All data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, GpS vs. control group). MCP: monocyte chemoattractant protein; sTNFRI: soluble tumor necrosis factor receptor I.
Figure 6.16 IHC staining of IL-4 in the small intestine and colon with or without GpS treatment. Small intestine and colon tissues of the WT and $Apc^{Min/+}$ mice were collected after 8 weeks of treatment. Positive expression is indicated by the brown color staining. Nuclear is stained and appeared in blue color that was done by hematoxylin staining. Arrows indicate the representative staining of the positive cells.
Figure 6.17 IHC staining of iNOS (I) and Arginase I (II) in the small intestine and colon with or without GpS treatment. Small intestine and colon tissues of the WT and $Apc^{Min/+}$ mice were collected after 8 weeks of treatment. Positive expression is indicated by the brown color staining. Nuclear is stained and appeared in blue color that was done by hematoxylin staining. Arrows indicate the representative staining of the positive cells.
6.3 Discussion

The aims of this study are three-fold: 1) Investigate how gut microflora would be affected by the growth of tumor in the gut in the presence and absence of treatment with anti-cancer herbal saponins; 2) Elucidate the changes of the host’s intestinal mucosal barrier under the influence of herbal treatment; and 3) Assess the potential role of microflora for the efficacy of the herbal medicines. We then first investigated the effect of GpS on the gut microbiota. ERIC-PCR and 16S rRNA pyrosequencing revealed that GpS treatment altered the fecal microbial composition in both the ApcMin/+ mice and their WT littermates. GpS treatment seemed to create more favorable growth environment for the beneficial microbes. For example, GpS treatment showed an increased trend in the abundance of Bifidobacterium pseudolongum, which are the beneficial inhabitants in the intestine and known as probiotics (Abe et al., 1995). These species of bacteria produce conjugated linoleic acid and linolenic acid isomers from the free linoleic acid and alpha-linolenic acid. The converted products have well-known health benefits (Gorissen et al., 2010). Streptococcus thermophilus is an essential lactic acid bacterium, and commonly used in the production of yogurt (Boyle et al., 2006, Kilic et al., 1996). Interestingly, Streptococcus thermophilus can only be detected in the GpS-treated ApcMin/+ mice (Fig. 6.11). Oral administration of bacterial
components derived from *Parabacteroides distasonis* has been reported to reduce intestinal inflammation (Kverka *et al.*, 2011). Likewise, *Parabacteroides distasonis* can only be observed in the GpS-treated $Apc^{Min/+}$ mice. In the previous Chapter on the study of xenograft nude mice, we found that GpS treatment increased *Clostridium cocleatum* and *Bacteroides acidifaciens*, which have several well-documented beneficial effects to the host. Here, the increase of these two bacterial species can also be found in the GpS-treated $Apc^{Min/+}$ mice. IgA is known to reduce intestinal proinflammatory signaling, and has a key role in gut homeostasis (Peterson *et al.*, 2007). *Bacteroides acidifaciens* can promote IgA production, which may contribute to maintain the intestinal homeostasis (Yanagibashi *et al.*, 2012). Attene-Ramos *et al.* (2010) found sulfate- and sulfur-reducing gut microbes can produce hydrogen sulfide and contribute to the cancer progression trigged by the genotoxic insult to the colonic epithelium. In our study, we found GpS treatment can reduce the sulfate- and sulfur-reducing bacteria lineage (*Deltaproteobacteria, Desulfovibrionacea* and LE30) and potential opportunistic pathogens like *Acinetobacter lwoffii*, which may avoid their certain deleterious effects to the host. The reduced microbial diversity in GpS-treated mice may somehow reflect such changes, indicating that the microbial communities could be shaped upon GpS treatment.
The gut barrier function plays a vital role in the homeostasis of intestinal environment and the gut microbial ecosystem. The intestinal epithelium is covered by a protective mucus layer, which is mostly comprised of mucin glycoproteins. These mucin glycoproteins are largely synthesized and secreted by goblet cells, constituting the first line of immune defense. Goblet cells can respond to the gut microbiota and host-derived inflammatory factors (Deplancke and Gaskins, 2001, Dharmani et al., 2009, Kim and Ho, 2010). Lack of mucin can lead to a defective mucus barrier and result in increased pathogenic bacterial adhesion and penetration into surface epithelial cells, and increase intestinal permeability. As a consequence, the incidence of intestinal inflammation and injury increase (Kim and Ho, 2010). In this connection, we tried to further explore the beneficial roles of GpS by examining the gut mucosal structure and its functions. The IHC staining experiments revealed that both goblet and Peneth cells were increased in number in the Apc\textsuperscript{Min/+} mice treated with GpS. It has been reported that mucin can stimulate the growth of sulfate-reducing bacteria and subsequently generate toxic substances to the intestinal epithelium (Attene-Ramos \textit{et al.}, 2010, Gibson \textit{et al.}, 1988). This might account for why lower population of mucin-producing goblet cells was coincident with more abundant sulfate- and sulfur-reducing bacteria in the control Apc\textsuperscript{Min/+} mice. Paneth cells secrete
substantial quantities of α-defensins, which are predominant antimicrobial peptides in the human intestine (Bevins and Salzman, 2011). Paneth cell dysfunction may affect the secretion of antimicrobials, predisposing the host to intestinal inflammation or other diseases. Study showed that Paneth cell dysfunction affect the secretion of antimicrobials and cause the microbial imbalance (Menendez and Brett Finlay, 2007). The Paneth cell with α-defensin deficiency can also cause a decrease in the relative abundance of Bacteroidetes but an increase in Firmicutes (Salzman et al., 2010). Compared with the control mice in our study, an increased population of Paneth cells occurred in the GpS-treated Apc<sup>Min/+</sup> mice, coinciding with the increased Bacteroidetes/Firmicutes ratio. Furthermore, impaired expression of E-cadherin has been linked to a disturbance of intestinal homeostasis and barrier function. E-cadherin plays a key role in the maturation of Paneth and goblet cells (Schneider et al., 2010), and STAT3 can negatively regulate E-cadherin and positively modulate N-cadherin (Xiong et al., 2012). In our study, GpS treatment can increase E-cadherin but decrease N-cadherin in the Apc<sup>Min/+</sup> mice, which might attribute to their effect on the down-regulation of the phosphorylation of STAT3.

