

TOXIC AND HEALTH IMPACTS OF AFLATOXIN B₁ AND GREEN TEA
POLYPHENOLS ON THE GUT MICROBIOME IN RATS

by

JINCHENG WANG

(Under the Direction of JIA-SHENG WANG)

ABSTRACT

Commensal microorganisms are critical to host physiology and health. Extensive evidence has shown that they could modulate the kinetics and dynamics of xenobiotics in host system. It is largely unknown, however, how xenobiotics modulate commensal microbiota and impact the overall health. With a repeated dosing regimen, utilizing 16S rRNA gene survey and metagenomic analysis, this dissertation elaborates the dose-response relationship between the gut microbiome of rat models with two natural xenobiotics, aflatoxin B₁ (AFB₁) and green tea polyphenol (GTP) respectively. We found that male young F344 rats orally exposed to different concentrations of AFB₁ (0, 5, 25, and 75 µg/kg respectively) for 4 weeks significantly decreased phylogenetic diversity, but increased the evenness, of the gut microbiota in a dose-dependent manner. In addition to a dose-dependent shift in the community structure, some beneficial lactic acid bacteria were significantly depleted. In contrast, administering 0, 0.5% and 1.5% (g/ml) of GTP in water to female middle-aged Sprague-Dawley rats for 6 months significantly decreased both the phylogenetic diversity and evenness of the gut microbiota and induced a dose-dependent shift in community structure. *Bacteroidetes* and *Oscillospira*, previously

linked to lean phenotype, were enriched; and *Peptostreptococcaceae*, previously linked to colorectal cancer, were depleted. Correspondingly, among many microbial genes impacted by GTP, genes related to energy production and conversion were consistently enriched with increasing doses. These results demonstrate that AFB₁ and GTP induced compositional and functional changes in the gut microbiome, which could be critical to the overall health of the experimental rats. In addition, a standard protocol for preparing DNA libraries for microbiome studies and analyzing next-generation sequencing data for the microbiome has been established. Further studies are warranted to explore the phenotypical modification of the gut microbiota to confirm the specific functions and pathways that were modified, and to elucidate the interactions between other types of xenobiotics and the commensal microbiota and their potential roles in modulating health.

INDEX WORDS: Microbiome, aflatoxin B₁, green tea polyphenols, next-generation sequencing, 16S rRNA gene survey, metagenomic analysis

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DEDICATION

To my wife, parents, and grandparents!

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CHAPTER 1

INTRODUCTION

1.1 Background

It has long been recognized that commensal microorganisms (microbiota) residing on and inside humans and animals are critical to host physiology and health (Hooper and Gordon, 2001). Advances in high-throughput technologies in the past decade or so, especially next-generation sequencing (NGS) technologies, have shifted the study of commensal microorganisms from focusing on functions of individual species to the whole microbial community living in a particular niche. *Microbiome* has been used to represent this newest advance in the subject of interests. The earliest usage of “Microbiome” in the English literature appeared in a book chapter published in 1988 (Eisen, 2015), which defined “microbiome” as "a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physic-chemical properties" (Whipps *et al.*, 1988). A more widely accepted definition appeared later in which “microbiome” was described as "the collective genome of our indigenous microbes (microflora)" (Hooper and Gordon, 2001), probably in consonance with the human genome project completed earlier in that year. Since then, studies employing transcriptomics, metabolomics, and other high-throughput tools have emerged and been brought into the field of the microbiome.

Approximately 38 trillion bacteria, consisting of 500-1,000 different species, reside in human colon, carrying genetic information approximately 100 times more than

human genome (Sender *et al.*, 2016). Extensive evidence which dates to late 19 century have demonstrated that the gut microbiota could assist host metabolism, shape gastrointestinal tract directly and indirectly, influence homeostasis and functioning of local immune systems (Leser and Mølbak, 2009; DuPont and DuPont, 2011). Recent progress further revealed possible associations between the disturbance of the gut microbiome with diseases such as obesity (Ley *et al.*, 2006), inflammatory bowel disease (Manichanh *et al.*, 2006; Qin *et al.*, 2010), type II diabetes (Qin *et al.*, 2012), colorectal cancer (Sobhani *et al.*, 2011), and some other metabolic syndromes (Walters *et al.*, 2014) in human and animals.

1.2 Significance

The gut microbiota is among the first to be exposed to any xenobiotics that have been ingested by humans or animals. The interactions between gut microbiota and xenobiotics could lead to at least two consequences that may be critical to the overall health of the host: (1) the gut microbiota could metabolize, activate, or deactivate a xenobiotic, which may significantly change the kinetics and dynamics of the xenobiotics in the host system; (2) the gut microbiota could be modified by the xenobiotic structurally or functionally, which may impact their own homeostasis in the host system (Nicholson *et al.*, 2005). The impact of gut microbiota on the host response to xenobiotics has long been overlooked, largely due to the limitations of available technologies. Filling this gap would refresh our understanding of the effects of drugs or toxicants in the target system, shed light on the variations in individual susceptibility to xenobiotics, and most importantly develop novel methods toward personalized medicine.

Aflatoxins are a group of mycotoxins attracting consistent interest because the toxigenic fungus, *Aspergillus*, contaminates a large fraction of the world's crops (Lizárraga-Paulín *et al.*, 2011). Approximately 4.5 billion people in developing countries are chronically exposed to uncontrolled amounts of aflatoxins from foods (Williams *et al.*, 2004). Aflatoxin B₁ (AFB₁), one of the most potent natural toxins, has been categorized as a Group 1 human carcinogen by International Agency for Cancer Research (IARC, 1993). Despite extensive toxicological studies, differential species-specific sensitivity to AFB₁ has yet been fully explained, and it is possible that the gut microbiota plays a role in its effects on the host system. Green tea polyphenol (GTP) is a mixture of catechins naturally contained in tea, apple, and other plants. Multiple *in vitro* and animal studies have shown that GTP is beneficial to health. A previous clinical trial conducted by our group has demonstrated its chemopreventative capability within a human population (Wang *et al.*, 2003). Although multiple mechanisms have been suggested to explain the beneficial effects of GTP towards human health, the interplay between GTP and gut microbiota may be critical because most of the GTP ingested by human and animals have relatively low bioavailability (Del Rio *et al.*, 2010; Clifford *et al.*, 2013). This dissertation reveals some of the impacts of AFB₁, as a model toxin, and GTP, as a model beneficial compound, on the gut microbiota of rat models and could serve as a proof of concept study to demonstrate that xenobiotics may influence host health through modulation of the gut microbiota.

1.3 Objectives, Specific Goals, and Hypotheses

The overall objective of this dissertation is to evaluate how AFB₁ and GTP would interact with the gut microbiota by examining the structural and functional modification of the gut microbiota of rat models. The following specific goals are:

- (I) To develop a reliable experimental and data analysis protocol for gut microbiome studies in toxicology
- (II) To evaluate how xenobiotics modulate the community composition of gut microbiome of model rats
- (III) To evaluate how xenobiotics modulate the functional gene profile of gut microbiome of model rats
- (IV) To evaluate the dynamics of the gut microbiome in response to xenobiotic exposure at multiple sampling times

The dissertation is guided by two overall hypotheses: (I) administering AFB₁ or GTP to experimental animals could induce dose-dependent modification on their gut microbiome; (II) modifications on the gut microbiome are consistent throughout the duration of experiments.

References

- Clifford, M.N., van der Hooft, J.J., and Crozier, A. (2013) Human studies on the absorption, distribution, metabolism, and excretion of tea polyphenols. *Am. J. Clin. Nutr.*, **98**, 1619S–1630S.
- Del Rio, D., Costa, L.G., Lean, M.E.J., and Crozier, A. (2010) Polyphenols and health: what compounds are involved? *Nutr. Metab. Cardiovasc. Dis.*, **20**, 1–6.
- DuPont, A.W. and DuPont, H.L. (2011) The intestinal microbiota and chronic disorders of the gut. *Nat. Rev. Gastroenterol. Hepatol.*, **8**, 523–531.
- Eisen, J. “What does the term microbiome mean? And where did it come from? A bit of a surprise”. The Winnower. <https://thewinnower.com/papers/what-does-the-term->

microbiome-mean-and-where-did-it-come-from-a-bit-of-a-surprise. 2015.
Accessed July 26, 2016.

- Hooper, L.V. and Gordon, J.I. (2001) Commensal host-bacterial relationships in the gut. *Science*, **292**, 1115–1118.
- IARC. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Lyon, France: International Agency for Research on Cancer; 1993:245-395. *IARC monographs on the evaluation of carcinogenic risks to humans*; vol 56.
- Leser, T.D. and Mølbak, L. (2009) Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environ. Microbiol.*, **11**, 2194–2206.
- Ley, R.E., Turnbaugh, P.J., Klein, S., and Gordon, J.I. (2006) Microbial ecology: human gut microbes associated with obesity. *Nature*, **444**, 1022–1023.
- Lizárraga-Paulín, E.G., Moreno-Martínez, E., and Miranda-Castro, S.P. (2011) Aflatoxins and their impact on human and animal health: an emerging problem. In, Guevara-González, R.G. (ed), Aflatoxins - Biochemistry and Molecular Biology. Intech, Rijeka, Croatia, pp. 255–282.
- Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., Nalin, R., Jarrin, C., Chardon, P., Marteau, P., *et al.* (2006) Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut*, **55**, 205–211.
- Nicholson, J.K., Holmes, E., and Wilson, I.D. (2005) Opinion: Gut microorganisms, mammalian metabolism and personalized health care. *Nat. Rev. Microbiol.*, **3**, 431–438.
- Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., *et al.* (2010) A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*, **464**, 59–65.
- Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W., Guan, Y., Shen, D., *et al.* (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*, **490**, 55–60.
- Sender, R., Fuchs, S., and Milo, R. (2016) Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLOS Biology*, **14**, e1002533.
- Sobhani, I., Tap, J., Roudot-Thoraval, F., Roperch, J.P., Letulle, S., Langella, P., Corthier, G., Van Nhieu, J.T., and Furet, J.P. (2011) Microbial dysbiosis in colorectal cancer (CRC) patients. *PloS One*, **6**, e16393.

- Walters, W.A., Xu, Z., and Knight, R. (2014) Meta-analyses of human gut microbes associated with obesity and IBD. *FEBS Lett.*, **588**, 4223–4233.
- Wang, J.-S., Huang, T., Yu, J., Wei, Z., Liang, Y., Li, Y., Wang, K., Shi, J., Cui, Y., Luo, H., *et al.* (2003) Chemoprevention trial of green tea polyphenols in high-risk population of liver cancer. *Proc. Annu. Meet. Am. Assoc. Cancer Res.*, **44**.
- Whipps, J.M., Lewis, K., and Cooke, R.C. (1988) Mycoparasitism and plant disease control. In, Burge, M.N. (ed), *Fungi in biological control systems*. Manchester University Press, pp. 161–187.
- Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M., and Aggarwal, D. (2004) Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am. J. Clin. Nutr.*, **80**, 1106–1122.

CHAPTER 2

LITERATURE REVIEW

2.1 Green Tea Polyphenol

2.1.1 Absorption, Distribution, Excretion of Green Tea Polyphenols

Even though tea has been a popular drink for thousands of years and ascribed for various beneficial effects on human health, research to scientifically evaluate and quantify GTP's effects truly began since 1995 thanks to the availability of necessary analytical instruments (Scalbert *et al.*, 2005; Wang *et al.*, 2015). Since then, much research has documented the pharmacokinetics of GTP constituents after oral intake. The polyphenols contained in green tea are primarily catechins, which belong to a subclass of flavonoids, flavanol. Structurally, flavonoids are distinguished from other phenolic compounds by having two phenyl rings (A and B) that are connected by an oxygenated heterocycle (ring C) (Figure 2-1) (Manach *et al.*, 2004). Different subclasses of flavonoid vary mainly by whether there is a ketone structure or a hydroxyl group on the C ring. Catechins are monomeric flavanols with a hydroxyl group at position 3 of C ring, phloroglucinol substitution on A ring, two or three hydroxyl group at position 3',4',5' of B ring. Primarily four catechins are found in fresh tea leaves: (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (EG), and (-)-epigallocatechin gallate (EGCG) (Figure 2-1) (Higdon and Frei, 2003).

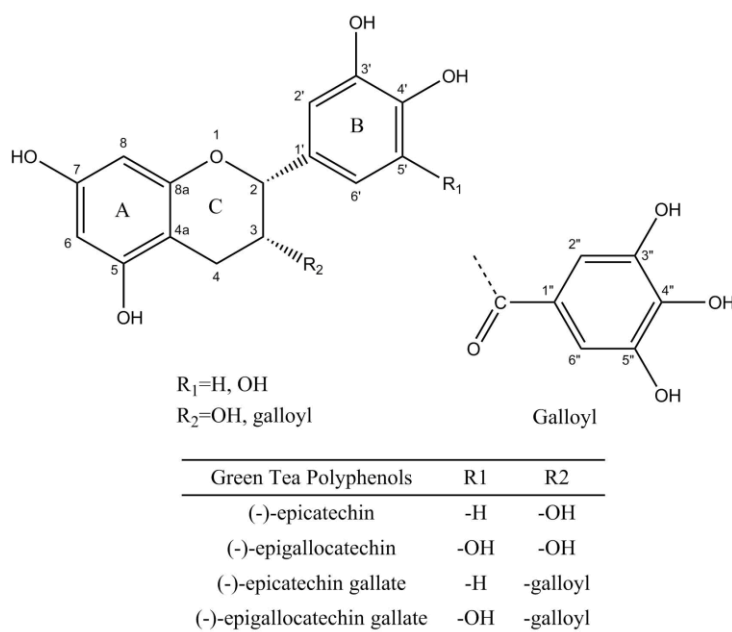


Figure 2-1 Chemical structure of four major catechins in green tea

Many studies have quantitatively addressed the pharmacokinetics of GTP since mid-1990s. Upon oral administration, the GTP are absorbed in upper gastrointestinal tract (Auger *et al.*, 2008; Stalmach *et al.*, 2010). Unlike other types of flavonoids, the four catechins could be absorbed from intestinal tract into hepatic portal vein in their original forms without degalloylation in rats (Okushio *et al.*, 1995, 1996). It is still inconclusive whether the catechins could be extensively conjugated via methylation, sulfation, or glucuronidation in intestine while they are absorbed (Piskula and Terao, 1998; Vaidyanathan and Walle, 2002; Crespy *et al.*, 2004). However, both animal and human studies have shown that, in plasma, EGC, EC presented (if not exclusively) in conjugated form, whereas EGCG and ECG mainly in its original forms (Lee *et al.*, 1995; Kim *et al.*, 2000; Chow *et al.*, 2001; Van Amelsvoort *et al.*, 2001; Stalmach *et al.*, 2010; Clifford *et al.*, 2013). The overall absorption of these catechins after oral intake can be estimated based on dosing experiments among ileostomists, which suggested that about 70-90% of

EC and EGC, whereas only 40-50% of EGCG or ECG were absorbed in intestine (Auger *et al.*, 2008; Stalmach *et al.*, 2010). It worth mentioning that some human studies provided in-direct evidence suggesting limited degalloylation may occur in oral cavity and intestine (Yang *et al.*, 1999; Van Amelsvoort *et al.*, 2001; Stalmach *et al.*, 2010), although some studies reported opposite findings (Chen *et al.*, 1997; Auger *et al.*, 2008).

Animal and human studies have shown a rapid distribution of GTP once absorbed. Most studies showed that EC, EGC would reach their peak concentration, measured as total catechins, in plasma in 1.5 to 2.5 hours after a single oral dose of either GTP mixtures or the two catechins separately (Yang *et al.*, 1998; van het Hof *et al.*, 1999; Van Amelsvoort *et al.*, 2001), and EGCG would reach its peak concentration in 2 to 4 hours depending on the dose administered (Yang *et al.*, 1998; Chow *et al.*, 2001). The peak concentrations and total recoveries from plasma were higher for EC and EGC than EGCG in most studies (Yang *et al.*, 1998; Van Amelsvoort *et al.*, 2001). A study administered a mixture of GTP containing EC, EGC, EGCG intravenous (i.v.) showed that ECG and EC were absorbed faster than EGCG which tended to distribute to peripheral compartment (Chen *et al.*, 1997). Corresponding to their fast absorption, EC, EGC and EGCG tend to be rapidly eliminated from the blood stream suggested by a half-life generally within 6 hours (Yang *et al.*, 1998; Van Amelsvoort *et al.*, 2001) and would not be detected after 24 hours of a single dose (Van Amelsvoort *et al.*, 2001; Stalmach *et al.*, 2010). Relatively few studies reported the disposition of ECG; one human study reported that ECG took longer to reach a peak concentration after a single oral dose, took longer to be eliminated, and existed in blood stream even after 24 hours (Van Amelsvoort *et al.*, 2001).

GTP and their metabolites are excreted either via urine or bile acids. The current consensus is that urine is the major excretion route for EGC and EC, but exclusively in conjugated forms (Lee *et al.*, 1995; Yang *et al.*, 1998; van het Hof *et al.*, 1999; Kim *et al.*, 2000; Chow *et al.*, 2001; Auger *et al.*, 2008; Clifford *et al.*, 2013). However, the proportion of urine excretion that accounts for the ingestion varied from 2% to as high as 28.5% in different studies (Lee *et al.*, 1995; van het Hof *et al.*, 1999; Clifford *et al.*, 2013). Because of the facts that EGCG would be rapidly eliminated from blood circulation but could not be detected in urine, it is speculated that EGCG would be mainly excreted via bile acids (Chen *et al.*, 1997; Clifford *et al.*, 2013), possibly EGC and EC as well (Chen *et al.*, 1997). One study which analyzed the bile acids composition after administered EGCG to experimental rats orally showed that EGCG and its metabolites in bile acids only accounted for about 3% of overall intake (Kida *et al.*, 2000). On the contrary, another study dosed radiolabeled EGCG i.v. to experimental rats showed that nearly 77% of intake was recovered in bile (Kohri *et al.*, 2001).

2.1.2 Metabolism of Green Tea Polyphenols

Metabolism of a xenobiotic compound by an organism typically comprises two phases. During phase I, oxidation, reduction and hydrolysis typically occur to convert the compound to be more polar and active. And during phase II, conjugation with other more water soluble moieties likely occurs so that the compound can be easily excreted. Most mammals, including humans, are only capable of metabolizing the catechins via phase II reaction, more specifically, methylation, glucuronidation, and sulfation (Feng, 2006). Nevertheless, phase II metabolites of catechins are numerous due to the many substitution sites on the aromatic rings. With the help of microorganisms commensally

resident within the gastrointestinal tract of human and animals, catechins could be broken down into smaller molecules via phase I reactions. Feng has provided a thorough review on the metabolism of green tea catechins (Feng, 2006), related contents of which is summarized below and updated with new information after that publication.

2.1.2.1 Phase II reactions

Studies of the activity of conjugative enzymes using liver, intestinal microsomes and cytosols *in vitro* and analysis of metabolites in urine or plasma of rat and humans *in vivo* have identified many catechin phase II metabolites although structures of some metabolites (e.g. specific substitution site) are still unknown.

2.1.2.1.1 EC and EGC

The metabolism of EC and EGC is quite similar, probably due to their structural similarity. In rats, after oral ingestion of green tea catechins, glucuronide, sulfate, along with methyl-glucuronide or methyl-sulfate conjugates of EC or EGC could be detected in plasma and urine (Feng, 2006). Methylation-only metabolites were not detected, suggesting methylation might probably occur prior glucuronidation or sulfation (Clifford *et al.*, 2013). Monoglucuronide, or methyl-glucuronide appears to be the major metabolites for EC, and monoglucuronide, and monosulfate were the major metabolites for EGC in rat urine (Li *et al.*, 2001). Methylation and glucuronidation may occur at the small intestine and liver in rat as shown in *in vitro* studies (Kuhnle *et al.*, 2000; Donovan *et al.*, 2001) and a recent *in vivo* study using radiolabeled C¹⁴ (Borges *et al.*, 2016). Sulfation may occur in liver (Donovan *et al.*, 2001), however the recent *in vivo* study did not detect sulfated EC in liver tissues (Borges *et al.*, 2016), which might not be

unexpected given a previous *in vitro* study has shown that sulfation is less efficient in rat (Vaidyanathan and Walle, 2002).

In humans, EC could be readily conjugated with glucuronide, sulfate and methyl group, or their combination; and EGC-3'-O-glucuronide is the major metabolite for EGC (Li *et al.*, 2001; Feng, 2006). It worth mentioning that an *in vitro* study initially reported no glucuronidation of EC or EGC in humans despite the fact that human liver and intestine tissues could express some UDP-glucuronosyltransferase (UGT) isoenzymes, and suggested that sulfation is the major phase II reaction of EC in humans (Vaidyanathan and Walle, 2002). However multiple reports identified glucuronides of EC and EGC in human urine (Li *et al.*, 2001, 2000) and plasma (Actis-Goretta *et al.*, 2013) in *in vivo* studies.

Although there are four hydroxyl group on EC, to which the conjugating moiety can bind, O-methylation has only been reported C3' and C4' on B ring, and O-glucuronidation on C5 and C7 on A ring and C3' on B ring. It is interesting that so far glucuronic conjugation was only detected on C7 of EGC A ring and C3' of B ring, and methylation was only detected on C4' of B ring (Feng, 2006). It is still unclear which position sulfate would bind to.

2.1.2.1.2 ECG and EGCG

EGCG is the most abundant constitute in green tea, accounting for more than 50% of the total catechins (Feng, 2006). The metabolism of EGCG and ECG is more complicated due to the addition of a gallic acid at C3 of C ring of catechin backbone. Surprisingly, there have not been many reports on the metabolic fate of ECG after oral intake in rats. One study dosed the rats with ECG i.v. and found mono- and di-

methyated ECG in the bile (Kohri *et al.*, 2003). Of note, these results were obtained after treatment with glucuronidase/sulfatase. In this same study, the authors dosed the rats using gastric gavage and analyzed the metabolites of ECG in urine after treatment with glucuronidase/sulfatase. No original, or methyated ECG was detected in urine (Kohri *et al.*, 2003). A similar study on EGCG from the same research group showed that after administering EGCG i.v., mono- and di- methyated EGCG were identified in bile (Kohri *et al.*, 2001). Di-methyated EGCG was also detected in the plasma of rats which were dosed with 0.6% green tea preparation orally (Meng *et al.*, 2002). Glucuronidation and sulfation of ECG and EGCG have not been studied explicitly in rats, although several reports indicated the existence of glucuronide and sulfate conjugates of ECG and EGCG (Kida *et al.*, 2000; Lu *et al.*, 2003; Feng, 2006).

In human, no metabolites of ECG were observed in either plasma or urine (Feng, 2006), probably due to degalloylation mentioned previously. Currently, only di-methyated EGCG were reported in human plasma and urine explicitly after oral intake of tea catechins (Meng *et al.*, 2002). Again, glucuronidation of EGCG probably occurs in human given results from *in vitro* study of enzyme activity (Lu *et al.*, 2003).

Multiple positions on ECG and EGCG could be conjugated. O-methylation is mostly reported on C3', C4', C5' on ring B and C4'' on ring D; O-glucuronidation on C7 of ring A, C3' of ring B, and C3'', C4'' of ring D.

2.1.2.2 Phase I reaction

Currently, reports of Phase I metabolism by human and animals are very limited, except one human study suggested human saliva esterase could hydrolyze EGCG to EGC (Yang *et al.*, 1999). Although mammalian genomes do not possess the capability of

degrading green tea catechins, the microorganisms residing in the intestine could readily metabolize the catechins through Phase I reactions (Schantz *et al.*, 2010), the importance of which has been increasingly recognized. The microbial metabolism of the four catechins is described below combining the thorough summary provided by (Feng, 2006) and a recent report of EGCG (Takagaki and Nanjo, 2010) (Figure 2-2):

- (I) ECG and EGCG would firstly be degalloylated to produce gallic acid and EC or EGC. Multiple studies have shown that the conjugated form of pyrogallol, which is produced by decarboxylation of gallic acid, is the major microbial metabolites in plasma and urine for ECG and EGCG (Takizawa *et al.*, 2003; Kohri *et al.*, 2003).
- (II) Cleavage of the ether bond between C1 and C2 on C ring by hydrogenation would yield hydroxylphenyl-phloroglucinol-propan-2-ol, which may be partially dehydroxylated on B ring to produce various hydroxylphenyl moiety.
- (III) Ring fission of the phloroglucinol moiety (ring A) would produce phenolic 4-hydroxyl valeric acids, which might be converted to a phenolic dihydrofuran through lactonization reaction.
- (IV) Phenolic 4-hydroxyl valeric acids could be transformed to phenolic valeric acids by dihydroxylation.
- (V) Phenolic valeric acid could then be converted to phenolic propionic acid and to hydroxybenzoic acid, in a fashion similar to β -oxidation in fatty acid metabolism. The resulting benzoic acid might be bind to glycine to form hippuric acid.

During the process, valerolactone, valeric, and propionic acid metabolites could be readily conjugated with methyl, glucuronide, and sulfate moieties to form phase II metabolites, which are commonly detected in human and animal urines (Feng, 2006; Li *et al.*, 2001).

2.1.3 Beneficial effects of GTP

Oxidative damage has been the central to the pathology of many chronic diseases (Higdon and Frei, 2003). The antioxidative potential of GTP has attracted many research to examine its effects on disease prevention, mostly focusing on cardiovascular disease and cancer.

2.1.3.1 *Cardiovascular disease*

Many human epidemiological studies have evaluated the relationship between tea consumption and the risk of cardiovascular disease (CVD). An thorough summarization of studies prior to 2003 could be found in a comprehensive review by Hidgon and Frei (Higdon and Frei, 2003). These authors suggested that the protective effects of tea consumption (mostly black tea) was still inconclusive given most studies failed to establish an inverse relationship (Higdon and Frei, 2003). However, the only three epidemiological studies focusing on green tea consumption consistently revealed a protect effect of green tea on general CVD risk (Sasazuki *et al.*, 2000; Nakachi *et al.*, 2000) and on incidents of stroke (Sato *et al.*, 1989). A protective effect from green tea consumption but not black tea was also confirmed by two recent meta-analysis (Wang *et al.*, 2011; Pang *et al.*, 2016). The main mechanism for the protective effects of tea catechins on CVD is suggested related to the inhibition of oxidation of low density lipoprotein (LDL) (Scalbert *et al.*, 2005). Some other mechanisms might also contribute

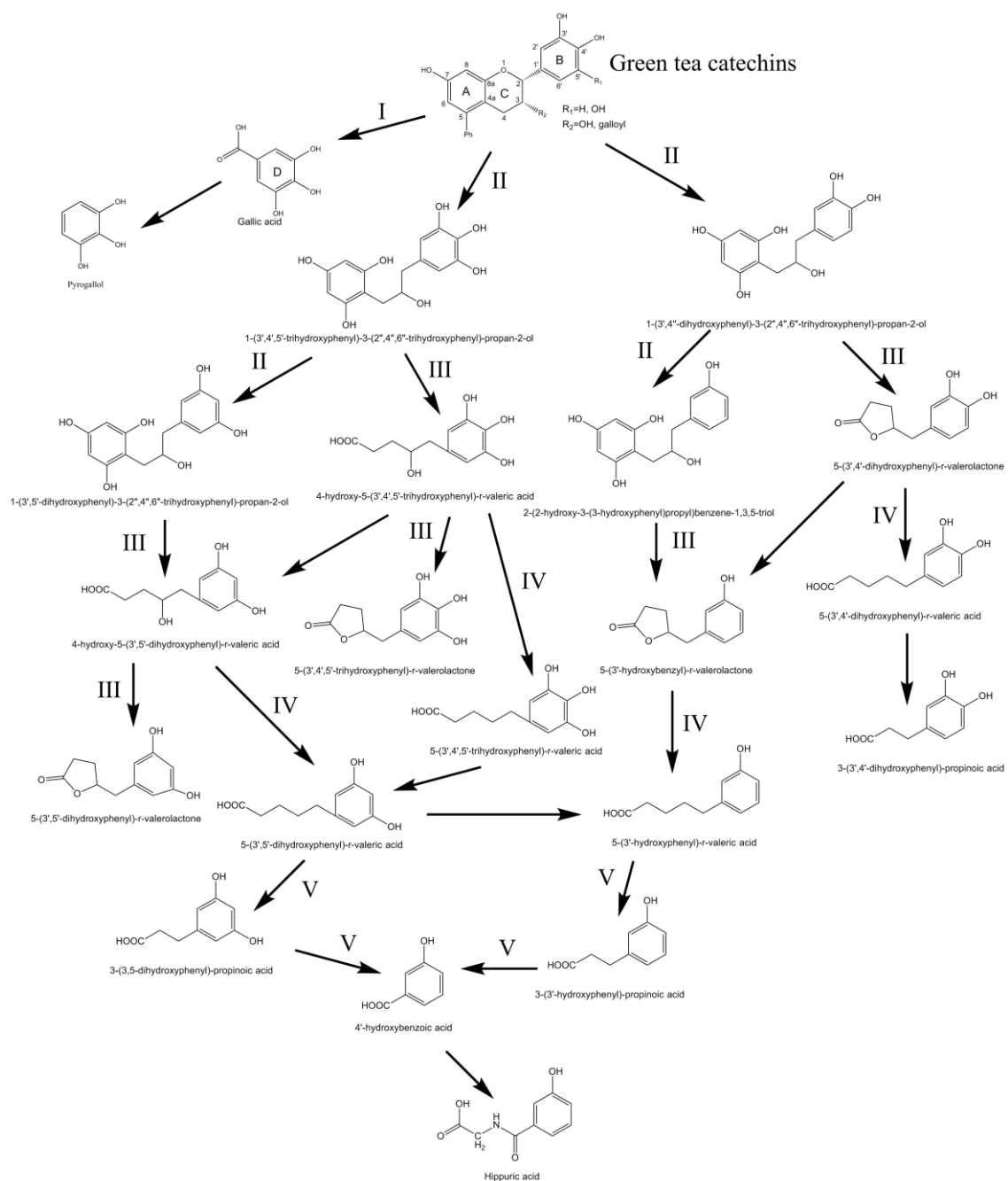


Figure 2-2 Microbial metabolism of green catechins

such as inhibiting the invasion and proliferation of the smooth muscle cells in the arterial wall, antithrombotic effects, improving endothelial function and effects of lipid metabolism (Scalbert *et al.*, 2005).

2.1.3.2 Cancer

Comparing to CVD, the relationship between tea consumption and cancer risk is far less conclusive, despite multiple animal studies have shown an anticarcinogenic effects of polyphenols (Scalbert *et al.*, 2005). The dominant amount of epidemiological studies has been focused on black tea, the consumption of which might increase, decrease, or not affect the risks of many types of cancer (Higdon and Frei, 2003). The discrepancy of these studies might be due to the confounding factors, especially life-style factors (e.g. smoking). The studies on green tea most often yield non-significant effects on cancer risks (Higdon and Frei, 2003). However, a series of human perspective studies from our lab have been successfully established a protective effect of GTP on liver cancer incorporating biomarkers as surrogate endpoints, and therefore are summarized below (Wang *et al.*, 2015).

2.1.3.2.1 Phase II clinical trial with green tea polyphenols in Southern Guangxi, China

A randomized, double blinded, and placebo controlled, phase IIa chemoprevention trial was conducted in Guangxi, China to validate GTP biomarkers in human body fluids, study the modulative effects of GTPs on carcinogen biomarkers, and to directly examine the possible adverse effect of GTPs in human subjects (Wang *et al.*, 2003; Huang *et al.*, 2004). The study screened 1,200 blood samples for hepatitis B virus (HBV) and aflatoxin (AF) biomarkers and recruited 124 residents who were both HBsAg and AF-albumin adducts positive, aged 20-55 with normal liver function test, serum alpha-fetoprotein negative, no personal history of cancer, and no use of prescribed medications. These subjects were regarded to have higher risk of liver cancer. Two doses of GTP (500 mg and 1000 mg, equivalent to two and four cups of tea drink, respectively)

and placebo were administrated to three groups. Initial studies did not find significant differences on adverse effects and parameters representing liver and kidney function among three groups, indicating the relative safety of GTP in human subjects subjects (Wang *et al.*, 2003; Huang *et al.*, 2004).

2.1.3.2.2 Validation of green tea polyphenols biomarkers in human population levels

Several follow-up studies (Luo *et al.*, 2004; Luo, Cox, *et al.*, 2006; Wang *et al.*, 2008) were conducted to validate the use of GTP biomarkers in human studies based on previously mentioned phase II clinical trial. A total of 340 urine samples collected at day 0 (baseline), 1- and 3-month of the clinical trial were analyzed. Urinary excretion of GTPs was adjusted by creatinine levels. Trace amounts of GTP components were detectable for all 3 groups at baseline with no statistical significance ($p=0.92$). Analysis of urine samples collected at 1- and 3-month revealed that levels of urinary EGC and EC were dose-dependently elevated in GTP-treated groups. Significant differences between times and groups of treatment ($p<0.05$) were also found. A total of 343 plasma samples collected at the same time points were analyzed. Trace amounts of GTP components were detectable for all 3 groups at baseline with no statistical significance ($p>0.33$). Analysis of samples collected at 1- and 3-month revealed a similar pattern as in urine sample: plasma levels of EGCG and ECG were dose-dependently elevated in GTP-treated groups. Significant differences between times and groups of treatment ($p<0.05$) were also found. These results demonstrated that urinary excretion of EGC and EC and plasma levels of EGCG and ECG can serve as valid biomarkers for green tea consumption, and may be applicable in future intervention and epidemiologic studies (Luo *et al.*, 2004). Luo *et al* further confirmed previous results by a metabolomics study in which 106 metabolites of

GTP were identified, and 56 of them were chosen to construct discriminant functions (DFs) based on the data at 1- and 3- months. Overall this study found metabolic profiles effective in discriminating different GTP dosages (Luo, Cox, *et al.*, 2006).

2.1.3.2.3 Modulation of carcinogen biomarkers by green tea polyphenols

Reactive oxygen species (ROS), produced in the process of carcinogen metabolism, inflammation, and aerobic respiration, can attack all macromolecules including lipids, proteins and DNA. Urinary 8-hydroxydeoxyguanosine (8-OHdG) generally reflects the whole body's oxidative DNA damage and repair, and becomes a putative biomarker for oxidative stress (Pilger *et al.*, 2001). Detection of urinary 8-OHdG provides a sensitive and non-invasive means to evaluate the efficacy of chemoprevention. Urinary excretion of GTPs and 8-OHdG were therefore analyzed in samples collected from the phase II clinical trial (Luo, Tang, *et al.*, 2006). At the end of 3-month intervention, 8-OHdG levels decreased significantly in both GTP-treated groups, suggesting that clinical intervention with GTPs was effective in diminishing oxidative DNA damage (Luo, Tang, *et al.*, 2006).

The mediation effects of GTP among subjects with HBsAg and AF-albumin adducts positive (high liver cancer risk subjects) were also assessed in above phase II clinical trial (Tang *et al.*, 2008). The AF-albumin adduct levels in serum samples collected at 1-month of the intervention was significantly decreased in the high-dose group ($p < 0.05$) as compared with level in the placebo group. The levels of AF-albumin adducts in serum samples collected at 3-month of the intervention showed significant decrease in the low- and the high-dose groups as compared with the levels of the baseline values ($p < 0.05$). Levels of AFM₁ and AFB₁-mercapturic acid (AFB₁-NAC) in urine

samples collected at 3-month of the intervention showed that treatment of GTP for up to three months significantly decreased levels of AFB₁ in both low-dose and high-dose intervention groups. Treatment of GTP elevated levels of AFB₁-NAC in GTP-treated groups (Tang *et al.*, 2008). These results demonstrate that treatment with GTP effectively inhibits phase I metabolic enzyme activities and induces phase II metabolic enzyme activities.

2.1.3.2.4 Long-term clinical trial with green tea polyphenols in Southern Guangxi, China

Current short-term evidence showed that taking GTPs does not have obvious negative effects, elevates plasma and urinary GTP components, and mediates oxidative stress and biomarkers of high liver cancer risk. Long-term clinical trial was therefore warranted. Yu *et al.* initiated a 3-year randomized, double blinded and placebo control study in 2004 (Yu *et al.*, 2006). This study screened 10,000 adult people and recruited 1,826 HBsAg positive adults with normal liver function test, serum alpha-fetoprotein negative, no personal history of cancer, and no use of prescribed medications, in Guangxi, China. Two capsules (500 mg GTP, or placebo), were instructed to be taken twice daily after meal. The efficacy of the trial is measured as the ability of GTPs to decrease the levels of serum and urinary AF biomarkers and urinary 8-OHdG within the first year. The efficacy of GTPs will be eventually measured as the ability to reduce actual incidence of hepatocellular carcinoma (HCC) in the studied population at the end of three years of intervention. The HCC incidence rate for years 2 and 3 was significantly reduced in GTPs-treated group (443.46/100,000 person year) as compared to that of placebo group (1092.39/100,000 person year) (p-one-sided=0.039) (Yu *et al.*, 2006).

2.1.3.3 *Body weight control*

Extensive studies have evaluated the weight loss and weight management effects of green tea as a natural diet supplements. One meta-analysis of 11 human studies revealed that green tea catechin has a small positive effects on weight loss and weight management and the effects might be modified by ethnicity and caffeine consumption (Hursel *et al.*, 2009). Another meta-analysis of 15 studies later showed that the positive effects on weight loss of tea catechins were only present when caffeine was co-administered and caffeine acted as a synergistic agent to catechins because the co-administration of catechin and caffeine resulted in a larger weight loss compared to caffeine alone (Phung *et al.*, 2010). However, it is worth pointing out that the conclusion that GTP alone did not have an effect on weight loss were drawn from only 2 studies with opposite results (Takeshita *et al.*, 2008; Hill *et al.*, 2007). A closer look at the study which reported a gain of body weight after tea catechin consumption (EGCG to be exact) (Hill *et al.*, 2007) showed that there was an insignificant gain of total body mass (0.08 kg) compared to baseline in group given a commercial product of EGCG, but an insignificant but larger loss of total body mass (0.45 kg) compared to baseline in the placebo group. Moreover, in fact, among the 11 articles that were included in the previous meta-analysis (Hursel *et al.*, 2009), all 8 studies which were conducted among Asian population with low caffeine intake reported an loss of body weight due to tea catechins. A recent systematic review evaluated studies conducted in Japan and outside separately and found that studies outside Japan revealed a small but insignificant favorable effect of GTP as compared to a larger effect found in studies among Japanese (Jurgens *et al.*, 2012). Two recent studies in Europe reported that GTP did not have

significant effect on body weight management (Mielgo-Ayuso *et al.*, 2014; Janssens *et al.*, 2015), even though GTP extract was administered with caffeine in one study (Janssens *et al.*, 2015). The two recent studies both administered GTP to the volunteer for 12 weeks which might not be sufficient given the low dose that were given and the diversified human diet. Further studies are need to further clarify the weight management effect of GTP.