The cytokine environment, including proinflammatory and antiinflammatory
cytokines in the intestinal mucosa, may affect the CRC progression (Csizsar et al., 2004). In this study, the level of mucosal IL-4 in the GpS treated Apc\(^{Min/+}\) mice was significantly higher than that in the control mice. Endogenous IL-4 is usually produced by Th2 cells, Natural killer T cells, mast cells, basophils as well as eosinophils (Li et al., 2009, Nelms et al., 1999, Rochman et al., 2009). Gut inflammation can cause a decreased IL-4 production in the mucosa (Karttunnen et al., 1994). A continuous expression of IL-4 may provide an effective therapy for various diseases, including cancers and immunologic disorders (Okada and Kuwashima, 2002). IL-4 is known as an antiinflammatory cytokine. In early report, IL-4 was suggested to inhibit colon cancer cell growth (Lahm et al., 1994, Toi et al., 1992) as well as cell invasion and migration (Uchiyama et al., 1996). Reduced type II IL-4R signaling has been suggested to drive CRC initiation and associate with increased CRC risk (Ingram et al., 2013). In an AOM-induced CRC model, deletion of the IL-4R\(\alpha\) gene was associated with an increased number of aberrant crypt focus, indicating the protective role of IL-4R\(\alpha\) signaling in CRC carcinogenesis (Ko et al., 2008). In addition, IL-4 has been reported to function as a potent inhibitor of angiogenesis (Lee et al., 2002, Volpert et al., 1998). As early as 1993, recombinant human IL-4 (rhIL-4) was applied to patients with advanced cancer to investigate the toxicity and pharmacokinetics in clinical trials. However,
rhIL-4 administration by intravenous and subcutaneous routes exhibited certain clinical side-effects (Prendiville et al., 1993). In our study, GpS treatment can induce the endogenous production of IL-4 in the intestinal mucosa, which might avoid the side-effect caused by exogenous intake of IL-4. Furthermore, IL-4 can regulate the mucus production secreted by goblet cell (Fallon et al., 2002).

Lamina propria effector T cells are known to be involved in the maintenance of mutualistic response to the commensal microbiota by producing cytokines such as IL-4, which can help IgA production and promote epithelial repair (Murphy et al., 2011). Moreover, the process of macrophage activation is tightly modulated by various cytokines. IL-4 can promote alternative activation of macrophages into M2 cells and inhibit M1 cell phenotype transformation. An increase in M2 cells (repair macrophages) can contribute to an enhanced tissue repair and a reduced pathological inflammation (Luzina et al., 2012, Novak and Koh, 2013). Therefore, we supposed that GpS treatment might exert one of the protective effects in the gut mucosal barrier through the induction of IL-4 secretion, which can contribute to the mucus and IgA production, as well as the tissue repair associated with M2 macrophage.

In addition to IL-4, GpS significantly increased MCP-1 and MCP-5 that can
activate tumoricidal activity of macrophages *in vivo*. MCP-1 has been recognized as IL-4-associated macrophage gene signature (Wang and Joyce, 2010), and IL-4 functions as a potent stimulator for MCP-1 expression (Kikuchi *et al.*, 1994). MCP-1 can recruit monocytes, T-lymphocytes and dendritic cells to the inflammatory sites of tissue injury or infection (Carr *et al.*, 1994, Xu *et al.*, 1996). Epithelial injury can result in MCP-1 production, which initiates the repair process (Lundien *et al.*, 2002). Recent studies have revealed that MCP-1 is mainly produced by goblet and Paneth cells (Lau *et al.*, 2012, McGhee and Fujihashi, 2012). In our study, the GpS-treated *Apc*^Min/+^ mice presented an increase in goblet and Paneth cells and concurrently with an increased MCP-1 expression in intestinal mucosa. MCP-5 and MCP-1 are structural and functional homologues, and the expression of MCP-5 can also be induced in macrophages (Sarafi *et al.*, 1997). Moreover, elevated circulating level of sTNFRI is considered to be a marker of a proinflammatory state (Parsons *et al.*, 2005). sTNFRI is increased in tumor-bearing hosts (Selinsky and Howell, 2000), and the serum level of sTNFRI has been suggested to be a promising biomarker and an early diagnostic method of colorectal adenoma (Hosono *et al.*, 2012). Removal or inactivation of sTNFRI may become a strategy for cancer immunotherapy (Selinsky *et al.*, 1998). In this study, in addition to increasing IL-4, MCP-1 and MCP-5, GpS treatment can also
significantly decrease the level of sTNFRI in the intestinal mucosa in the $Apc^{Min/+}$ mice. Together, GpS might act as an immunomodulatory agent, which could strengthen its chemopreventive effect in CRC development.

We also tried to find out the possible link between the regulatory effects on the gut microbiota and the mucosal cytokines upon GpS treatment. In contrast to the controls, the GpS-treated $Apc^{Min/+}$ mice showed a reduction in Coprococcus. The increased level of MCP-1 has been reported to be negatively correlated with the abundance of Coprococcus (Bailey et al., 2011). Coincidentally, in our study, the up-regulation of MCP-1 was observed in the intestinal mucosa of the GpS-treated $Apc^{Min/+}$ mice. It has been reported that the resident intestinal bacteria is associated with the stability of beta-catenin in intestinal epithelial cells. For example, *Aeromonas veronii* was found to promote the accumulation of cytoplasmic beta-catenin in the intestinal epithelium (Cheesman et al., 2010). The gut microbiota has been shown to enhance tumor burden in $Apc^{Min/+}$ mice via STAT3 phosphorylation (Li et al., 2012). Tyrosine-phosphorylated STAT3 is a poor prognostic marker for various cancers including CRC (Kusaba et al., 2006). It is also a molecular bridge between chronic inflammation and tumorigenesis (Lin, 2010). Blockade of STAT3 activation can slow down tumor development of CRC.
(Corvinus et al., 2005). Compared with the controls, the down-regulation of beta-catenin and the phosphorylation of STAT3 were observed in both the GpS-treated WT and \(Apc^{Min/+}\) mice. However, additional studies are needed to figure out the potential association between the alteration in the gut microbiota and the two signaling pathways in the future. Altogether, GpS could affect the host-microbe interactions by regulating the gut microbial ecosystem and modulating the intestinal mucosal environment, which might contribute to its chemopreventive efficacy against the tumorigenesis in \(Apc^{Min/+}\) mice.
CHAPTER 7

THE PREBIOTIC-LIKE EFFECTS OF HERBAL SAPONINS ON GUT MICROBIAL COMPOSITIONS IN C57/B6J MICE
7.1 Introduction

Microbiota plays a vital role in health and disease (Neish, 2009, Nicholson et al., 2005) and is essential for ensuring the proper functioning of metabolic reactions, immune regulation, epithelial development, and protection against pathogens. Recent findings have revealed that gut microbiota plays an even greater role in modulating human metabolic phenotypes and the drug responses of humans than previously believed. Microbial composition is affected by the dietary and drug uptake of the host (Ley et al., 2006, Nicholson et al., 2005). Conversely, microbes influence the bioavailability and bioactivity of ingested products, including functional foods and herbal medicines (Akao et al., 2000, Wang et al., 2005). Recent findings have indicated that a certain ratio of two predominant gut bacterial phyla, Firmicutes and Bacteroidetes, is related to obesity in humans and mice (Ley et al., 2005, Ley et al., 2006). Studies have also shown that gut microbiota affects the bioavailability of consumable natural products. For instance, the flavone baicalin, isolated from the Radix Scutellariae, is hydrolyzed by gut microbes to form aglycone, which is then absorbed and subsequently conjugated back to baicalin (Akao et al., 2000). Another study showed that chamomile tea, a functional food, altered metabolites and bacterial composition in the gut (Wang et al., 2005). The metabolic activation of ginseng saponins (ginsenosides) by
intestinal bacteria are also being investigated extensively (Bae et al., 2004, Hasegawa et al., 1997, Hasegawa et al., 1996, Lee et al., 2009, Shin et al., 2003).

Accumulating evidence has shown that dietary prebiotics, including whole plant foods, polyphenols, and fiber, are able to up-regulate beneficial intestinal microbiota and may contribute toward the health effects of these dietary supplements (Tuohy et al., 2012). Studies showed, for example, that fecal bifidobacterium was selectively present in human subjects under oligofructose and inulin diets (Bouhnik et al., 2007, Ramirez-Farias et al., 2009). Few cases of animal and humans studies also revealed similar observations. For instance, feeding of resveratrol, a polyphenolic compound found in grapes, berries and various plants, increased lactobacilli and bifidobacteria as well as decrease enterobacteria in colitis rats (Larrosa et al., 2009). Biotransformation by human intestinal bacteria of dietary phenolic compounds such as daidzein to equol, and flaxseeds to enterodiol were also reported (Matthies et al., 2008, Wang et al., 2010, Woting et al., 2010).