2.1.3.4 *Other health benefits*

Many studies have shown a preventative role of tea consumption on osteoporosis (Shen *et al.*, 2013). Several *in vitro* studies revealed that components of tea polyphenols could inhibit the formation ,differentiation and enhance the apoptosis of osteoclast cell which is responsible for bone resorption, and enhance the survival, proliferation, differentiation and mineralization of osteoblast cell which is responsible for bone formation (Shen *et al.*, 2013). These findings were further supported by multiple animal studies using various bone-loss models (Shen *et al.*, 2013). However, the results of human epidemiological studies have been inconsistent which are mixed with positive relationship between tea consumption and bone mineral density and generally insignificant findings (Higdon and Frei, 2003; Shen *et al.*, 2013).

2.2 Aflatoxin B₁

2.2.1 Absorption, Distribution, Excretion of Aflatoxin B₁

The toxicokinetics of AFB₁ have been extensively studied in various species since 1960s when the specific structure of AFB₁ was elucidated and confirmed via total synthesis (Wogan *et al.*, 2012). A literature in 1994 has provided a thorough review on

the fates of AFB₁ in humans and animals (Cullen and Newberne, 1994), which was summarized below with updated information.

AFB₁ is a relatively small and lipophilic molecule, making it efficiently absorbed through gastrointestinal (GI) tract after oral administration, mainly in small intestine through passive diffusion and most efficient from duodenum (Kumagai, 1989; Cullen and Newberne, 1994). The absorption of AFB₁ in small intestine is largely complete because multiple reports on various species have shown similar toxicokinetics of AFB₁ after oral administration as compared to intraperitoneal (i.p.) or i.v. (Wogan *et al.*, 1967; Cullen and Newberne, 1994). Although being efficient, the passage of AFB₁ through GI tract is relatively slow (Wogan *et al.*, 1967), which was likely due to extensive metabolism of AFB₁ in intestine tissues (Cullen and Newberne, 1994).

Absorbed AFB₁ and its metabolites would be transported into liver where further metabolism occurs. A fraction of AFB₁ metabolites would then be excreted via bile acid back into the small intestine, completing the enterohepatic circulation, and would not be reabsorbed (Cullen and Newberne, 1994). The rest of AFB₁ and metabolites in liver would then either enter the systematic circulation to distribute into other organs and tissues, or deposit into liver tissue. Liver is the major organ for concentrating AFB₁, followed by kidney to a lesser extent (Cullen and Newberne, 1994). AFB₁ can stay in the liver for an extended period, mainly irreversibly bound to tissue macromolecules (Cullen and Newberne, 1994). Several studies have shown that considerable amounts of AFB₁ metabolites, up to 13.6% of administered dose in monkey, could be detected in liver about 100 hours after the initial dose (Wong and Hsieh, 1980; Dalezios and Wogan, 1972).

In plasma, AFB₁ would be rapidly eliminated; the biological half-life among monkey, rat, and mouse ranged from 36.5 minutes to 12.9 minutes (Wong and Hsieh, 1980). The excretion of AFB₁ and its metabolites, however being relative slow, occurs mainly through biliary and urinary pathway (Cullen and Newberne, 1994). Wong and Hsieh (1980) reported that the total excretion of dosed AFB₁ i.v. ranged from 80% to 72% in monkey, rat and mouse, 100 hours after the initial dose. Multiple studies have shown that biliary excretion accounts for about 40-50% of ingested AFB₁ doses in conjugated forms in various species (Coulombe and Sharma, 1985; Wong and Hsieh, 1980). The major AFB₁ metabolite excreted in bile is AFB₁-glutathione (GSH) conjugated in SD rats (Holeski *et al.*, 1987). Urinary excretion accounts for about 10-20% of ingested AFB₁ (Raj and Lotlikar, 1984; Groopman *et al.*, 1988; Cullen and Newberne, 1994), among which AFM₁ is the major metabolites. Other urinary metabolites include AFP₁, AFB₁-N⁷-guanine adduct, and less amount of thiol metabolites, AFB₁-GSH, AFB₁-Cys-Gly adduct (Cullen and Newberne, 1994). Besides biliary and urinary pathway, AFB₁ metabolites may be excreted through milk, which prompts significant food safety concerns (Cullen and Newberne, 1994). Most studies reported the amount of AFM₁ excreted in milk was about 1% of ingested AFB₁; the percentage could range from 0-4% (Cullen and Newberne, 1994).

2.2.2 Metabolism of AFB₁

Metabolism of AFB₁ follows the classic two phase scheme. The phase I reaction, as previously mentioned in metabolism of GTP, makes the AFB₁ more polar by oxidization, reduction and hydrolysis, and the phase II reaction conjugates AFB₁ with some water soluble moieties. Metabolism of AFB₁ could occur in small intestine, liver,

and kidney. As mentioned in the previous section, the biotransformation of AFB₁ in small intestine appears to be an important pathway for AFB₁ detoxification.

2.2.2.1 *Monohydroxylation*

In phase I, AFB₁ could be readily hydroxylated by cytochromes P450 into three major metabolites (see Figure 2-3): AFQ₁, AFB_{2a}, and AFM₁. AFQ₁ is formed by 3 α -hydroxylation, which is mainly catalyzed by CYP1A2 and 3A4 (Ueng *et al.*, 1998; Diaz and Murcia, 2011). Although AFQ₁ is a minor metabolite of AFB₁, this transformation is an important detoxification pathway because AFQ₁ is much less toxic than AFB₁ and is not mutagenic (Diaz and Murcia, 2011). AFB_{2a} could be formed by hydration of the double bond between C8 and C9 on AFB₁, probably catalyzed by a CYP isoform because this reaction was identified by incubating of aflatoxins with liver microsomal preparations from avian and mammalian species (Patterson and Roberts, 1970). It is generally considered as a detoxification metabolite because AFB_{2a} is unstable and tends to form Schiff bases with protein, which is less active (Patterson and Roberts, 1970; Diaz and Murcia, 2011). AFM₁ is the major metabolite excreted in bile, urine, and milk. First isolated as an AFB₁ metabolite from milk of cows and rats fed an AFB₁-containing diet (de Jongh *et al.*, 1964) with its structure elucidated shortly after (Holzapfel *et al.*, 1966), In human, AFM₁ is formed by hydroxylation of C9a on AFB₁, catalyzed by CYP1A2 (Ueng *et al.*, 1998). This reaction is also found in many non-mammal

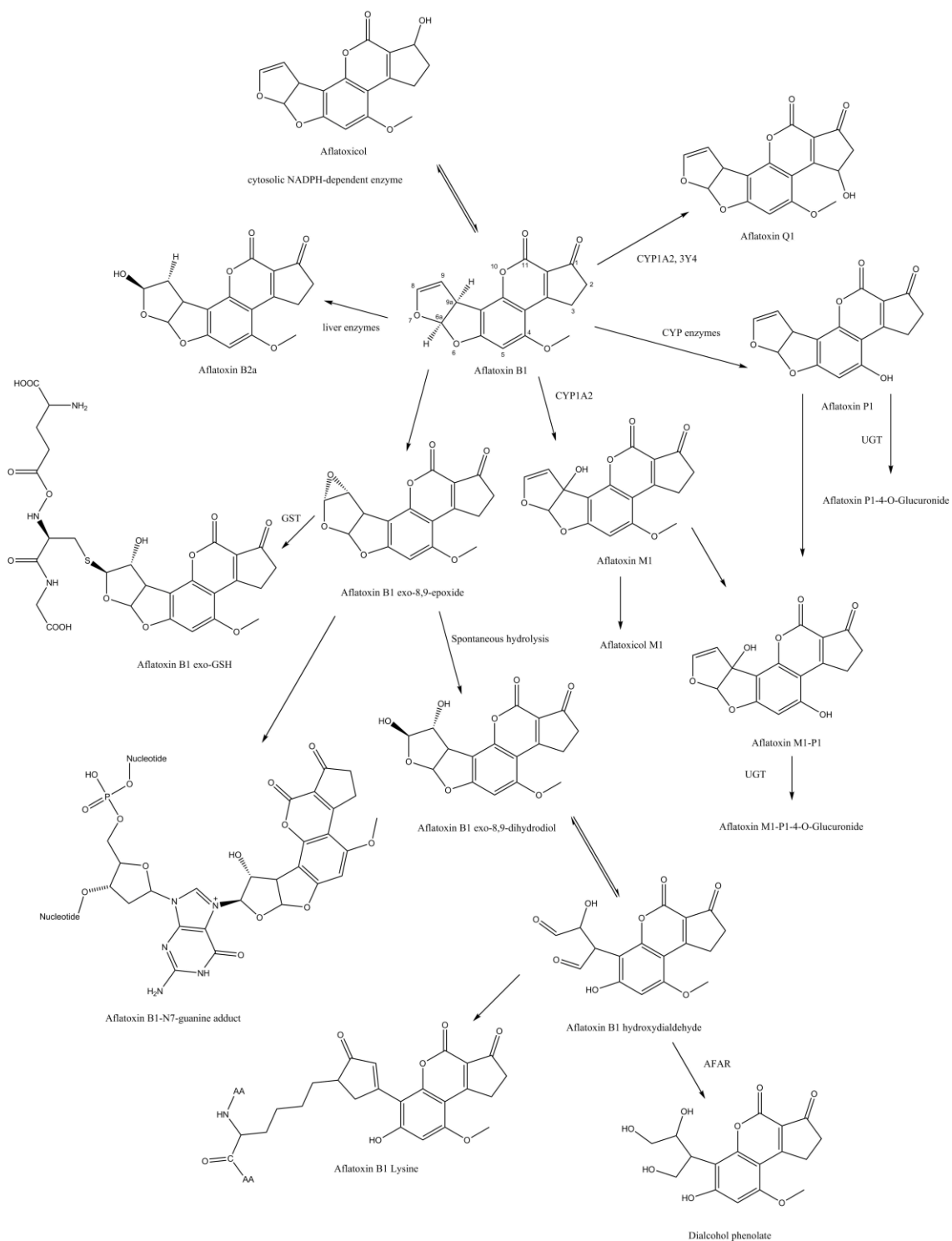


Figure 2-3 AFB₁ metabolism pathways

species (Diaz and Murcia, 2011). The hydroxylation of AFB₁ into AFM₁ is not necessarily a detoxification pathway because extensive studies have shown AFM₁ is a

relatively potent carcinogen and only slightly less toxic than AFB₁ in acute toxicity (Eaton *et al.*, 1994; Diaz and Murcia, 2011).

2.2.2.2 Reduction

The C1 keto group of AFB₁ can be reduced to a hydroxyl group to form cyclopentol aflatoxicol (AFL) (Eaton *et al.*, 1994), catalyzed by a cytosolic NADPH-dependent enzyme in liver of many avian and mammalian species except for mouse (Patterson and Roberts, 1972). Since AFL can be oxidized back to AFB₁ by liver cytosol or red blood cells from several species (Diaz and Murcia, 2011), its formation is not a significant detoxification pathway (Eaton *et al.*, 1994).

2.2.2.3 Demethylation

AFP₁ is formed by 4-O-demethylation of AFB₁ catalyzed by liver cytochrome P450 from many mammalian species (Dalezios *et al.*, 1971; Diaz and Murcia, 2011). It is still unclear which CYP isoenzyme is responsible for this reaction, but one study has shown that CYP321A1 in an earworm is capable of this biotransformation (Niu *et al.*, 2008). AFP₁ could be readily conjugated by UGT to form AFP₁-4-O-glucuronide and therefore considered a detoxification metabolite (Diaz and Murcia, 2011).

2.2.2.4 Epoxidation

The most critical step in AFB₁ metabolism is the formation of AFB₁-exo-8,9-epoxide (AFBO) because AFBO is electrophilic, unstable, highly active, and the active form for the carcinogenic and toxic activity of AFB₁ (Diaz and Murcia, 2011). The formation of AFBO is catalyzed by CYP1A2, 3A4, and possibly 3A5, 3A7 in human (Kamdem *et al.*, 2006; Dohnal *et al.*, 2014), CYP2A4 and 2A5 in mouse (Yanagimoto *et al.*, 1994), or by prostaglandin synthase, or lipoxygenases (Guengerich *et al.*, 1998). The

unstable AFBO may be bind to the N7 in the guanine base to form DNA adduct (Guengerich *et al.*, 2001), which is the main culprit for the carcinogenicity of AFB₁. In addition to AFBO, one study suggested that all aflatoxin derivatives with an unsaturated bond on the furofuran ring on AFB₁ can bind to DNA through epoxidation and DNA adducts of AFM₁ and AFP₁ have been identified in liver extracts of AFB₁ dosed rats (Essigmann *et al.*, 1983).

Epoxide group can also be hydrolyzed to form AFB₁ exo-8,9-dihydrodiol, which could undergo reversible ring-opening rearrangement of the furofuran on AFB₁ to form a hydroxyldialdehyde (Guengerich *et al.*, 1998; Diaz and Murcia, 2011). This dialdehyde could bind to the lysine residues in protein to form stable adducts (Guengerich *et al.*, 1998). Albumin is the only protein in serum that could be bind to AFB₁ metabolites to form aflatoxin-lysine adduct (Sabbioni *et al.*, 1987) which is a common biomarker for AFB₁ exposure in human epidemiological studies (Wang *et al.*, 1996). The AFB₁-dialdehyde could be further reduced by a liver AFB₁ aldehyde reductase (AFAR) to dialcohol and excreted (Guengerich *et al.*, 2001), serving as a detoxification pathway (Eaton *et al.*, 1994).

2.2.2.5 Conjugation

Phase II metabolism of AFB₁ is relatively simple: only two pathways have been identified so far (Diaz and Murcia, 2011). Activated AFBO can be bind to glutathione (GSH) by glutathione S transferase (GST), which is the major detoxication metabolites excreted in bile (Eaton *et al.*, 1994). AFP₁ and AFM₁-P₁ can be conjugated with glucuronic acid by UGT, a common and ubiquitous phase II reaction and served as a detoxification pathway (Diaz and Murcia, 2011).

2.2.3 Toxicity of AFB₁

The risk of AFB₁ exposure among living organisms mainly arise from chronic exposure to elevated AFB₁ levels in contaminated food. Nevertheless, AFB₁ is highly toxic for most animal species or extreme toxic for some susceptible species (Diaz and Murcia, 2011), and incidence of being accidentally exposed to lethal level of AFB₁ among human and animals occurred in history. In fact, AFB₁ was initially identified after an outbreak of acute hepatotoxic disease leading to the death of 100,000 turkeys near London, UK (Wannop, 1961). A recent outbreak of acute aflatoxicosis in 2003 claimed 120 lives in Kenya (Wogan *et al.*, 2012). Since identification and purification in 1960s, extensive literature has studied the toxicity of AFB₁, which revealed that animals of different species vary in its susceptibility to acute and chronic toxicity (Diaz and Murcia, 2011). In this section, current evidence regarding the acute and chronic toxicity of AFB₁ is summarized with a focus on human and rat. The dose (concentration of exposure), duration, targets and specific endpoints are provided whenever these information is available.

2.2.3.1 *Acute toxicity*

The acute toxicity of AFB₁ measured by LD50 varies from 0.3 mg/kg for duckling and rabbits as the most susceptible species to 9-17.9 mg/kg in rodent species (i.e. mouse, rat, hamster) (Newberne and Butler, 1969; Patterson, 1973). It is interesting that, comparing to duckling, chick is more resistant to AFB₁ acute toxicity; its LD50 ranged 6.5-16.5 mg/kg (Patterson, 1973). Besides, in rats, younger animals appeared to be more susceptible to AFB₁ toxicity with an LD50 around 0.56 mg/kg (Newberne and

Butler, 1969). Acute exposure of lethal level of AFB₁ would cause lesions in periportal zone accompanied by biliary proliferation (Newberne and Butler, 1969).

Acute aflatoxicosis among human has been reported several times in history although not very common. Clinical manifestation of aflatoxicosis among humans include vomiting, jaundice, fever, abdominal pain and ascites, pulmonary edema, fatty infiltration, oedema of the feet, and necrosis of liver (IARC, 1993; Kensler *et al.*, 2011). In India, an incidence in 1974 associated with consumption of contaminated maize, which contained 6.25-15.6 mg/kg aflatoxins, claimed 106 fatalities among 397 patients (Krishnamachari *et al.*, 1975). An estimated 2-60 mg aflatoxins were consumed daily by the impacted population over the period of a month (Krishnamachari *et al.*, 1975). In Kenya, an outbreak of aflatoxicosis in 1981 caused 12 death out of 20 patients with hepatitis after consuming contaminated maize which contained 12 mg/kg AFB₁ (Ngindu *et al.*, 1982). The recent outbreak of aflatoxicosis in Kenya in 2004 claimed 125 deaths among 317 poisoned patients (Lewis *et al.*, 2005). In the maize and maize products samples collected from the markets in the affected regions, only AFB₁ was found (Wogan *et al.*, 2012). The geometric mean of total aflatoxin level was 52.91 µg/kg and the daily exposure was estimated to be 50 mg per person (Wogan *et al.*, 2012).

2.2.3.2 Chronic toxicity

The chronic toxicity of AFB₁ mainly lies on its carcinogenicity. AFB₁ is a procarcinogen which could be activated to the 8,9-epoxide, the ultimate carcinogen (Dragan and Pitot, 1994). As confirmed by both animal and human studies, the epoxide may bind to the N7-guanine to form DNA adduct which cause genotoxicity and induce tumor (Wogan *et al.*, 2012). Numerous studies have shown that aflatoxins exposure

would induce hepatic carcinoma since even before aflatoxin was isolated (Newberne and Butler, 1969; Kensler *et al.*, 2011). Using purified aflatoxin, Barnes and Butler firstly reported that a dose of 1.75 mg/kg AFB₁ in diets induced liver cancer in 3 rats after 89 days (Barnes and Butler, 1964). Another study showed that feeding rats at a dose as low as 15 µg/kg induced hepatocellular carcinoma in all male Fisher rats after 68 weeks and all female rats after 80 weeks (Wogan and Newberne, 1967). In addition to liver cancer, tumors at colon, glandular stomach, and kidney have also been reported (Dragan and Pitot, 1994). In human, several epidemiological studies have established a link between AFB₁ exposure and liver cancer (Ross *et al.*, 1992; Wu *et al.*, 2005). Hepatitis B virus infection is typically presented with elevated AFB₁ exposure, which exerted an additive effect to hepatocellular carcinoma risk (Wu *et al.*, 2005). In addition to cancer, chronic exposure to AFB₁ has also been reported to cause growth suppression (Kensler *et al.*, 2011), immunosuppression (Bondy and Pestka, 2000; Qian *et al.*, 2014) in animal and humans.

2.2.3.3 *Species-dependent toxicity*

Responses of living organisms to acute and chronic toxicity of AFB₁ present a species-dependent pattern (Wogan, 1992; Cullen and Newberne, 1994). The range of sensitivity to AFB₁ exposure is wide even among genetically close-related species such as poultry species (Rawal *et al.*, 2010) and rodents (Dycaico *et al.*, 1996). Several factors have been summarized to potentially affect acute AFB₁ toxicity, including diet and nutrition, gender, some specific biomolecules, and other environmental factors (Cullen and Newberne, 1994). However, these factors could explain the variations observed

between different studies of the same species, but might not be able to fully explain the wide specific-dependent sensitivity.

Regarding the species-specific response of AFB₁, most studies suggested metabolism may be critical: the amount of AFB₁ being activated to AFBO other than other less toxic metabolites (e.g. AFP₁), and the amount of AFBO being conjugated by phase II enzymes (e.g. GST) (Cullen and Newberne, 1994; Diaz and Murcia, 2011). An *in vitro* study of the activity of liver microsomal enzymes of four poultry species showed that metabolism of AFB₁ could only partially explain the variation in species-specific sensitivity (Lozano and Diaz, 2006). In addition to metabolism, factors related to absorption of AFB₁ might also contribute to the variation. For example, one study showed that intestinal absorption rate of AFB₁ was considerably higher in suckling than in older rats, probably due to high lipid level in the intestines of suckling (Hsieh and Wong, 1994), which may be used to explain the much higher sensitivity to AFB₁ in suckling than older rats. In fact, young animals of all species are more sensitive to AFB₁ than their adults (Cullen and Newberne, 1994). The intestinal microbial community (microbiota) has gained increasingly attention due to their critical roles towards host physiology. The work in our group has shown that AFB₁ could modify the microbiota in a rat model (Wang *et al.*, 2016), which we speculated might also contribute to its species-specific toxicity.

2.3 Current evidence on impacts of xenobiotics on the commensal microbiome

The impacts of xenobiotics on commensal microbiota are believed to be critical to host physiology. A recent review article provided an overview of the potential interactions between xenobiotics and intestinal microbiome (Lu *et al.*, 2015). In this

section, the current evidence on impacts of xenobiotics on the commensal microbiome are summarized in three loosely defined categories, potential environmental toxicants, pharmaceutical compounds (including antibiotic agents), and probiotic/prebiotic agents.

2.3.1 Potential Environmental Toxicants

2.3.1.1 Alcohol

Excessive alcohol consumption may lead to liver damage in which intestinal endotoxemia is a critical cofactor for the alcohol-induced pathogenesis (Nolan 1989; Mutlu *et al.* 2009). Multiple mechanisms have been proposed to cause endotoxemia, among which a dysbiosis of gut microbiota has been increasingly recognized (Mutlu *et al.* 2009). Among the earliest studies, Mutlu *et al.* gavaged adult male Sprague-Dawley (SD) rats with alcohol at a stable dose of 8 g/kg body weight (b.w.) /day for 10 weeks and analysed ileum and colon tissue-associated microbiota using a length-heterogeneity PCR of 16S rRNA genes from samples taken at 4, 6, and 10 weeks (Mutlu *et al.* 2009). A significant decrease of microbial community biodiversity was observed in colonic samples taken at 10 weeks, in accordance with a modification of microbial community compositions comparing to controls and samples collected in other times, as shown in principle coordinate analysis (PCoA). Their study suggested a dysbiosis of the colonic microbiota after 10 weeks of alcohol dosing. In the same study, the effect of a probiotic strain (*Lactobacillus rhamnosus* GG ATCC 53103 (LGG), 2.5×10^7 CFU/day) or a potential prebiotic (oats, 10 g/kg/day) together with alcohol were evaluated which showed no dysbiosis was present in colonic microbiota at 10 weeks (Mutlu *et al.* 2009). Alcohol-induced dysbiosis and the protective effects of LGG in gut microbiota were potentiated by a similar study which dosed male C57BL/6 mice (8-10 weeks old) with

5% (v/v) of alcohol in a liquid diet for 8 weeks using 16S rRNA gene sequencing analysis (Bull-Otterson *et al.* 2013). The study found that alcohol induced a significant increase in the gram-negative *Alcaligenes* and gram-positive *Corynebacterium* (Bull-Otterson *et al.* 2013).

2.3.1.2 Metals or Metalloid

Toxic metals are among the longest known toxicants due to its early application in human history. The fact that they are mostly nature occurring and ubiquitous distributed both in the environment and within anthropogenic products makes them the first to be evaluated when new information related to health endpoints is available. At present, most studies regarding toxic metals and microbiome focused on cadmium (Cd) and lead (Pb) exposure in animal models. One study dosed male Balb/c mice (8-9 weeks old) daily with 5 different doses of CdCl₂, from 23 mg/kg to 50 mg/kg, for 45 days in drinking water, and performed quantitative culture to assess bacterial counts of selected species using intestinal contents and biopsy samples collected at the end of the study (Fazeli, Hassanzadeh, and Alaei 2011). Reduced bacterial counts of all tested species in all dosing groups were recorded and the reduction was more profound in samples from small intestine than large intestine. Nonetheless, no dose-response relationship was established despite multiple doses were used (Fazeli, Hassanzadeh, and Alaei 2011). Similar results was also found in another study using quantitative PCR (qPCR) of 16S rRNA genes extracted from fecal pellets of female Balb/c mice (8 weeks old) dosed with 20 mg/kg or 100 mg/kg CdCl₂ in drinking water for 3 weeks (Y. Liu *et al.* 2014). The treatments reduced the ratio of *Firmicutes/Bacteroidetes*, several genes related to short-chain fatty acid (SCFA) production, and colonic concentrations of SCFA (Y. Liu *et al.* 2014). Both

Cd and Pb exposure were examined in a study using 16S gene sequencing analysis (Breton *et al.* 2013), in which 20 or 100 ppm of CdCl₂, or 100 or 500 ppm of PdCl₂ (equivalent to 3 or 15 mg/kg b.w. for Cd, and 15 or 75 mg/kg b.w. for Pd) were spiked into the drinking water of female Balb/c mice (6 weeks old) for 8 weeks. No significant effects of these two metals on the microbial community biodiversity were found at the end of the study. However, significant alternations of several bacterial families and genus were found in treated groups, although no alternations were found in any bacterial phylum (Breton *et al.* 2013). Much lower levels of CdCl₂ (1 to 10 mg/L, equivalent to 0.15 mg/kg b.w. to 1.2 mg/kg b.w.) were tested in drinking water of male C57BL/6 mice of 5 weeks old for 10 weeks (S. Zhang *et al.* 2015). Significant reductions of phylum *Firmicutes* and *γ-proteobacteria* starting from 4 weeks till the end of the study were recorded using qPCR analysis of 16S rRNA genes extracted from fecal pellets. However, only *Firmicutes* was significantly reduced in samples from cecal contents at the end of the study. Randomly selected cecal samples were analysed by 16S rRNA sequencing analysis and confirmed the decreases of *Firmicutes* and *γ-proteobacteria* in metal treated groups and a modification of community composition in the highest dosing group through PCoA analysis. These results highlighted the influence of experimental methods and sample matrix to the final results.

Arsenic (As) was the only metalloid that has been evaluated so far. In a study using 16S gene sequencing analysis, Lu *et al.* added 10 ppm of NaAsO₂ (equivalent to 1.5 mg/kg b.w./day) into the drinking water of female C57BL/6 mice of 6 weeks old for 4 weeks and found that the treatment significantly perturbed gut microbiota shown in PCoA analysis using fecal pellets collected at the end of the study (Lu *et al.* 2014).

2.3.1.3 Metal Nanoparticles

Metal nanoparticles have been widely used in consumer products mainly because of its large surface to mass ratio. Similar as bulk metals, concerns have been raised on the potential health impacts due to inhalation, ingestion or direct contact with these chemicals. The impacts of nanoparticles on the microbiome was also among the earliest to be examined among all potential toxicants. A study dosed 10-day old quails with hydrocolloidal Ag-nanoparticle (AgNP) at 0 to 25 mg/kg in drinking water for 12 days (the highest dose was equivalent to 10-25 mg/kg b.w./day) and collected cecal contents of the animals for quantitative culture of selected gut bacteria (Sawosz *et al.* 2007). Significant increases of gram-positive bacteria including some lactic acid bacteria, *Lactobacillus* spp., were only found in the highest dosing group. The promoting effect of lactic acid bacteria by AgNP was also documented in another study targeting fruit fly (*Drosophila melanogaster*) larvae cultured on a medium containing 450 µg/ml AgNP (Han *et al.* 2014). The microbiota in the midgut contents of fruit fly larvae grown on AgNP medium was dominated by *Lactobacillales* (mainly *L. brevis*) and had less diversity as compared to control group via 16S rRNA gene sequencing analysis (Han *et al.* 2014). Another study dosed adult zebrafish with AgNP or CuNP at 500 mg/kg in the feed (equivalent to 15 mg NP/kg b.w./day) for 14 days and collected digestive tract contents for 16S rRNA gene based denaturing gradient gel electrophoresis (DGGE) analysis (Merrifield *et al.* 2013). The microbial community composition of CuNP treated fish, but not AgNP treated fish, was different from control fish as measured by Bray-Curtis similarity.

2.3.1.4 Anthropogenic Chemicals

Some anthropogenic chemicals where were specifically designed to kill microorganisms were also examined due to concern of accidental human exposure through contaminated food or water source. One study dosed female SD rats (8 weeks old) with 40 or 100 mg/kg b.w./day of epoxiconazole, a fungicide, in feed for 90 days and showed an alternated gut microbiota with a decrease in *Firmicutes*, and an increase in *Bacteroidetes* and families *Lachnospiraceae* and *Enterobacteriaceae*, by analysing collected fecal samples using 16S rRNA sequencing analysis (Xu *et al.* 2015). Another study dosed male ICR mice (10 weeks old) with trichloroacetamide, a disinfection byproduct in drinking water treatment, at 3 different doses up to 5 mg/L (corresponding to a human equivalent dose of 968 mg/kg/day) for 90 days (Y. Zhang *et al.* 2015). Metagenomics sequencing and metabolomics were performed in samples collected at the end of the study, recording a decrease of biodiversity and *Firmicutes/Bacteroidetes* ratio following increasing doses. Similar experiment strategies were adopted in another study which dosed C57BL/6 mice (7 weeks old) with 4 mg/L of diazinon (equivalent to 0.6 mg/kg b.w./day), an organophosphate pesticide, in drinking water for 13 weeks (B. Gao *et al.* 2016). In addition to alternations in gut microbiota as revealed by 16S rRNA sequencing analysis, a decrease in the family of *Lachnospiraceae*, a possible short-chain fatty acids producers, and a decrease of *Firmicutes/Bacteroidetes* ratio were found in treated mice. A gender-specific response of gut microbiota after exposure to diazinon was suggested.

2.3.1.5 Natural Toxins

Mycotoxins are fungal secondary metabolites harmful to human and animals. Mycotoxin contamination has been a leading public health problem because a large world population, especially in developing countries, are exposed to uncontrolled amount of mycotoxins via contaminated foods (Bennett and Klich 2003; Wang *et al.* 2016). Wache *et al.* was among the earliest who assessed the effect of mycotoxins on the gut microbiome of animals (Waché *et al.* 2008). White pigs of 9 weeks old were fed for 4 weeks, in which deoxynivalenol (DON) was the major mycotoxin contained in the feed with a concentration of 2.8 mg/kg (Waché *et al.* 2008). DON had a moderate effect on cultivable bacteria from quantitative bacterial culture of fecal samples. But their effects on the community biodiversity indexes (dominance and richness) based on capillary electrophoresis single-stranded conformation polymorphism analysis were generally stable over the studying period, although several individual peaks from the gel (indicating certain microbial species) showed significant different peak areas between dosing and control groups. Another study inoculated germ-free SD rats (male, 8 weeks old) with fresh human fecal flora and treated these rats with 100 µg/kg b.w. of DON via gavage for 4 weeks (Saint-Cyr *et al.* 2013). Changes of several bacterial groups in the gut microbiota, such as *Bacteroides-Prevotella* and *Escherichia coli*, were recorded along the experiment period via qPCR analyses. But a lack of control group made it difficult to assess the effects of DON on their findings explicitly. A pilot study on the effects of aflatoxin B₁ (AFB₁) on the gut microbiota of male F344 rats of 5 weeks old were conducted (Wang *et al.* 2016), in which rats were dosed with 3 different concentrations of AFB₁ up to 75 µg/kg b.w. via gavage for 4 weeks and fecal samples were collected at the

end of the study. A significantly decrease of phylogenetic diversity and the number of identified operational taxonomic unit (OTU) but an increase of Shannon index were found in a dose-dependent manner via 16S rRNA sequencing analysis. These results may suggest that AFB₁ had depleted less abundance and rare species in the rat intestine, and the gut microbiota adapted to the toxin by maintaining the abundant species. Several phylotypes were modified by AFB₁ in a dose-dependent manner including two *Lactobacillales* species which had largest decrease while increasing AFB₁ doses (Wang *et al.* 2016).

Microcystin-LR, a toxic compound produced by cyanobacteria during toxic algae bloom, was evaluated in male SD rats (6 weeks old) at 1.0 mg/kg b.w., orally 4 times in one week (Lin *et al.* 2015). A microarray-based GeoChip analysis of bacterial and fungal functional genes was performed using fecal samples collected at the end of the study. Significantly modified microbial communities in treated group were found based on the similarity analyses of phylogenetic classification of the genes. However, the biodiversities were not different comparing treatment and control groups. Several gene abundances were modified, among which genes related to carbon and aromatics degradation were enriched (Lin *et al.* 2015).

2.3.2 Therapeutic Agents

2.3.2.1 Antibiotics

Humans are exposed to antibiotics not only for medical treatment but also through daily diet in which residuals remain from their use in agriculture (Francino 2015). The critical role of commensal microbiota in host physiology and health has prompted concerns over the interactions between antibiotics and commensal microbiota, which

mainly lie on two aspects: the disruption of commensal microbiota due to usage of antibiotics in early life stage may increase susceptibility to autoimmune disease in later life (the hygiene hypothesis) (Dethlefsen and Relman 2011), and the possible horizontal transfer of antibiotic resistance genes from commensals to opportunistic pathogens (van Schaik 2015). Consequently, the effects of antibiotics on the commensal microbiota are the most extensive and thorough studied in the field.

Animal Studies. Back *et al.* were among the first to assess the effects of antibiotics on gut microbiota using quantitative cultures of intestinal contents of male Wistar rats (Back *et al.* 1980). They dosed the rats orally with ampicillin sodium, neomycin sulphate, a mixture of neomycin and lincomycin, or rifampicin at 200 mg/kg b.w./day for 4 days, and found that ampicillin preferentially suppressed anaerobic microorganisms, neomycin suppressed aerobic flora, and the mixture of neomycin and lincomycin suppressed both aerobes and anaerobes. However, rifampicin did not reduce any components of the gut flora after 4 days of treatment. A time series analysis of gut flora contents collected each day after the beginning of rifampicin dosing revealed that the aerobic flora was in fact greatly suppressed at the first day but recovered in the following dosing days, suggesting that the rats developed aerobic flora resistant against rifampicin within the dosing period. On the other hand, time series analysis of the gut flora of ampicillin dosing group post the antibiotic treatment showed a recovery of gut aerobic flora. These results underscored the complexity of gut microbiome dynamics and importance of time series of analysis (Back *et al.* 1980).

The dynamics of microbiome were evaluated in a series of studies employing molecular biology techniques. One study dosed C57BL/6 IL-10^{-/-} mice (4-6 weeks old)

with a mixture of antibiotics (amoxicillin, 150 mg/kg b.w./day; metronidazole, 34.5 mg/kg b.w./day; bismuth, 9.25 mg/kg b.w./day) added into their chow for 10 days (Antonopoulos *et al.* 2009). A subset of mice was followed for another 2 weeks after completion of antibiotic treatment. The gut microbiota from mice dosed with antibiotics were significantly enriched with *Proteobacteria* (71% in treatment vs. 1% in control) measured by 16S rRNA sequencing analysis. After the 2-week recovery, the gut microbiota returned to a state highly reassembled that in controls as measured by Bray-Curtis similarity indexes (Antonopoulos *et al.* 2009). In another set of experiments in the same study, 75 mg/kg b.w./day of cefoperazone were added into drinking water and provided to the same strain of mouse for 10 days (Antonopoulos *et al.* 2009). After completion of antibiotic treatment, the authors evaluated the recovery process with or without adding a “donor” mouse from the control group into the cage of treated mouse. As expected, antibiotic treatment significantly lowered the bacterial load, biodiversity (measured by OTU counts) and altered the gut microbiota (measured by pairwise comparison using Bray-Curtis similarity index). However, after 6 weeks of recovery, the biodiversity and microbial compositions were quite different as compared to control samples, although bacterial loads of gut microbiota from the group without “donor” mouse returned to a level comparable to controls. On the contrary, the gut microbiota of mice with “donor” mouse highly reassembled that of the control mice (Antonopoulos *et al.* 2009). Another study treated male Lewis rat with a 1:1 mixture of Vancomycin and Imipenem in drinking water at a dose of 100 mg/kg b.w./day for 3 days, which drastically decreased bacterial load in the gut microbiota as measured by qPCR of partial 16S rRNA genes and altered the microbial community (C. Manichanh *et al.* 2010). After suspension

of antibiotics, the microbiota recovered after one month but did not fully return to a community similar to control even after 3 months post treatment, i.e. the recovered microbiota shared only a few phylotypes with controls (C. Manichanh *et al.* 2010). Similar results were present in another study investigating the effects of antibiotic treatment on the host susceptibility to *C. difficile* infection (CDI) (Theriot *et al.* 2014). C57BL/6 mice were treated with 75 mg/kg b.w./day of cefoperazone in drinking water for 10 days, and cecal contents were collected at 2 days and 6 weeks after the treatment. Before antibiotic treatment, the gut ecology was resistant to CDI, but the gut microbiota was significantly changed in samples collected at 2 days post treatment, and was fully susceptible to CDI; 6-week recovery made the gut microbiota of the mice returned to be more similar (but still significantly different) to the baseline and resistant to CDI. Alone with changes of microbiota, the metabolic environment was shifted to be more favorable to germination and growth of *C. difficile* after the treatment (Theriot *et al.* 2014). These results corroborated with a similar report using hamster as a model animal (Peterfreund *et al.* 2012). Besides rodent models, larval fathead minnow was studied as the model animal to evaluate the effects of environmental levels of triclosan on the fish gut microbiome (Narrowe *et al.* 2015). The larval fish were exposed to either 100 ng/L or 1000 ng/L of triclosan in tank water for 7 days, and in control solutions for another 14 days of depuration. Exposure of triclosan at environmental levels could disrupt fish gut microbiota as shown by significantly changed biodiversities and community composition in treatment groups; the effects diminished after 14 days of depuration, suggesting a recovery from antibiotic exposure. However, the measured biodiversity in samples

collected immediately after exposure (7th day) showed an increasing trend with dose, contrary to most of the previous findings (Narrowe *et al.* 2015).