Saponin is a family of phytochemicals commonly found in many medicinal and edible plants. They are a group of amphiphilic glycosides containing one or more sugar chains bound to a nonpolar triterpene (Fig.1-I) or steroid aglycone (Fig.1-II)
skeleton. Herbal saponins exhibit diverse biological and pharmacological effects, including antitumor, immunomodulatory, antiinflammatory, cardiovascular, hepatoprotective, cholesterol-lowering and antiviral activities (Francis et al., 2002, Lacaille-Dubois and Wagner, 1996, Sparg et al., 2004). However, the effects of saponins on modulation of the intestinal microbiota are still poorly understood. Remarkably few studies are linked to the influence of herbal saponins on the composition of the gut microbial community, indicating a serious gap in our understanding of the saponins-microbe interactions. In this study, we aim to understand the impact of herbal saponins on the composition of gut microbiota and the metabolic profiles of the host. The saponins from the four commonly used dietary herbs, *Gynostemma pentaphyllum* (GpS), *Radix notoginseng* (NGS), *Radix ginseng* (GS) and red ginseng (RGS), were included in this study.

7.2 Results

7.2.1 Preparation and standardization of total saponins

**Gp saponins (GpS):** Authentication and chemical profiling of each batch were monitored accordingly. Detailed chemical figure printings of GpS can be found in our recent report (Wu et al., 2011b).
Saponins from *Radix notoginseng* (NGS), *Radix ginseng* (GS) and red ginseng (RGS): Standardization of the saponins was performed by UPLC-MS using ginsenosides as standard markers. In brief, UPLC was performed with a Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA) which was equipped with a binary solvent delivery system and coupled to a microTOF-Q mass spectrometer (Bruker) with an ESI source. All the operations and analysis of data were conducted using the Hystar software (Bruker Daltonik GmbH, Germany). The chromatography was performed on an Acquity BEH C<sub>18</sub> column (2.1 x 100mm, 1.7µm). The mobile phase consisted of 0.1% formic acid in deionized water (A) and 0.1% formic acid in acetonitrile (B). A gradient elution procedure was used: 0-23 min, 10-40%B; 23-27 min, 40-85%B; 27-29 min, 85-100%B; 29-32 min, 100%B; 32-36 min, 10%. The flow rate was kept at 0.35 ml/min, and the injection volume was 2 µl. The ESI-MS data were acquired in negative mode, and the conditions of MS analysis were as follows: end plate offset, -500 V; capillary voltage, 4500 V; collision energy, 10 eV; nebulizing gas (N<sub>2</sub>) pressure, 2.5 bar; drying gas (N<sub>2</sub>) flow rate, 8.0 l/min; drying gas temperature 180 °C; mass range, *m/z* 100 -3000; spectra rate, 3.0 Hz. The peaks in the chromatogram of the ginsenoside were identified by comparing the retention time values and the mass spectra of reference standards. Extracted ion chromatograms
(EICs) at m/z 997.50 for the [M + HCOO]\(^{-}\) ion of R\(_{1}\), m/z 845.49 for the [M + HCOO]\(^{-}\) ion of Rg\(_{1}\), Rg\(_{6}\), F\(_{4}\), Rk\(_{1}\) and Rg\(_{5}\), m/z 991.55 for the [M + HCOO]\(^{-}\) ion of Re, m/z 829.50 for the [M + HCOO]\(^{-}\) ion of S-Rg\(_{2}\) and R-Rg\(_{2}\), m/z 1153.6 for the [M + HCOO]\(^{-}\) ion of Rb\(_{1}\), m/z 683.44 for the [M + HCOO]\(^{-}\) ion of Rh\(_{1}\), m/z 1123.59 for the [M + HCOO]\(^{-}\) ion of Re, Rb\(_{2}\) and Rb\(_{3}\), m/z 991.55 for the [M + HCOO]\(^{-}\) ion of Rd, m/z 665.43 for the [M + HCOO]\(^{-}\) ion of Rk\(_{3}\), Rh\(_{4}\), m/z 829.50 for the [M + HCOO]\(^{-}\) ion of S-Rg\(_{3}\) and R-Rg\(_{3}\) were integrated (Fig. 7.1).
Figure 7.1 Extracted ion chromatograms of notoginseng saponins (I), ginseng saponins (II) and red ginseng saponins (III) by UPLC-MS method.
7.2.2 Different herbal saponins differently influence on the profiles of fecal microbiota

In order to investigate the dynamic changes of gut microbiota of mice upon feeding with different herbal saponins, fecal samples were collected from the control and four experimental groups at D0, D5, D10 and D15 (Fig. 7.2-Ia). Genomic DNA isolated from the fecal samples was analyzed by ERIC-PCR. Among all the treated mice, the fecal microbial fingerprints showed an average of 19 bands per sample, ranging from approximately 100 to 3000 bp with various intensities (Fig. 7.2-I). When compared the diversity among groups using the Shannon-Wiener diversity index plot of the ERIC-PCR data, no significant difference were found among groups (Fig. 2-II). However, when the ERIC-PCR data of each treatment group was plotted against the control by PLS-DA plots, with the exception of the NGS, all other groups formed distinct clusters, apart from the control mice (Fig. 7.3). The NGS group showed no clear difference from the control (Fig. 7.3-II). The PLS-DA was also applied to identify four differential ERIC-PCR fragments (1200, 950, 230 and 210 bp), presumably representing the subset of the gut microbiome between the treated and the control (Fig. 7.4). Overall, GpS and GS treatments significantly enriched the 1200 bp fragment. GpS along with RGS and GS also increased the abundance of the 210 bp PCR
fragment which was particularly obvious in the D10 and D15 treatments with GpS.

Additionally, prolong treatment with GS decreased the intensity of 950 bp fragment, while GpS significantly decreased the 230 bp fragment. These discriminative ERIC-PCR fragments further revealed the differential response of the gut microbiota to different herbal saponins, and might be the main contributors to the distinct ERIC-PCR profiles among different treatment groups.
Figure 7.2 A time course study of fecal microbiota of mice from different herbal saponins treatment groups. I. ERIC-PCR fingerprints of the fecal microbiota of individual C57BL/6 mice fed with water control (b), GpS (c), NGS (d), RGS (e), and GS (f). ERIC-PCR data of fecal samples obtained at D0, D5 & D10 of three representative mice were showed in the charts. A total of ten mice were used for each group. The treatment scheme was illustrated in I-a. II. Shannon-Wiener diversity index (H' index). All data are presented as mean ± SEM; n=10/group.
Figure 7.3 PLS-DA score plots of ERIC-PCR data. Gel images were digitized by Image Lab 3.0 system (Bio-Rad). Based on the distance and the intensity of each DNA bands, SIMCA-P 12.0 tool was applied to obtain the PLS-DA score plots. I. Control vs. GpS; II. Control vs. NSG; III. Control vs. RGS; IV. Control vs. GS. n=10/group.
Figure 7.4 The discriminative fragments of ERIC-PCR identified from the control and four herbal saponins treatment groups. **I.** ~1200 bp fragment; **II.** ~950 bp fragment; **III.** ~230 bp fragment; **IV.** ~210 bp fragment. Arrows in Fig. 2-I indicate the corresponding fragments. All data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001 versus D0 samples); n=10/group.
7.2.4 Major bacterial genera identified from different treatment groups

The intestinal microbiota contains thousands of different cultivable and non-cultivable bacteria. However, few bacterial genera are commonly found in the gut as commensal microbes. To get insight of the compositions of the fecal microbiota, we used 16S rRNA specific primers for *Clostridium* spp., *Enterococcus* spp. and *Lactobacillus* spp. belonged to the Gram-positive Firmicutes Phylum; *Bacteroides* spp. belonged to the Gram-negative Bacteroidetes Phylum; and *Bifidobacterium* spp. to detect the presence of the bacteria in the fecal samples. We found that all four herbal saponins can significantly enhance the level of *Bacteroides* (Fig. 7.5-I); GpS and NGS also effectively increased *Bifidobacterium, Lactobacillus*, as well as *Enterococcus*, and no obvious changes in the level of *Clostridium* in all treatment groups (Fig. 7.5-II~V).
Figure 7.5 Relative abundance of major bacterial genera in the fecal samples upon treatment with the tested herbal saponins. The presence of the bacteria genera in fecal samples from the controls, and the four treatment groups were determined by PCR reaction with each genus-specific 16S rRNA primers. The acquired specific bands were normalized against the intensity of the bands amplified using the universal 16S rRNA primers for eubacteria to generate the relative intensity of each mouse sample. The intensity of each specific band was expressed as the fold change over the D0 control of each mouse. All data are presented as mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001 versus Day 0 samples); n=10/group.
7.2.5 Herbal saponins altered the fecal metabolites