Several studies assessed the effects of antibiotic treatment on the predisposition of obesity and diabetes. Effects of vancomycin and a mixture of broad-spectrum antibiotics (including streptomycin, colistin and ampicillin) on the gut microbiota of non-obese diabetic (NOD) mice were compared (Candon *et al.* 2015). The antibiotics were administered *in utero* to NOD mice until 40 weeks postnatal in drinking water at the dose of 0.2 mg/ml for vancomycin, and 7 mg/ml for the mixture, equivalent to 30, and 1050 mg/kg b.w./day for adult mice. Analyses of fecal samples collected at 8 week-of-age showed that antibiotic mixture significantly depleted the gut microbiota and altered the microbial composition. Vancomycin had a less severe but significant effects as well. Accelerated and increased incidence in type 1 diabetes in both treatment groups were recorded despite that vancomycin only partially depleted gut microbiota; however, this observation could only be found in male mice. The incidence in female mice did not deviate from controls, although the gut microbiota was similarly altered by antibiotics as in male mice (Candon *et al.* 2015). Another study compared the effects of vancomycin and ceftazidime using diet-induced obese (DIO) C57BL/6 mice (4 weeks old) (Rajpal *et al.* 2015), which were dosed with either vancomycin, or ceftazidime at 50, 150, and 500 mg/kg, daily in food for 2 weeks. Ceftazidime dosing groups significantly depleted gut microbiota upon the completion of antibiotic treatments, while vancomycin did not have an observable effect deviating from vehicle. Both antibiotics modified the microbial compositions, however, only mice in ceftazidime dosing groups had a favorable glycemic profile of glucose, changed the fat proportion, and reduced body weight. It requires

caution to interpret the results from this study because another treatment was included with 10% oligofructose saccharide (a potential prebiotic), which had a similar weight control effect as ceftazidime, yet distinct gut microbiota and comparable biodiversity as vancomycin and vehicle groups. Therefore, gut microbiota was the key modulator of obesity in this rodent model might be questionable.

In addition to obesity, some other diseases were investigated for linkage with microbiome. One study showed that 3-week treatment of enrofloxacin at approximately 40 mg/kg b.w./day in drinking water depleted both aerobic and anaerobic bacteria in 10-12 week old male DBA1 mice (a rheumatoid arthritis mouse model) via quantitative bacterial culture (Dorożyńska *et al.* 2014). The effects persisted even 18 days post-treatment until the end of the entire experiment. The depletion of the gut microbiota was correlated with an increased severity of collagen-induced arthritis and several proinflammatory cytokines. A cystic fibrosis (CF) model mouse Balb/c Cfr^{tm1UNC} was exposed to 0.2 mg/ml of streptomycin *in utero* until 12 weeks old to evaluate the role of gut microbiome on the development of CF phenotypes (Bazett, Bergeron, and Haston 2016). Treatment with streptomycin for 12 weeks significantly lowered the bacterial load than untreated controls as measured by qPCR of 16S rRNA genes. However, no antibiotic effect was found among wild-type mice. It was surprising that untreated wild-type mice had the lowest bacterial load, yet were significantly more diverse than Cfr^{tm1UNC} mice. The composition of the gut microbiota was modified by antibiotic treatment, which was ascribed to the changes in airway hyperresponsiveness and lymphocyte profile observed in transgenic mice.

Low dose antibiotic addition in feed is a common practice in agriculture to increase production of livestock. The effects of multiple broad-spectrum antibiotics including penicillin, vancomycin, the mixture of penicillin and vancomycin, or chlortetracycline at a sub-therapeutic dose used in agriculture (1 mg/kg b.w./day total antibiotics) in drinking water for 7 weeks were compared on the gut microbiota of weaning C57BL/6J mice (Cho *et al.* 2012). Antibiotics did not alter the overall microbial counts measured by either 16S rRNA or ITS gene copies from qPCR analysis. However, the ratio of *firmicutes* and *bacteroidetes* was higher in antibiotic treated groups as compared to control, alongside with increased adiposity and changes in key genes involved in metabolism of short-chain fatty acid. Another study also investigated low dose antibiotics use among piglets and documented shifts in gut microbial composition (Looft *et al.* 2012; Looft *et al.* 2014). Especially, an enrichment of *Escherichia coli* as well as many functional genes attributed to *E. coli* physiology were documented in the gut microbiota of piglets eating antibiotic-containing feeds (Looft *et al.* 2014).

Human studies. Several studies examined the effects of antibiotics on commensal microbiota while treating various diseases. The effects of 7-day treatment (twice per day) with a mixture of antibiotics (metronidazole (400 mg), clarithromycin (250 mg), and omeprazole (20 mg)) for *Helicobacter pylori* infection were examined among six patients (52-72 years old) with either gastric or duodenal ulcer (Jakobsson *et al.* 2010). The analyses of throat swab and fecal samples from treated patients using both 16S rRNA gene sequencing analysis and terminal-restriction fragment length polymorphism showed altered microbial community and decreased biodiversity in both sites shortly after the treatment was finished. The analyses of samples taken 1 year and 4 years after the

treatment recorded partial return of microbiota to their pre-treatment state in some cases (Jakobsson *et al.* 2010). It is of note that the community compositions varied significantly among the six individuals at the baseline, and it was unclear how similar the microbiota were among individuals after the treatment and following dates given the information provided in the article. The effects of two antibiotics, vancomycin (125 mg, 4 times per day for 10 days) and fidaxomicin (200 mg, 2 times per day for 10 days) as treatment for *Clostridium difficile* infection (CDI) were compared (Louie *et al.* 2012). Using qPCR of selected bacterial species, the total bacterial loads in CDI patients were significantly lower than healthy controls. And interestingly, only vancomycin but not fidaxomicin reduced the counts of most tested bacteria in addition to *C. difficile*. However, the CDI recurrence rate was significantly lower in fidaxomicin-treated patients as compared to vancomycin, suggesting that although both antibiotics effectively eradicated *C. difficile*, the microbiota maintaining effect of fidaxomicin during the treatment period accounted for the low recurrence rate (Louie *et al.* 2012). In addition to gut microbiota, one study evaluated the effects of levofloxacin on the nasal microbiome among patients accepted for acute exacerbations of chronic rhinosinusitis (Merkley *et al.* 2015). Aspirate and swab samples were collected before and after oral treatment with 500 mg or nebulized topical treatment with 90 mg levofloxacin, twice daily for 2 weeks. The bacterial loads decreased after the treatment as measured by qPCR of 16S rRNA genes. However, an increase in biodiversity was observed from swab samples, but not aspirate samples. This discrepancy was suggested to be due to the difference in treatment modalities for the patient (Merkley *et al.* 2015). Again, similar to what was seen in other human studies, large inter-individual variations in community compositions existed before and after treatment.

In addition, healthy human volunteers have been recruited to evaluate the specific effects of antibiotics on human microbiome in several studies. Among one of the earliest effort, Dethlefsen *et al.* conducted a comprehensive evaluation of the dynamics of gut microbiota in response to antibiotic treatment by recruiting three adult healthy human volunteers and followed for 10 months (Dethlefsen and Relman 2011). Two 5-day antibiotic treatment sessions were conducted, during which the volunteers took 1 grams of ciprofloxacin per day (~12.5 mg /kg b.w./day). Fecal samples were collected daily during the treatment sessions and less frequent between sessions, on which 16S rRNA sequencing analysis were performed. Ciprofloxacin treatment reduced the biodiversity and modified the compositions of gut microbiota in each dosing session. However, the baseline gut microbiota was quite different among subjects, and the direction of microbial composition changes were not congruent among subjects and even during the two sessions within same subjects. To make it more complicated, although the gut microbiota of each subject partially recovered during interim periods, but the recovery varied among subjects and was not congruent after the two sessions within same subjects (Dethlefsen and Relman 2011). The impact of postnatal antibiotics on the gut microbiome of preterm infants was evaluated in another study by taking two cross-sectional samples (10 and 30 days after birth) via fecal swaps (Dardas *et al.* 2014). Comparing infants with a history of 2-day to 7-10 days antibiotic administration, the gut microbiota of infants with the longer post-natal antibiotic treatment had a significantly lower biodiversity. However, the difference was not seen in samples taken at 30 days after birth, which was explained as continuing inhabiting of neonatal gut microbiota (Dardas *et al.* 2014). Long-term effects of multiple antibiotics on gut and salivary microbiome were assessed in one study using

16S rRNA sequencing analysis (Zaura *et al.* 2015). The persistence and magnitude of effects on gut microbiome varies significantly according to different antibiotics, among which clindamycin had the most aggregated and prolonged effects. Meantime, effects of the same exposure on saliva microbiome were transient and superficial, suggesting salivary microbiome was stable in response to antibiotic stress, which was consistent with a previous report (Jakobsson *et al.* 2010). It also worth noting that whichever antibiotics were administered, the gut and salivary microbiome eventually returned to reassemble pre-treatment states. A new antibiotic, GSK1322322, targeting bacterial peptide deformylase, was tested in a phase I clinical trial (Arat *et al.* 2015). Healthy human volunteers were administered this drug either intravenously (i.v.) or i.v. combined with oral administration. The comparisons of fecal samples taken before and at the completion of the treatment showed that only i.v. combined with oral administration, not i.v. alone, showed effects on gut microbiota. The biodiversity was decreased and the community composition were altered. This result, potentially linked to the routes of drug administration, may suggest that the changes in gut microbiome was a direct effect of antibiotics, not a result of a systematic changes. These findings will further justify the idea of modulating the microbiome to improve the health.

2.3.2.2 Other Drugs

In addition to antibiotics, a few other pharmaceutical compounds have been investigated. The effects of orthodontic treatment in combination with fluoride mouthwash on oral microbiota were studied among teenager volunteers (Koopman *et al.* 2015). A triple-blind clinical trial was conducted by assigning fluoride mouthwash containing a mixture of 100 ppm amine-fluoride and 150 ppm sodium-fluoride, or

placebo to subjects received orthodontic fixation. Supragingival plaque samples were collected before, during, and after the orthodontic appliances. Fluoride mouthwash had little effect on the biodiversity of oral microbiota during the study period as compared to the placebo group and across time, which coincided with the findings from several previous studies (Jakobsson *et al.* 2010; Zaura *et al.* 2015). Another study tested the effects of proton pump inhibitors on the predisposition of *C. difficile* infection among healthy human volunteers using 16S rRNA gene sequencing analysis (Freedberg *et al.* 2015). Treatment of 40 mg omeprazole (twice daily) for 4 weeks did not change the biodiversity or modify community composition of the gut microbiota in fecal samples. However, the families *Enterococcaceae* and *Streptococcaceae*, which were associated with increased risk for CDI, were increased by omeprazole.

2.3.3 Prebiotics or Probiotics

Scientific evidence on the beneficial effects of probiotics dates back to the early 1900s when lactic acid bacteria was reported to be associated with enhanced longevity among several human populations (Bron, van Baarlen, and Kleerebezem 2011). In a report from a joint working group of World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO), probiotic was defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO and WHO 2006). Although the application of prebiotics might date even longer, its concept was initially formalized in a 1995 article (Gibson and Roberfroid 1995) and recently refined as “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). Both definitions

reflect that the nature of beneficial effects of pro/prebiotics closely involve interactions with gut microbiota and the host intestinal microenvironment. Consequently, it is not surprising to find a large volume of reports studying the impacts of these compounds on gut microbiota.

Animal studies. An early study inoculated female germ-free C3H mice (6 weeks old) with 7 strains of bacteria isolated from a 20-day-old female infant as human baby flora (HBF) and tested effects of two *Lactobacillus* strains, *L. paracasei* NCC 2461 and *L. rhamnosus* NCC 4007, on HBF and metabolomic responses of the mice (F.-P. J. Martin *et al.* 2008). Each *Lactobacillus* strain (10^8 CFU) was provided *ad libitum* in a saline drink daily for 2 weeks. Both probiotic groups significantly decreased CFUs of *Bifidobacteria longum* and *Staphylococcus aureus* among the 7 strains of HBF via quantitative culture of fecal pellets collected at the end of the study. Additionally, *L. rhamnosus* group lowered the counts of *B. breve*, *S. epidermidis*, and *Clostridium perfringens*, but elevated *E. coli* counts (F.-P. J. Martin *et al.* 2008). It was unclear for the implications of these observations because strains such as *B. longum* and *B. breve* are common probiotic strains. A later study assessed the effects of a mixture of probiotic strains on gut flora and disease prevention using an animal model of colitis (Nanda Kumar *et al.* 2008). Male Swiss albino mice (unknown age) received either intra-gastric phosphate buffered saline (PBS) or a mixture of 8 probiotic strains (4 from *Lactobacillus* and 4 from *Bifidobacterium*) at 10^8 - 10^{10} CFU/day for 14 days. Starting from Day 8, 4% dextran sodium sulfate were added to the feed to induce colitis (Nanda Kumar *et al.* 2008). The administration of probiotics attenuated chemical-induced colitis as indicated by histological assessment and reduced mRNA expression of several pathological

cytokines in distal colon tissues. The relative abundances of *Bifidobacterium*, *Bacteroides*, and *L. acidophilus* groups were restored in probiotic intervention group shown by qPCR analysis of feces (Nanda Kumar *et al.* 2008).

In addition to probiotics, several studies evaluated the effects of several prebiotic compounds. One study tested a common prebiotic, oligofructose (FOS), on the gut microbiome of a diet-induced obesity (DIO) mouse model (Everard *et al.* 2014). C57BL/6 mice (10 weeks old) under either control or high fat diet were provided with 0.3 g/mice/day (~15 g/kg b.w./day) of FOS in drinking water for 8 weeks. FOS treatment increased several proteins associated with improving intestinal epithelial functions as measured by qPCR analysis of expressed RNA in jejunum tissues, and altered the gut microbial composition and functional profiles shown in metagenomic sequencing analysis of cecal contents, implying a reversal of effects induced by high fat diet (Everard *et al.* 2014). Another study tested the effects of FOS and a seaweed, *Chondrus crispus* (rich in water soluble polysaccharide carrageenan), on the gut microbiota of male SD rats (3 weeks old) (J. Liu *et al.* 2015). Potential prebiotics were added into the rat diet (0.5% or 2.5% for each) for 21 days and 16S rRNA gene microarray analysis were performed using colon contents collected at the end of the study. Principle component analysis of the microarray data did not show alternations of community composition due to prebiotic treatment. Moreover, no shift of microorganism at phylum and family level in treatment groups was observed. However, certain species, such as *B. breve*, was elevated in a dose-dependent manner in both prebiotic groups. SCFA concentrations were elevated in fecal samples in a dose-dependent manner but only in *C. crispus* treated group (J. Liu *et al.* 2015). The effects of potato fiber on the gut microbiome of adult dogs were tested

(Panasevich *et al.* 2015). Female hound, averaging 6.13 years old, were fed with five doses of potato fiber (up to 6%; or ~0.85 g/kg bw/day) in diets in a Latin square design with each dosing period of 14 days. No positive effects on either biodiversity or microbial community composition were associated with prebiotic treatment as shown in 16S rRNA gene sequencing analyses of fecal samples collected in the last 4 days of each dosing period. Species such as *Faecalibacterium spp.* were enriched with increasing prebiotic doses, which was also positively correlated to butyrate production (Panasevich *et al.* 2015). A 6-month dosing experiment was performed using mid-aged female SD rats dosed by different concentrations of green tea polyphenols (GTP) extracts (up to 400 mg/kg b.w.) in drinking water (under review). The gut microbiota was evaluated via 16S gene sequencing and metagenomic sequencing analysis using mid-gut contents collected at 2 time points. GTP induced a dose-dependent decrease of biodiversity after 3 months of treatment and maintained the pattern until 6-month. The microbial composition of animals from the highest dose group was significantly different from other animals (control and lower dose group) at both sampling dates. Several species that were previously linked to lean phenotype in obesity studies were enriched in a dose-dependent manner by GTP. This finding was further corroborated by our results showed that several genes related to energy metabolism were also elevated in treatment groups.

One study tested the effects of both probiotic strains and prebiotic compound in broilers under chronic heat stress (Sohail *et al.* 2015). Either 0.1% of the probiotic mixture (7 strains, total 2×10^9 CFU/g of feed), or 0.5% mannan-oligosaccharides (MOS), or a combination of the probiotic mixture and MOS, in diets were provided to 55-day-old chickens *ad libitum* for 42 days. DGGE of 16S rRNA and 16S rRNA

sequencing analysis were conducted to evaluate trachea and cecal microbiota. Despite distinct differences in the trachea and cecal microbiota, no effects were observed in either biodiversity or microbial composition due to the prebiotic or probiotic treatment within each site. Large inter-individual variations were noted in this study, which was common in human studies but not in animal studies.

Human studies. Several studies focused on the effects of probiotic/prebiotic on infant microbiota and potential implications to later life stage. In a randomized clinical trial, newborns were fed with LGG (previously mentioned *L. rhamnosus* GG ATCC 53103) at 10^9 CFU/day from birth to 6 month, which promoted several potential beneficial species and yield a more even microbial community in infants guts as revealed by 16S rRNA gene microarray analysis (Cox *et al.* 2010). In another cross-sectional study, hospitalized infants of 3 months old, who received *L. reuteri* DSM 17938 at 10^8 CFU/day in the previous month, were recruited with age and sex matched controls (without probiotic administration) (Savino *et al.* 2015). The quantitative bacterial culture of fecal samples collected on the day of admission into hospital showed lower total anaerobic gram-negative bacterial counts, higher total anaerobic gram-positive counts and lower *Enterobacteriaceae* and *Enterocci* in probiotic group (Savino *et al.* 2015).

In terms of potential prebiotics, one study assessed the effects of bovine α -lactalbumin (α -lac) and casein glycomacropeptide (GMP) enriched infant formula on gut microbiota using fluorescent in-situ hybridization (FISH) analysis of several potential beneficial strains (Brück *et al.* 2006). Different dose combinations of α -lac and GMP were tested on healthy full-term infants of 6 weeks old until 6 months. No major differences in the potential beneficial strains, *Bifidobacterium* and *Lactobacillus*, were

observed as compared to control groups. However, once again, large inter-individual variations were observed, which may have masked any effects of the prebiotics. A similar study evaluated the effects of inulin, a type of FOS, on selected gut bacteria (Kapiki *et al.* 2007). Placebo or 0.4 g/100 ml inulin was added to the formula of infants within 0-14 days after birth and fed for 14 days. Quantitative culture of infant stool collected at 7 days after starting the experiment showed higher *Bifidobacteria* and *Bacteroids* and lower *E. coli* and *Enterococci* in samples taken from the prebiotic group.

The effects of a combination of a probiotic strain (*L. rhamnosus* GR-1, 10^{10} CFU/day) and a local nutritional plant (4.3 g *Moringa oleifera* dried leaves) on the overall health among pregnant women and their newborns were evaluated in one study among a rural Tanzanian population (Bisanz *et al.* 2015). Analysis of the oral, fecal and vaginal microbiome using 16S rRNA sequencing analysis in multiple sampling dates during the ~88 days of intervention did not present any observable effects in microbiota ascribed to the supplements as compared to controls. However, analyses of infant fecal samples showed that infants who were born to women under probiotic intervention group had a higher *Bifidobacterium* and lower *Enterobacteriaceae* abundance as compared to infants born to women in control group.

In addition to infants, effects of probiotics or prebiotics on healthy adults were investigated in several studies. Adult female monozygotic twins were assessed for the effects of a commercial fermented milk products (FMP) on their gut microbiota (McNulty *et al.* 2011). The study consisted of a pre-treatment period of 4 weeks, followed by a consumption period of 7 weeks, during which the volunteers took two 4-oz servings of FMP daily (containing 3.2×10^7 CFU *Bifidobacterium animalis subsp. lactis*

CNCM I-2494/g and 6.3×10^7 CFU *Lactobacillus delbrueckii* subsp. *bulgaricus* (CNCM I-1623, CNCM I-1519)/g FMP), and a post-treatment period of 4 weeks. No significant changes in microbial community composition as measured by 16S rRNA sequencing analysis, or in the abundances of genes encoding known enzymes as measured by metagenomic sequencing analysis were observed (McNulty *et al.* 2011). It is worth mentioning that the sequencing depth (on average 1,644 sequences/sample for 16S rRNA analysis, and 60,863 sequences/sample for metagenomic analysis) in this study was relatively low, which might contribute to their insignificant results. In the same study, the authors inoculated 15 microbial species derived from human gut into 6- to 8-week-old germ-free C57BL/6 male mice and assessed the impacts of FMP on this microbial consortium (McNulty *et al.* 2011). After about 2-week adaptation period, the mice were gavaged with either a single dose of 2×10^7 total CFU of FMP or multiple doses of the same amount of FMP at day 0, 7 and 21. Minimal changes in microbial community composition were noticed in both single and multiple dosing regimens. However, RNA-Seq analysis of fecal samples and mass spectrometry of urinary metabolites showed that FMP strains resulted in significant changes in expression of microbiota-encoded enzymes involved in numerous metabolic pathways, most prominently those related to carbohydrate metabolism (McNulty *et al.* 2011). Healthy elder volunteers (65-80 years old) were assessed for the effects of LGG on the gut microbiome by integrating 16S rRNA, metagenomic, and transcriptomic sequencing analysis (Eloe-Fadrosh *et al.* 2015). LGG at 10^{10} CFU were consumed by subjects twice daily for 28 days. Despite large inter-individual variations in microbial community composition, the same individual maintained a relative stable microbial community and no observable effects of probiotic

consumption were found by comparing microbial community before and after probiotic consumption. However, the transcriptome analyses revealed effects of LGG on a variety of genes which might be linked to some beneficial effects (Eloe-Fadrosh *et al.* 2015).

Isomalt, a common sugar replacer, was evaluated for its potential prebiotic capabilities via a placebo-control, crossover experiment (Gostner *et al.* 2006). A stable dose of 30 g/day isomalt was consumed by healthy volunteers (21-53 years old) for 4 weeks. An increase in *Bifidobacteria* via quantitative culture and FISH targeting 16S/23S rRNA genes, and reduced β -glucosidase activity were observed in fecal samples collected at the end of the dosing period (Gostner *et al.* 2006). Healthy male adults (21-28 years old) were tested for the effects polydextrose and soluble corn fiber on gut microbiota in another study (Holscher *et al.* 2015). Microbial metagenomic analyses of fecal samples collected after the subjects consumed polydextrose or soluble corn fiber (21 g/day each for 21 days) showed an increase in the ratio of *Bacteroidetes/Firmicutes* and several functional genes associated with carbohydrate, amino acid, and lipid metabolism, as well as metabolism of cofactors and vitamins (Holscher *et al.* 2015).

As studies with antibiotics, efforts have been made to evaluate effects of probiotics or prebiotics among diseased patients. The effects of a probiotic beverage on the gut microbiota of alcoholic liver cirrhosis patients (~53 years old on average) and their disease outcomes were evaluated in a placebo-control experiment (Koga *et al.* 2013). The beverage, which contained about 8^{10} CFU of *L. casei* Shirota YIT 9029, was provided to the patients in treatment group daily for two weeks. Patients who took the probiotic beverage showed an improved transthyretin levels along the course of the trial based on serum sample analysis, suggesting a restoration of liver function. qPCR showed

that several microbial species returned to levels comparable to an outside reference of gut microbiota from healthy humans, suggesting a restoration of healthy gut microbiota due to the probiotic strain (Koga *et al.* 2013). A placebo-control trial using a crossover design was conducted to evaluate the effects of a mixture of two *Lactobacillus* strains on vaginal microbiome of post-menopausal women with intermediate bacterial vaginosis (Bisanz *et al.* 2014). *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (2.5×10^9 CFU each) were administered twice daily for 3 days via a vaginal insert. No significant improvement of bacterial vaginosis in probiotic treatment periods were noted. By analysing vaginal swab samples using 16S rRNA gene sequencing, the changes of microbial community were not obvious given the UPGMA clustering and PCoA analysis, although an increase of relative abundance of *Lactobacillus* and a possible decrease of *Atopobium* were recorded (Bisanz *et al.* 2014). Patients of cirrhosis with minimal hepatic encephalopathy (~58 years old on average) were recruited in a placebo-control trial to assess the effects of LGG treatment (5×10^{10} CFU/g for 8 weeks) (Bajaj *et al.* 2014). Probiotic treatment improved dysbiosis in gut microbiota as shown by increases in abundance of autochthonous species and decrease in species associated with worse disease (Bajaj *et al.* 2014). Another study recruited patients of cystic fibrosis and tested the effects of *L. reuteri* DSM 17938 in a placebo-control, crossover experiments (del Campo *et al.* 2014). The patients (8-44 years old) received 10^8 CFU/day of the probiotic strain in a chewable tablet for 6 months and their gut microbiota was analysed by 16S rRNA sequencing analysis. Probiotic treatment drastically modified the gut microbiota by reducing the average abundance of γ -*Proteobacteria* and increasing the average abundance of *Firmicutes* and *Bacteroidetes* across all samples towards proportions that commonly seen

in healthy human gut microbiota. A significant improvement of a clinical gastrointestinal health indicator, GIOLI, was also found with the probiotic treatment.

Similar in animal studies, many researchers evaluated the effects of a mixture of probiotic strains. One study assessed the effects of a commercial probiotic mixture, VSL #3, among irritable bowel disease patients (31-56 years old) (Ng *et al.* 2013). The patients received VSL #3 (consist of 3 *Lactobacillus* strains, 2 *Bifidobacterium* strains, and 1 *Streptococcus* strains) with a total dose of 1.8×10^{12} CFU/day for 4 weeks. Rectal tissue samples collected via colonoscopy were analysed by 16S rRNA sequencing analysis. The analysis of biodiversity comparing samples taken before and after probiotic treatment showed different trends using different indexes. PCoA analysis did not show clear differentiation of microbiota in response to probiotic treatment as well, although several phylotypes were significantly changed after probiotic treatment compared to baseline (Ng *et al.* 2013). The same probiotic mixture was tested in another study of constipation patients (20-59 years old) who received VSL #3 at a level of 9×10^{11} CFU/day of for 2 weeks (Kim *et al.* 2015). Although the study recorded an improvement of symptom relief of functional constipation after probiotic treatment, the 5 bacteria (*Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli* spp., *Clostridium* spp., and *Bacteroides* spp.) did not change after treatment as compared to baseline as shown in qPCR analysis (Kim *et al.* 2015). Effects of mixtures of probiotics were also tested among a group of colorectal cancer patients in a placebo-control trial (Z. Gao *et al.* 2015). Recruited patients (40-75 years old) received capsules of probiotics (*B. longum*, *L. acidophilus*, *E. faecalis* in 1:1:1 ratio) at a total dose of 6×10^7 CFU/day for five days. Intestinal tissue-associated microbiota was assessed by 16S rRNA sequencing analysis. A

significant increase of Chao1 indexes, but decrease of Shannon and Simpson indexes in the intervention group were observed, although large inter-individual variation existed.

Among many species, *Fusobacterium*, a genus that was previously linked with tumorigenesis, was reduced in prebiotic treated patients (Z. Gao *et al.* 2015).

References

- Actis-Goretta, L., Lévêques, A., Rein, M., Teml, A., Schäfer, C., Hofmann, U., Li, H., Schwab, M., Eichelbaum, M., and Williamson, G. (2013) Intestinal absorption, metabolism, and excretion of (-)-epicatechin in healthy humans assessed by using an intestinal perfusion technique. *Am. J. Clin. Nutr.*, **98**, 924–933.
- Antonopoulos, D.A., Huse, S.M., Morrison, H.G., Schmidt, T.M., Sogin, M.L., and Young, V.B. (2009) Reproducible Community Dynamics of the Gastrointestinal Microbiota following Antibiotic Perturbation. *Infect. Immun.*, **77**, 2367–2375.
- Arat, S., Spivak, A., Van Horn, S., Thomas, E., Traini, C., Sathe, G., Livi, G.P., Ingraham, K., Jones, L., Aubart, K., *et al.* (2015) Microbiome changes in healthy volunteers treated with GSK1322322, a novel antibiotic targeting bacterial peptide deformylase. *Antimicrob. Agents Chemother.*, **59**, 1182–1192.
- Auger, C., Mullen, W., Hara, Y., and Crozier, A. (2008) Bioavailability of polyphenon E flavan-3-ols in humans with an ileostomy. *J. Nutr.*, **138**, 1535S–1542S.
- Back, D.J., Breckenridge, A.M., Crawford, F.E., Cross, K.J., Orme, M.L., Percival, A., and Rowe, P.H. (1980) Reduction of the enterohepatic circulation of norethisterone by antibiotics in the rat: Correlation with changes in the gut flora. *J. Steroid Biochem.*, **13**, 95–100.
- Bajaj, J.S., Heuman, D.M., Hylemon, P.B., Sanyal, A.J., Puri, P., Sterling, R.K., Luketic, V., Stravitz, R.T., Siddiqui, M.S., Fuchs, M., *et al.* (2014) Randomised clinical trial: Lactobacillus GG modulates gut microbiome, metabolome and endotoxemia in patients with cirrhosis. *Aliment. Pharmacol. Ther.*, **39**, 1113–1125.
- Barnes, J.M. and Butler, W.H. (1964) Carcinogenic Activity of Aflatoxin to Rats. *Nature*, **202**, 1016–1016.
- Bazett, M., Bergeron, M.-E., and Haston, C.K. (2016) Streptomycin treatment alters the intestinal microbiome, pulmonary T cell profile and airway hyperresponsiveness in a cystic fibrosis mouse model. *Sci. Rep.*, **6**, 19189.
- Bennett, J.W. and Klich, M. (2003) Mycotoxins. *Clin. Microbiol. Rev.*, **16**, 497–516.
- Bisanz, J.E., Enos, M.K., PrayGod, G., Seney, S., Macklaim, J.M., Chilton, S., Willner, D., Knight, R., Fusch, C., Fusch, G., *et al.* (2015) Microbiota at Multiple Body

- Sites during Pregnancy in a Rural Tanzanian Population and Effects of Moringa-Supplemented Probiotic Yogurt. *Appl. Environ. Microbiol.*, **81**, 4965–4975.
- Bisanz, J.E., Seney, S., McMillan, A., Vongsa, R., Koenig, D., Wong, L., Dvoracek, B., Gloor, G.B., Sumarah, M., Ford, B., *et al.* (2014) A systems biology approach investigating the effect of probiotics on the vaginal microbiome and host responses in a double blind, placebo-controlled clinical trial of post-menopausal women. *PloS One*, **9**, e104511.
- Bondy, G.S. and Pestka, J.J. (2000) Immunomodulation by Fungal Toxins. *J. Toxicol. Environ. Health Part B*, **3**, 109–143.
- Borges, G., van der Hooft, J.J.J., and Crozier, A. (2016) A comprehensive evaluation of the [2-14C](–)-epicatechin metabolome in rats. *Free Radic. Biol. Med.*, **99**, 128–138.
- Breton, J., Massart, S., Vandamme, P., De Brandt, E., Pot, B., and Foligné, B. (2013) Ecotoxicology inside the gut: impact of heavy metals on the mouse microbiome. *BMC Pharmacol. Toxicol.*, **14**, 62.
- Bron, P.A., van Baarlen, P., and Kleerebezem, M. (2011) Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. *Nat. Rev. Microbiol.*
- Brück, W.M., Redgrave, M., Tuohy, K.M., Lönnerdal, B., Graverholt, G., Hernell, O., and Gibson, G.R. (2006) Effects of bovine α -lactalbumin and casein glycomacropeptide-enriched infant formulae on faecal microbiota in healthy term infants. *J. Pediatr. Gastroenterol. Nutr.*, **43**, 673–679.
- Bull-Otterson, L., Feng, W., Kirpich, I., Wang, Y., Qin, X., Liu, Y., Gobejishvili, L., Joshi-Barve, S., Ayvaz, T., Petrosino, J., *et al.* (2013) Metagenomic Analyses of Alcohol Induced Pathogenic Alterations in the Intestinal Microbiome and the Effect of Lactobacillus rhamnosus GG Treatment. *PLoS ONE*, **8**, e53028.
- del Campo, R., Garriga, M., Pérez-Aragón, A., Gualarte, P., Lamas, A., Máiz, L., Bayón, C., Roy, G., Cantón, R., Zamora, J., *et al.* (2014) Improvement of digestive health and reduction in proteobacterial populations in the gut microbiota of cystic fibrosis patients using a Lactobacillus reuteri probiotic preparation: A double blind prospective study. *J. Cyst. Fibros.*, **13**, 716–722.
- Candon, S., Perez-Arroyo, A., Marquet, C., Valette, F., Foray, A.-P., Pelletier, B., Milani, C., Ventura, M., Bach, J.-F., and Chatenoud, L. (2015) Antibiotics in early life alter the gut microbiome and increase disease incidence in a spontaneous mouse model of autoimmune insulin-dependent diabetes. *PLoS ONE*, **10**, e0125448.
- Chen, L., Lee, M.-J., Li, H.E., and Yang, C.S. (1997) Absorption, distribution, and elimination of tea polyphenols in rats. *Drug Metab. Dispos.*, **25**, 1045–1050.

- Cho, I., Yamanishi, S., Cox, L., Methé, B.A., Zavadil, J., Li, K., Gao, Z., Mahana, D., Raju, K., Teitler, I., *et al.* (2012) Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature*, **488**, 621–626.
- Chow, H.S., Cai, Y., Alberts, D.S., Hakim, I., Dorr, R., Shahi, F., Crowell, J.A., Yang, C.S., and Hara, Y. (2001) Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. *Cancer Epidemiol. Prev. Biomark.*, **10**, 53–58.
- Clifford, M.N., van der Hooft, J.J., and Crozier, A. (2013) Human studies on the absorption, distribution, metabolism, and excretion of tea polyphenols. *Am. J. Clin. Nutr.*, **98**, 1619S–1630S.
- Coulombe, R.A. and Sharma, R.P. (1985) Clearance and excretion of intratracheally and orally administered aflatoxin B1 in the rat. *Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc.*, **23**, 827–830.
- Cox, M.J., Huang, Y.J., Fujimura, K.E., Liu, J.T., McKean, M., Boushey, H.A., Segal, M.R., Brodie, E.L., Cabana, M.D., and Lynch, S.V. (2010) Lactobacillus casei Abundance Is Associated with Profound Shifts in the Infant Gut Microbiome. *PLoS ONE*, **5**, e8745.
- Crespy, V., Nancoz, N., Oliveira, M., Hau, J., Courtet-compondu, M.-C., and Williamson, G. (2004) Glucuronidation of the Green Tea Catechins, (-)-epigallocatechin-3-gallate and (-)-epicatechin-3-gallate, by Rat Hepatic and Intestinal Microsomes. *Free Radic. Res.*, **38**, 1025–1031.
- Cullen, J.M. and Newberne, P.M. (1994) Acute hepatotoxicity of aflatoxins. In, Eaton, D.L. and Groopman, J.D. (eds), *The Toxicology of Aflatoxins. Human Health, Veterinary, and Agricultural Significance*. Academic Press, Inc., San Diego, California, pp. 3–27.
- Dalezios, J., Wogan, G.N., and Weinreb, S.M. (1971) Aflatoxin P-1: a new aflatoxin metabolite in monkeys. *Science*, **171**, 584–585.
- Dalezios, J.I. and Wogan, G.N. (1972) Metabolism of Aflatoxin B1 in Rhesus Monkeys. *Cancer Res.*, **32**, 2297–2303.
- Dardas, M., Gill, S.R., Grier, A., Pryhuber, G.S., Gill, A.L., Lee, Y.-H., and Guillet, R. (2014) The impact of postnatal antibiotics on the preterm intestinal microbiome. *Pediatr. Res.*, **76**, 150–158.
- Dethlefsen, L. and Relman, D.A. (2011) Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc. Natl. Acad. Sci.*, **108**, 4554–4561.
- Diaz, G.J. and Murcia, H.W. (2011) Biotransformation of Aflatoxin B1 and Its Relationship with the Differential Toxicological Response to Aflatoxin in

- Commercial Poultry Species. In, Guevara-González, R.G. (ed), *Aflatoxins - Biochemistry and Molecular Biology*. Intech, Rijeka, Croatia, pp. 3–20.
- Dohnal, V., Wu, Q., and Kuča, K. (2014) Metabolism of aflatoxins: key enzymes and interindividual as well as interspecies differences. *Arch. Toxicol.*, **88**, 1635–1644.
- Donovan, J.L., Crespy, V., Manach, C., Morand, C., Besson, C., Scalbert, A., and Rémésy, C. (2001) Catechin is metabolized by both the small intestine and liver of rats. *J. Nutr.*, **131**, 1753–1757.
- Dorożyńska, I., Majewska-Szczepanik, M., Marcińska, K., and Szczepanik, M. (2014) Partial depletion of natural gut flora by antibiotic aggravates collagen induced arthritis (CIA) in mice. *Pharmacol. Rep.*, **66**, 250–255.
- Dragan, Y.P. and Pitot, H.C. (1994) Aflatoxin carcinogenesis in the context of the multistage nature of cancer. In, Eaton, D.L. and Groopman, J.D. (eds), *The Toxicology of Aflatoxins. Human Health, Veterinary, and Agricultural Significance*. Academic Press, Inc., San Diego, California, pp. 179–206.
- Dycaico, M.J., Stuart, G.R., Tobal, G.M., De Boer, J.G., Glickman, B.W., and Provost, G.S. (1996) Species-specific differences in hepatic mutant frequency and mutational spectrum among lambda/lacI transgenic rats and mice following exposure to aflatoxin B1. *Carcinogenesis*, **17**, 2347–2356.
- Eaton, D.L., Ramsdell, H.S., and Neal, G.E. (1994) Biotransformation of aflatoxins. In, Eaton, D.L. and Groopman, J.D. (eds), *The Toxicology of Aflatoxins. Human Health, Veterinary, and Agricultural Significance*. Academic Press, Inc., San Diego, California, pp. 45–72.
- Eloe-Fadrosh, E.A., Brady, A., Crabtree, J., Drabek, E.F., Ma, B., Mahurkar, A., Ravel, J., Haverkamp, M., Fiorino, A.-M., Botelho, C., *et al.* (2015) Functional dynamics of the gut microbiome in elderly people during probiotic consumption. *mBio*, **6**, e00231-15.
- Essigmann, J.M., Green, C.L., Croy, R.G., Fowler, K.W., Büchi, G.H., and Wogan, G.N. (1983) Interactions of aflatoxin B1 and alkylating agents with DNA: Structural and Functional Studies. *Cold Spring Harb. Symp. Quant. Biol.*, **47**, 327–337.
- Everard, A., Lazarevic, V., Gaïa, N., Johansson, M., Ståhlman, M., Backhed, F., Delzenne, N.M., Schrenzel, J., François, P., and Cani, P.D. (2014) Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity. *ISME J.*, **8**, 2116–2130.
- FAO and WHO. Probiotics in food: health and nutritional properties and guidelines for evaluation. Rome, Italy: World Health Organization/Food and Agriculture Organization of the United Nations; 2006. *FAO food and nutrition paper*; vol 85. Available at: <ftp://ftp.fao.org/docrep/fao/009/a0512e/a0512e00.pdf>. Accessed March 20, 2017.