UHPLC-Q-TOF mass spectrometry was performed on the aforementioned fecal samples of the experimental animals to view the metabolic profiles at Day 0, 5, 10 and 15. The acquired data were subjected to principal component analysis (PCA) using the MPP B.02.00 software. As shown in Fig. 7.6-I, the control mice at D5, D10 and D15 time points clustered together and separated from the D0, suggesting that feeding with solvent control by gavage might alter gut microbiota. On the other hand, the fecal metabolites in the treatment groups displayed a continued shift over the period of 15 days. The base peak intensity chromatograms showed that the control along with the GpS and NGS were close to each other, than to the RGS and GS profiles (Fig. 7.6-II). Red ginseng is the processed ginseng and supposedly would generate new active saponin derivatives through the process. In order to further reveal the general pattern of discriminative metabolites between these two groups, a heatmap (Fig. 7.6-III) was generated using MPP software. Compared to the control group, roughly half of the discriminative metabolites (b & d regions) showed similar changes in the RGS and GS groups. Meanwhile, some metabolites showed different degrees of alteration (a) or an opposite alteration (c). The heatmap also revealed the marked differences between the untreated and treated groups as demonstrated in the PCA mappings (Fig. 7.6-I).
Figure 7.6 Comparison of fecal metabolic profiles among different treatment groups in ESI positive mode. I. Time course study of fecal metabolic profiles of different treatment groups. The fecal metabolites were extracted and analyzed using UHPLC-Q-TOF/MS from the treatment groups. The resulting metabolite profiles at different time points were subjected for PCA plotting analysis. Red color: Day 0; Gray color: Day 5; Blue color: Day 10; Brown color: Day 15. Ctrl (n=5); GpS (n=6); NGS (n=5); RGS (n=5); GS: (n=5). II. Representative base peak intensity (BPI) chromatograms of the fecal metabolites. III. Heatmap analysis of discriminative metabolites in fecal samples from the control mice, RGS and GS treated mice.
7.3 Discussion

Triterpene saponins have been recognized as the main constituents contributing to the health benefits of many dietary and medicinal plants. Ginseng, raw and processed, notoginseng and jiao-gu-lan (G. pentaphyllum) are among the most common saponin-rich herbs used in China and Southeastern Asian. These four herbs share some common saponins, however, are different from one another by having its own unique profile of saponins, which explain their overlapping biological activities, yet each herb with its own health benefits and pharmacological functions.

Prebiotics are considered as nondigestible food ingredients that can stimulate the growth of beneficial intestinal bacteria, including bifidobacteria and lactic acid bacteria to the benefit of host health (Roberfroid, 2007, Tuohy et al., 2012, Vulevic et al., 2004). In the past 10 years, prebiotic research has mainly focused on fiber and polyphenolic compounds. The potential role of triterpenoid saponins as prebiotics has been by and large oversight. The results presented here is to address the interaction between gut microbiota and herbal saponins, and whether the herbal triterpenoid saponins can act as prebiotics by influencing the host intestinal microbiota.
In this study, we followed the changes of fecal microbiota of mice over a period of 15 days upon the herbal treatments. The responses of the gut microbiota to the feedings are different among the four herbs. Interestingly, among the four herbal saponins, RGS and GS which share similar chemical profiles (Kim et al., 2000), also displayed relatively similar profiles of microbial composition revealed by the PLS-DA charts (Fig. 7.3-III & IV) and similar fecal metabolic profiles (Fig. 7.6-II).

Species of *Bifidobacterium* and *Lactobacillus* have been consumed as probiotics (Yang, 2008). Increased level of gut *Bacteroides* is often linked to various health benefits in many recent reports. For example, *Bacteroides* alleviated obesity-associated metabolic phenotypes (Ridaura et al., 2013). Certain species from *Bacteroides* such as *B. acidifaciens* can promote IgA production (Yanagibashi et al., 2012). In our study, we found that the level of *Bacteroides*, a major genus within the phylum Bacteroidetes, was increased by all herbal saponins (Fig. 5). We also observed that GpS and NGS showed much stronger effects than the GS and RGS on the beneficial bacteria, including *Bifidobacterium* and *Lactobacillus*. One of the beneficial effects of *Bacteroides* and *Bifidobacterium*, as revealed in the recent reports, is able to metabolize ginsenoside Rb1 to compound K that exhibits potent pharmacological effects in
antitumor, anti-inflammatory, and anti-allergic actions (Bae et al., 2000, Kim et al., 2013). Our results seem to echo the effects of polyphenol-rich green tea and black tea extracts on enhancing the bifidobacterium species and the associated bifidogenic effects, suggesting that both GpS and NGS may possess the prebiotic-like potential, at least in this aspect (Jin et al., 2012, van Duynhoven et al., 2013). In summary, this study provides a systemic analysis for the interaction between the herbal saponins and the gut microbial ecosystem based on the microbial and metabolic profiles as the consequences of saponins intake of the animals. Altogether, these tested saponins exerted prebiotic-like effects on the gut microbial ecosystem, suggesting that herbal saponins might be applied for manipulating the microbiota for the purpose of good health or the combinational therapy for microbiota-associated diseases. However, further studies are needed to investigate the association between such changes in the gut microbiota and improvements in specific health outcomes.
CHAPTER 8

CONCLUSIONS AND

GENERAL DISCUSSION
8.1 Conclusions and general discussion

In this thesis project, we postulated that Gp saponins may change the gut microbial composition, which in turn affect drug efficacy through host-microbe interactions. Athymic nude mice and $Apc^{Min/+}$ mice were employed for investigating the relationship between gut microbiota and cancer that occur at sites distant to the gut and inside the gut, respectively, as well as the impact of Gp saponins on the different tumorigenic models. In addition to the optimizations of the experimental designs (Chapter 3) and the baseline study of the $Apc^{Min/+}$ mouse model (Chapter 5) for the thesis project, there are three main themes involved the interplays of the host, microflora and the herbal saponins (Chapter 4, 6, & 7). The key findings are summarized belows:

1. The impact of Gp saponins on gut microbiota in normal and tumor-bearing nude mice:

Athymic nude mice have been employed for tumorigenic research for decades, however, the relationships between the gut microbiome and host’s response to the grafted tumors and drug treatments are unexplored. In this study, we analyzed the fecal microbiome of the nonxenograft and xenograft nude mice under the treatment of phytosaponins from a medicinal plant *Gynostemma pentaphyllum*
(GpS). Analysis of ERIC-PCR data showed that microbiota profile of xenograft nude mice departed from that of the nonxenograft mice. On the other hand, 10 day treatment with GpS was able to shape the microflora closer to the fecal microflora from mice at Day 0 time point. Data from 16S pyrosequencing of the fecal samples reiterates the differences in microbiome between the normal and xenograft nude mice. In addition to the increased ratio of Bacteroidetes/Firmicutes, GpS also markedly induced the relative abundance of Clostridium cocleatum and Bacteroides acidifaciens, for which the beneficial effects on the host have well documented. This study, for the first time, characterizes the properties of gut microbiome in nude mice responding to tumor implant and drug treatment. We also demonstrate that dietary saponins such as GpS can potentially regulate the gut microbial ecosystem by increasing the number of symbionts which might contribute to the anticancer effect of GpS (Fig. 8.1).
2. The impact of Gp saponins on gut microbiota in Ap<sub>c</sub><sup>Min/+</sup> mice and their WT littermates:

Xenograft nude mice study was our first example for the cancer that occurred at distant sites to the gut, then we moved to the Ap<sub>c</sub><sup>Min/+</sup> mouse model, a common model for colorectal tumorigenesis, for the study of cancer raised inside the gut. In Ap<sub>c</sub><sup>Min/+</sup> mice model, we hypothesized that the polyp formation might induce the imbalance of symbiosis to dysbiosis of gut microbiota and the treatment of GpS could modulate the microbial composition to the benefit of the host disease status. Using ERIC-PCR and 16S pyrosequencing analysis, we revealed that GpS treatment altered the overall fecal microbiome and increased
Bacteroidetes/Firmicutes ratio. Interestingly, it seemed that GpS treatment might create more favorable growth conditions for the beneficial microbes and simultaneously inhibit the deleterious microbes. On the other hand, we found GpS treatment increased the levels of anti-inflammatory and epithelial repair-related cytokines, such as IL-4, MCP-1 and MCP-5, while significantly reduced sTNFRI in the intestinal mucosa of the treated \( Apc^{Min/+} \) mice when compared with the controls. In addition, GpS increased the number of Paneth cells and goblet cells, down-regulated the phosphorylation of Src and STAT3 as well as N-cadherin but up-regulated E-cadherin in the intestinal mucosa. GpS treatment might exert a protective effect in the gut mucosal barrier via induction of IL-4 secretion, which can contribute to the mucus and IgA production, as well as the polarization of antiinflammatory M2 type macrophages (tissue repair-related type). In summary, GpS might affect the host-microbe interactions by regulating the gut microbial ecosystem and improving the gut barrier function, which could strengthen its chemopreventive efficacy against colorectal cancer development in \( Apc^{Min/+} \) mice (Fig. 8.2).
Figure 8.2 The impact of Gp saponins on gut microbiota in Apc<sup>Min/+</sup> mice.

Differentiated structures of fecal microbiome were revealed between tumor-bearing mice and their healthy counterparts, including xenograft versus nonxenograft nude mice and Apc<sup>Min/+</sup> mice versus WT littermates. Our results might indicate the characteristic features of cancer-induced dysbiotic microflora composition. On the other hand, Gp saponins exerted significant anticancer effects in both xenograft nude mice and Apc<sup>Min/+</sup> mice. Interestingly, Gp saponins showed a much stronger impact on the phylotypes with known beneficial (e.g., *Clostridium cocleatum*, *Bacteroides acidifaciens*) or deleterious effects (e.g., sulfate- and sulfur-reducing bacteria) in the tumor-bearing mice than their healthy
counterparts. The modulatory effect of Gp saponins on the gut mucosal environment (e.g., Paneth cells, Goblet cells, gut barrier function markers, cytokine profiles) was more apparent in the Apc\(^{Min/+}\) mice than the WT. It seemed that the pharmacological activity of Gp saponins were more evident under pathological condition like tumor-bearing status.