- Fazeli, M., Hassanzadeh, P., and Alaei, S. (2011) Cadmium chloride exhibits a profound toxic effect on bacterial microflora of the mice gastrointestinal tract. *Hum. Exp. Toxicol.*, **30**, 152–159.
- Feng, W.Y. (2006) Metabolism of green tea catechins: an overview. *Curr. Drug Metab.*, **7**, 755–809.
- Francino, M.P. (2016) Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. *Front. Microbiol.*, **6**.
- Freedberg, D.E., Toussaint, N.C., Chen, S.P., Ratner, A.J., Whittier, S., Wang, T.C., Wang, H.H., and Abrams, J.A. (2015) Proton pump inhibitors alter specific taxa in the human gastrointestinal microbiome: a crossover trial. *Gastroenterology*, **149**, 883–885.
- Gao, B., Bian, X., Mahbub, R., and Lu, K. (2017) Sex-specific effects of organophosphate diazinon on the gut microbiome and its metabolic functions. *Environ. Health Perspect.*, **125**, 198–206.
- Gao, Z., Guo, B., Gao, R., Zhu, Q., Wu, W., and Qin, H. (2015) Probiotics modify human intestinal mucosa-associated microbiota in patients with colorectal cancer. *Mol. Med. Rep.*, **12**, 6119–6127.
- Gibson, G. and Roberfroid, M. (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr*, **125**, 1401–1412.
- Gostner, A., Blaut, M., Schäffer, V., Kozianowski, G., Theis, S., Klingenberg, M., Dombrowski, Y., Martin, D., Ehrhardt, S., Taras, D., *et al.* (2006) Effect of isomalt consumption on faecal microflora and colonic metabolism in healthy volunteers. *Br. J. Nutr.*, **95**, 40.
- Groopman, J.D., Cain, L.G., and Kensler, T.W. (1988) Aflatoxin exposure in human populations: measurements and relationship to cancer. *Crit. Rev. Toxicol.*, **19**, 113–145.
- Guengerich, F.P., Cai, H., McMahon, M., Hayes, J.D., Sutter, T.R., Groopman, J.D., Deng, Z., and Harris, T.M. (2001) Reduction of Aflatoxin B1 Dialdehyde by Rat and Human Aldo-keto Reductases. *Chem. Res. Toxicol.*, **14**, 727–737.
- Guengerich, F.P., Johnson, W.W., Shimada, T., Ueng, Y.-F., Yamazaki, H., and Langouët, S. (1998) Activation and detoxication of aflatoxin B1. *Mutat. Res. Mol. Mech. Mutagen.*, **402**, 121–128.
- Han, X., Geller, B., Moniz, K., Das, P., Chippindale, A.K., and Walker, V.K. (2014) Monitoring the developmental impact of copper and silver nanoparticle exposure in *Drosophila* and their microbiomes. *Sci. Total Environ.*, **487**, 822–829.

- Higdon, J. and Frei, B. (2003) Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit. Rev. Food Sci. Nutr.*, **43**, 89–143.
- Hill, A.M., Coates, A.M., Buckley, J.D., Ross, R., Thielecke, F., and Howe, P.R.C. (2007) Can EGCG reduce abdominal fat in obese subjects? *J. Am. Coll. Nutr.*, **26**, 396S–402S.
- van het Hof, K.H., Wiseman, S.A., Yang, C.S., and Tijburg, L.B.M. (1999) Plasma and Lipoprotein Levels of Tea Catechins Following Repeated Tea Consumption. *Exp. Biol. Med.*, **220**, 203–209.
- Holeski, C.J., Eaton, D.L., Monroe, D.H., and Bellamy, G.M. (1987) Effects of phenobarbital on the biliary excretion of aflatoxin P1-glucuronide and aflatoxin B1-S-glutathione in the rat. *Xenobiotica Fate Foreign Compd. Biol. Syst.*, **17**, 139–153.
- Holscher, H.D., Caporaso, J.G., Hooda, S., Brulc, J.M., Fahey, G.C., and Swanson, K.S. (2015) Fiber supplementation influences phylogenetic structure and functional capacity of the human intestinal microbiome: follow-up of a randomized controlled trial. *Am. J. Clin. Nutr.*, **101**, 55–64.
- Holzapel, C.W., Steyn, P.S., and Purchase, I.F. (1966) Isolation and structure of aflatoxins M1 and M2. *Tetrahedron Lett.*, **25**, 2799–2803.
- Hsieh, D.P. and Wong, J.J. (1994) Pharmacokinetics and excretion of aflatoxins. In, Eaton, D.L. and Groopman, J.D. (eds), *The Toxicology of Aflatoxins. Human Health, Veterinary, and Agricultural Significance*. Academic Press, Inc., San Diego, California, pp. 73–88.
- Huang, T., Yu, J., Tang, L., Luo, H., Billam, M., Wei, Z., Liang, Y., Li, Y., Wang, K., Shi, J., *et al.* (2004) Phase IIa chemoprevention trial of green tea polyphenols in high-risk population of liver cancer: I. Design, clinical outcome, and baseline biomarker data. *Int. J. Cancer Prev.*, **1**, 269–280.
- Hursel, R., Viechtbauer, W., and Westerterp-Plantenga, M.S. (2009) The effects of green tea on weight loss and weight maintenance: a meta-analysis. *Int. J. Obes.*, **33**, 956–961.
- IARC. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Lyon, France: International Agency for Research on Cancer; 1993:245-395. *IARC monographs on the evaluation of carcinogenic risks to humans*; vol 56.
- de Iongh, H., Vles, R.O., and van Pelt, J.G. (1964) Milk of Mammals Fed an Aflatoxin-Containing Diet. *Nature*, **202**, 466–467.

- Jakobsson, H.E., Jernberg, C., Andersson, A.F., Sjölund-Karlsson, M., Jansson, J.K., and Engstrand, L. (2010) Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS ONE*, **5**, e9836.
- Janssens, P.L., Hursel, R., and Westerterp-Plantenga, M.S. (2015) Long-Term Green Tea Extract Supplementation Does Not Affect Fat Absorption, Resting Energy Expenditure, and Body Composition in Adults. *J. Nutr.*, **145**, 864–870.
- Jurgens, T.M., Whelan, A.M., Killian, L., Doucette, S., Kirk, S., and Foy, E. (2012) Green tea for weight loss and weight maintenance in overweight or obese adults. *Cochrane Database Syst. Rev.*, **12**, CD008650.
- Kamdem, L.K., Meineke, I., Gödtel-Armbrust, U., Brockmöller, J., and Wojnowski, L. (2006) Dominant contribution of P450 3A4 to the hepatic carcinogenic activation of aflatoxin B1. *Chem. Res. Toxicol.*, **19**, 577–586.
- Kapiki, A., Costalos, C., Oikonomidou, C., Triantafyllidou, A., Loukatou, E., and Petrohilou, V. (2007) The effect of a fructo-oligosaccharide supplemented formula on gut flora of preterm infants. *Early Hum. Dev.*, **83**, 335–339.
- Kensler, T.W., Roebuck, B.D., Wogan, G.N., and Groopman, J.D. (2011) Aflatoxin: A 50-Year Odyssey of Mechanistic and Translational Toxicology. *Toxicol. Sci.*, **120**, S28–S48.
- Kida, K., Suzuki, M., Matsumoto, N., Nanjo, F., and Hara, Y. (2000) Identification of Biliary Metabolites of (–)-Epigallocatechin Gallate in Rats. *J. Agric. Food Chem.*, **48**, 4151–4155.
- Kim, S., Lee, M.-J., Hong, J., Li, C., Smith, T.J., Yang, G.-Y., Seril, D.N., and Yang, C.S. (2000) Plasma and tissue levels of tea catechins in rats and mice during chronic consumption of green tea polyphenols. *Nutr. Cancer*, **37**, 41–48.
- Kim, S.-E., Choi, S.C., Park, K.S., Park, M.I., Shin, J.E., Lee, T.H., Jung, K.W., Koo, H.S., and Myung, S.-J. (2015) Change of Fecal Flora and Effectiveness of the Short-term VSL#3 Probiotic Treatment in Patients With Functional Constipation. *J. Neurogastroenterol. Motil.*, **21**, 111–120.
- Koga, H., Tamiya, Y., Mitsuyama, K., Ishibashi, M., Matsumoto, S., Imaoka, A., Hara, T., Nakano, M., Ooeda, K., Umezaki, Y., *et al.* (2013) Probiotics promote rapid-turnover protein production by restoring gut flora in patients with alcoholic liver cirrhosis. *Hepatol. Int.*, **7**, 767–774.
- Kohri, T., Nanjo, F., Suzuki, M., Seto, R., Matsumoto, N., Yamakawa, M., Hojo, H., Hara, Y., Desai, D., Amin, S., *et al.* (2001) Synthesis of (–)-[4-³H] epigallocatechin gallate and its metabolic fate in rats after Intravenous administration. *J. Agric. Food Chem.*, **49**, 1042–1048.

- Kohri, T., Suzuki, M., and Nanjo, F. (2003) Identification of metabolites of (–)-epicatechin gallate and their metabolic fate in the rat. *J. Agric. Food Chem.*, **51**, 5561–5566.
- Koopman, J.E., van der Kaaij, N.C.W., Buijs, M.J., Elyassi, Y., van der Veen, M.H., Crielaard, W., ten Cate, J.M., and Zaura, E. (2015) The effect of fixed orthodontic appliances and fluoride mouthwash on the oral microbiome of adolescents – a randomized controlled clinical trial. *PLoS ONE*, **10**, e0137318.
- Krishnamachari, K.A., Bhat, R.V., Nagarajan, V., and Tilak, T.B. (1975) Hepatitis due to aflatoxicosis. An outbreak in Western India. *Lancet Lond. Engl.*, **1**, 1061–1063.
- Kuhnle, G., Spencer, J.P.E., Schroeter, H., Shenoy, B., Debnam, E.S., Srai, S.K.S., Rice-Evans, C., and Hahn, U. (2000) Epicatechin and Catechin are O-Methylated and Glucuronidated in the Small Intestine. *Biochem. Biophys. Res. Commun.*, **277**, 507–512.
- Kumagai, S. (1989) Intestinal absorption and excretion of aflatoxin in rats. *Toxicol. Appl. Pharmacol.*, **97**, 88–97.
- Lee, M.-J., Wang, Z.-Y., Li, H., Chen, L., Sun, Y., Gobbo, S., Balentine, D.A., and Yang, C.S. (1995) Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiol. Prev. Biomark.*, **4**, 393–399.
- Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Lubber, G., Kieszak, S., Nyamongo, J., Backer, L., Dahiye, A.M., Misore, A., *et al.* (2005) Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. *Environ. Health Perspect.*, **113**, 1763–1767.
- Li, C., Lee, M.-J., Sheng, S., Meng, X., Prabhu, S., Winnik, B., Huang, B., Chung, J.Y., Yan, S., Ho, C.-T., *et al.* (2000) Structural identification of two metabolites of catechins and their kinetics in human urine and blood after tea ingestion. *Chem. Res. Toxicol.*, **13**, 177–184.
- Li, C., Meng, X., Winnik, B., Lee, M.-J., Lu, H., Sheng, S., Buckley, B., and Yang, C.S. (2001) Analysis of urinary metabolites of tea catechins by liquid chromatography/electrospray ionization mass spectrometry. *Chem. Res. Toxicol.*, **14**, 702–707.
- Lin, J., Chen, J., He, J., Chen, J., Yan, Q., Zhou, J., and Xie, P. (2015) Effects of microcystin-LR on bacterial and fungal functional genes profile in rat gut. *Toxicon*, **96**, 50–56.
- Liu, J., Kandasamy, S., Zhang, J., Kirby, C.W., Karakach, T., Hafting, J., Critchley, A.T., Evans, F., and Prithiviraj, B. (2015) Prebiotic effects of diet supplemented with the cultivated red seaweed *Chondrus crispus* or with fructo-oligo-saccharide on

- host immunity, colonic microbiota and gut microbial metabolites. *BMC Complement. Altern. Med.*, **15**.
- Liu, Y., Li, Y., Liu, K., and Shen, J. (2014) Exposing to cadmium stress cause profound toxic effect on microbiota of the mice intestinal tract. *PLoS ONE*, **9**, e85323.
- Looft, T., Allen, H.K., Cantarel, B.L., Levine, U.Y., Bayles, D.O., Alt, D.P., Henrissat, B., and Stanton, T.B. (2014) Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. *ISME J*, **8**, 1566–1576.
- Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., Sul, W.J., Stedtfeld, T.M., Chai, B., Cole, J.R., *et al.* (2012) In-feed antibiotic effects on the swine intestinal microbiome. *Proc. Natl. Acad. Sci.*, **109**, 1691–1696.
- Louie, T.J., Cannon, K., Byrne, B., Emery, J., Ward, L., Eyben, M., and Krulicki, W. (2012) Fidaxomicin preserves the intestinal microbiome during and after treatment of *Clostridium difficile* infection (CDI) and reduces both toxin reexpression and recurrence of CDI. *Clin. Infect. Dis.*, **55**, S132–S142.
- Lozano, M.C. and Diaz, G.J. (2006) Microsomal and cytosolic biotransformation of aflatoxin B1 in four poultry species. *Br. Poult. Sci.*, **47**, 734–741.
- Lu, H., Meng, X., Li, C., Sang, S., Patten, C., Sheng, S., Hong, J., Bai, N., Winnik, B., Ho, C.-T., *et al.* (2003) Glucuronides of tea catechins: enzymology of biosynthesis and biological activities. *Drug Metab. Dispos. Biol. Fate Chem.*, **31**, 452–461.
- Lu, K., Abo, R.P., Schlieper, K.A., Graffam, M.E., Levine, S., Wishnok, J.S., Swenberg, J.A., Tannenbaum, S.R., and Fox, J.G. (2014) Arsenic exposure perturbs the gut microbiome and its metabolic profile in mice: an integrated metagenomics and metabolomics analysis. *Environ. Health Perspect.*
- Luo, H., Cox, S., Gao, W.-M., Yu, J., Tang, L., and Wang, J.-S. (2006a) Metabolic profiling in validation of plasma biomarkers for green tea polyphenols. *Metabolomics*, **2**, 235–241.
- Luo, H., Tang, L., Tang, M., Billam, M., Huang, T., Yu, J., Wei, Z., Liang, Y., Wang, K., Zhang, Z., *et al.* (2004) Validation of green tea polyphenol biomarkers in high-risk population of liver cancer. *Proc. Am. Assoc. Cancer Res.*, **45**, 931.
- Luo, H., Tang, L., Tang, M., Billam, M., Huang, T., Yu, J., Wei, Z., Liang, Y., Wang, K., Zhang, Z.Q., *et al.* (2006b) Phase IIa chemoprevention trial of green tea polyphenols in high-risk individuals of liver cancer: modulation of urinary excretion of green tea polyphenols and 8-hydroxydeoxyguanosine. *Carcinogenesis*, **27**, 262–268.
- Manach, C., Scalbert, A., Morand, C., Rémésy, C., and Jiménez, L. (2004) Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.*, **79**, 727–747.

- Manichanh, C., Reeder, J., Gibert, P., Varela, E., Llopis, M., Antolin, M., Guigo, R., Knight, R., and Guarner, F. (2010) Reshaping the gut microbiome with bacterial transplantation and antibiotic intake. *Genome Res.*, **20**, 1411–1419.
- Martin, F.-P.J., Wang, Y., Sprenger, N., Yap, I.K.S., Lundstedt, T., Lek, P., Rezzi, S., Ramadan, Z., van Bladeren, P., Fay, L.B., *et al.* (2008) Probiotic modulation of symbiotic gut microbial–host metabolic interactions in a humanized microbiome mouse model. *Mol. Syst. Biol.*, **4**.
- McNulty, N.P., Yatsunenko, T., Hsiao, A., Faith, J.J., Muegge, B.D., Goodman, A.L., Henrissat, B., Oozeer, R., Cools-Portier, S., Gobert, G., *et al.* (2011) The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. *Sci. Transl. Med.*, **3**, 106ra106–106ra106.
- Meng, X., Sang, S., Zhu, N., Lu, H., Sheng, S., Lee, M.-J., Ho, C.-T., and Yang, C.S. (2002) Identification and characterization of methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats. *Chem. Res. Toxicol.*, **15**, 1042–1050.
- Merkley, M.A., Bice, T.C., Grier, A., Strohl, A.M., Man, L.-X., and Gill, S.R. (2015) The effect of antibiotics on the microbiome in acute exacerbations of chronic rhinosinusitis: Antibiotics and the microbiome in sinusitis. *Int. Forum Allergy Rhinol.*, **5**, 884–893.
- Merrifield, D.L., Shaw, B.J., Harper, G.M., Saoud, I.P., Davies, S.J., Handy, R.D., and Henry, T.B. (2013) Ingestion of metal-nanoparticle contaminated food disrupts endogenous microbiota in zebrafish (*Danio rerio*). *Environ. Pollut.*, **174**, 157–163.
- Mielgo-Ayuso, J., Barrenechea, L., Alcorta, P., Larrarte, E., Margareto, J., and Labayen, I. (2014) Effects of dietary supplementation with epigallocatechin-3-gallate on weight loss, energy homeostasis, cardiometabolic risk factors and liver function in obese women: randomised, double-blind, placebo-controlled clinical trial. *Br. J. Nutr.*, **111**, 1263–1271.
- Mutlu, E., Keshavarzian, A., Engen, P., Forsyth, C.B., Sikaroodi, M., and Gillevet, P. (2009) Intestinal dysbiosis: A possible mechanism of alcohol-induced endotoxemia and alcoholic steatohepatitis in rats. *Clin. Exp. Res.*, **33**, 1836–1846.
- Nakachi, K., Matsuyama, S., Miyake, S., Suganuma, M., and Imai, K. (2000) Preventive effects of drinking green tea on cancer and cardiovascular disease: epidemiological evidence for multiple targeting prevention. *BioFactors Oxf. Engl.*, **13**, 49–54.
- Nanda Kumar, N.S., Balamurugan, R., Jayakanthan, K., Pulimood, A., Pugazhendhi, S., and Ramakrishna, B.S. (2008) Probiotic administration alters the gut flora and attenuates colitis in mice administered dextran sodium sulfate. *J. Gastroenterol. Hepatol.*, **23**, 1834–1839.

- Narrowe, A.B., Albuthi-Lantz, M., Smith, E.P., Bower, K.J., Roane, T.M., Vajda, A.M., and Miller, C.S. (2015) Perturbation and restoration of the fathead minnow gut microbiome after low-level triclosan exposure. *Microbiome*, **3**.
- Newberne, P.M. and Butler, W.H. (1969) Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: a review. *Cancer Res.*, **29**, 236–250.
- Ng, S.C., Lam, E.F., Lam, T.T., Chan, Y., Law, W., Tse, P.C., Kamm, M.A., Sung, J.J., Chan, F.K., and Wu, J.C. (2013) Effect of probiotic bacteria on the intestinal microbiota in irritable bowel syndrome: mucosal microbiota in IBS. *J. Gastroenterol. Hepatol.*, 1624-1631.
- Ngindu, A., Kenya, P., Ocheng, D., Omondi, T., Ngare, W., Gatei, D., Johnson, B., Ngira, J., Nandwa, H., Jansen, A., *et al.* (1982) Outbreak of acute hepatitis caused by aflatoxin poisoning in Kenya. *The Lancet*, **319**, 1346–1348.
- Niu, G., Wen, Z., Rupasinghe, S.G., Zeng, R.S., Berenbaum, M.R., and Schuler, M.A. (2008) Aflatoxin B1 detoxification by CYP321A1 in *Helicoverpa zea*. *Arch. Insect Biochem. Physiol.*, **69**, 32–45.
- Nolan, J.P. (1989) Intestinal endotoxins as mediators of hepatic injury—an idea whose time has come again. *Hepatology*, **10**, 887–891.
- Okushio, K., Matsumoto, N., Kohri, T., Suzuki, M., Nanjo, F., and Yukihiro, H. (1996) Absorption of tea catechins into rat portal vein. *Biol. Pharm. Bull.*, **19**, 326–329.
- Okushio, K., Matsumoto, N., Suzuki, M., Nanjo, F., and Hara, Y. (1995) Absorption of (-)-epigallocatechin gallate into rat portal vein. *Biol. Pharm. Bull.*, **18**, 190.
- Panasevich, M.R., Kerr, K.R., Dilger, R.N., Fahey, G.C., Guérin-Deremaux, L., Lynch, G.L., Wils, D., Suchodolski, J.S., Steer, J.M., Dowd, S.E., *et al.* (2015) Modulation of the faecal microbiome of healthy adult dogs by inclusion of potato fibre in the diet. *Br. J. Nutr.*, **113**, 125–133.
- Pang, J., Zhang, Z., Zheng, T., Bassig, B.A., Mao, C., Liu, X., Zhu, Y., Shi, K., Ge, J., Yang, Y., *et al.* (2016) Green tea consumption and risk of cardiovascular and ischemic related diseases: A meta-analysis. *Int. J. Cardiol.*, **202**, 967–974.
- Patterson, D.S. (1973) Metabolism as a factor in determining the toxic action of the aflatoxins in different animal species. *Food Cosmet. Toxicol.*, **11**, 287–294.
- Patterson, D.S. and Roberts, B.A. (1972) Aflatoxin metabolism in duck-liver homogenates: the relative importance of reversible cyclopentenone reduction and hemiacetal formation. *Food Cosmet. Toxicol.*, **10**, 501–512.
- Patterson, D.S. and Roberts, B.A. (1970) The formation of aflatoxins B2a and G2a and their degradation products during the in vitro detoxification of aflatoxin by livers of certain avian and mammalian species. *Food Cosmet. Toxicol.*, **8**, 527–538.

- Peterfreund, G.L., Vandivier, L.E., Sinha, R., Marozsan, A.J., Olson, W.C., Zhu, J., and Bushman, F.D. (2012) Succession in the gut microbiome following antibiotic and antibody therapies for *Clostridium Difficile*. *PLoS ONE*, **7**, e46966.
- Phung, O.J., Baker, W.L., Matthews, L.J., Lanosa, M., Thorne, A., and Coleman, C.I. (2010) Effect of green tea catechins with or without caffeine on anthropometric measures: a systematic review and meta-analysis. *Am. J. Clin. Nutr.*, **91**, 73–81.
- Pilger, A., Germadnik, D., Riedel, K., Meger-Kossien, I., Scherer, G., and Rudiger, H.W. (2001) Longitudinal study of urinary 8-hydroxy-2'-deoxyguanosine excretion in healthy adults. *Free Radic Res*, **35**, 273–280.
- Piskula, M.K. and Terao, J. (1998) Accumulation of (-)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues. *J. Nutr.*, **128**, 1172–1178.
- Qian, G., Tang, L., Guo, X., Wang, F., Massey, M.E., Su, J., Guo, T.L., Williams, J.H., Phillips, T.D., and Wang, J.-S. (2014) Aflatoxin B₁ modulates the expression of phenotypic markers and cytokines by splenic lymphocytes of male F344 rats: Immunotoxicity of aflatoxin B₁ in F344 rats. *J. Appl. Toxicol.*, **34**, 241–249.
- Raj, H.G. and Lotlikar, P.D. (1984) Urinary excretion of thiol conjugates of aflatoxin B₁ in rats and hamsters. *Cancer Lett.*, **22**, 125–133.
- Rajpal, D.K., Klein, J.-L., Mayhew, D., Boucheron, J., Spivak, A.T., Kumar, V., Ingraham, K., Paulik, M., Chen, L., Van Horn, S., *et al.* (2015) Selective spectrum antibiotic modulation of the gut microbiome in obesity and diabetes rodent models. *PloS One*, **10**, e0145499.
- Rawal, S., Kim, J.E., and Coulombe Jr., R. (2010) Aflatoxin B₁ in poultry: Toxicology, metabolism and prevention. *Res. Vet. Sci.*, **89**, 325–331.
- Roberfroid, M. (2007) Prebiotics: the concept revisited. *J. Nutr.*, **137**, 830S–837S.
- Ross, R.K., Yuan, J.M., Yu, M.C., Wogan, G.N., Qian, G.S., Tu, J.T., Groopman, J.D., Gao, Y.T., and Henderson, B.E. (1992) Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet Lond. Engl.*, **339**, 943–946.
- Sabbioni, G., Skipper, P.L., Büchi, G., and Tannenbaum, S.R. (1987) Isolation and characterization of the major serum albumin adduct formed by aflatoxin B₁ in vivo in rats. *Carcinogenesis*, **8**, 819–824.
- Saint-Cyr, M.J., Perrin-Guyomard, A., Houée, P., Rolland, J.-G., and Laurentie, M. (2013) Evaluation of an oral subchronic exposure of deoxynivalenol on the composition of human gut microbiota in a model of human microbiota-associated rats. *PLoS ONE*, **8**, e80578.

- Sasazuki, S., Kodama, H., Yoshimasu, K., Liu, Y., Washio, M., Tanaka, K., Tokunaga, S., Kono, S., Arai, H., Doi, Y., *et al.* (2000) Relation between green tea consumption and the severity of coronary atherosclerosis among Japanese men and women. *Ann. Epidemiol.*, **10**, 401–408.
- Sato, Y., Nakatsuka, H., Watanabe, T., Hisamichi, S., Shimizu, H., Fujisaku, S., Ichinowatari, Y., Ida, Y., Suda, S., and Kato, K. (1989) Possible contribution of green tea drinking habits to the prevention of stroke. *Tohoku J. Exp. Med.*, **157**, 337–343.
- Savino, F., Fornasero, S., Ceratto, S., De Marco, A., Mandras, N., Roana, J., Tullio, V., and Amisano, G. (2015) Probiotics and gut health in infants: A preliminary case–control observational study about early treatment with *Lactobacillus reuteri* DSM 17938. *Clin. Chim. Acta*, **451**, 82–87.
- Sawosz, E., Binek, M., Grodzik, M., Zielińska, M., Sysa, P., Szmidt, M., Niemiec, T., and Chwalibog, A. (2007) Influence of hydrocolloidal silver nanoparticles on gastrointestinal microflora and morphology of enterocytes of quails. *Arch. Anim. Nutr.*, **61**, 444–451.
- Scalbert, A., Manach, C., Morand, C., Rémésy, C., and Jiménez, L. (2005) Dietary Polyphenols and the Prevention of Diseases. *Crit. Rev. Food Sci. Nutr.*, **45**, 287–306.
- van Schaik, W. (2015) The human gut resistome. *Philos. Trans. R. Soc. B Biol. Sci.*, **370**.
- Schantz, M., Erk, T., and Richling, E. (2010) Metabolism of green tea catechins by the human small intestine. *Biotechnol. J.*, **5**, 1050–1059.
- Shen, C.-L., Chyu, M.-C., and Wang, J.-S. (2013) Tea and bone health: steps forward in translational nutrition. *Am. J. Clin. Nutr.*, **98**, 1694S–1699S.
- Sohail, M.U., Hume, M.E., Byrd, J.A., Nisbet, D.J., Shabbir, M.Z., Ijaz, A., and Rehman, H. (2015) Molecular analysis of the caecal and tracheal microbiome of heat-stressed broilers supplemented with prebiotic and probiotic. *Avian Pathol.*, **44**, 67–74.
- Stalmach, A., Mullen, W., Steiling, H., Williamson, G., Lean, M.E.J., and Crozier, A. (2010) Absorption, metabolism, and excretion of green tea flavan-3-ols in humans with an ileostomy. *Mol. Nutr. Food Res.*, **54**, 323–334.
- Takagaki, A. and Nanjo, F. (2010) Metabolism of (–)-Epigallocatechin Gallate by Rat Intestinal Flora. *J. Agric. Food Chem.*, **58**, 1313–1321.
- Takeshita, M., Takashima, S., Harada, U., Shibata, E., Hosoya, N., Takase, H., Otsuka, K., Meguro, S., Komikado, M., and Tokimitsu, I. (2008) Effects of long-term consumption of tea catechins-enriched beverage with no caffeine on body composition in humans. *Yakuri Chiryo*, **36**, 767–776.

- Takizawa, Y., Morota, T., Takeda, S., and Aburada, M. (2003) Pharmacokinetics of (–)-Epicatechin-3-*O*-gallate, an Active Component of Onpi-to, in Rats. *Biol. Pharm. Bull.*, **26**, 608–612.
- Tang, L., Tang, M., Xu, L., Luo, H., Huang, T., Yu, J., Zhang, L., Gao, W., Cox, S.B., and Wang, J.-S. (2008) Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention. *Carcinogenesis*, **29**, 411–417.
- Theriot, C.M., Koenigsknecht, M.J., Carlson, P.E., Hatton, G.E., Nelson, A.M., Li, B., Huffnagle, G.B., Z. Li, J., and Young, V.B. (2014) Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to *Clostridium difficile* infection. *Nat. Commun.*, **5**.
- Ueng, Y.F., Shimada, T., Yamazaki, H., and Peterguengerich, F. (1998) Aflatoxin B₁ oxidation by human cytochrome P450s. *J. Toxicol. Sci.*, **23 Suppl 2**, 132–135.
- Vaidyanathan, J.B. and Walle, T. (2002) Glucuronidation and sulfation of the tea flavonoid (–)-epicatechin by the human and rat enzymes. *Drug Metab. Dispos.*, **30**, 897–903.
- Van Amelsvoort, J., Van Het Hof, K., Mathot, J., Mulder, T., Wiersma, A., and Tijburg, L. (2001) Plasma concentrations of individual tea catechins after a single oral dose in humans. *Xenobiotica*, **31**, 891–901.
- Waché, Y.J., Valat, C., Postollec, G., Bougeard, S., Burel, C., Oswald, I.P., and Fravallo, P. (2008) Impact of deoxynivalenol on the intestinal microflora of pigs. *Int. J. Mol. Sci.*, **10**, 1–17.
- Wang, J., Tang, L., Glenn, T.C., and Wang, J.-S. (2016) Aflatoxin B₁ induced compositional changes in gut microbial communities of male F344 rats. *Toxicol. Sci.*, **150**, 54–63.
- Wang, J., Tang, L., and Wang, J.-S. (2015) Biomarkers of dietary polyphenols in cancer studies: current evidence and beyond. *Oxid. Med. Cell. Longev.*, **2015**, 1–14.
- Wang, J.-S., Huang, T., Yu, J., Wei, Z., Liang, Y., Li, Y., Wang, K., Shi, J., Cui, Y., Luo, H., *et al.* (2003) Chemoprevention trial of green tea polyphenols in high-risk population of liver cancer. *Proc. Am. Assoc. Cancer Res.*, **44**.
- Wang, J.S., Luo, H., Wang, P., Tang, L., Yu, J., Huang, T., Cox, S., and Gao, W. (2008) Validation of green tea polyphenol biomarkers in a phase II human intervention trial. *Food Chem. Toxicol.*, **46**, 232–240.
- Wang, J.S., Qian, G.S., Zarba, A., He, X., Zhu, Y.R., Zhang, B.C., Jacobson, L., Gange, S.J., Muñoz, A., and Kensler, T.W. (1996) Temporal patterns of aflatoxin-albumin adducts in hepatitis B surface antigen-positive and antigen-negative residents of Daxin, Qidong County, People's Republic of China. *Cancer Epidemiol. Biomark. Prev.*, **5**, 253–261.

- Wang, Z.-M., Zhou, B., Wang, Y.-S., Gong, Q.-Y., Wang, Q.-M., Yan, J.-J., Gao, W., and Wang, L.-S. (2011) Black and green tea consumption and the risk of coronary artery disease: a meta-analysis. *Am. J. Clin. Nutr.*, **93**, 506–515.
- Wannop, C.C. (1961) The Histopathology of Turkey ‘X’ Disease in Great Britain. *Avian Dis.*, **5**, 371–381.
- Wogan, G.N. (1992) Aflatoxin carcinogenesis: interspecies potency differences and relevance for human risk assessment. *Prog. Clin. Biol. Res.*, **374**, 123–137.
- Wogan, G.N., Edwards, G.S., and Shank, R.C. (1967) Excretion and tissue distribution of radioactivity from aflatoxin B1-14C in rats. *Cancer Res.*, **27**, 1729–1736.
- Wogan, G.N., Kensler, T.W., and Groopman, J.D. (2012) Present and future directions of translational research on aflatoxin and hepatocellular carcinoma. A review. *Food Addit. Contam. Part A*, **29**, 249–257.
- Wogan, G.N. and Newberne, P.M. (1967) Dose-response characteristics of aflatoxin B1 carcinogenesis in the rat. *Cancer Res.*, **27**, 2370–2376.
- Wong, Z.A. and Hsieh, D.P. (1980) The comparative metabolism and toxicokinetics of aflatoxin B1 in the monkey, rat, and mouse. *Toxicol. Appl. Pharmacol.*, **55**, 115–125.
- Wu, L., Estrada, O., Zaborina, O., Bains, M., Shen, L., Kohler, J.E., Patel, N., Musch, M.W., Chang, E.B., Fu, Y.-X., *et al.* (2005) Recognition of Host Immune Activation by *Pseudomonas aeruginosa*. *Science*, **309**, 774–777.
- Xu, C., Liu, Q., Huan, F., Qu, J., Liu, W., Gu, A., Wang, Y., and Jiang, Z. (2015) Changes in gut microbiota may be early signs of liver toxicity induced by epoxiconazole in rats. *Chemotherapy*, **60**, 135–142.
- Yanagimoto, T., Itoh, S., Sawada, M., Hashimoto, H., and Kamataki, T. (1994) Molecular cloning and functional expression of a mouse cytochrome P-450 (Cyp3a-13): examination of Cyp3a-13 enzyme to activate aflatoxin B1 (AFB1). *Biochim. Biophys. Acta*, **1201**, 405–410.
- Yang, C.S., Chen, L., Lee, M.-J., Balentine, D., Kuo, M.C., and Schantz, S.P. (1998) Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. *Cancer Epidemiol. Prev. Biomark.*, **7**, 351–354.
- Yang, C.S., Lee, M.J., and Chen, L. (1999) Human salivary tea catechin levels and catechin esterase activities: implication in human cancer prevention studies. *Cancer Epidemiol. Biomark. Prev.*, **8**, 83–89.
- Yu, J., Huang, T., Zhang, L., Wei, Z., Liang, Y., Li, Y., Wang, K., Shi, J., Luo, H., Tang, L., *et al.* (2006) Chemoprevention trial of green tea polyphenols in high-risk

population of liver cancer in southern Guangxi, China. *Proc. Am. Assoc. Cancer Res.*, **2006**, 1148.

- Zaura, E., Brandt, B.W., Teixeira de Mattos, M.J., Buijs, M.J., Caspers, M.P.M., Rashid, M.-U., Weintraub, A., Nord, C.E., Savell, A., Hu, Y., *et al.* (2015) Same exposure but two radically different responses to antibiotics: resilience of the salivary microbiome versus long-term microbial shifts in feces. *mBio*, **6**, e01693-15-e01693-15.
- Zhang, S., Jin, Y., Zeng, Z., Liu, Z., and Fu, Z. (2015a) Subchronic Exposure of Mice to Cadmium Perturbs Their Hepatic Energy Metabolism and Gut Microbiome. *Chem. Res. Toxicol.*, **28**, 2000–2009.
- Zhang, Y., Zhao, F., Deng, Y., Zhao, Y., and Ren, H. (2015b) Metagenomic and Metabolomic Analysis of the Toxic Effects of Trichloroacetamide-Induced Gut Microbiome and Urine Metabolome Perturbations in Mice. *J. Proteome Res.*, **14**, 1752–1761.

CHAPTER 3

METHODOLOGY DEVELOPMENT

The measurement of a microbiome from a particular niche could be roughly divided into descriptions of the microbial community structure/composition and constructions of the microbial functional profile. Thanks to the maturation of next-generation DNA sequencing (NGS) technologies, the former measurement can be achieved by 16S rRNA based gene surveys and the latter by whole genome shotgun (WGS) sequencing based metagenomic analyses. Therefore, research in the microbiome has grown exponentially and achieved significant progress. With the accumulation of data, it is increasingly evidenced that the experimental and data analysis pipelines used in microbiome measurement can introduce considerable variations to the results and influence the inferences (Gohl *et al.*, 2016; Sinha *et al.*, 2015). Consequently, a standard experimental and data analysis protocol is critical to the validity, reproducibility, and comparability of a study, and therefore has been developed during the completion of this dissertation.

Currently, a typical microbiome analysis from a specific sample consists of several steps: (I) DNA extraction from original sample, (II) sequencing library preparation, (III) sequencing, (IV) sequencing data quality filtering, (V) constructing features table, and (VI) statistical analyses. While completing this dissertation, the analysis of the impacts of AFB₁ on the gut microbiome of F344 rats was our first attempt on this topic, which served as a pilot project mainly for methods development. Upon

completing the evaluation of GTP on the gut microbiome of Sprague-Dawley rats, the experimental and data analysis methods have been gradually formalized. In this chapter, the final protocol for experimental and data analysis pipelines is presented.

3.1 DNA extraction

The samples collected for the microbiome analysis for our projects were frozen fecal contents. Microbial DNA was extracted mainly using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Valencia, CA) following manufacture protocols with necessary modification and an extra beads-beating step using MIBIO 0.1 mm glass beads tubes (MOBIO, Carlsbad, CA) to improve the stability and reproducibility.

Immediately after removal from the freezer, about 90 mg of frozen fecal samples were weighed into the 2 ml MIBIO glass beads tubes and placed on ice. InhibitEX buffer (1000 μ l) was added into the tube and vortexed continuously for 15 minutes at maximum level. The inhibitEX buffer mainly contains cell lysis reagents SDS and the QIAGEN proprietary reagent inhibitEX which can absorb substances that degrade DNA and inhibit downstream experiments. The suspension was then placed into a 70 °C waterbath for 10 minutes, followed by vortexing for another 10 minutes. These steps were performed to ensure homogeneous lysis of the microbial cells in fecal samples.

The following steps followed the manufacture's protocol which includes digesting the lysate with proteinase K, binding the DNA in the solution to a silica based membrane spin column with guanidinium chloride, washing out the impurities with alcohol, and eluting the DNA from the spin column by using a Tris-EDTA buffer. A total of 200 μ l of DNA in concentrations ranging from 25-500 ng/ μ l could be obtained. The concentration and purity could be measured using a NanoDrop spectrophotometers. Bovine serum

albumin (BSA) could be added into the extracts to reach a concentration of 0.1 µg/µl to stabilize downstream PCR reaction. Two aliquots of 20 µl extracted DNA which were diluted to around 20 ng/µl were prepared and stored in -20 °C freezer for library preparations.

3.2 16S rRNA sequencing library preparation

16S ribosomal RNA (16S rRNA) is a component of the 30S small subunit of prokaryotic rRNA, existing in multiple copies in all prokaryotic cells. The gene that encodes 16S rRNA is 1542 base pairs (bp) in *Escherichia coli* and is highly conserved among all bacteria. Due to its ubiquity and functional stability, the 16S rRNA gene is the most commonly used housekeeping genetic markers in studies of bacterial phylogeny and taxonomy (Janda and Abbott, 2007). Within the 16S rRNA gene, 10 regions of highly conserved sequences are separated by 9 relatively variable regions which could be used to identify a bacterial species and to assess its phylogenetic status (Baker *et al.*, 2003). There are advantages and weakness of using different variable regions of 16S rRNA genes for bacterial identification (Di Bella *et al.*, 2013); in this dissertation, a primer-pair that amplifies the V3-V4 regions of 16S rRNA genes was selected to minimize amplification bias and maximize taxonomic resolution (Klindworth *et al.*, 2013).

A two-step quadruple-index PCR method was used to prepare the 16S rRNA gene libraries. In the primary PCR reactions, locus-specific primers covering the V3 and V4 regions of 16S rRNA [S-D-Bact-0341-b-S-17 (Bakt_341F):

CCTACGGGNGGCWGCAG, and S-D-Bact-0785-a-A-21 (Bakt_805R):

GACTACHVGGGTATCTAATCC (Klindworth *et al.*, 2013)] were fused with Illumina TruSeq Read1 and Read2 sequences. Internal tag sequences of 5 bases long (Faircloth

and Glenn, 2012) with 0-3 base spacers were inserted between the Illumina and locus-specific portions of the oligonucleotides. The diluted DNA aliquots were amplified using KAPA HiFi HotStart PCR kits (Kapa Biosystems, Inc., Boston, MA). Reactions included 1 µl of DNA, 1.5 µl of each primer (at 5 µM), 6.25 µl of master mix and 14.75 µl PCR-grade water in a 25 µl reaction with the following thermal cycling conditions: one cycle of pre-denaturation at 95 °C for 3 minutes, 20 cycles of denaturation at 95 °C for 20 seconds, annealing at 60 °C for 30 seconds, elongation at 72 °C for 30 seconds, and one cycle of post-elongation at 72 °C for 5 minutes. Three replicate PCR reactions per sample with different tag sequences were performed in the primary PCR reaction and pooled for the secondary limited-cycle PCR to reduce potential PCR bias (Nocker *et al.*, 2007). Following generation of the primary amplicons, another pair of fusion oligonucleotides was used as primers to generate full-length dual-indexed Illumina TruSeqHT style libraries (Glenn *et al.* 2016). The resultant amplicons were normalized, purified 1:1 with Speedbeads (Faircloth and Glenn, 2014), and stored at -20°C prior to sequencing. Sequencing of these 16S rRNA fragment libraries was performed in the Georgia Genomic Facility (University of Georgia, Athens, GA) using the Illumina MiSeq with v2 600 cycle chemistry, targeting to obtain ~100,000 paired-end (PE) reads of 300 nucleotides (nt) from each end per sample. A flowchart for 16S rRNA gene sequencing library preparation was presented in Figure 3-1.

3.3 16S rRNA Sequencing Data Analysis

The Illumina MiSeq platform was selected for 16S rRNA sequencing because firstly it could produce reasonably long sequencing reads which could cover the entire length of our target 16S rRNA gene fragment and secondly the throughput is sufficient

for our purpose. A typical MiSeq run can produce a total of 15 million paired reads, which can sequence 150 samples simultaneously if targeting 100,000 reads per sample.

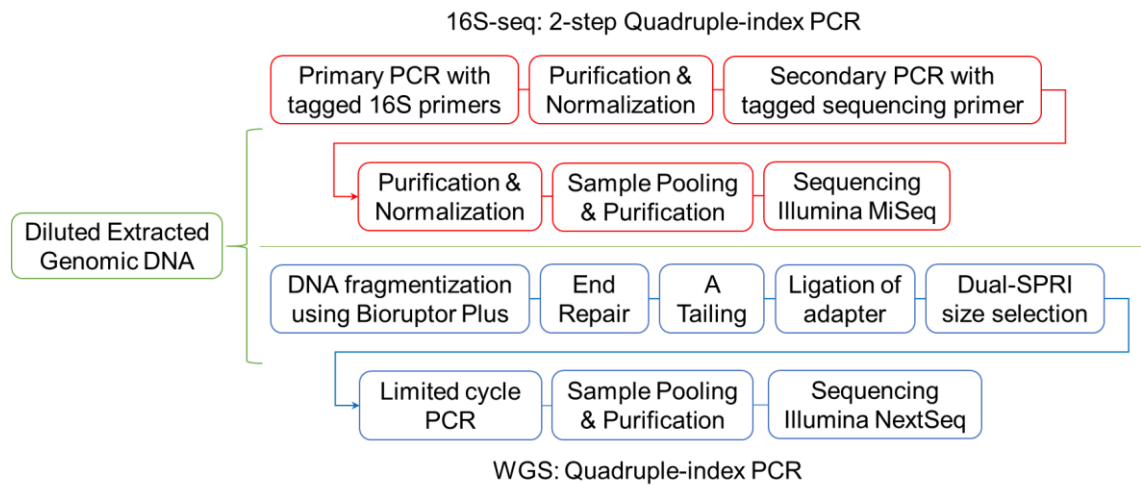


Figure 3-1 The experimental workflows for whole genome shotgun sequencing (WGS) and partial 16S rRNA gene sequencing (16S-seq) library preparation.

The MiSeq platform automatically performs base calling and demultiplexing based on the indexes sequences on the sequencing adapters, and therefore outputs the demultiplexed PE reads with quality scores for the users. If samples are pooled based on inner tags, then software such as Mr. Demuxy (https://pypi.python.org/pypi/Mr_Demuxy) is used for a second round of demultiplexing. Processing the sequencing reads typically involves two stages: in the first stage, raw sequencing data are quality filtered and processed to build a feature table; in the second stage, statistical analysis and scientific inferences are performed based on the feature table. A feature table in the microbiome studies is a two-dimensional table, in which the column names are sample IDs, the row names are different features (e.g. microbial species, functional genes), and the values are the occurrence/abundance of each feature in each sample. The feature table is produced

based on the raw sequencing reads using bioinformatic analysis and is further analyzed using statistical methods, that are consequently essential in the microbiome analysis.

Demultiplexed PE sequencing reads from 16S rRNA sequencing were quality filtered by trimming low-quality bases ($Q < 20$) from 3' end of each read so that the remaining reads did not contain more than 3 low-quality bases. The PE reads were then merged with a minimum overlap of 10 NTs, no dovetail overlap, and maximum mismatch density of 0.05. Merged reads were processed so that any inner tags, spacers, and locus-specific primers were trimmed off from each end and limited to 350-550 nt in length. The resultant reads were analyzable reads that could be deposited into public sequence archive databases and input into the QIIME pipeline (Caporaso, Kuczynski, *et al.*, 2010) for further processing. Briefly, the analyzable sequences from each sample were clustered into Operational Taxonomic Unit (OTU) by uclust algorithm (Edgar, 2010) using a *de novo* strategy with the similarity threshold of 97%. Representative sequences for each OTU were compared against the Greengene 16S rRNA gene database 13-8 release (DeSantis *et al.*, 2006) using uclust algorithm with the similarity threshold of 90%. The top 3 database hits that matched the above representative sequences for each OTU were selected; the taxonomic information assigned to an OTU was set to be the most detailed lineage description which was shared by at least 51% of these three database hits. A phylogenetic tree was constructed by firstly aligning the representative sequences of each OTU using PyNAST algorithm (Caporaso, Bittinger, *et al.*, 2010) against Greengene reference sequences and then using FastTree software with GTR model and Gamma 20 approximation (Price *et al.*, 2010). The OTU table (the feature table) and phylogenetic tree were the major elements that would be used for later

statistical analysis. The procedure for 16S rRNA data analysis was presented in Figure 3-2.

3.4 Analysis of standard mock communities to gauge the 16S rRNA gene survey

To evaluate the validity of the library preparation and data analysis procedure in the 16S rRNA sequencing analysis, two standard mock communities (HM-276D and HM-277D) were processed and analyzed. HM-276D and HM-277D are artificially generated microbial communities containing the genome of 20 microbial species

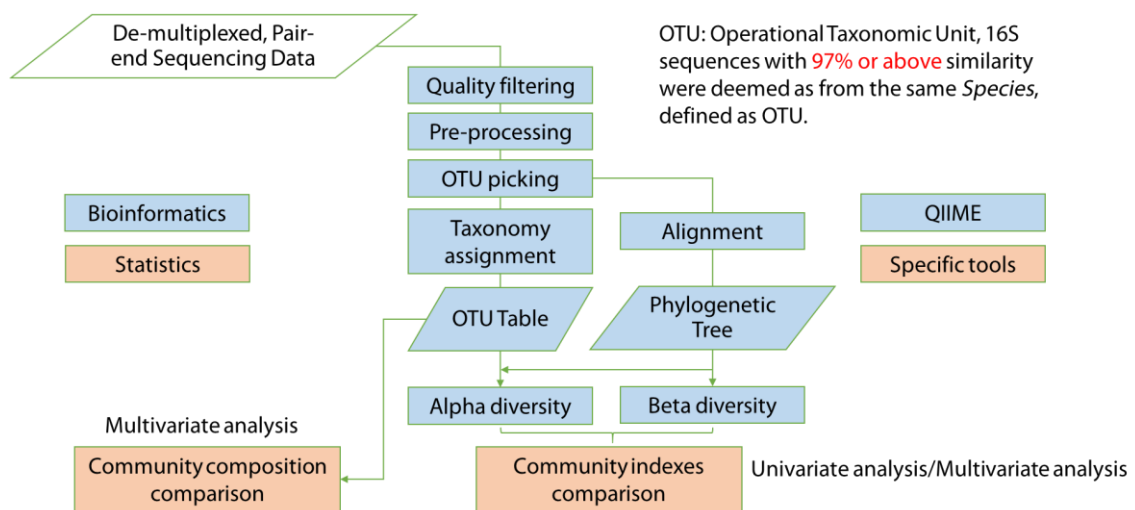


Figure 3-2 The procedures for analyzing 16S rRNA sequencing reads

developed by Human Microbiome Project (NIH HMP Working Group *et al.*, 2009); HM-276D contains even rRNA operon counts whereas HM-277D contains staggered rRNA operon counts from 20 microbial species (Table 3-1).

Genomic DNAs from both standard mock communities were diluted so that equal amounts of 50,000 16S rRNA gene operons from the 20 species in HM-276D (1:20 dilution) and 1,000 to 1,000,000 operons from the same 20 species in HM-277D (1:10 dilution) were input into the primary PCR (1 µl input DNA). The library preparation and sequencing data analysis followed the procedures described in section 3.2-3.3. A total of

24,723 and 25,507 quality filtered reads were available for HM-276D and HM-277D respectively. A total of 197 OTUs were identified in HM-276D, including all of the 20 stated species (abundances ranging from 0.76% to 11.87%). In comparison, 153 OTUs were identified in HM-277D, but only including 18 of the 20 species (abundances ranged from 0.0039% to 49.68%). Figure 3-3 A-B presented the measured abundances of the 20 species from HM-276D and 277D (columns) against their expected abundance (dash

Table 3-1 Composition of microbial mock community HM-276D and HM-277D

Organism	NCBI Reference Sequence	Input Materials (operons/μl)	
		HM-276D*	HM-277D*
<i>Acinetobacter baumannii</i> strain 5377	NC_009085	1,000,000	100,000
<i>Actinomyces odontolyticus</i> strain 1A.21	NZ_AAYI02000000	1,000,000	10,000
<i>Bacillus cereus</i> strain NRS 248	NC_003909	1,000,000	1,000,000
<i>Bacteroides vulgatus</i> strain NCTC 11154	NC_009614	1,000,000	10,000
<i>Clostridium beijerinckii</i> strain NCIMB 8052	NC_009617	1,000,000	1,000,000
<i>Deinococcus radiodurans</i> strain R1 (smooth)	NC_001263, NC_001264	1,000,000	10,000
<i>Enterococcus faecalis</i> strain OG1RF	NC_017316	1,000,000	10,000
<i>Escherichia coli</i> strain K12 substrain MG1655	NC_000913	1,000,000	10,000,000
<i>Helicobacter pylori</i> strain 26695	NC_000915	1,000,000	100,000
<i>Lactobacillus gasseri</i> strain 63 AM	NC_008530	1,000,000	100,000
<i>Listeria monocytogenes</i> strain EGDe	NC_003210	1,000,000	100,000
<i>Neisseria meningitidis</i> strain MC58	NC_003112	1,000,000	100,000
<i>Propionibacterium acnes</i> strain KPA171202	NC_006085	1,000,000	100,000
<i>Pseudomonas aeruginosa</i> strain PAO1-LAC	NC_002516	1,000,000	1,000,000
<i>Rhodobacter sphaeroides</i> strain ATH 2.4.1	NC_007493, NC_007494	1,000,000	10,000,000
<i>Staphylococcus aureus</i> strain TCH1516	NC_010079	1,000,000	1,000,000
<i>Staphylococcus epidermidis</i>	NC_004461	1,000,000	10,000,000

FDA strain PCI 1200			
<i>Streptococcus agalactiae</i>			
strain 2603 V/R	NC_004116	1,000,000	1,000,000
<i>Streptococcus mutans</i>			
strain UA159	NC_004350	1,000,000	10,000,000
<i>Streptococcus pneumoniae</i>			
strain TIGR4	NC_003028	1,000,000	10,000

*The following reagent was obtained through BEI Resources, NIAID, NIH as part of the Human Microbiome Project: Genomic DNA from microbial mock community B (Even, High Concentration), v5.1H, for Whole Genome Shotgun Sequencing, HM-276D; Genomic DNA from microbial mock community B (Staggered, High Concentration), v5.2H, for Whole Genome Shotgun Sequencing, HM-277D

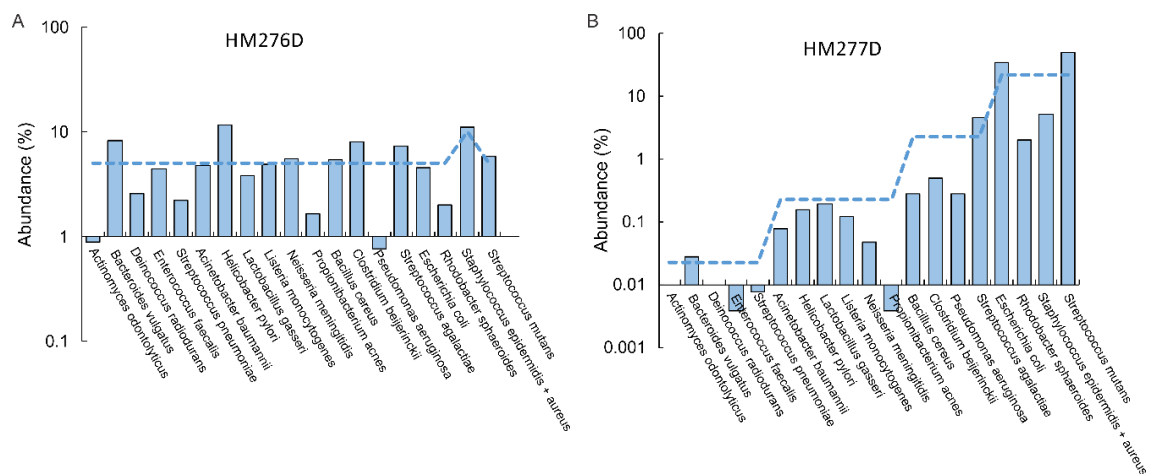


Figure 3-3 Measured (columns) and expected (dash lines) abundances of 20 stated species in (A) HM-276D and (B) HM-277D.

lines). Because the V3 and V4 region of 16S rRNA cannot differentiate *Staphylococcus epidermidis* and *S. aureus*, their abundances are plotted together. Comparing the measured to theoretical abundances the average over- or under-estimation of expected abundance was only 1.7 folds (geometric mean was used to limit the impact of a few extreme values) for HM-276D and 3.7 folds for HM-277D, which are similar to a recent publication (Gohl *et al.*, 2016).

OTUs other than the 20 stated species captured in the analysis all existed in very low abundance (on average less than 0.02% and no greater than 0.65%), suggesting that

filtering of OTUs based on their abundance is necessary to avoid spurious findings. Consequently, a receiver operating characteristic (ROC) analysis was performed to select the threshold abundance for filtering. In ROC analysis, the threshold was defined as the proportion, the OTU whose abundance above which was considered as existence in the sample (test positive), otherwise, as spurious findings (test negative). The sensitivity of the test was defined as the proportion of the 20 stated species in the test positive and the specificity of the test was defined as the proportion of the captured OTUs other than the 20 stated species in the test negative. An ROC curve was plot for HM-276D and HM-277D respectively (Figure 3-4), based on which an abundance of 0.1% was suggested to filter OTUs in the 16S rRNA analysis of real samples.

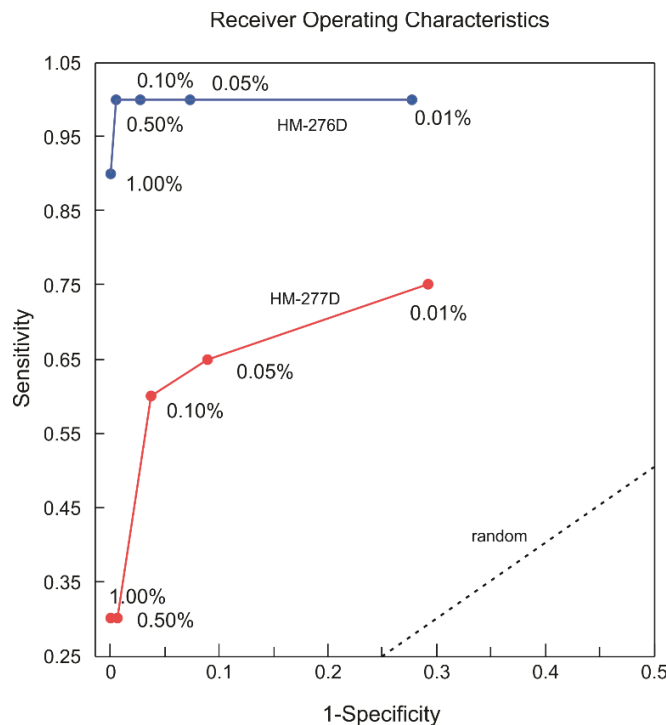


Figure 3-4 Receiver operating characteristics analysis to determine the OTU abundance filtering threshold.

3.5 Whole genome shotgun sequencing library preparation

Although 16S rRNA technique is relatively easy to implement and is widely accepted, it only provides compositional information with some intrinsic pitfalls (Janda

and Abbott, 2007). Considering the microbial functional redundancy and horizontal gene transfer, compositional information might not be very useful in evaluating the functions of the gut microbiota. Consequently, a WGS based metagenomics analysis could provide valuable information to implement the microbiome study.

A double-index single step PCR reaction was used to prepare the WGS sequencing library. One aliquot of diluted DNA was fragmentized using Bioruptor Plus (Diagenode, Denville, NJ) to obtain a collection of DNA fragments with a dominant size of ~500 (bp), which was checked by a gel electrophoresis. Fragmented DNA was then processed using KAPA LTP library preparation kit (Kapa Biosystems, Inc., Boston, MA) according to the manufacturer's protocol. Briefly, the DNA fragments were end-repaired for blunt ends, followed by adding an adenosine on 3' end (A-tailing). Using this A tail, a Y-yoke adapter with partial sequences of the iTru PCR primer-binding site was ligated on each side of the DNA fragments and subsequently amplified with an iTru primer to generate full-length dual-indexed Illumina TruSeqHT style library in a limited-cycle PCR (Glenn *et al.* 2016). Prepared WGS libraries were sequenced using the Illumina NextSeq platform to obtain ~2,000,000 PE reads of 150 bp per sample. The flowchart of WGS sequencing library preparation was also presented in Figure 3-1.

3.6 Metagenomic analysis

The Illumina NextSeq platform was selected for WGS because metagenomic analysis requires much more sequences per sample (deeper sequencing depth) as compared to 16S rRNA gene survey. A typical run of NextSeq produces 260 million read pairs, which if targeting 2 million read pairs per sample could sequence 130 samples simultaneously. Metagenomic analysis was performed following a similar strategy in Li

et al. (2014) and Nielson *et al.* (2014). Raw de-multiplexed PE reads from each sample was quality filtered using Trimmomatic-v0.3.6 (Bolger *et al.*, 2014) so that the remaining sequences did not contain more than 3 consecutive low quality bases ($Q < 20$) and had a minimum length of 75 nt. The filtered reads were then fed to MEGAHIT-v1.0.2 (Li *et al.*, 2016) for metagenome assembly. The proportion of quality filtered reads that could be mapped to MEGAHIT assemblies were checked using Bowtie2 (Langmead and Salzberg, 2012). MEGAHIT assemblies for each sample were processed by Prodigal-v2.6.3 (Hyatt *et al.*, 2010) for microbial gene prediction. Predicted genes from each sample that were longer than 100 bp were pooled, de-replicated according to sequence prefix, and clustered with 99% identity using vsearch-v1.11.1 (<https://github.com/torognes/vsearch>), producing a non-redundant microbial meta-gene catalog (MGC). The corresponding amino acid sequences for MGC were annotated according to Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (genus_prokaryotes section) using GhostKOALA (Kanehisa *et al.*, 2016), and Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups (eggNOG) v4.5 database (Huerta-Cepas *et al.*, 2016) (bacteria section, bactNOG), using in-house scripts. The quality filtered reads from each sample were subsequently mapped to the annotated MGC using Bowtie2 to generate gene abundances which were further normalized using the gene length (Qin *et al.*, 2012). Normalized gene abundance tables (the feature table) for each sample were the products of metagenomic analysis and subjected to further statistical comparisons. The procedure for WGS metagenomic data analysis is presented in Figure 3-5.

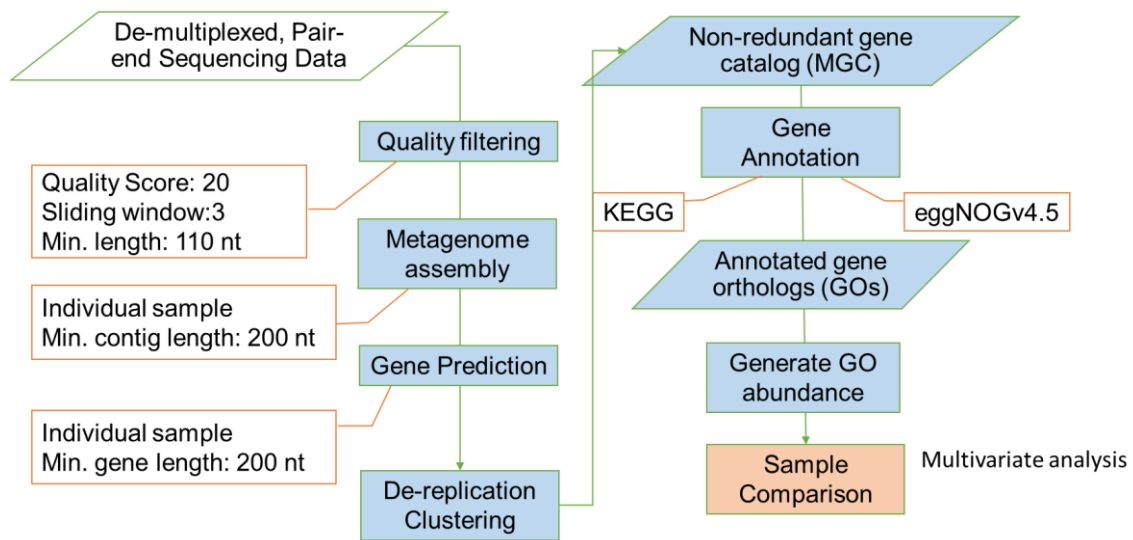


Figure 3-5 The procedures for analyzing WGS sequencing reads

References

- Baker, G.C., Smith, J.J., and Cowan, D.A. (2003) Review and re-analysis of domain-specific 16S primers. *J. Microbiol. Methods*, **55**, 541–555.
- Bolger, A.M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, **30**, 2114–2120.
- Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., and Knight, R. (2010a) PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinforma. Oxf. Engl.*, **26**, 266–267.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., *et al.* (2010b) QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods*, **7**, 335–336.
- DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., and Andersen, G.L. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.*, **72**, 5069–5072.
- Di Bella, J.M., Bao, Y., Gloor, G.B., Burton, J.P., and Reid, G. (2013) High throughput sequencing methods and analysis for microbiome research. *J. Microbiol. Methods*, **95**, 401–414.
- Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, **26**, 2460–2461.

- Faircloth, B.C. and Glenn, T.C. (2012) Not all sequence tags are created equal: designing and validating sequence identification tags robust to indels. *PLOS ONE*, **7**, e42543.
- Faircloth, B.C. and Glenn, T.C. (2014) Protocol: Preparation of an AMPure XP substitute (AKA Serapure). DOI: 10.6079/J9MW2F26.
- Glenn, T.C., Nilsen, R., Kieran, T.J., Finger, J.W., Pierson, T.W., Bentley, K.E., Hoffberg, S., Louha, S., Garcia-De-Leon, F.J., Portilla, M.A. del R., *et al.* (2016) Adapterama I: Universal stubs and primers for thousands of dual-indexed Illumina libraries (iTru & iNext). *bioRxiv*, 049114.
- Gohl, D.M., Vangay, P., Garbe, J., MacLean, A., Hauge, A., Becker, A., Gould, T.J., Clayton, J.B., Johnson, T.J., Hunter, R., *et al.* (2016) Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies. *Nat. Biotechnol.*, **34**, 942–949.
- Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M.C., Rattei, T., Mende, D.R., Sunagawa, S., Kuhn, M., *et al.* (2016) eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res.*, **44**, D286–D293.
- Hyatt, D., Chen, G.-L., LoCascio, P.F., Land, M.L., Larimer, F.W., and Hauser, L.J. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics*, **11**, 1.
- Janda, J.M. and Abbott, S.L. (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J. Clin. Microbiol.*, **45**, 2761–2764.
- Kanehisa, M., Sato, Y., and Morishima, K. (2016) BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J. Mol. Biol.*, **428**, 726–731.
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., and Glockner, F.O. (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.*, **41**, e1.
- Langmead, B. and Salzberg, S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nat. Methods*, **9**, 357–359.
- Li, D., Luo, R., Liu, C.-M., Leung, C.-M., Ting, H.-F., Sadakane, K., Yamashita, H., and Lam, T.-W. (2016) MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. *Methods*, **102**, 3–11.

- Li, J., Jia, H., Cai, X., Zhong, H., Feng, Q., Sunagawa, S., Arumugam, M., Kultima, J.R., Prifti, E., Nielsen, T., *et al.* (2014) An integrated catalog of reference genes in the human gut microbiome. *Nat. Biotechnol.*, **32**, 834–841.
- Nielsen, H.B., Almeida, M., Juncker, A.S., Rasmussen, S., Li, J., Sunagawa, S., Plichta, D.R., Gautier, L., Pedersen, A.G., Le Chatelier, E., *et al.* (2014) Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. *Nat. Biotechnol.*, **32**, 822–828.
- NIH HMP Working Group, Peterson, J., Garges, S., Giovanni, M., McInnes, P., Wang, L., Schloss, J.A., Bonazzi, V., McEwen, J.E., Wetterstrand, K.A., *et al.* (2009) The NIH Human Microbiome Project. *Genome Res.*, **19**, 2317–2323.
- Nocker, A., Burr, M., and Camper, A.K. (2007) Genotypic Microbial Community Profiling: A Critical Technical Review. *Microb. Ecol.*, **54**, 276–289.
- Price, M.N., Dehal, P.S., and Arkin, A.P. (2010) FastTree 2 – Approximately maximum-likelihood trees for large alignments. *PLOS ONE*, **5**, e9490.
- Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W., Guan, Y., Shen, D., *et al.* (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*, **490**, 55–60.
- Sinha, R., Abnet, C.C., White, O., Knight, R., and Huttenhower, C. (2015) The microbiome quality control project: baseline study design and future directions. *Genome Biol.*, **16**, 276.

CHAPTER 4

AFLATOXIN B1 INDUCED COMPOSITIONAL CHANGES IN GUT MICROBIAL COMMUNITIES OF MALE F344 RATS ¹

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Abstract

Aflatoxins are a group of potent foodborne toxicants naturally occurring in maize and groundnuts. Differential species-specific sensitivity to aflatoxins has been documented but cannot be fully explained by the differences in metabolism of these toxicants among animal species. Commensal microbial communities (microbiota) are critical to human and animal health, but few studies have assessed interactions between xenobiotic toxins and these microbiota, and its potential effects to humans and animals. Here, an exploratory dosing experiment was conducted to explore effects of Aflatoxin B₁ (AFB₁) on the gut microbiota in a commonly used rat model. Male F344 rats were randomly divided into groups and treated with different concentrations of AFB₁. Microbial communities in fecal samples were assessed using 16S rRNA sequence analysis. We found that samples from the control group had a phylogenetically diverse community, and that increasing AFB₁ doses decreased this diversity but increased evenness of community composition. In addition, the gut microbiota from different samples were clustered according to their dosing regimens. There is no community shift at the phylum level but some lactic acid bacteria were significantly depleted by AFB₁. These findings suggested that AFB₁ could modify the gut microbiota in a dose-dependent manner.

4.1 Introduction

Commensal microbial communities (microbiota), especially those that reside in the gastrointestinal tract of animals are critical to the healthy development of some important internal structures, e.g., villous structures, immune organs (DuPont and DuPont, 2011; Nicholson *et al.*, 2005). Recently animal and human studies have shown

that perturbed gut microbiota are associated with multiple health problems such as obesity and inflammatory bowel disease (Ley *et al.*, 2006; Qin *et al.*, 2010; Turnbaugh *et al.*, 2006). Researchers have become increasingly interested in how xenobiotic exposure would impact these crucial communities.

Aflatoxins are a group of mycotoxins attracting consistent research and public interests because the toxigenic fungus, *Aspergillus*, contaminates a large fraction of the world's crops (Lizarraga-Paulin *et al.*, 2011). Williams *et al.* (2004) estimated that approximately 4.5 billion people in developing countries are chronically exposed to uncontrolled amounts of aflatoxins from foods. Extensive studies have documented that aflatoxins, especially the most potent congener, aflatoxin B₁ (AFB₁), can be metabolically activated in the liver to cause acute hepatic toxicity and immune toxicity, form DNA adducts, and induce gene mutations (Qian *et al.*, 2014; Wogan *et al.*, 2012). Furthermore, chronic exposure to low levels of aflatoxins is a risk factor for hepatocellular carcinoma and AFB₁ has been categorized as a Group 1 human carcinogen by International Agency for Cancer Research (IARC) (IARC, 2012).

The effects of only a few types of xenobiotic toxicants on gut microbiota have been studied so far: two heavy metals (Cd and Pb) (Breton *et al.*, 2013b; Breton *et al.*, 2013a), metal nano-particles (Han *et al.*, 2014; Merrifield *et al.*, 2013), arsenic (Lu *et al.*, 2014a; Lu *et al.*, 2013; Lu *et al.*, 2014b), alcohol (Bull-Otterson *et al.*, 2013; Xie *et al.*, 2013), and a few other toxic organics (Choi and Toborek, 2012; Kish *et al.*, 2013; Kohl *et al.*, 2014; Peterfreund *et al.*, 2012; Swann *et al.*, 2009; Yip *et al.*, 2014). Two major findings have been revealed from these studies: firstly, gut microbiota contributes to host resistance to xenobiotic toxicants; and secondly, xenobiotics can modify the composition

of microbial communities. Results from these studies begin to shed light on this important issue, but much work remains to be done. Reliable standard experimental protocols are needed to perform such toxicological experiments, including better data analysis procedures to assess the effects and interactions of toxins/toxicants on microbiota.

Differential species-specific sensitivity to AFB₁ has been documented but cannot be fully explained by the differences in metabolism of AFB₁ among animal species (Rawal *et al.*, 2010). We therefore hypothesize that the gut microbiota play a role in modulation of species responses to AFB₁ toxicity. Accordingly, the rationale underlying this study is that AFB₁ could induce changes in gut microbiota of its host and therefore eventually influence the effects of this toxin. This hypothesis was tested via community compositional assessment of gut microbiota in a rat model dosed with different concentrations of AFB₁. 16S rRNA gene sequence analysis was used to assess the microbiota. The objectives of this study were designed to: (1) establish reliable experiment protocols for preparing microbial 16S rRNA gene libraries and data preparation/analysis procedures for assessing the effects of AFB₁ on commensal microbiota, and (2) use these protocols to determine if there is a dose-response relationship between AFB₁ exposure and the observed microbial communities of the exposed rats.

4.2 Materials and Methods

4.2.1 Chemicals, animals and experiment design.

This study used a design that was similar to a previous toxicity study in which detailed procedures on chemicals and animal preparations were described (Qian *et al.*,

2014). Briefly, 20 male Fischer 344 rats (5 weeks old, 120-130g) were acclimatized for 1-week before the experiment, randomly allocated into four dosing groups: control, low (5 µg AFB₁/kg body weight (b.w.)), medium (25 µg/kg b.w.), high (75 µg/kg b.w.); upon starting of the experiment, gavaged with control vehicle (DMSO) or AFB₁ in DMSO, 5 days per week for a duration of 4 weeks. DMSO was used due to the hydrophobicity of AFB₁ and its minimum effects on animals (< 50 µL/animal). Rat feces were collected at the end of the fourth week in metabolic cages and stored at -80°C.

4.2.2 16S rRNA gene sequencing.

Genomic DNA of gut microbiota was extracted from fecal samples collected from each individual using QIAamp DNA stool mini kits (QIAGEN, Valencia, CA) following manufacture protocols with minor modification. Briefly, immediately after removal from the freezer, about 90 mg of frozen feces was weighed into a 2 ml tube and placed on ice with 500 µl of stool lysis buffer added. A disposable spatula or pipette tip was used to help homogenize the sample. Another 900 µl of stool lysis buffer was added after sufficient homogenization was obtained. We then followed the manufacturer's protocols, resulting in 200 µl of DNA in buffer from each sample with concentrations ranged from about 70 ng/µl to 150 ng/µl and stored at -20°C.

A two-step Quadruple-index PCR method was used to prepare the 16S rRNA gene libraries. Briefly, locus-specific primers to the V3 and V4 regions of 16S rRNA (Klindworth *et al.*, 2013): S-D-Bact-0341-b-S-17 (Bakt_341F) CCTACGGGNGGCWGCAG and S-D-Bact-0785-a-A-21 (Bakt_805R) GACTACHVGGGTATCTAATCC) were fused with Illumina TruSeq Read1 and Read2 sequences. Internal tag sequences 5 bases long (Faircloth and Glenn, 2012) with 0-3 base

spacers were inserted between the Illumina and locus-specific portions of the oligonucleotides. The DNA samples were diluted to approximately 20 ng/μl and PCR amplified using Kapa HiFi HotStart PCR kits (Kapa Biosystems, Inc., Boston, MA). 1 μl of DNA was added to the 25 μl reaction with following thermal cycling conditions: one cycle of pre-denaturation at 95 °C for 3 minutes, 20 cycles of denaturation at 95 °C for 20 seconds, annealing at 60 °C for 30 seconds, elongation at 72 °C for 30 seconds, and one cycle of post-elongation at 72 °C for 5 minutes. Following generation of the primary amplicons, another pair of fusion oligonucleotides was used as primers to generate full-length dual-indexed Illumina TruSeqHT style libraries. The resultant amplicons were normalized, purified 1:1 with Speedbeads (Faircloth and Glenn, 2014), and stored at -20°C prior to sequencing. Sequencing of these 16S rRNA fragment libraries was performed in the Georgia Genomic Facility (University of Georgia, Athens, GA) using the Illumina MiSeq with v2 500 cycle chemistry, resulting in paired-end 250 base reads to obtain ~30,000 reads per sample.

4.2.3 Data preparation.

The raw paired-end, demultiplexed sequence reads for each sample from sequencing facility were merged using FLASH 1.2.9 (Magoc and Salzberg, 2011) available through Geneious 8.1 software (Biomatters Inc, San Francisco, CA). Internal tags, base spacers, and locus-specific primers from each end of merged sequences were trimmed and sequences outside the length range 400-450 base-pairs were discarded using Geneious 8.1. Outputs from Geneious 8.1 were quality filtered using QIIME pipeline (Quantitative Insights Into Microbial Ecology) (Caporaso *et al.*, 2010b) with following settings: sequences were trimmed from each end to obtain the shortest sequences without

3 consecutive low-quality base calls which were defined as having a Phred quality score lower than 20 and without any ambiguous base calls; the resultant sequences that were shorter than 75% of their original length were discarded (Bokulich *et al.*, 2013). The resultant analyzable sequence reads were deposited into Sequence Read Archive database with the accession number PRJNA291923.

Analyzable sequences from each sample were clustered into Operational Taxonomic Unit (OTU) by uclust algorithm (Edgar, 2010) using a *de novo* strategy with the similarity threshold of 97%. Representative sequences for each OTU were compared against the Greengene 16S rRNA gene database 13-8 release (DeSantis *et al.*, 2006) using uclust algorithm with the similarity threshold of 90%. The top 3 database hits that matched the above representative sequences for each OTU were selected; the taxonomic information assigned to an OTU was set to be the most detailed lineage description which was shared by at least 51% of these three database hits. A phylogenetic tree was constructed by firstly aligning the representative sequences of each OTU using PyNAST algorithm (Caporaso *et al.*, 2010a) against Greengene reference sequences and then using FastTree software with GTR model and Gamma 20 approximation (Price *et al.*, 2010). Above mentioned procedures were implemented through QIIME with customized commands. The OTU table and phylogenetic tree were the major elements for data analysis.

4.2.4 Data analysis.

Complementary divergence-based and taxon-based methods were used to compare samples among different treatment groups. Divergence-based methods are more powerful in certain circumstances because not all species in one sample are equally

related; samples containing species that are phylogenetically closely related should be considered to be more similar as compared to samples containing species that are phylogenetically distant from each other (Lozupone and Knight, 2008). Three methods were employed to facilitate data analysis: α -diversity analysis, β -diversity analysis, and direct comparisons of community compositions in samples from different group. All analysis used 0.05 as significance level.

4.2.4.1 *α -diversity analysis.*

α -diversity describes the diversity within a community (i.e. a sample in this study). A divergence-based metric, phylogenetic diversity (PD) (Faith, 1992), and a taxon-based metric, Shannon index (Magurran, 2004) were computed for each sample using QIIME. Mean value and 95% confidence interval were calculated for each dosing group. To avoid sequencing depth bias, the sample sizes from different sample were equalized by randomly jackknifing 24000 sequences per sample (approximately the number of sequences of the sample with least analyzable reads). One-way ANOVA test were conducted to analyze dosing effect on alpha diversity and Duncan's test were conducted for post-hoc comparisons using R 3.1.2 (R Core Team, 2014).