3. The impact of different herbal saponins on gut microbiota in C57BL/6 mice:

Herbal saponins have raised considerable interest because of their health-promoting effects. With the encouraging results obtained from the previous studies, we extended the study to saponins from few other commonly consumed dietary and medicinal herbs, namely the saponins from Radix Notoginseng, Radix Ginseng and red ginseng (processed ginseng). We compared the impacts of these four different herbal saponins on the host’s microbial and metabolic profiles in C57BL/6 mice. We demonstrated that these tested saponins exerted prebiotic-like effects on the gut microbial ecosystem, suggesting that herbal saponins might be used for manipulating the microbiota for the purpose of good health or the combination therapy for some microbiota-associated diseases.
It has been reported that the gut microbiota can influence the drug metabolism, which can be manipulated to alter the outcomes of interventions (Jia et al., 2008). As is known, Compound K is more effective than ginsenoside Rb1 in certain aspects. Recently, another report has demonstrated that the ability to metabolize ginsenoside Rb1 to compound K is different among individuals. 16S rRNA gene pyrosequencing revealed that this variation may be attributed to the different composition of gut microbiota of the individuals. When compared to the non-responsive samples, the human fecal samples with the metabolic activity transforming ginsenoside Rb1 to compound K showed higher abundance of Bacteroidetes and Tenericutes, but lower abundance of Firmicutes and Proteobacteria. The Bacteroides and Bifidobacterium, which have been suggested to potently metabolize ginsenoside Rb1 to compound K were dramatically increased in these response samples (Kim et al., 2013). In our study, we found increased Bacteroides and Bifidobacterium in saponins-treated mice, which might also attribute to the drug metabolism. Further studies are needed to explore the relationship among the chemical constituents, microbial composition and host metabolites, which may provide evidence for the personalized healthcare.
Conclusion:

More and more diseases are demonstrated to be associated with the disruption of the symbiosis, which refers to the homeostasis between the microbiota and the host, resulting in a state known as dysbiosis (Chow et al., 2010, Hawrelak and Myers, 2004, Neish, 2009). In this study, we demonstrate that tumor growth affects gut microbiota. GpS treatment can alter the microbiome by increasing beneficial microbes and decreasing harmful microbes, restoring eubiosis from dysbiosis. The regulation of gut microbiota may contribute to maintain the homeostasis of gut microbial ecosystem in the host, thus curing the disease or at least alleviating its symptoms. In conclusion, our study characterizes the dysbiotic microflora in tumor-bearing animals and clarifies the impact of herbal saponins on the gut microbiota, which may provide evidence for manipulating microbiota for improving the health of the host.
8.2 Future plan

Verification of the beneficial effects of the identified signature bacterial strains

In the current study, pyrosequencing of 16S rRNA gene has revealed the impact of Gp saponins on the microbial composition. We like to further conduct a functional analysis of the gut microbiome. Metatranscriptomics enables the investigation on the actively transcribed RNA, which gives valuable information on the genes expressed within a certain community. On the other hand, we have demonstrated that *Clostridium cocleatum* increased considerably in GpS-treated tumor-bearing mice. In addition to the reported beneficial effects, *Clostridium cocleatum* may also take part in the metabolism of Gp saponins through glucosidase activities (Clavel *et al.*, 2007). *Clostridium cocleatum* also showed resistance against the colonization of *Clostridium difficile*, a notorious deleterious microbe (Boureau *et al.*, 1993). Therefore, we plan to investigate the direct effect of GpS on *Clostridium cocleatum*, *Clostridium difficile* as well as two common probiotics including *Bifidobacterium pseudolongum* and *Bifidobacterium breve*. We are also interested in the possible effect of *Clostridium cocleatum* itself on tumorigenesis.
Interaction between the GpS and the microbial sensors

Toll-like receptor 4 (TLR4), an important microbial sensor, is crucial in host-microbe interaction. For better understanding of the possible interaction between Gp saponins and TLR4, molecular docking simulation of different Gp saponins to TLR4 was performed. It seemed that Gp saponins showed high scores for TLR4 binding (Fig. 8.3). Among these saponins, Gypenoside XLIX has been shown to exert inhibitory effect on NF-κB activation (Huang et al., 2006). Fig. 8.4 illustrated the binding modes of Top 5 chemicals (in the ranking of Fig. 8.3) as well as Gypenoside XLIX. All these preliminary docking results provide a hint for the study of the effect of Gp saponins on TLR4 signaling pathway.

**Figure 8.3** Ranking list of drug-binding cavity of TLR4.
Figure 8.4 Molecular docking results show the most energetically favorable binding modes of chemicals against TLR4 protein.

In summary, in the future, we plan to (i) perform metatranscriptomics analysis of the microbiome upon GpS treatment and characterize functional capacity; (ii) investigate the direct effect of GpS on the strains from the following species including *Clostridium cocleatum*, *Clostridium difficile*, *Bifidobacterium pseudolongum* and *Bifidobacterium breve in vitro*; (iii) examine the *in vivo* effect of *Clostridium cocleatum* on tumorigenesis in xenograft nude mice and *Apc^{Min/+}* mice; (iv) explore the effect of GpS on TLR4 signaling pathway.
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2. **Chen L., Tai W.C.S., Hsiao, W.L.W**, The prebiotic-like effects of herbal saponins on gut microbial compositions in C57/B6J mice. (Submitted)

3. **Chen L., Singh M., Leung F.C.C., Hsiao, W.L.W**, The impacts of herbal triterpenoid saponins on gut microbiota and cancer in \(Apc^{Min/+}\) mice. (Manuscript in preparation)
PRESENTATIONS AND ABSTRACTS


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May 2014