4.2.4.2 *β -diversity analysis.*

β -diversity describes the relationship between two communities (or one community at different time points). The unweighted UniFrac index describes divergence distance between two samples, which measures the fraction of branches in a phylogenetic trees, which lead to an OTU that only appears in either one of the two communities but not both (Lozupone and Knight, 2005). Unweighted Unifrac index was computed for all pairs of samples to construct distance matrix. To avoid sequencing depth bias, calculation

of unweighted UniFrac index was performed on subsampled samples as previously described. The principle coordinate analysis (PCoA) was performed using the distance matrix constructed by unweighted UniFrac index to examine sample clustering. In order to measure the uncertainty of β -diversity analysis, each sample was randomly jackknifed 18,000 sequences per sample and analyzed following the above procedures; the jackknifing procedure was repeated 10 times. All above procedures were implemented in QIIME.

4.2.4.3 Direct *comparisons of community compositions*.

α -diversity and β -diversity analysis target the gut microbial community. From the perspective of toxicology, the specific gut bacteria that were significantly impacted by exposure to AFB₁ may also be important. Consequently, direct comparisons of community compositions were performed using MetagenomeSeq software (Paulson *et al.*, 2013) implemented in R 3.1.2. Briefly, MetagenomeSeq uses normalized OTU counts from each sample as input to avoid bias due to preferentially sampling associated with sequencing yields and a Zero-Inflated Gaussian model to account for the zero OTU counts; a maximum-likelihood model and Empirical Bayes were used to generate significance of dosing effects (Paulson *et al.*, 2013). This analysis compares differentiated abundance of each OTU in different dosing groups. Before performing the analysis, the original datasets were subset to include only OTUs that had counts in at least six samples (half the total) to improve statistical capacity. The p-values generated from the Empirical Bayes for each OTU were further corrected to adjust for multiple-comparison using *Bonferroni* method implemented in R 3.1.2.

4.3 Results

4.3.1 16S rRNA gene sequencing and data preparation.

Among the initial 20 rats, fecal samples from 3 rats in each group were available, making a total of 12 samples for analysis in this study. Microbial DNA from fecal samples were extracted, 16S rRNA gene libraries were prepared using a two-step quadruple-index PCR methods before sending out for sequencing. An average of 32786 pairs of raw paired-end sequences (ranged from 27293 to 38113, Table 4-1) were obtained from the sequencing facility for each sample. The length distributions of the target 16S rRNA gene fragment from each sample after merging and trimming are plotted in Figure 4-1. More than 80% of sequences from each sample passed quality filtering, indicating that the sequencing technique is robust and efficient.

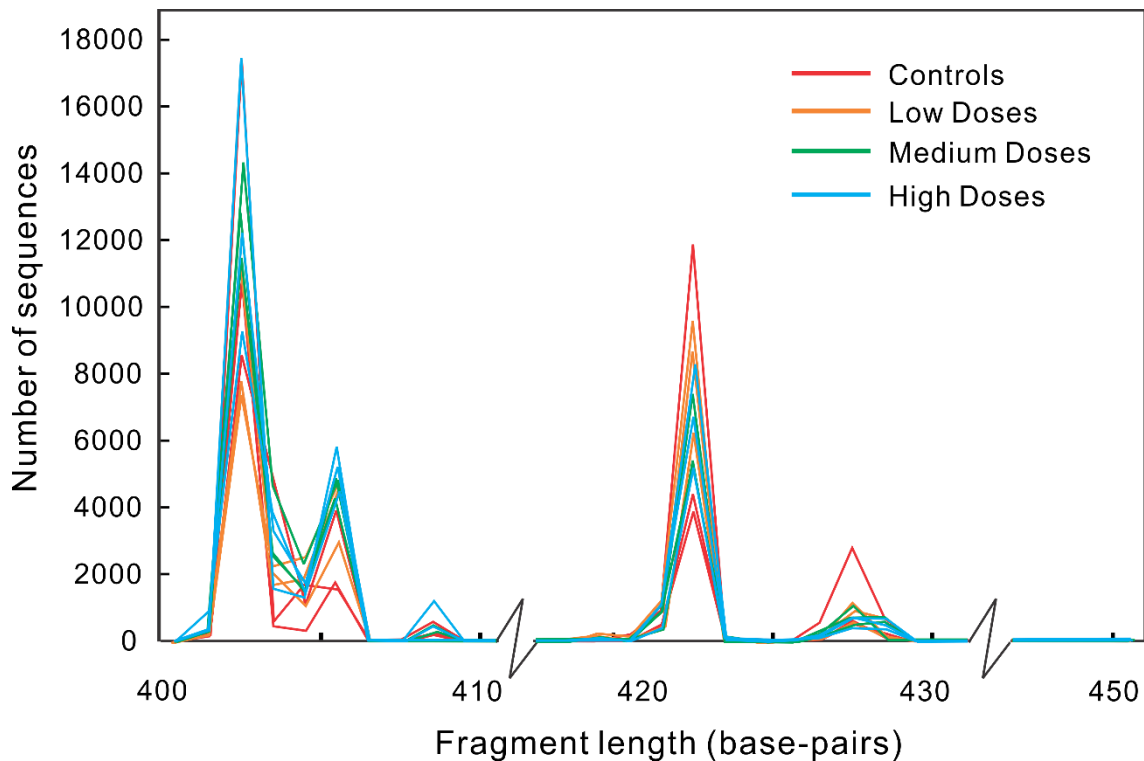


Figure 4-1 Fragment length distribution of sequences from each sample after merging and trimming. The target fragment is 427 base-pairs long in *Escherichia coli* [from site 358 to 784, excluding primers (Klindworth *et al.*, 2013)].

4.3.2 α -diversity analysis.

PD reflects the genetic diversity of a microbial community. The more distantly related species a community has, the higher is PD for the community. As illustrated in Figure 4-2A, increasing AFB₁ doses decreased the PD; one-way ANOVA test suggested a significant AFB₁ dosing effect on PD (F-value = 123.24, p -value < 0.05). Duncan's post-hoc test was used to adjust for experiment-wise error rate, which showed significant differences in all pairwise comparisons except that between medium and high dosing group.

Shannon index simultaneously considers species richness and evenness. The more species (regardless of the genetic relationship between species) a community has and the more even a community is distributed by each species, the higher Shannon index is for the community. As illustrated in Figure 4-2B, Shannon index had an exact opposite pattern as presented for PD. That is, increasing AFB₁ doses increased Shannon index, although the one-way ANOVA test failed to suggest a significant dosing effect. As for comparison, the number of unique OTUs averaged across each dosing group is also plotted in Figure 4-2C. This measurement directly presents the number of different microbial species within a community. One-way ANOVA test suggested a significant dosing effect on unique OTUs. The control group had an average of 689 OTUs and was significantly higher than the other groups as confirmed by Duncan's test; the low, medium and high dose had decreased number of unique OTUs, 556, 453 and 455 respectively.

Detailed information regarding α -diversity, observed OTUs, and taxonomic information for each sample can be found in the Table 4-2.

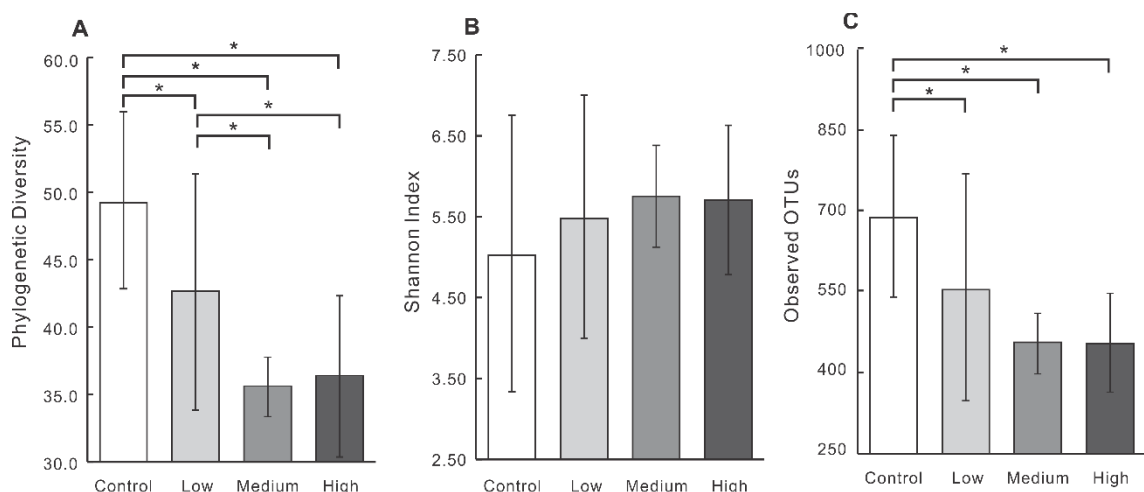


Figure 4-2 Alpha diversity indexes based on 16S rRNA gene sequence analysis among aflatoxin B₁ (AFB₁) treatment groups with three replicate individuals per treatment group and 24,000 sequences per sample. Error bars indicate 95% confidence interval. * indicates significant difference between means of different treatment groups determined by Duncan's post-hoc test. (A) Phylogenetic diversity decreased significantly with increasing AFB₁ in a dose-dependent manner (one-way ANOVA $p < 0.05$). (B) Shannon index increased with increasing of AFB₁ in a dose-dependent manner, but was not significant (one-way ANOVA $p > 0.05$). (C) Observed operational taxonomic units (OTUs) decreased with increasing of AFB₁ in a dose-dependent manner (one-way ANOVA $p < 0.05$).

4.3.3 β -diversity analysis.

Ranging from 0 to 1, the two communities that have closer related species will have a smaller Unweighted UniFrac Index; vice versa. Pair-wise comparisons will produce an N by N Unifrac Index matrix, where N is the number of total samples in all groups. This matrix has the property of a distance matrix and therefore could be analyzed by PCoA. As illustrated in Figure 4-3, the first three principle components could readily cluster the samples according to their dosing regimens. Ellipsoids around each sample point generated via jackknifing analysis suggested low uncertainties. Samples plotted in PCoA also appeared to locate in a dose-dependent manner: samples from low dose group located in between the control samples and higher dose groups. In addition, control samples had relatively large variation, increasing doses brought samples from the same

dosing group closer together. It is worth noticing that similar as in α -diversity analysis, samples from medium and high dosing groups had relatively small intragroup variation and cannot be differentiated.

4.3.4 Direct comparison of community compositions.

Despite advantages of divergence-based methods, above analyses cannot recognize the species (OTUs) or lineages that drive the clustering in association with different AFB₁ doses. Consequently, a direct comparison of community compositions could complement these analysis. Through initial screening, among 2479 unique OTUs in all samples, 253 OTUs appeared in at least 6 samples; following analysis was performed on these 253 OTUs. After fitting the Zero-Inflated Gaussian model, Empirical Bayes testing, and *Bonferroni* corrections, 191 OTUs appeared to have a corrected *p*-value below pre-defined significance level, among which the top 20 consistent OTUs were plotted in Figure 4-4 and listed in Table 4-3. Figure 4-4 plotted the model estimated Log_2 fold-change of normalized OTU abundance in low, medium, and high dosing groups as compared to the control groups. Among the 20 OTUs, the increases in treatment groups ranged from 1.13 fold (OTU:denovo27) to as high as 19 fold increase (OTU: denovo207) as compared to controls; the decreases ranged from 78% (OTU: denovo1046) to as low as 2% (OTU: denovo510) of what was in controls. Given taxonomic information of these top 20 OTUs in Table 2, there was no consistent pattern of increase or decrease at phylum level and at least down to order level. Three *Clostridiales* species had the largest increase while two *Lactobacillales* species had the largest decrease; the two species *Lactobacillales* belongs to the widely known probiotic lactic acid bacteria (de Vries *et al.*, 2006).

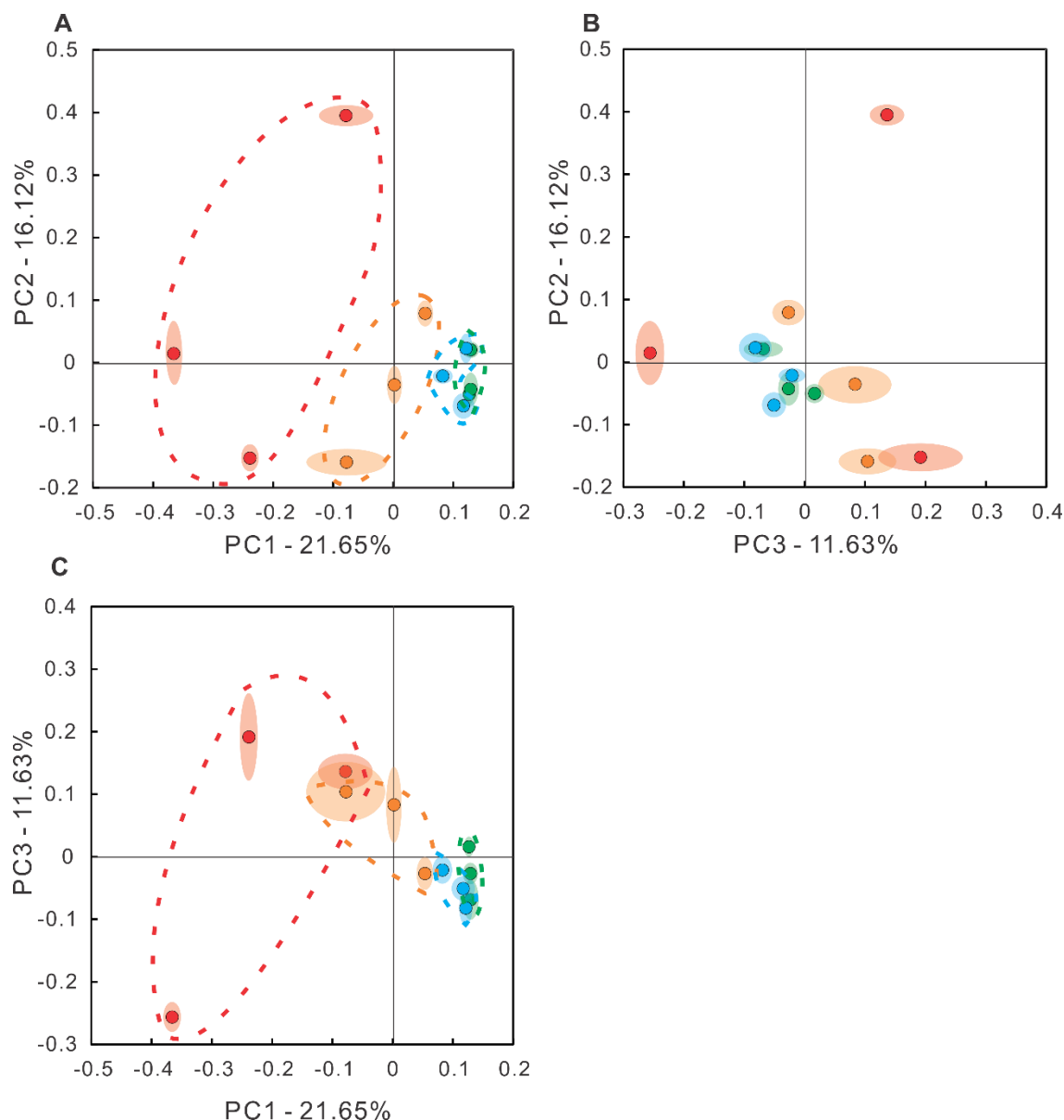


Figure 4-3 Beta diversity analysis based on the unweighted Unifrac index. (A) – (C) 2-dimension plots for the top three principle components (PC1, PC2, PC3). Each dot represents a sample point. Ellipsoids around each dot represent the uncertainty of each sample point based on jackknifing analysis. Red, orange, green and blue dots represent control, low, medium, and high dosing groups respectively. Coordinates in axis are for illustration purpose only and selected arbitrary and therefore do not have clear biological meanings. Percentage associated with each PC is the proportion of an eigenvalue for the respective PC in the sum of eigenvalues for all PCs.

Table 4-1 Sequences obtained from genomic sequencing facility and processing for 12 samples.

Data Processing	Control1	Control2	Control3	Low1	Low2	Low3	Medium1	Medium2	Medium3	High1	High2	High3
Original Data (Pairs)	29502 ^a	30370	29575	30832	27293	31420	34726	31083	36886	38113	37382	36254
Merging & Trimming ^b	27595 (93.5)	28578 (94.1)	27390 (92.6)	28597 (92.8)	25479 (93.4)	29470 (93.8)	31584 (91.0)	28435 (91.5)	34618 (93.9)	34043 (89.3)	32901 (88.0)	30750 (84.8)
Quality Filtering ^c	26228 (88.9)	27127 (89.3)	25907 (87.6)	27107 (87.9)	24199 (88.7)	28010 (89.1)	30014 (86.4)	27145 (87.3)	32996 (89.5)	32369 (84.9)	31328 (83.8)	29179 (80.5)

^a Data value indicates the number of sequences in respective samples and percentages in original counts in parentheses.

^b Merging is a process of combining pair-end Illumina reads of the same fragment to be a single nucleotide sequence. Trimming is a process of cutting inner tags, locus specific primers, and extracting sequences between 400-450 base pairs.

^c Quality filtering is a process in which merged sequences were cut from each end to obtain the shortest sequences without consecutive 3 bases with a quality score lower than 20 and without any ambiguous base calls. Sequences shorter than 75% the original length after cutting were excluded

Table 4-2 Detailed α -diversity metrics and abundance of detected Operational Taxonomic Units (OTUs) in each sample

	Con1 _a	Con2	Con3	Low 1	Low 2	Low 3	Med 1	Med 2	Med 3	High 1	High 2	High 3
<i>α-diversity metrics</i>												
Phylogenetic Diversity	52.5	47.6	48.3	40.7	46.9	40.8	34.9	36.7	35.5	37.3	33.7	38.4
Shannon Index	5.8	4.7	4.6	5.7	6	4.8	5.6	6.1	5.7	5.7	5.4	6.1
Observed OTUs	745	624	699	523	653	493	434	479	447	481	412	474

<i>Taxonomic information for detected OTUs^b</i>		Abundance in original samples (%)											
<i>p_Actinobacteria;</i>													
c_Actinobacteria; o_Actinomycetales; f_Corynebacteriaceae; g_Corynebacterium		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Actinobacteria; o_Actinomycetales; f_Micrococcaceae; g_Rothia		0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Actinobacteria; o_Bifidobacteriales; f_Bifidobacteriaceae; g_Bifidobacterium		0.0	0.2	0.0	0.0	0.0	0.2	0.0	0.1	0.0	0.0	0.0	0.0
c_Coriobacteriia; o_Coriobacteriales; f_Coriobacteriaceae; g_		0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Coriobacteriia; o_Coriobacteriales; f_Coriobacteriaceae; g_Adlercreutzia		0.2	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
<i>p_Bacteroidetes;</i>													
c_Bacteroidia; o_Bacteroidales; f_;	g_	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Bacteroidia; o_Bacteroidales; f_;	g_	0.2	0.4	0.0	0.1	0.2	0.0	0.1	0.1	0.1	0.1	0.0	0.2
c_Bacteroidia; o_Bacteroidales; f_Bacteroidaceae; g_Bacteroides		13.0	40.8	15.2	30.0	22.3	29.9	22.1	17.6	14.5	22.3	14.6	18.9
c_Bacteroidia; o_Bacteroidales; f_Porphyromonadaceae; g_Parabacteroides		0.4	0.3	0.1	0.4	0.3	0.4	0.4	0.2	0.4	0.4	0.3	0.3
c_Bacteroidia; o_Bacteroidales; f_Prevotellaceae; g_Prevotella		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Bacteroidia; o_Bacteroidales; f_Rikenellaceae; g_		0.1	0.2	0.1	0.6	0.4	0.7	0.5	0.2	0.2	0.4	0.2	0.4
c_Bacteroidia; o_Bacteroidales; f_Rikenellaceae; g_AF12		0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.1	0.0	0.1	0.0	0.0

[illegible]

[illegible]

c_Clostridia; o_Clostridiales; f_Dehalobacteriaceae; g_Dehalobacterium	0.0	0.1	0.0	0.2	0.3	0.1	0.2	0.2	0.1	0.2	0.2	0.2
c_Clostridia; o_Clostridiales; f_Lachnospiraceae; Other	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_	8.3	3.4	2.8	8.3	2.8	0.9	14.3	15.6	10.7	12.5	19.1	11.3
c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_Blautia	4.1	0.4	1.2	2.4	0.0	0.0	3.4	1.6	2.3	2.9	1.4	1.0
c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_Coproccoccus	0.2	14.0	5.1	0.7	6.6	1.0	2.3	1.6	16.8	2.2	10.4	2.0
c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_Dorea	9.0	0.3	0.6	3.2	1.6	0.1	2.9	6.9	2.7	2.2	2.4	3.8
c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_Roseburia	0.0	0.1	0.2	3.4	0.6	0.2	0.9	0.0	0.7	0.8	0.7	0.2
c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_[Ruminococcus]	0.0	0.1	0.1	0.3	0.9	0.2	0.5	0.4	0.2	0.4	0.3	0.2
c_Clostridia; o_Clostridiales; f_Peptococcaceae; g_	0.0	0.1	0.4	0.1	0.9	0.3	0.3	0.1	0.1	0.5	0.1	0.3
c_Clostridia; o_Clostridiales; f_Peptococcaceae; g_rc4-4	0.1	0.2	0.0	0.2	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.1
c_Clostridia; o_Clostridiales; f_Peptostreptococcaceae; g_	0.0	0.1	0.0	0.0	0.2	0.2	0.0	0.1	0.1	0.0	0.3	2.0
c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_	19.1	18.8	20.3	11.8	10.6	30.4	11.5	14.0	11.3	13.1	11.2	12.8
c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Anaerotruncus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Oscillospira	1.0	0.7	1.3	3.1	4.0	0.9	3.1	6.9	2.4	3.5	3.3	2.9
c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Ruminococcus	5.2	1.1	29.1	3.1	5.1	2.4	6.0	1.8	6.7	6.5	8.7	7.1

[illegible]

c_Betaproteobacteria; o_Rhodocyclales; f_Rhodocyclaceae; g_Dechloromonas	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Deltaproteobacteria; o_Desulfovibrionales; f_Desulfovibrionaceae; g_	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0
c_Deltaproteobacteria; o_Desulfovibrionales; f_Desulfovibrionaceae; g_Bilophila	2.2	0.2	0.6	2.2	0.3	0.1	1.8	0.0	1.9	1.2	0.9	2.0
c_Deltaproteobacteria; o_Desulfovibrionales; f_Desulfovibrionaceae; g_Desulfovibrio	0.0	0.0	0.0	0.1	0.2	0.1	0.0	0.3	0.1	0.1	0.1	0.1
c_Gammaproteobacteria; o_Enterobacteriales; f_Enterobacteriaceae; g_	0.5	0.1	0.1	0.2	0.0	0.1	0.0	0.0	0.1	0.1	0.0	0.1
c_Gammaproteobacteria; o_Enterobacteriales; f_Enterobacteriaceae; g_Proteus	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Gammaproteobacteria; o_Pasteurellales; f_Pasteurellaceae; g_	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Gammaproteobacteria; o_Pasteurellales; f_Pasteurellaceae; g_Haemophilus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Gammaproteobacteria; o_Pseudomonadales; f_Pseudomonadaceae; g_Pseudomonas	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>p_TM7</i> ;												
c_TM7-3; o_CW040; f_F16; g_	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>p_Tenericutes</i> ;												
c_Mollicutes; o_Anaeroplasmatales; f_Anaeroplasmataceae; g_Anaeroplasma	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
c_Mollicutes; o_RF39; f_ ; g_	0.0	0.0	0.0	0.0	0.2	0.0	0.3	0.0	0.6	0.3	0.3	0.2

c_RF3; o_ML615J-28; f_; g_	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
p_Verrucomicrobia;												
c_Verrucomicrobiae; o_Verrucomicrobiales; f_Verrucomicrobiaceae; g_Akkermansia	2.2	0.7	0.8	0.1	0.0	0.0	0.8	1.6	0.0	1.5	0.0	4.2

^a Con indicates control group; Low indicates low dosing group (5 µg AFB1/kg body weight (bw)); Med indicates medium dosing group (25 µg/kg bw); High indicates high dosing group (75 µg/kg bw).

^b p_ indicates phylum name; c_ indicates class name; o_ indicates order name; f_ indicates family name; g_ indicates genus name.

Table 4-3 Taxonomic information for the top 20 Operational Taxonomic Units (OTUs) with highest F-test values.

OTU ^a	Phylum	Class	Order	Family	Genus	Species	Control	Low	Medium	High
<i>Significantly increased</i>							Average abundance (%) ^b			
denovo207	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>				0.53	5.28	2.28	3.14
denovo1074	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>				0.02	0.14	0.30	0.3
denovo290	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>				1.41	2.09	2.84	3.85
denovo760	<i>Bacteroidetes</i>	<i>Bacteroidia</i>	<i>Bacteroidales</i>	<i>Bacteroidaceae</i>	<i>Bacteroides</i>	<i>uniformis</i>	0.82	0.6	0.52	0.68
denovo13	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Ruminococcaceae</i>	<i>Oscillospira</i>		0.05	0.11	0.19	0.23
denovo383	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>				0.09	0.82	1.07	0.56
denovo1095	<i>Bacteroidetes</i>	<i>Bacteroidia</i>	<i>Bacteroidales</i>	<i>S24-7</i>			0.89	0.94	0.55	0.56
denovo1190	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Ruminococcaceae</i>	<i>Oscillospira</i>		0.06	0.36	0.38	0.35
denovo1212	<i>Bacteroidetes</i>	<i>Bacteroidia</i>	<i>Bacteroidales</i>	<i>Bacteroidaceae</i>	<i>Bacteroides</i>		3.61	5.37	2.5	4.51
denovo621	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Lachnospiraceae</i>	<i>Coproccoccus</i>		0.08	0.47	0.87	0.59

denovo1100	<i>Bacteroidetes</i>	<i>Bacteroidia</i>	<i>Bacteroidales</i>	<i>Bacteroidaceae</i>	<i>Bacteroides</i>	12.21	15.36	11.6	11.36
denovo187	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Ruminococcaceae</i>	<i>Oscillospira</i>	0.21	0.78	1.42	0.92
denovo27	<i>Bacteroidetes</i>	<i>Bacteroidia</i>	<i>Bacteroidales</i>	<i>Rikenellaceae</i>		0.07	0.31	0.11	0.14
<i>Significantly decreased</i>									
denovo510	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Lactobacillales</i>	<i>Streptococcaceae</i>	<i>Streptococcus</i>	1.83	0.65	0.11	0.14
denovo1580	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Lactobacillales</i>	<i>Streptococcaceae</i>	<i>Lactococcus</i>	0.71	0.59	0.34	0.26
denovo1910	<i>Bacteroidetes</i>	<i>Bacteroidia</i>	<i>Bacteroidales</i>			0.18	0.11	0.07	0.09
denovo1888	<i>Proteobacteria</i>	<i>Deltaproteobacteria</i>	<i>Desulfovibrionales</i>	<i>Desulfovibrionaceae</i>	<i>Bilophila</i>	0.98	0.85	1.20	1.34
denovo1762	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Ruminococcaceae</i>	<i>Ruminococcus</i>	0.98	0.43	1.13	0.94
denovo1389	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>			0.49	0.25	0.81	0.95
denovo1046	<i>Firmicutes</i>	<i>Clostridia</i>	<i>Clostridiales</i>	<i>Ruminococcaceae</i>		0.30	0.26	0.31	0.67

^a OTUs are ordered in descending of estimated fold changes in respective categories (significant increased/decreased).

^b Data values indicate the percentages of each species in original samples averaged over each group

4.4 Discussion

This study was prompted by the increasing interests on the effects of xenobiotic agent on organism's commensal gut microbiota. We hypothesized that a xenobiotic toxin/toxicant could induce compositional and/or functional changes in gut microbiota, and these changes will eventually influence the toxic effects of these agents. The current study analyzed fecal samples from groups of male F344 rats continuously dosed by different sub-lethal concentrations of AFB₁, a Group 1 human carcinogen, using 16S rRNA gene sequencing analysis, and developed a standard protocol for future studies. We found that AFB₁ treatment induced the microbial community compositional changes in a dose-dependent manner.

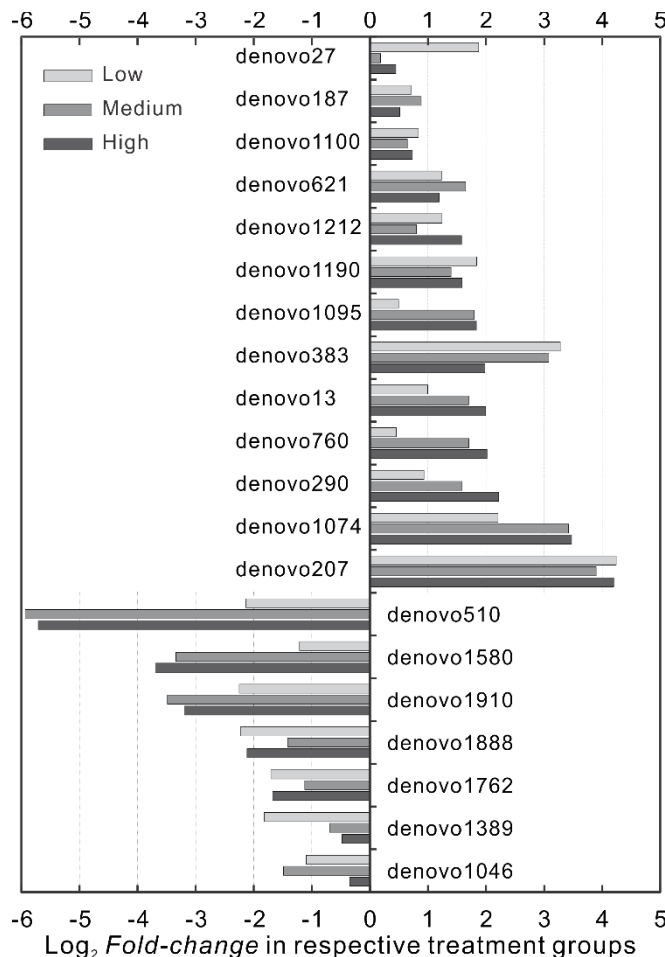


Figure 4-4 Log₂ fold-change in respective treatment groups as compared to control group among top OTUs with highest F-test value, estimated from Zero-Inflated Gaussian model. “denovo” followed with a number represents a specific OTU detected within the gut microbial community

Doses selected in this study was equivalent to 0.03 to 0.45 mg/kg (30-450 ppb) of AFB₁ in food. It is in the range commonly found in contaminated foods consumed daily by several populations especially in developing countries (Daniel *et al.*, 2011; Li *et al.*, 2001; Sharma and Sumbali, 2000) and was in the category of realistic dose as suggested by a recent publication (Grenier and Applegate, 2013). The repeated dosing protocol adopted in this study is the standard protocol we developed for continuously dosing animal experiments. The protocol may have some influence on short-term metabolites, such as urinary metabolites, but no influence on blood and fecal metabolites as well as aflatoxin biomarkers.

To our knowledge, this study is the first study to attempt to establish a dose-response relationship between a toxin/toxicant and responses of microbial community. It was surprising to observe that PD decreased while Shannon index increased with increasing AFB₁ doses. We speculate that there were more rare species (in terms of both phylogenetic relationship and abundance) in the control samples, which drove the PD high. Increasing AFB₁ exposure depleted low tolerant species (probably those rare species), resulting in a decrease of PD but an increase in evenness of community compositions, and therefore increased the Shannon index. This explanation was further backed up by a decrease of unique OTU numbers found in samples while increasing doses as seen in Figure 4-2C; approximately 1/3 of unique OTUs was depleted in high dosing group as compared to control samples. Similar findings have been observed in many other studies, for example, a species of fruit fly larvae dosed with Ag nano-particle had a lower diversity in mid-gut microbiota as compared to controls (Han *et al.*, 2014);

inflammatory bowel disease patients were documented to have fewer species (Manichanh *et al.*, 2006), fewer genes (Qin *et al.*, 2010) as compared to healthy controls; individuals with obesity was associated with a significant decrease in phylogenetic diversity as compared to lean individuals from a twin-pairs study (Turnbaugh *et al.*, 2009). A number of studies that focused on antibiotics effects on human gut microbiome also documented the depletion of gut microbiota in human volunteers, too, and the effects persisted after the cease of antibiotics treatment for an extended period (Jakobsson *et al.*, 2010; Jernberg *et al.*, 2010). It would be good for future studies to investigate the effects of xenobiotic toxicants on gut microbiome to include a time-course analysis, which may have important implications regarding the temporal role of microbiota in response to exposure to toxicants.

It is also worth noting that samples from medium- and high-dosing groups had similar α -diversity and clustered together in β -diversity analysis despite a large increase in doses (from 25 $\mu\text{g/kg}$ b.w. to 75 $\mu\text{g/kg}$ b.w.). The implications of this finding are unclear because of lack of previous evidences. One explanation may be that increased doses of AFB₁ had screened the resistant microbial species; the remaining species were toxic tolerant and could survive this toxin. This explanations may have important implications in that if the gut microbiota contain toxic resistant species, its interaction with this toxin may reduce the toxicity to its host. From another perspective, results from this study showed the potential that the gut microbiota of a host could be intentionally modified to accumulate toxin-tolerant species, a potential natural biological intervention strategy for mitigation of AFB₁-induced toxic effects. As previously mentioned, the toxicity resistance functions of gut microbiota have been documented in multiple studies.

For example, Kohl *et al.* (2014) has showed that the gut microbiota was responsible for the toxic-resistance ability to a toxic Creosote resin of a Desert Woodrat strain. Series of studies by Lu (Lu *et al.*, 2013; Lu *et al.*, 2014b) reported that perturbed gut microbiota in laboratory mice modified the host response to arsenic toxicity. Breton *et al.* (2013b) reported that after multiple oral doses of Cadmium and Lead up to 100 ppm, germ-free mice had significantly higher tissue metal concentrations than conventional mice. Swann *et al.* (2009) reported that toxicity effects, in terms of loss of vitality, of sub-toxic dosing of hydrazine, was more severe in germ-free rats than conventional and control rats.

Direct comparisons on community compositions of samples from different groups may not be informative in some cases because different microbial species may share similar genes and functions, and therefore, might not be appropriate to be treated as different. This idea is evidenced in that different individuals may share the same “core microbiome” which were identifiable at the gene level instead of at the organismal lineage level (Turnbaugh *et al.*, 2009). We did this comparison here, however, to complement divergence-based methods and provide further insights on the effects of AFB₁. An overview of community taxonomic compositions at phylum level have showed that *Firmicutes* and *Bacteroidetes* constituted more than 90% of the microbiota from all samples, consistent to what have been found previously in mice and human gut microbiota (Turnbaugh *et al.*, 2006); however the relative abundance of these two phylum was not appeared to be impacted by AFB₁ dosing (results not shown). The microbial community compositional changes have been documented in all previously mentioned toxicological studies. However, considering the large number of species (500~1000) resides in gut, false positive should be carefully avoided. In this study,

control of false positive was accomplished by multiple steps: only OTUs that appeared in more than half of the all samples were included (reducing the comparisons to 253 OTUs); the conservative *Bonferroni* method was used to correct for the multiple comparisons; only consistent OTUs (OTUs consistently increased or decreased in all three treatment groups as compared to control groups) were selected; only the top 20 consistent OTUs were selected. Species whose compositions significantly increased among the top 20 OTUs were from *Clostridiales* of *Firmicutes* and *Bacteroidales* of *Bacteroidetes* (Table 2). Species whose compositions significantly decreased among the top 20 OTUs were all from *Firmicutes* except one species of *Bacteroidales* from *Bacteroidetes* and one unknown *Bilophila* species from *Proteobacteria*. The two *Lactobacillales* from *Firmicutes*, *Streptococcus sp.* and *Lactococcus sp.* had the largest decrease, appearing in a dose-dependent manner. These species are members of lactic acid bacteria (LAB) (Stiles and Holzapfel, 1997) which have been shown to bind and remove AFB₁ via surface protein in several studies (Haskard *et al.*, 2001; Peltonen *et al.*, 2001). It was difficult to speculate the specific functions, the importance of other species which were also significantly changed and their interactions with AFB₁, partly due to the lack of previous information, except that antimicrobial activities of AFB₁ to gram-negative and -positive bacteria have been revealed decades ago (Arai *et al.*, 1967). In addition, many different microbial species have been previously reported to be impacted by xenobiotic toxicants or by disease status. However, there have not been a consensus on the linkage between specific species with a toxicant. The ratio between *Bacteroidetes* and *Firmicutes* has been previously reported in several studies, which was linked to certain disease status such as obesity, although a recent meta-analysis (Walters *et al.*, 2014) suggested that the

ratio between these two phyla might not be indicative after reviewing all current studies. From the present study, species that were either significantly increased or decreased were from both *Firmicutes* and *Bacteroidetes*, suggesting that changes at that phylum level might not be a good indicator to assess compositional changes.

The 16S rRNA fragment amplified in this study is from site 358 to 784 under *Escherichia coli* system of nomenclature, a 427 base-pairs long fragment (Klindworth *et al.*, 2013). As shown in Figure 4-1, in the present rat gut microbiota, this gene fragment peaked in five different length including 427. The fact of all 12 samples have highly consistent distributions further verified the precision of current data. A recent study (Sinclair *et al.*, 2015) has shown multiple peaks of the same fragment in sediment samples and demonstrated that the peaks were the result of different microbial species. We did not, however, differentiate different species according to the fragment length because it is irrelevant to the scope of current study.

The strength of current study mainly falls in three aspects. Firstly, this is the first study as far as we know that successfully established a dose-response relationship between xenobiotic exposure and gut microbiome alternations. Different from traditional toxicological research, study on microbiota generates multi-dimensional data which caused some theoretical difficulties to assess response (effect). This study employed α - and β -diversity matrix, describing the response at the whole community level to conquer the difficulties. Secondly, this study adopted reliable phylogenetic and statistical methods to assess the community compositional alternations of the model animal using both divergence-based and taxon-based based methods. Last but not the least, this study developed a complete protocol from sample collection, DNA library preparation, data

preparation and analysis for conducting a 16S rRNA gene survey based microbiome study in toxicology. This procedure and parameters adopted in this study could serve as a framework for any future similar study.

The study is exploratory, and primarily limited by its small-scale nature. Consequently, the relative small sample size may limit our ability to assess the statistical power of current analysis and to perform more sophisticated analysis. However, results presented in this study did provide solid evidence of AFB₁ treatment effect. The fact that increasing doses decreased variations within treatment group as seen in β -diversity analysis suggested that AFB₁, not the sample size, may be accounted for the intragroup variation. Regarding the sequence depth, a previous study suggested that 20,000 sequences per individual were sufficient to stabilize individual's microbiome functional profile (Turnbaugh *et al.*, 2009). In rarefaction analysis, our data showed that α -diversity of an individual sample plateaued after around 30,000 reads (results not shown). Consequently, we deemed targeting at 30,000 reads per sample was sufficient for current study scope. Male F344 rats are more sensitive to AFB₁ induced effects compared to female rats (Prince and Campbell, 1982). Consequently, we only included male rats to avoid confounding effects due to rat sex in this exploratory study.

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References

- Arai, T., Ito, T., and Koyama, Y. (1967). Antimicrobial activity of aflatoxins. *J. Bacteriol.* **93**, 59-64.
- Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A., and Caporaso, J. G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* **10**, 57-59.
- Breton, J., Massart, S., Vandamme, P., De Brandt, E., Pot, B., and Foligne, B. (2013a). Ecotoxicology inside the gut: impact of heavy metals on the mouse microbiome. *BMC Pharmacol. Toxicol.* **14**, 62.
- Breton, J., Daniel, C., Dewulf, J., Pothion, S., Froux, N., Sauty, M., Thomas, P., Pot, B., and Foligne, B. (2013b). Gut microbiota limits heavy metals burden caused by chronic oral exposure. *Toxicol. Lett.* **222**, 132-138.
- Bull-Otterson, L., Feng, W., Kirpich, I., Wang, Y., Qin, X., Liu, Y., Gobejishvili, L., Joshi-Barve, S., Ayvaz, T., Petrosino, J., Kong, M., Barker, D., McClain, C., and Barve, S. (2013). Metagenomic analyses of alcohol induced pathogenic alterations in the intestinal microbiome and the effect of *Lactobacillus rhamnosus* GG treatment. *Plos One* **8**, e53028.
- Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26**, 266-267.
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Tumbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**, 335-336.
- Choi, J. J., and Toborek, M. (2012). Exercise alters the abundance and composition of gut microflora and attenuates PCB-induced changes in gut microbiome. *J. Neuroimmune. Pharmacol.* **7**, S33-S34.

- Daniel, J. H., Lewis, L. W., Redwood, Y. A., Kieszak, S., Breiman, R. F., Flanders, W. D., Bell, C., Mwihia, J., Ogana, G., Likimani, S., Straetemans, M., and McGeehin, M. A. (2011). Comprehensive Assessment of Maize Aflatoxin Levels in Eastern Kenya, 2005-2007. *Environ. Health Perspect.* **119**, 1794-1799.
- de Vries, M. C., Vaughan, E. E., Kleerebezem, M., and de Vos, W. M. (2006). *Lactobacillus plantarum*-survival, functional and potential probiotic properties in the human intestinal tract. *Int. Dairy J.* **16**, 1018-1028.
- DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., and Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* **72**, 5069-5072.
- DuPont, A. W., and DuPont, H. L. (2011). The intestinal microbiota and chronic disorders of the gut. *Nat. Rev. Gastroenterol. Hepatol.* **8**, 523-531.
- Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460-2461.
- Faircloth, B. C., and Glenn, T. C. (2012). Not all sequence tags are created equal: designing and validating sequence identification tags robust to indels. *PLoS One* **7**, e42543.
- Faircloth, B. C., and Glenn, T. C. Protocol: Preparation of an AMPure XP substitute (AKA Serapure). doi: 10.6079/J9MW2F26.
- Faith, D. P. (1992). Conservation Evaluation and Phylogenetic Diversity. *Biol. Conserv.* **61**, 1-10.
- Grenier, B., and Applegate, T. J. (2013). Modulation of intestinal functions following mycotoxin ingestion: meta-analysis of published experiments in animals. *Toxins* **5**, 396-430.
- Han, X., Geller, B., Moniz, K., Das, P., Chippindale, A. K., and Walker, V. K. (2014). Monitoring the developmental impact of copper and silver nanoparticle exposure in *Drosophila* and their microbiomes. *Sci. Total Environ.* **487**, 822-829.
- Haskard, C. A., El-Nezami, H. S., Kankaanpaa, P. E., Salminen, S., and Ahokas, J. T. (2001). Surface binding of aflatoxin B(1) by lactic acid bacteria. *Appl. Environ. Microbiol.* **67**, 3086-3091.
- International Agency for Research on Cancer (IARC) (2012). Chemical agents and related occupations. *IARC monographs on the evaluation of carcinogenic risks to humans.* **100F**, 225-248.

- Jakobsson, H. E., Jernberg, C., Andersson, A. F., Sjolund-Karlsson, M., Jansson, J. K., and Engstrand, L. (2010). Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One* **5**, e9836.
- Jernberg, C., Lofmark, S., Edlund, C., and Jansson, J. K. (2010). Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology* **156**, 3216-3223.
- Kish, L., Hotte, N., Kaplan, G. G., Vincent, R., Tso, R., Gaenzle, M., Rioux, K. P., Thiesen, A., Barkema, H. W., Wine, E., and Madsen, K. L. (2013). Environmental particulate matter induces murine intestinal inflammatory responses and alters the gut microbiome. *PLoS One* **8**, e62220.
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., and Glockner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **41**, e1.
- Kohl, K. D., Weiss, R. B., Cox, J., Dale, C., and Dearing, M. D. (2014). Gut microbes of mammalian herbivores facilitate intake of plant toxins. *Ecol. Lett.* **17**, 1238-1246.
- Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology - Human gut microbes associated with obesity. *Nature* **444**, 1022-1023.
- Li, F. Q., Yoshizawa, T., Kawamura, O., Luo, X. Y., and Li, Y. W. (2001). Aflatoxins and fumonisins in corn from the high-incidence area for human hepatocellular carcinoma in Guangxi, China. *J. Agric. Food Chem.* **49**, 4122-4126.
- Lizarraga-Paulin, E. G., Moreno-Martinez, E., and Miranda-Castro, S. P. (2011). Aflatoxins and their impact on human and animal health: an emerging problem. In *Aflatoxins - biochemistry and molecular biology* (R. G. Guevara-González, Ed.) Eds.), pp. 255-282. InTech, Rijeka, Croatia.
- Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* **71**, 8228-8235.
- Lozupone, C. A., and Knight, R. (2008). Species divergence and the measurement of microbial diversity. *FEMS Microbiol. Rev.* **32**, 557-578.
- Lu, K., Cable, P. H., Abo, R. P., Ru, H., Graffam, M. E., Schlieper, K. A., Parry, N. M. A., Levine, S., Bodnar, W. M., Wishnok, J. S., Styblo, M., Swenberg, J. A., Fox, J. G., and Tannenbaum, S. R. (2013). Gut microbiome perturbations induced by bacterial infection affect arsenic biotransformation. *Chem. Res. Toxicol.* **26**, 1893-1903.
- Lu, K., Abo, R. P., Schlieper, K. A., Graffam, M. E., Levine, S., Wishnok, J. S., Swenberg, J. A., Tannenbaum, S. R., and Fox, J. G. (2014a). Arsenic exposure perturbs the gut microbiome and its metabolic profile in mice: an integrated

- metagenomics and metabolomics analysis. *Environ. Health Perspect.* **122**, 284-91.
- Lu, K., Mahbub, R., Cable, P. H., Ru, H., Parry, N. M. A., Bodnar, W. M., Wishnok, J. S., Styblo, M., Swenberg, J. A., Fox, J. G., and Tannenbaum, S. R. (2014b). Gut microbiome phenotypes driven by host genetics affect arsenic metabolism. *Chem. Res. Toxicol.* **27**, 172-174.
- Magoc, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27**, 2957-2963.
- Magurran, A. E. (2004). Measuring biological diversity. Blackwell Publishing, Malden, MA.
- Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., Nalin, R., Jarrin, C., Chardon, P., Marteau, P., Roca, J., and Dore, J. (2006). Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* **55**, 205-211.
- Merrifield, D. L., Shaw, B. J., Harper, G. M., Saoud, I. P., Davies, S. J., Handy, R. D., and Henry, T. B. (2013). Ingestion of metal-nanoparticle contaminated food disrupts endogenous microbiota in zebrafish (*Danio rerio*). *Environ. Pollut.* **174**, 157-163.
- Nicholson, J. K., Holmes, E., and Wilson, I. D. (2005). Gut microorganisms, mammalian metabolism and personalized health care. *Nat. Rev. Microbiol.* **3**, 431-438.
- Paulson, J. N., Stine, O. C., Bravo, H. C., and Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nat. Methods* **10**, 1200-1202.
- Peltonen, K., El-Nezami, H., Haskard, C., Ahokas, J., and Salminen, S. (2001). Aflatoxin B-1 binding by dairy strains of lactic acid bacteria and bifidobacteria. *J. Dairy Sci.* **84**, 2152-2156.
- Peterfreund, G. L., Vandivier, L. E., Sinha, R., Marozsan, A. J., Olson, W. C., Zhu, J., and Bushman, F. D. (2012). Succession in the gut microbiome following antibiotic and antibody therapies for *Clostridium difficile*. *PLoS One* **7**, e46966.
- Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2--approximately maximum-likelihood trees for large alignments. *PLoS One* **5**, e9490.
- Qian, G., Tang, L., Guo, X., Wang, F., Massey, M. E., Su, J., Guo, T. L., Williams, J. H., Phillips, T. D., and Wang, J. S. (2014). Aflatoxin B-1 modulates the expression of phenotypic markers and cytokines by splenic lymphocytes of male F344 rats. *J. Appl. Toxicol.* **34**, 241-249.
- Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D.,

- Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.-M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H. B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Dore, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Bork, P., Ehrlich, S. D., Wang, J., and Meta, H. I. T. C. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**, 59-65.
- R Core Team (2014). R: A language and environment for statistical computing. Vienna, Austria.
- Rawal, S., Kim, J. E., and Coulombe, R., Jr. (2010). Aflatoxin B1 in poultry: toxicology, metabolism and prevention. *Res. Vet. Sci.* **89**, 325-331.
- Sharma, Y. P., and Sumbali, G. (1999). Incidence of aflatoxin producing strains and aflatoxin contamination in dry fruit slices of quinces (*Cydonia oblonga* Mill.) from the Indian State of Jammu and Kashmir. *Mycopathologia* **148**, 103-107.
- Sinclair, L., Osman, O. A., Bertilsson, S., and Eiler, A. (2015). Microbial community composition and diversity via 16S rRNA gene amplicons: evaluating the illumina platform. *Plos One* **10**, e0116955.
- Stiles, M. E., and Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microbiol.* **36**, 1-29.
- Swann, J., Wang, Y., Abecia, L., Costabile, A., Tuohy, K., Gibson, G., Roberts, D., Sidaway, J., Jones, H., Wilson, I. D., Nicholson, J., and Holmes, E. (2009). Gut microbiome modulates the toxicity of hydrazine: a metabonomic study. *Mol Biosyst* **5**, 351-355.
- Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027-1031.
- Turnbaugh, P. J., Hamady, M., Yatsunencko, T., Cantarel, B. L., Duncan, A., Ley, R. E., Sogin, M. L., Jones, W. J., Roe, B. A., Affourtit, J. P., Egholm, M., Henrissat, B., Heath, A. C., Knight, R., and Gordon, J. I. (2009). A core gut microbiome in obese and lean twins. *Nature* **457**, 480-484.
- Walters, W. A., Xu, Z., and Knight, R. (2014). Meta-analyses of human gut microbes associated with obesity and IBD. *FEBS Lett.* **588**, 4223-4233.
- Williams, J. H., Phillips, T. D., Jolly, P. E., Stiles, J. K., Jolly, C. M., and Aggarwal, D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am. J. Clin. Nutr.* **80**, 1106-1122.

- Wogan, G. N., Kensler, T. W., and Groopman, J. D. (2012). Present and future directions of translational research on aflatoxin and hepatocellular carcinoma. A review. *Food Addit. Contam., Part A* **29**, 249-257.
- Xie, G., Zhong, W., Zheng, X., Li, Q., Qiu, Y., Li, H., Chen, H., Zhou, Z., and Jia, W. (2013). Chronic ethanol consumption alters mammalian gastrointestinal content metabolites. *J. Proteome Res.* **12**, 3297-3306.
- Yip, L. Y., Ku, H. C., Aw, C. C., Chen, W. S., Browne, E., Mahendran, R., Lee, Y. K., and Chan, E. C. Y. (2014). Investigation of host-gut microbiome interaction in Tacrine-dosed Lister-hooded rats through fecal metabolic profiling. *Drug Metab. Rev.* **45**, 147-147.

CHAPTER 5

LONG-TERM TREATMENT WITH GREEN TEA POLYPHENOLS MODIFIED THE GUT MICROBIOME OF FEMALE SPRAGUE-DAWLEY RATS ²

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Abstract

Background. Green tea polyphenols (GTP) has been shown to exert a spectrum of health benefits to humans. It is plausible that the beneficial effects of GTP are a result of its interaction with the gut microbiota.

Objective. The current study evaluated the effect of long-term treatment with GTP on the gut microbiota of experimental rats and the linkage between changes of the gut microbiota with the beneficial effects of GTP.

Methods. 72 female, middle-aged Sprague-Dawley rats were randomly allocated into 3 dosing groups and followed for 6 months. Either distilled water or water mixed with 0.5% or 1.5% GTP (g/ml) was freshly prepared daily and provided to the rats *ad libitum*. At the end of either the 3rd month or 6th month, half of the animals from each group were sacrificed and their fecal contents in colon were collected for microbiome analysis using 16S ribosomal RNA and shotgun metagenomic community sequencing.

Results. GTP treatment significantly decreased the biodiversity and modified the microbial community in a dose-dependent manner; similar patterns were observed at both 3-months and 6-months. Multiple operational taxonomic units (OTUs) and phylotypes were modified: the phylotypes *Bacteroidetes* and *Oscillospira*, previously linked to a lean phenotype in human and animal studies, were enriched; and *Peptostreptococcaceae* previously linked to colorectal cancer phenotype was depleted in GTP treatment group in a dose-dependent manner. Several microbial gene orthologs were modified, among which genes related to energy production and conversion were consistently enriched in samples from 6-months.

Conclusions. Long-term treatment with GTP induced a dose-dependent modification of the gut microbiome in experimental rats. The significantly modified microbial species and functional genes might be linked to beneficial effects of GTP. We speculate that the modulation of gut microbiota is one of the main mechanisms for GTP effects.

5.1 Introduction

Green tea polyphenols (GTP), a mixture of natural flavonoids found in brewed green tea, exert a spectrum of health benefits to humans, including protections from cardiovascular disease (Higdon and Frei, 2003), cancer (Luo *et al.*, 2006; Tang *et al.*, 2008), neurodegenerative disease (Higdon and Frei, 2003), and improving bone health (Shen *et al.*, 2013). The mechanisms of these beneficial effects mainly root on GTP's antioxidative properties (Higdon and Frei, 2003; Qian *et al.*, 2012). However, abundant research has shown that major GTP constituents are poorly absorbed in humans and exist primarily as conjugates which may not be bioactive in blood circulation once absorbed (Auger *et al.*, 2008; Stalmach *et al.*, 2010; Del Rio *et al.*, 2010; Clifford *et al.*, 2013a). Gut microbiota has recently been the spotlight of research in human health due to its great potential in influencing host physiology and health (Hooper and Gordon, 2001; Leser and Mølbak, 2009; Clemente *et al.*, 2012). Given that significant portion of unabsorbed GTP and GTP metabolites excreted through bile acid would pass from small intestine to large intestine (Chen *et al.*, 1997; Auger *et al.*, 2008; Clifford *et al.*, 2013b), the potential interactions between GTP and the gut microbiota might be important to impact the overall health of their host (Del Rio *et al.*, 2010; Visioli *et al.*, 2011).

Even though tea has been a popular drink for thousands of years and various beneficial human health benefits have been attributed to drinking tea, research to scientifically evaluate and quantify GTP's effects have only been undertaken since 1995 when the necessary analytical approaches became available (Scalbert *et al.*, 2005; Wang *et al.*, 2015). Since then, a growing body of research has documented the pharmacokinetics of GTP constituents after oral intake. Catechins, a major group of flavanols (flavan-3-ols), are abundant in green tea. Four major catechins found in fresh tea leaves are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG) (Higdon and Frei, 2003). Studies in humans with ileostomy have shown that these catechins are primarily absorbed in the upper gastrointestinal tract (GIT) (Auger *et al.*, 2008; Stalmach *et al.*, 2010). Also, a considerable amount of unabsorbed EGCG and ECG (~ 50-60% of intake) and lower portions of EC and EGC (~10-30% of intake) would pass to the large intestine without being absorbed (Auger *et al.*, 2008; Stalmach *et al.*, 2010). Once absorbed, catechins would reach their peak concentrations in plasma within hours, and be rapidly eliminated, returning to undetectable levels within ~24 hours after a single oral dose (Yang *et al.*, 1998; Van Amelsvoort *et al.*, 2001; Stalmach *et al.*, 2010), except for ECG which takes longer to be distributed and eliminated (Van Amelsvoort *et al.*, 2001). Absorbed catechins mostly present in conjugated forms (i.e. glucuronidated, sulfated, and methylated) while circulating in the blood stream, except for EGCG which circulates in unchanged form (Lee *et al.*, 1995; Clifford *et al.*, 2013b). Only EGC and EC may be eliminated via urine and exclusively in conjugated forms; EGCG, and possibly the other

three catechins to a lesser extent, could be eliminated via bile acids (Chen *et al.*, 1997; Kida *et al.*, 2000; Clifford *et al.*, 2013b), which then pass into the large intestine.

Many studies have evaluated the effects of oral intake of potential beneficial agents, i.e. probiotics or prebiotics, on the gut microbiota in human and animals, including a variety of *Lactobacillus*, *Enterococcus*, *Bifidobacterium* strains, or their mixtures (Eloe-Fadrosh *et al.*, 2015; McNulty *et al.*, 2011), and certain polysaccharides (Everard *et al.*, 2014; Sohail *et al.*, 2015). It is unknown, however, whether these agents modify the gut microbiota, or whether the observed changes of microbiota are related to the beneficial effects of these agents, because previous research has produced conflicting results. Large variations in doses administered and durations of treatment, inter-individual variation (del Campo *et al.*, 2014; Eloe-Fadrosh *et al.*, 2015), lack of standard and appropriate experimental and data analysis methods (Kuczynski *et al.*, 2010; Huttenhower *et al.*, 2014; Sinha *et al.*, 2015), and a lack of understanding of the mechanisms for beneficial effects may all contribute to the difficulty in reaching a consistent or plausible conclusion.

Given GTP's relative low bioavailability and direct contact with the gut microbiota, it is plausible that GTP would interact with the gut microbiota which might influence the overall health of the host. Few studies, however, have been conducted that evaluate interactions between GTP and the gut microbiota. A recent study did not find a significant effect of GTP on human gut microbiota in a 12-week randomized clinical trial (Janssens *et al.*, 2016). However, the dosing scheme, the trial duration, and the selection of study subjects might make it difficult to reveal GTP's effect in this study, especially considering the uncontrolled and diverse human diet. In this study, we conducted a long-

term repeated dosing experiment by administering GTP in drinking water for 6 months to middle-aged female Sprague-Dawley rats. We investigated their functional genome profile by shotgun metagenomic community sequencing (SMC-seq) and their microbial community composition by 16S ribosomal RNA (rRNA) amplicon sequencing (16S-seq). The study aims are to: (1) evaluate how GTP would modulate the gut microbiome in response to different doses, (2) evaluate the changes of gut microbiome across time, and (3) develop a reliable experimental and data analysis protocol.

5.2 Methods

5.2.1 Animals, Chemicals, Experimental Design

This study was concurrent with a previous study of the chronic toxicity and tolerance of green tea polyphenols (GTP) extracts (decaffeinated) among middle-aged ovariectomized rats, in which the experimental design and procedures on chemicals and animal preparations were described (manuscript in revision) (Figure 5-1). Briefly, after acclimation, female ovariectomized Sprague-Dawley (SD) rats (6 months old, Harlan Laboratories, Indianapolis, IN) were randomly allocated into 6 groups (n=12/group) in 3 dosing regimens based on body weight stratification. The rats were fed with pelleted AIN-93M diet (Dyets, Bethlehem, PA) and had free access to either regular distilled water (as the control) or daily freshly prepared distilled water mixed with 0.5%, or 1.5% GTP (weight/volume or g/ml) (as two treatment groups) until the end of either 3rd month or 6th month after initiation of the experiment, when one group from each dosing regimen was anesthetized with isoflurane gas and euthanized by bilateral thoracotomy.

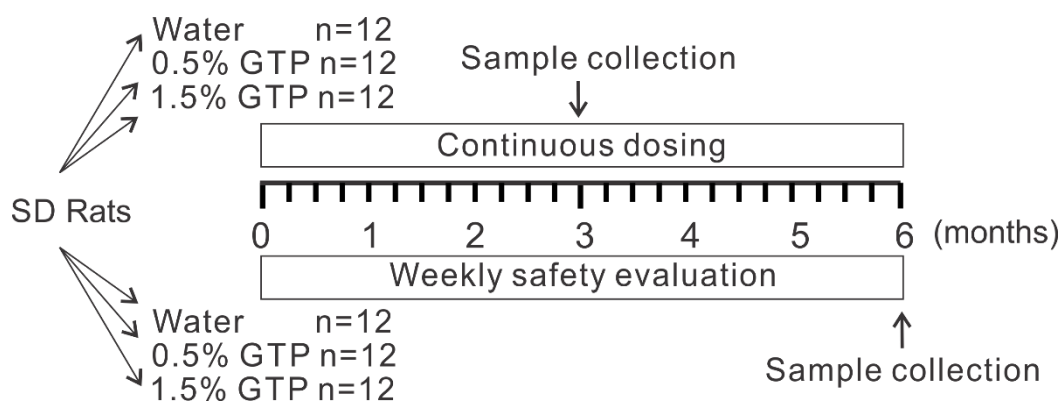


Figure 5-1 GTP dosing experimental design. 3-month old Sprague-Dawley (SD) rats were randomly allocated into 6 groups of 3 dosing regimens. At either 3rd or 6th month, mid-gut content were collected from sacrificed animals.

At each sampling, fecal samples from the mid-gut were collected and immediately stored in -80 °C freezer for further processing. Overnight fasting blood samples were also collected in the concurrent study for hematology and clinical chemistry analyses. Rats were housed in individual stainless steel cages and subjected to daily examination. Individual animal body weight, feeds and water consumption were recorded weekly. All procedures were approved by the Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee. No abnormal treatment-related findings in clinical observations were noted.

5.2.2 DNA Extraction, Library Preparation, and Sequencing

Microbial DNA was extracted from fecal contents using Qiagen Fast DNA Stool Mini Kit (QIAGEN, Valencia, CA) following the protocol published previously (Wang *et al.*, 2016). During initial homogenizing, the manual homogenizing was replaced with a beads-beating step using MIBIO 0.1 mm glass beads tubes (MOBIO, Carlsbad, CA) to improve the stability and reproducibility.

Extracted DNA from all samples was normalized into ~20 ng DNA/μl in PCR water for DNA library preparation for WGS and 16S-seq. One aliquot for WGS was

fragmentized using Bioruptor Plus (Diagenode, Denville, NJ) targeting a dominant fragment size of ~500 base pairs (bp), which was checked by gel electrophoresis, and subsequently processed using KAPA LTP library preparation kit (Kapa Biosystems, Inc., Boston, MA) according to manufacturer protocol. Another aliquot of the extracted DNA from selected samples for 16S-seq was processed using a 2-step Quadruple-index PCR procedure as previously described (Wang *et al.*, 2016) in which fragments spanning V3 and V4 regions of 16S rRNA gene were amplified. Three replicate PCR reactions per sample were performed in the primary PCR reaction and pooled for the secondary limited-cycle PCR to reduce potential PCR bias (Nocker *et al.*, 2007). The experiment flowchart for library preparation procedures is presented in Figure 5-2.

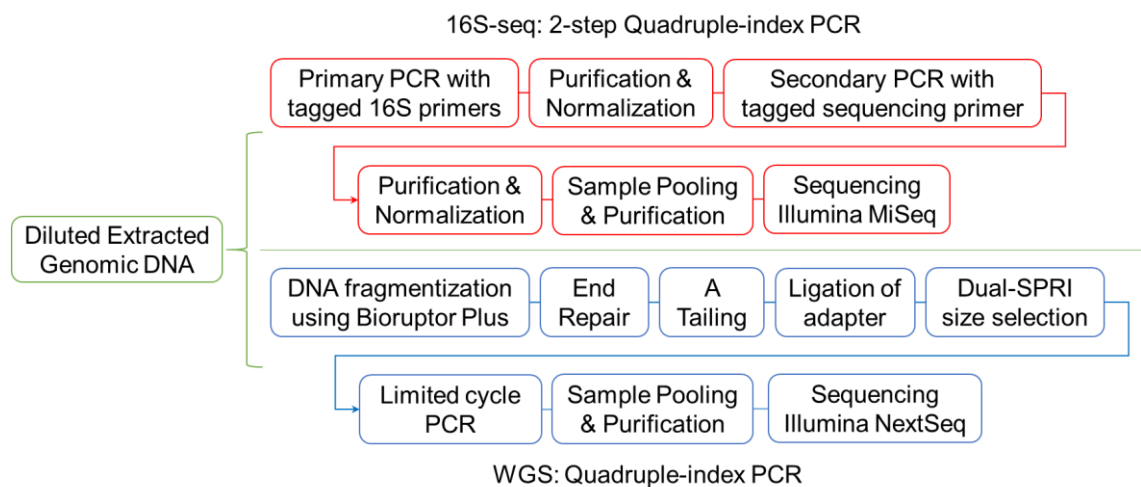


Figure 5-2 The experimental workflows for whole genome shotgun sequencing (WGS) and partial 16S rRNA gene sequencing (16S-seq) library preparation.

Genomic DNAs from two standard mock microbial communities (HM-276D and HM-277D, BEI Resources, Manassas, VA) were processed using the same 2-step Quadruple-index PCR procedure to gauge 16S-seq library preparation and data analysis procedure. HM-276D features 20 bacterial species at equimolar rRNA operon counts and

HM-277D features the same 20 bacterial species at staggering molar rRNA operon counts (The Human Microbiome Project Consortium, 2012; Jumpstart Consortium Human Microbiome Project Data Generation Working Group, 2012).

Prepared WGS libraries were sequenced using Illumina NextSeq platform to obtain ~2,000,000 Paired-End (PE) reads of 150 bp per sample; and 16S-seq libraries were sequenced using Illumina MiSeq platform to obtain ~100,000 PE reads of 300 bp per sample, at Georgia Genomic Facility (GGF, University of Georgia, Athens, GA). The sequencing depth was selected based on previous experiences to ensure an optimized cost-benefit.

5.2.3 Sequencing Data Analysis

Metagenomic analysis was performed using the sequencing reads obtained from WGS, following a similar strategy in Li *et al.* (2014) and Nielson *et al.* (2014). Raw paired-end, de-multiplexed sequencing reads from each sample were quality filtered using Trimmomatic-v0.3.6 (Bolger *et al.*, 2014), and fed to MEGAHIT-v1.0.2 (Li *et al.*, 2016) for metagenome assembling. The proportion of quality filtered reads that could be mapped to MEGAHIT assemblies was checked using Bowtie2 (Langmead and Salzberg, 2012). MEGAHIT assemblies for each sample were processed by Prodigal-v2.6.3 (Hyatt *et al.*, 2010) for microbial gene prediction. Predicted genes from each sample that were longer than 100 bp were pooled, de-replicated according to sequence prefix, and clustered with 99% identity using vsearch-v1.10.1(<https://github.com/torognes/vsearch>), producing a non-redundant microbial meta-gene catalog (MGC). The corresponding amino acid sequences for MGC were annotated according to Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (genus_prokaryotes section) using GhostKOALA (Kanehisa

et al., 2016), and Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups (eggNOG) v4.5 database (Huerta-Cepas *et al.*, 2016) (bacteria section, bactNOG), using in-house scripts. Both KEGG and eggNOG database group GO into functional categories with slightly different annotations. This study used the single letter functional categories as in eggNOG database to assure consistency. The quality filtered reads from each sample were subsequently mapped to the annotated MGC using Bowtie2 to generate gene abundances which were further normalized using the gene length (Qin *et al.*, 2012). Normalized gene abundance tables for each sample were the products of metagenomic analysis and subjected to further statistical comparisons.

The procedure for analyzing 16S-seq data was slightly modified based on our previous report (Wang *et al.*, 2016) due to an unexpected amount of low-quality reads in this batch of samples. Consequently, a trimming procedure was performed on the 3' end of each paired-end read initially, so that remaining reads did not contain more than 3 low quality (Q<20) or ambiguous nucleotides. Reads less than 200 bp in length were also filtered out. Above procedures were executed in Geneious R8.1.8 (Biomatters Inc, San Francisco, California). The remaining quality trimming procedures and community analysis using QIIME pipeline (Caporaso, Kuczynski, *et al.*, 2010) were similar as we previously reported (Wang *et al.*, 2016). The final OTU abundance table was filtered according to results from analysis of standard mock communities and subject to statistical comparisons.

5.2.4 Statistical Methods

The comparisons of gene abundances in metagenomic analysis and OTU abundances in 16S-seq in samples from different dosing groups were performed using

LEfSe (**L**inear discriminant analysis **E**ffect **S**ize)-v1.0 (Segata *et al.*, 2011). LEfSe incorporated a combination of univariate (Kruskal-Wallis rank sum test (KW test) and Wilcoxon rank-sum test (Wilcoxon test)) and multivariate (Linear Discrimination Analysis, LDA) statistical methods to determine the features (e.g. OTUs, Genes) most likely to explain differences between classes (e.g. Dosing regimens) and provide a measure of effect size (i.e. LDA score) (Segata *et al.*, 2011). The LEfSe analyses were performed in samples from two sampling dates separately, and the significance level was set to be 0.01.

The comparisons of biodiversity of the gut microbial community (α -diversity) in samples from different dosing groups were performed using a two-way ANOVA test, using the diversity indexes as the response variable, and dose and sampling date as the predictive variables. The β -diversity (Lozupone and Knight, 2008) between samples was measured using a weighted-Unifrac distance index, which integrated both the abundance and phylogenetic relationship of each OTU (Lozupone *et al.*, 2007). The weighted-Unifrac distance matrix constructed from each pair of samples were analyzed using a principal coordinate analysis (PCoA) and ADONIS tests to evaluate the effects of predictive variables with a significant level of 0.05.

The availability of gene abundance table, OTU abundance table, as well as the concurrent GTP tolerance experiment which measured multiple clinical endpoints of the tested animals, enabled the exploration of potential correlations between the gut microbiome with host physiology. **H**ierarchical **A**ll-against All **A**ssociation testing (HALLA)-v0.7.11 was used to find the multi-resolution associations among the three high-dimensional datasets (<http://huttenhower.sph.harvard.edu/halla>). HALLA employs a

hierarchical clustering of features (e.g. OTUs, Genes, or clinical measurements) in each dataset, dimensionality reduction, and iterative testing of associations between clusters, to provide well-powered association discoveries (<http://huttenhower.sph.harvard.edu/halla>). A false discovery rate of 0.1 using Benjamini–Hochberg–Yekutieli (BHY) correction was used to screen for candidate correlations.

5.3 Results

5.3.1 WGS and 16S amplicon sequencing data processing

Extracted DNA from a total of 72 samples from 6 groups (3 dose groups at 2 sampling times) were processed for WGS and metagenomic analysis. A total of 303,071,358 PE reads of 150 bp in length were obtained, among which 205,905,205 PE reads survived the stringent quality filtering process, making an average (Standard Deviation, SD) of 2,859,795 (1,019,602) PE reads per sample (average survival rate, 67.8%) for downstream analysis. Metagenome assembling was performed on each sample individually using MEGAHIT, achieving an average (SD) N50 length of 1,237 (314) nucleotides (nt), average maximum contig length of 126,871 (44,232) nt, and average contig counts of 87,730 (31,462), across all samples. The average mapping rate of quality filtered PE reads to the contigs assembled by MEGAHIT for each sample is 69.9% (7.6%) (range: 40.7% to 82.1%). Prodigal predicted an average of 139,878 (47,330) microbial genes per sample. After de-replication and clustering, the MGC constructed contained 2,367,134 genes, among which 829,036 and 1,748,024 genes respectively matched to gene orthologs (GOs) in KEGG database and eggNOG v4.5 database; a combining total of 1,760,017 genes were assigned a GO, constituting 74.3%

of the MGC. The mapping rate of quality filtered PE reads from each sample to MGC was on average 76.0% (0.05%).

The original genomic DNAs from both standard mock communities were diluted so that equal amounts of 50,000 16S rRNA gene operons from the 20 species in HM-276D (1:20 dilution) and 1,000 to 1,000,000 operons from the same 20 species in HM-277D (1:10 dilution) were input into the primary PCR (1 µl input DNA). 24,723 and 25,507 quality filtered reads were available for HM-276D and HM-277D respectively. A total of 197 OTUs were identified in HM-276D, including all of the 20 stated species (abundances ranging from 0.76% to 11.87%). In comparison, 153 OTUs were identified in HM-277D, but only including 18 of the stated 20 species (abundances ranged from 0.0039% to 49.68%). Given the results from the two standard mock communities, an abundance of 0.1% was selected based on an receiver operating characteristic (ROC) analysis to filter OTUs in the original 36 samples in GTP dosing experiments to prevent any spurious findings. If using 0.1%, 25 OTUs including all 20 supposed species were captured in HM-276D and 16 OTUs including 11 supposed species were captured in HM-277D.

Six samples from each GTP dosing group were randomly selected for 16S-seq. A total of 5,855,866 PE reads from 36 samples were obtained, among which 2,683,283 PE reads survived quality filtering (average survival rate, 47.0%). A total of 2,500,350 of sequences remained in the final OTU abundance table after abundance filtering on the individual sample in GTP dosing experiment, making an average (SD) of 69,454 (24,460) sequences per sample for analysis.

5.3.2 Long-term treatment with GTP modified the abundance of several gene orthologs

LEfSe was performed to detect differentially abundant GOs in samples collected from 3-month and 6-month separately. At each sampling time, LEfSe first employed KW test to assess the dosing effect of GTP on the abundance of each feature (in this case, GOs) independently. Features that were significantly different determined from KW test were further independently assessed by a pairwise Wilcoxon test. Identified features were then fitted into an LDA model and those with an LDA score larger than 2.0 were deemed differentially abundant in different dosing groups. A final manual screening was performed to select features that were modified in a dose-dependent manner.

At 3-month, 10 GOs were identified to be differentially abundant: 6 GOs decreased, and 4 GOs increased with increasing GTP doses (Table 5-1). GOs that involve “Replication, recombination and repair” (category [L]) constituted the most of the affected GOs (2 GOs), followed by those involve “Cell wall/membrane/envelope biogenesis” (category [M], 2 GOs), mostly decreased (Figure 5-3). Figure 5-4 presented the Log10 scaled normalized GO abundance for GOs identified by LEfSe in the order of decreasing LDA score.

Table 5-1 Significantly modified gene orthologs by green tea polyphenols.

Gene Ortholog ^a	Category	Function
<u>3-month treatment</u>		
Decreased		
ENOG4105D70	L	Integrase catalytic subunit
ENOG4105F5W	L	ATP-binding protein
ENOG4105EGG	V	ABC transporter
ENOG4105EXR	S	Unknown
ENOG4105CK0	M	Membrane bound o-acyl transferase
ENOG4108SJI	M	Hydrolase, family 25
Increased		
K01869	J	Leucyl-tRNA synthetase
ENOG4105WBW	S	Relaxase mobilization nuclease domain protein

ENOG4105EUN	L	Transposase
ENOG4105CCY	C	NADH flavin oxidoreductase, NADH oxidase

6-month treatment

Decreased

ENOG4105D2F	G	Ig domain protein, group 2 domain protein
ENOG4105CDX	S	Unknown
ENOG4105XN2	K	DNA-binding helix-turn-helix protein
ENOG4105C19	V	ABC transporter
ENOG4108KGH	K	Transcriptional regulator
ENOG410667J	S	Hydrolase
ENOG4108SJI	M	Hydrolase, family 25
ENOG4108VXD	M	Carboxypeptidase
ENOG4108UUM	L	Single-stranded DNA-binding protein
ENOG4105EF7	K	Transcriptional regulator
ENOG4105DAV	K	RNA polymerase sigma factor RpoD
ENOG4108M4B	M	Domain protein
ENOG4105DZ1	M	Carboxypeptidase
ENOG4105C5C	M	N-acetylmuramoyl-L-alanine amidase

Increased

ENOG4105F42	S	AAA-ATPase
ENOG4105K8S	S	Unknown
ENOG4108J2R	C	Molybdopterin oxidoreductase
ENOG4105FAP	L	Transposase
ENOG4107WWN	L	Transposase
ENOG41063CS	L	Replication Protein
ENOG4105FGY	P	TonB-dependent receptor, Plug Domain
K01869	J	Leucyl-tRNA synthetase
ENOG4105DAB	C	Fumarate reductase/succinate dehydrogenase, Flavoprotein domain protein
ENOG4105CGS	G	Alpha-glucosidase
ENOG4105D3S	C	4Fe-4S ferredoxin, iron-sulfur binding domain protein
ENOG4107VV4	S	Virulence-associated protein e
ENOG4105X7U	K	Transcriptional regulator, ARAC family
ENOG4106DHI	S	Cleaved adhesin domain protein
ENOG4105CCY	C	NADH flavin oxidoreductase, NADH oxidase
ENOG4105WBW	S	Relaxase mobilization nuclease domain protein

^a The gene ortholog ID, category, functions were assigned based on eggNOG v4.5 database except for K01869, which was assigned based on KEGG database.

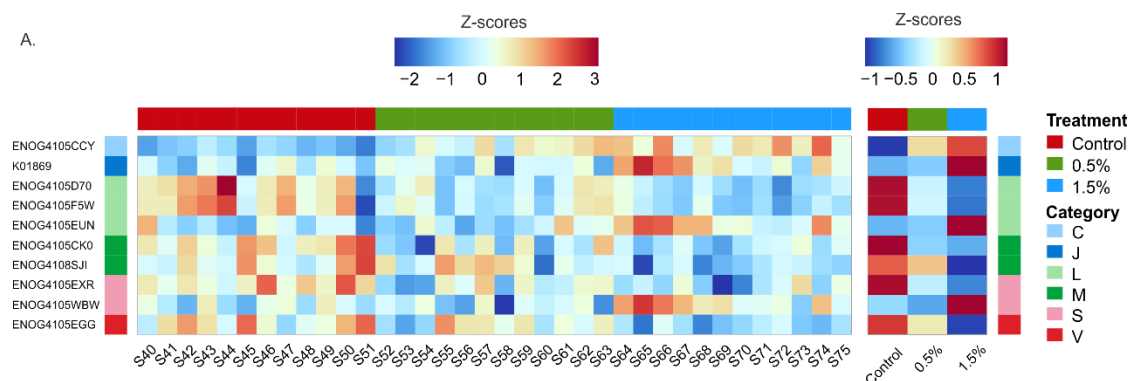


Figure 5-3 The heatmap of normalized abundance of GO that were significantly modified by 3-month treatment with GTP. Both individual samples and group average were presented.

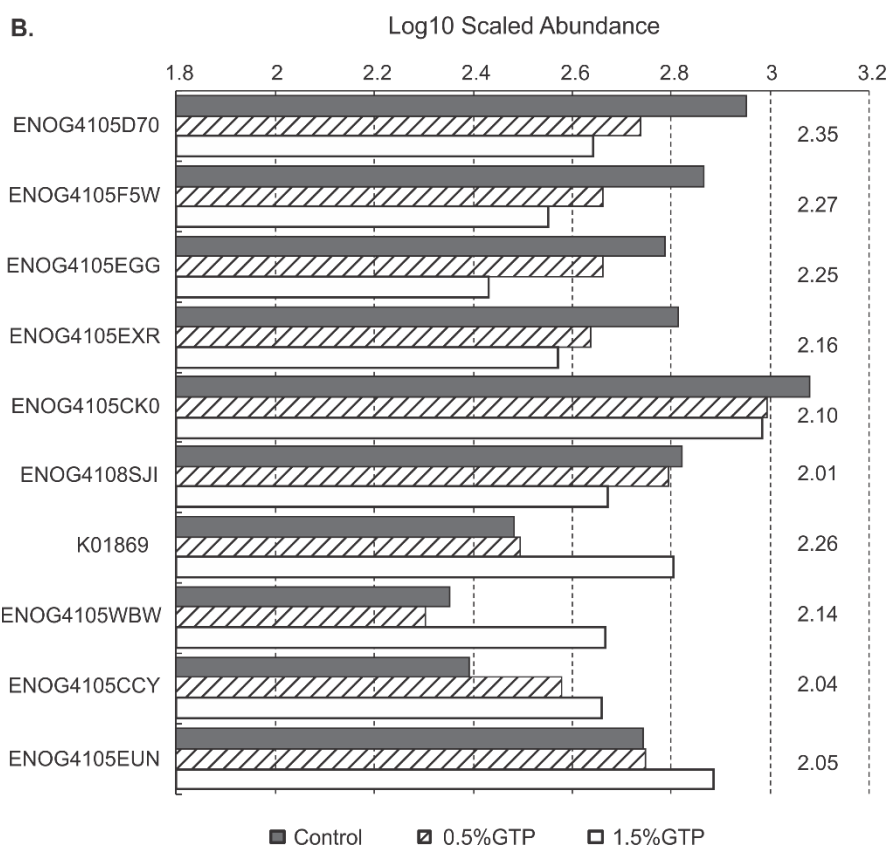


Figure 5-4 The abundance of GO that were significantly modified by 3-month treatment with GTP. Abundance has been scaled as if there were 1,000,000 reads in each sample.

At 6-month, 30 GOs were identified, among which GOs from category [M] and [K] (“transcription”) were the most (5 GOs each), followed by category [L] and [C]

(“Energy production and conversion”) (4 GOs each) (Table 5-1, Figure 5-5 - Figure 5-6).

It is surprising, however, that only a few GOs were consistently modified at 3-month and 6-month including K01869, ENOG4105WBW, and ENOG4105CCY, which were consistently enriched; and ENOG4108SJI, which was consistently depleted.

5.3.3 Long-term treatment with GTP modified the gut microbial community composition

Increasing doses of GTP among experimental rats decreased the gut community biodiversity as measured by Shannon index and Faith’s phylogenetic diversity(PD) (Faith, 1992) at the two sampling times, although an unexpected surge of biodiversity was observed at the dose of 0.5% GTP at 6-month (Figure 5-7). The two-way ANOVA test confirmed the significant dosing effect (p -value = 0.0041 for Shannon, and p -value = 0.0250 for PD), and suggested no significant effect from sampling time. A post-hoc Duncan’s test identified that samples from 1.5% GTP groups had significantly lower biodiversity indexes.

Correspondingly, the PCoA analysis of beta diversity using weighted Unifrac index revealed that different doses of GTP but not sampling date differentiated the gut microbial community (Figure 5-8A). An ADONIS test (Anderson, 2001) based on the weighted Unifrac distance matrix suggested a significant dosing effect, which could explain 29% (R^2) of overall variation (p -value<0.001). Plotting PC1 coordinates of each sample against the GTP doses showed a steady trend of samples moving from the negative side of the PC1 axis to positive while increasing doses (Figure 5-8B).

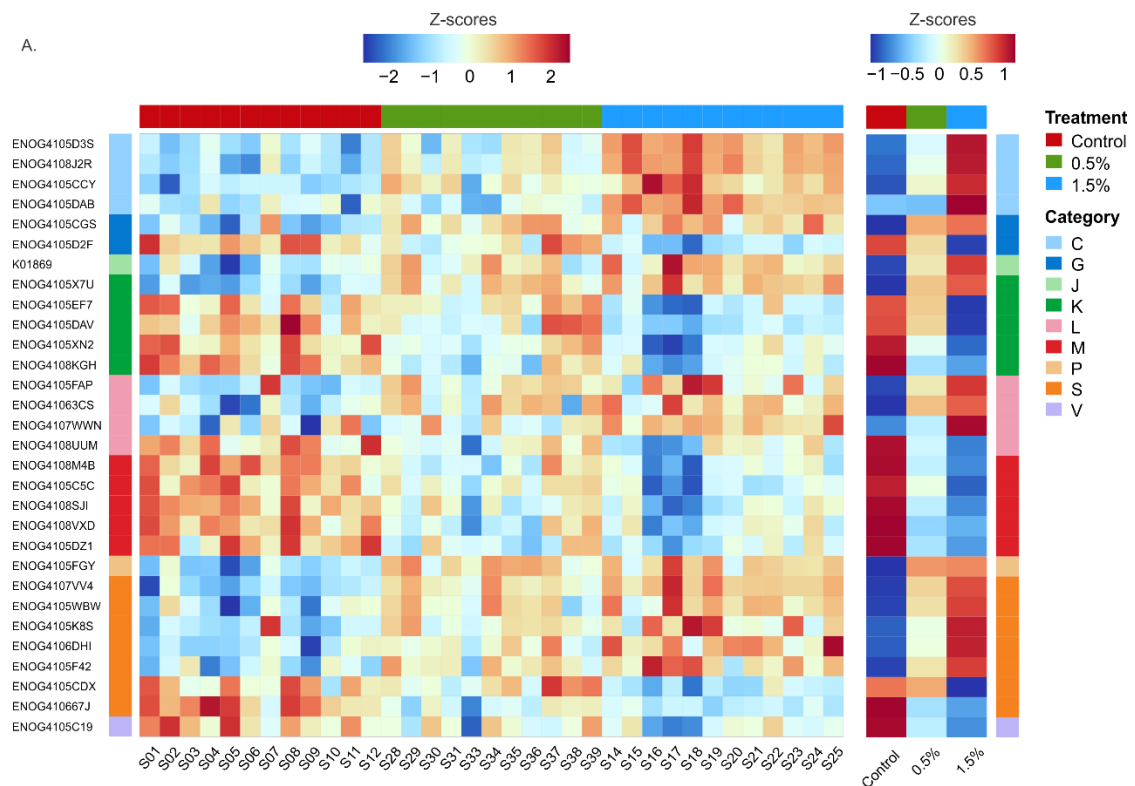


Figure 5-5 The heatmap of normalized abundance of GO that were significantly modified by 6-month treatment with GTP. Both individual samples and group average were presented.

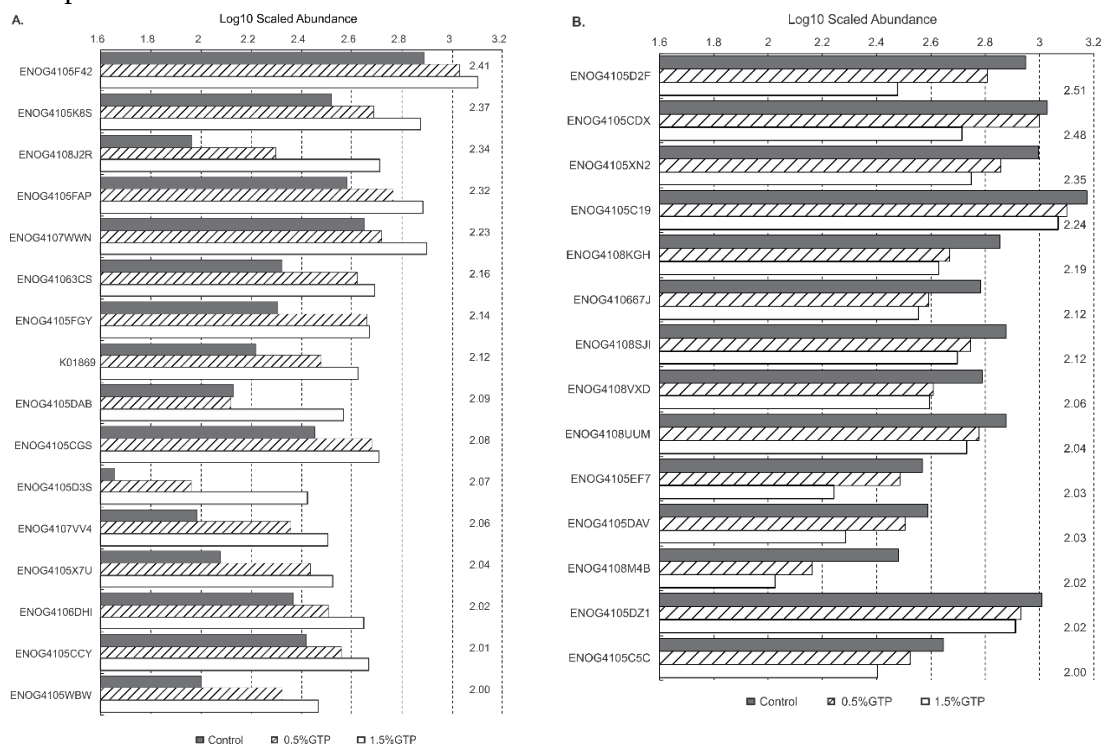


Figure 5-6 The abundance of GO that were significantly modified by 6-month treatment with GTP. (A) GO that were enriched; (B) GO that were depleted.

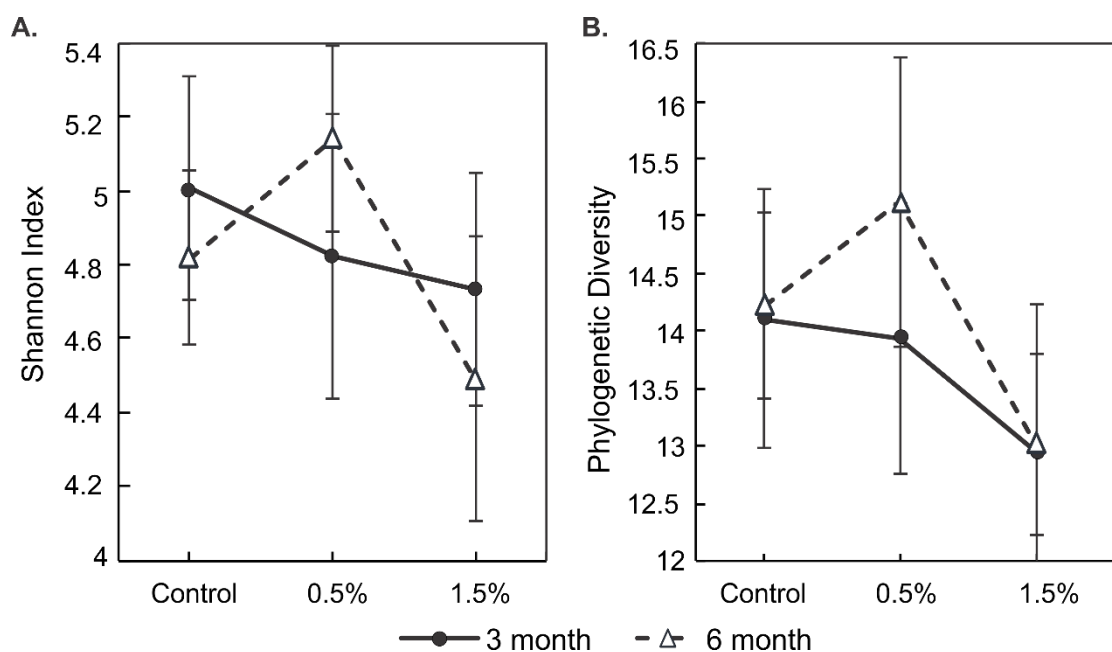


Figure 5-7 The alpha-diversity of samples collected at 3- and 6-month of GTP treatment. Group average and standard error was presented. Two-way ANOVA tests were performed for each index, which was used a response variable with dose and sampling time as predictive variable. Significant dosing effect ($p = 0.0041$ for Shannon and 0.0250 for PD) but insignificant sampling effect ($p > 0.05$) were obtained.

LEfSe was used to detect differentially abundant OTUs or bacterial phylotypes (from phylum to family level) in different GTP dosing groups. Again, two sampling times were analyzed separately. A manual scrutiny was then performed so that only features (either OTUs or phylotypes) that had at least 2.5 folds change of overall abundance in different dosing groups were identified. The threshold fold change was decided based on our analysis of standard mock community. Overall, all the OTUs or taxa selected had relatively high LDA score ranging from 3.39 to 5.10 with an average of 4.08.

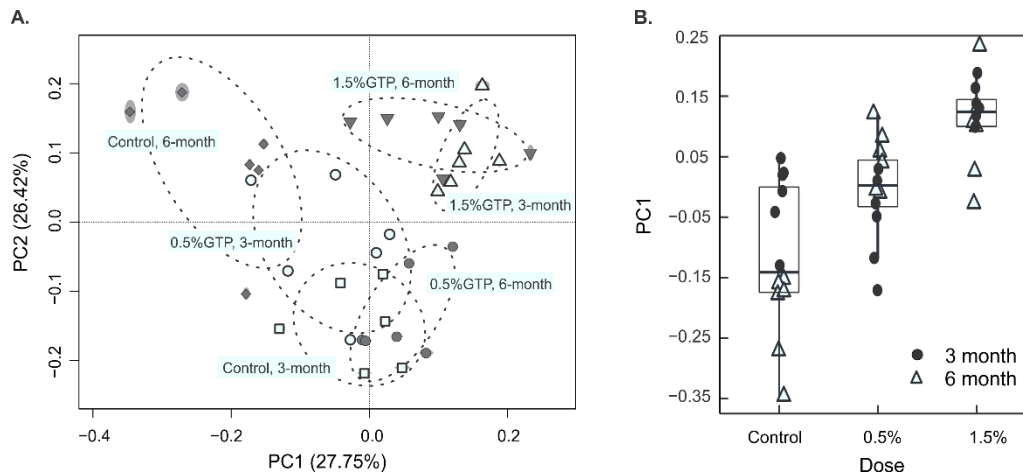


Figure 5-8 Principle Coordinate Analysis of beta-diversity measured by weighted UniFrac index. ADONIS test of dosing and sampling effect identified a significant dosing effect ($p < 0.001$, $R^2 = 0.29$) but not sampling effect.

At 3-month, 10 OTUs were differentially abundant in different dosing groups: 6 OTUs from *clostridiales* of *firmicutes* increased, and 2 OTUs from *bacteroidales* of *bacteroidetes* decreased with increasing GTP doses (Figure 5-9, Table 5-2). However, family *paraprevotellaceae* of *bacteroidetes* was the only phylotype differentially abundant in different dosing groups, which decreased with increasing GTP doses and may probably be due to a decrease in the genus *CF231* as one of the identified decreasing OTUs from *bacteroidales* (Table 5-2). Neither *clostridiales* nor *bacteroidales*, when testing as an overall order, was differentially abundant, suggesting that the changes of individual OTUs did not translate to the whole bacterial clade. At 6-month, 26 OTUs were differentially abundant including 5 *clostridiales* and 2 *erysipelotrichales* of *firmicutes*, 1 *bacteroidales* decreased with GTP doses, and 3 *bacteroidales*, 1 *clostridiales* (*oscillospira*), 1 *desulfovibrionales* of *proteobacteria* increased (Table 5-2). The phylum *bacteroidetes* and its descendant phylotype, *bacteroidia*, *bacteroidales*, and family *bacteroidaceae*, were all differentially abundant in different groups with a dose-

dependent increasing trend. One family *peptostreptococcaceae* of *firmicutes* decreased, which may be due to the decreasing of an OTU (denovo 2828) from this family recognized previously.

5.3.4 Correlations between the gut microbiome with clinical measurements

Information for 105 OTUs, 31,222 GOs, and 51 clinical endpoints were available in 36 samples from 3 different dosing regimens and 2 sampling times. The relationships between OTU abundance and clinical measurements, and between GO abundance and the clinical measurements, were checked using HALLA after filtering features that contain low information (entropy threshold was set to 1.5 in HALLA).

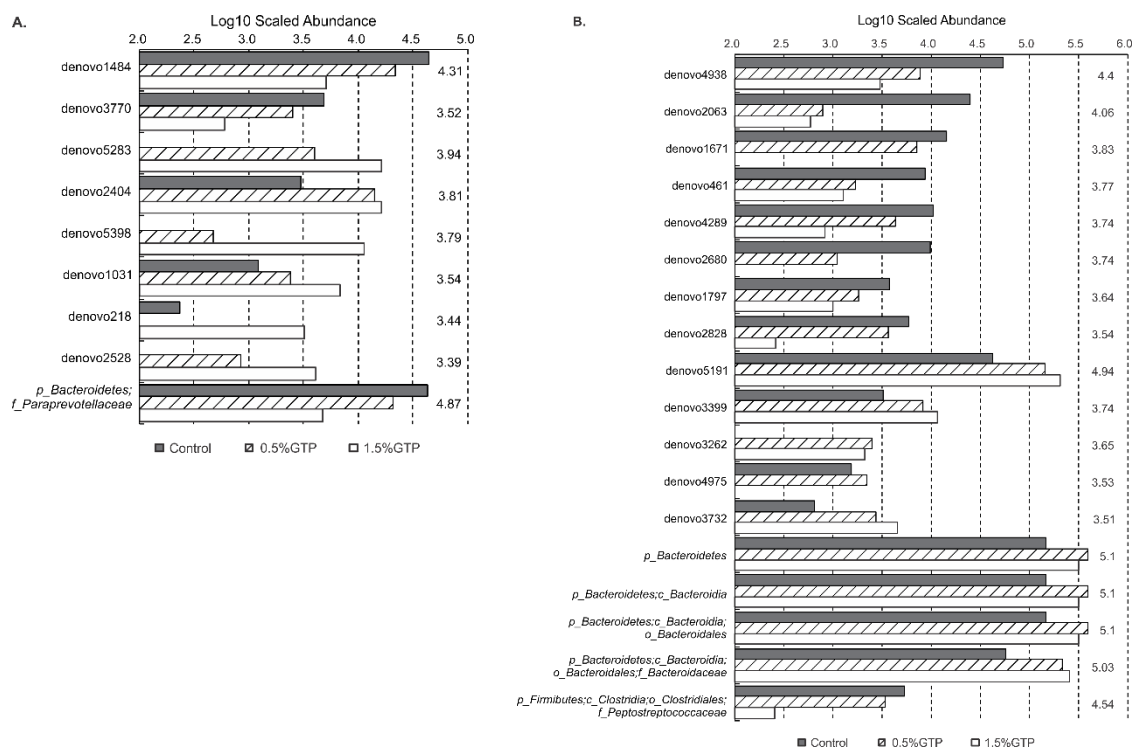


Figure 5-9 The abundance of OTUs that were significantly modified by GTP treatment: (A) 3-month; (B) 6-month.

The HALLA testing suggested 12 possible correlations between the abundance of specific OTUs and clinical measurements. Three of the OTUs were among those

significantly altered by GTP doses at 6th month: denovo5191, which was increased by GTP, was negatively correlated to mean corpuscular volume (MCV); denovo2828 (assigned to family *peptostreptococcaceae* of *firmicutes*), which was decreased by GTP, was positively correlated to a cluster of hematologic parameters consisted of total cholesterol, total bilirubin, and triglycerides concentrations; denovo1797 (assigned to family *ruminococcaceae* of *firmicutes*), which was decreased by GTP, was positively correlated to blood total cholesterol concentrations. However, HALLA did not identify any correlations between specific GOs and clinical measurements, even though a substantial amount of OTUs were correlated to GOs (data not shown).

Table 5-2 Significantly modified OTUs with their taxonomic assignments by green tea polyphenols.

OTU ^a	Phylum	Class	Order	Family	Genus
<u>3-month treatment</u>					
Decreased					
denovo1484	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	CF231
denovo3770 ^b	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Increased					
denovo5283	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
denovo2404	Firmicutes	Clostridia	Clostridiales		
denovo5398	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia
denovo1031	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
denovo218	Firmicutes	Clostridia	Clostridiales		
denovo2528	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
<u>6-month treatment</u>					
Decreased					
denovo4938	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Allobaculum
denovo2063	Firmicutes	Clostridia	Clostridiales		
denovo1671	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Allobaculum
denovo461	Firmicutes	Clostridia	Clostridiales		
denovo4289	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	
denovo2680	Firmicutes	Clostridia	Clostridiales		
denovo1797	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae Peptostreptococcaceae	
denovo2828	Firmicutes	Clostridia	Clostridiales	e	
Increased					
denovo5191	Bacteroidetes	Bacteroidia	Bacteroidales Bacteroidales	Bacteroidaceae	Bacteroides
denovo3399	Bacteroidetes	Bacteroidia		S24-7	

OTU ^a	Phylum	Class	Order	Family	Genus
			Desulfovibrionales		
denovo3262	Proteobacteria	Deltaproteobacteria		Desulfovibrionaceae	
denovo4975	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	
denovo3732	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira

^a OTU refers to operational taxonomic unit;

^b devono3770 was the only OTU with a species assignment as *Bacteroides acidifaciens*.

5.4 Discussion

Although extensive evidence suggesting GTPs are beneficial to health, it is still unclear how GTPs exert its beneficial effects. This study investigated the dynamics of the gut microbiome in a continuous, multiple-dosing experiment by evaluating both functional genomic and microbial compositional alternations of gut microbiota at two different sampling times, using next-generation sequencing (NGS) based technologies. We found that 3 months of continuous dosing of 1.5% GTP in drinking water induced significant changes in gut microbial composition as shown by both α -diversity and β -diversity analysis, which was persistent until the end of this experiment at 6th month. We identified several microbial species and functional genes which altered in accordance to GTP doses at 3rd month, but much more species and genes, and more profound changes have been observed in a GTP dose-dependent manner at 6th month.

It is counter intuitive at the beginning to observe a decrease of community biodiversity when increasing GTP doses, assuming its beneficial effects. Because in macro-ecosystems, biodiversity is crucial for conferring resilience of ecosystem functions to environmental perturbations (Lozupone *et al.*, 2012; Oliver *et al.*, 2015). In addition, multiple evidence has shown that decreased community biodiversity was associated with gut microbiota in patients with obesity, IBD, and other diseases (Lozupone *et al.*, 2012). However, when GTP exerted into this gut micro-ecosystem, certain species that may be more efficient with utilizing GTP or advantageous in GTP modified ecosystem would overgrowth, leading to a decrease in biodiversity overall. We speculate that a net gain of ecosystem functions that are beneficial to host physiology due to an increase of beneficial species or decrease of potentially pathogenic species, which appears as the case in our

study, could readily compensate potential negative impacts due to the decrease of biodiversity. Furthermore, it is common to observe a decrease in biodiversity in gut microbiota when treated with potential probiotic or prebiotic compounds. For example, Martin *et al.* noted that administering two *Lactobacillus* strains into female germ-free C3H mice inoculated with bacteria isolated from an infant significantly lowered the CFU counts of several interested bacterial species (Martin *et al.*, 2008). In a recent study employing 16S rRNA-based community composition analyses, Uronis *et al.* showed that a commercial probiotic mixture decreased the colonic microbial community diversity of colitis rats, which was significantly correlated with reduced clinical colitis scores (Uronis *et al.*, 2011).

Several microbial species or phylotypes were impacted by GTP dosing after 6 months of treatment. Intriguingly, the phylum *bacteroidetes* increased abundance in response to GTP in a dose-dependent manner. *Bacteroidetes* is a major phylum colonizing all the different parts of the gastrointestinal tract (GIT) in mammals regardless pH, nutrients, oxygen availability (Thomas *et al.*, 2011). Among many critical roles of *bacteroidetes* in host physiology, their main biological function is to degrade resistant biopolymers in the large intestine that mammals cannot digest by themselves (Thomas *et al.*, 2011). Recent advancements on the interactions between gut microbiota and obesity have shown that *bacteroidetes* were heavily depleted in both obese mice and obese humans (Ley *et al.*, 2005; Ruth E Ley *et al.*, 2006; Tilg, 2010), but fat-restricted or carbohydrate-restricted diet therapy could increase the abundance of *bacteroidetes*, which was correlated with the loss of body weight among obese human subjects (Ruth E Ley *et al.*, 2006). A metagenomic analysis of obese and lean twin pairs showed that a significant

portion of microbial genes that were enriched in lean phenotype were from *bacteroidetes* (Turnbaugh *et al.*, 2009). In addition to OTUs from *bacteroidetes*, one OTU that was assigned to genus *Oscillospira* from *firmicutes* was also increased in a dose-dependent manner. *Oscillospira* could not be grown in pure culture but was consistently captured by the 16S gene survey of human microbiome (Konikoff and Gophna, 2016). Many recent studies have shown a positive association between *Oscillospira* abundance and leanness (Tims *et al.*, 2013; Verdam *et al.*, 2013; Goodrich *et al.*, 2014; Escobar *et al.*, 2014). It is suggested to be a bile-resistant bacterium that may be able to degrade host glycans and contribute to the formation of secondary bile acids (Konikoff and Gophna, 2016). The only phylotype which decreased in a dose-dependent manner was the family *peptostreptococcaceae* of *fimicutes*, which was previously reported to be enriched in colorectal cancer patients in one study (Ahn *et al.*, 2013). The modifications of species and phylotypes mentioned above, or the gut microbiome in general, by GTP as seen in this study may explain the beneficial effects of GTP especially its weight management effects. Many mechanisms have been suggested for GTP's beneficial effects, among which antioxidant properties were the most prominent and widely studied (Scalbert *et al.*, 2005). However, the potential value of these mechanisms to explain the health benefits of GTP is limited due to their low bioavailability and fast metabolism (mostly conjugated) in the human physiological system (Scalbert *et al.*, 2005; Manach *et al.*, 2005; Wang *et al.*, 2015). For example, EGCG, the most abundant and major tea catechin accounting for GTP's anticarcinogenesis effect, has the least absorbability in human and rats as compared to other forms (Higdon and Frei, 2003; Wang *et al.*, 2015). Consequently, the hypothesis that GTPs benefit host physiological system by modifying its gut microbiome

is appealing and worth further verification, given the modifiable nature of gut microbiome. Only a few OTU were impacted by GTP, which did not translate into any phylotypes. It was also worth mentioning that at both 3- and 6- month, there were several OTUs differentially abundant, which did not follow a dose-dependent trend. At 6-month, 13 OTUs, almost exclusively from *bacteroidales* (except 2 *clostridiales*) increased significantly in 0.5% group but decreased in 1.5% GTP group. We were uncertain the underlying implication of this finding or if the fact that they were mostly *bacteroidales* was important. However, we suspect that these OTUs might be accounted for the surge of alpha diversity in 0.5% group at 6-month.

It may still be too early to suggest any implications from the changes of functional gene profile because the results did not present themselves in any particular pattern. It is interesting, though, that identified GOs from category [C] were all increased in response to GTP dosing. GOs in this category are responsible for energy production and conversion, which may be corresponding to the enrichment of *bacteroidetes*. An GO, ENOG4105CCY from [C] with a function description of “NADH Flavin oxidoreductase, NADH oxidase”, was identified in both 3-month and 6-month analysis. NADH is a key co-enzyme in the catabolic pathways for energy production: NADH Flavin oxidoreductase catalyses the reduction of NAD⁺ to NADH and oxidative degradation of complex organic compounds; NADH oxidase then catalyses the oxidation of NADH typically using O₂ as the ultimate electron acceptor; the energy released in this 2-step reactions was stored into ATPs (Garrett and Grisham, 2010). Consequently, an increase of this GO with increasing GTP might also be linked to the weight management effect of GTP. In addition, GOs from category [M] “cell wall/membrane/envelope biogenesis” and

all but one GOs from category [K] “transcription” were decreased with increasing GTP doses. One GO, ENOG4108SJI from [M] with a function description of “hydrolase, family 25”, was identified at both sampling times. The only enzyme in this family is lysozyme (EC:3.2.1.17), which could damage the bacterial cell wall. It is not clear, however, the reason that it was negatively correlated with an increase of GTP doses.

The sequencing and analysis of standard mock community in this study provided exciting and interesting results. The 2-step quadruple-index PCR amplification and the sequencing data processing procedure were highly sensitive in identifying targeting microbial organisms. However, an OTU abundance filtering procedure is critical to reducing the false discovery rate. The results from abundance estimation were encouraging: the average over- or under-estimation of expected abundance was only 1.7 folds (geometric mean was used to limit the impact of a few extreme values) for HM-276D and 3.7 folds for HM-277D. The amount of input materials (in this case 16S rRNA operons) was likely to be influential to abundance estimation because, in HM-277D, all species with an input of no more than 10,000 were underestimated, among which 2 species with 1000 operons were completely missed. Our results were general in line with a recent systematic evaluation of 16S rRNA gene survey analysis of standard mock community (Gohl *et al.*, 2016).

We noticed that a recent article has suggested that treatment of GTP does not change the gut microbiome using a 16S based gene survey in a human study (Janssens *et al.*, 2016). However, several key differences between that study and the current experiment may account for this discrepancy. The major difference is the dosage of GTP used. In this study, 1.5% GTP extract in drinking water was provided to the rats, which

was equivalent to ~400 mg/kg EGCG per day. The daily intake of EGCG by the human subject in Janssen *et al.* (Janssens *et al.*, 2016) is only equivalent to 8.9 mg/kg. Also, in the human study, the impurity (caffeine), the duration (12 weeks), the demographics (healthy adult European Caucasians, most female), the impacts of uncontrolled human diets, and the diversity indexes used (non-phylogenetic based) may all contribute to the differences. Furthermore, only using samples collected at the end of that human study might not be sufficient to capture the dynamic changes of the human gut microbiome. Consequently, we believe it is necessary to conduct well-designed longer term human clinical trials to prove the true relationships between GTP consumption and health benefits via the modifications of the gut microbiome.

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References

Ahn, J., Sinha, R., Pei, Z., Dominianni, C., Wu, J., Shi, J., Goedert, J.J., Hayes, R.B., and Yang, L. (2013) Human gut microbiome and risk for colorectal cancer. *J. Natl. Cancer Inst.*, **105**, 1907–1911.

- Anderson, M.J. (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecol.*, **26**, 32–46.
- Auger, C., Mullen, W., Hara, Y., and Crozier, A. (2008) Bioavailability of polyphenon E flavan-3-ols in humans with an ileostomy. *J. Nutr.*, **138**, 1535S–1542S.
- Bolger, A.M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, **30**, 2114–2120.
- del Campo, R., Garriga, M., Pérez-Aragón, A., Gualarte, P., Lamas, A., Máiz, L., Bayón, C., Roy, G., Cantón, R., Zamora, J., *et al.* (2014) Improvement of digestive health and reduction in proteobacterial populations in the gut microbiota of cystic fibrosis patients using a *Lactobacillus reuteri* probiotic preparation: A double blind prospective study. *J. Cyst. Fibros.*, **13**, 716–722.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods*, **7**, 335–336.
- Chen, L., Lee, M.-J., Li, H.E., and Yang, C.S. (1997) Absorption, distribution, and elimination of tea polyphenols in rats. *Drug Metab. Dispos.*, **25**, 1045–1050.
- Clemente, J.C., Ursell, L.K., Parfrey, L.W., and Knight, R. (2012) The impact of the gut microbiota on human health: an integrative view. *Cell*, **148**, 1258–1270.
- Clifford, M.N., van der Hooft, J.J., and Crozier, A. (2013) Human studies on the absorption, distribution, metabolism, and excretion of tea polyphenols. *Am. J. Clin. Nutr.*, **98**, 1619S–1630S.
- Del Rio, D., Costa, L.G., Lean, M.E.J., and Crozier, A. (2010) Polyphenols and health: what compounds are involved? *Nutr. Metab. Cardiovasc. Dis.*, **20**, 1–6.
- Eloe-Fadrosh, E.A., Brady, A., Crabtree, J., Drabek, E.F., Ma, B., Mahurkar, A., Ravel, J., Haverkamp, M., Fiorino, A.-M., Botelho, C., *et al.* (2015) Functional dynamics of the gut microbiome in elderly people during probiotic consumption. *mBio*, **6**, e00231-15.
- Escobar, J.S., Klotz, B., Valdes, B.E., and Agudelo, G.M. (2014) The gut microbiota of Colombians differs from that of Americans, Europeans and Asians. *BMC Microbiol.*, **14**, 311.
- Everard, A., Lazarevic, V., Gaïa, N., Johansson, M., Ståhlman, M., Backhed, F., Delzenne, N.M., Schrenzel, J., François, P., and Cani, P.D. (2014) Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity. *ISME J.*, **8**, 2116–2130.

- Faith, D.P. (1992) Conservation evaluation and phylogenetic diversity. *Biol. Conserv.*, **61**, 1–10.
- Garrett, R.H. and Grisham, C.M. (2010) *Biochemistry Brooks/Cole*, Cengage Learning, Belmont, CA.
- Gohl, D.M., Vangay, P., Garbe, J., MacLean, A., Hauge, A., Becker, A., Gould, T.J., Clayton, J.B., Johnson, T.J., Hunter, R., *et al.* (2016) Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies. *Nat. Biotechnol.*, **34**, 942–949.
- Goodrich, J.K., Waters, J.L., Poole, A.C., Sutter, J.L., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J.T., *et al.* (2014) Human genetics shape the gut microbiome. *Cell*, **159**, 789–799.
- Higdon, J. and Frei, B. (2003) Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit. Rev. Food Sci. Nutr.*, **43**, 89–143.
- Hooper, L.V. and Gordon, J.I. (2001) Commensal host-bacterial relationships in the gut. *Science*, **292**, 1115–1118.
- Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M.C., Rattei, T., Mende, D.R., Sunagawa, S., Kuhn, M., *et al.* (2016) eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res.*, **44**, D286–D293.
- Huttenhower, C., Knight, R., Brown, C.T., Caporaso, J.G., Clemente, J.C., Gevers, D., Franzosa, E.A., Kelley, S.T., Knights, D., Ley, R.E., *et al.* (2014) Advancing the microbiome research community. *Cell*, **159**, 227–230.
- Hyatt, D., Chen, G.-L., LoCascio, P.F., Land, M.L., Larimer, F.W., and Hauser, L.J. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics*, **11**, 1.
- Janssens, P.L.H.R., Penders, J., Hursel, R., Budding, A.E., Savelkoul, P.H.M., and Westerterp-Plantenga, M.S. (2016) Long-term green tea supplementation does not change the human gut microbiota. *PloS One*, **11**, e0153134.
- Jumpstart Consortium Human Microbiome Project Data Generation Working Group (2012) Evaluation of 16S rDNA-based community profiling for human microbiome research. *PLOS ONE*, **7**, e39315.
- Kanehisa, M., Sato, Y., and Morishima, K. (2016) BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J. Mol. Biol.*, **428**, 726–731.

- Kida, K., Suzuki, M., Matsumoto, N., Nanjo, F., and Hara, Y. (2000) Identification of biliary metabolites of (–)-epigallocatechin gallate in rats. *J. Agric. Food Chem.*, **48**, 4151–4155.
- Konikoff, T. and Gophna, U. (2016) *Oscillospira*: a central, enigmatic component of the human gut microbiota. *Trends Microbiol.*, **24**, 523–524.
- Kuczynski, J., Liu, Z., Lozupone, C., McDonald, D., Fierer, N., and Knight, R. (2010) Microbial community resemblance methods differ in their ability to detect biologically relevant patterns. *Nat. Methods*, **7**, 813–819.
- Langmead, B. and Salzberg, S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nat. Methods*, **9**, 357–359.
- Lee, M.-J., Wang, Z.-Y., Li, H., Chen, L., Sun, Y., Gobbo, S., Balentine, D.A., and Yang, C.S. (1995) Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiol. Prev. Biomark.*, **4**, 393–399.
- Leser, T.D. and Mølbak, L. (2009) Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environ. Microbiol.*, **11**, 2194–2206.
- Ley, R.E., Bäckhed, F., Turnbaugh, P., Lozupone, C.A., Knight, R.D., and Gordon, J.I. (2005) Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. U. S. A.*, **102**, 11070–11075.
- Ley, R.E., Turnbaugh, P.J., Klein, S., and Gordon, J.I. (2006) Microbial ecology: human gut microbes associated with obesity. *Nature*, **444**, 1022–1023.
- Li, D., Luo, R., Liu, C.-M., Leung, C.-M., Ting, H.-F., Sadakane, K., Yamashita, H., and Lam, T.-W. (2016) MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. *Methods*, **102**, 3–11.
- Li, J., Jia, H., Cai, X., Zhong, H., Feng, Q., Sunagawa, S., Arumugam, M., Kultima, J.R., Prifti, E., Nielsen, T., *et al.* (2014) An integrated catalog of reference genes in the human gut microbiome. *Nat. Biotechnol.*, **32**, 834–841.
- Lozupone, C.A., Hamady, M., Kelley, S.T., and Knight, R. (2007) Quantitative and Qualitative Diversity Measures Lead to Different Insights into Factors That Structure Microbial Communities. *Appl. Environ. Microbiol.*, **73**, 1576–1585.
- Lozupone, C.A. and Knight, R. (2008) Species divergence and the measurement of microbial diversity. *FEMS Microbiol. Rev.*, **32**, 557–578.
- Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., and Knight, R. (2012) Diversity, stability and resilience of the human gut microbiota. *Nature*, **489**, 220–230.

- Luo, H., Tang, L., Tang, M., Billam, M., Huang, T., Yu, J., Wei, Z., Liang, Y., Wang, K., Zhang, Z.Q., *et al.* (2006) Phase IIa chemoprevention trial of green tea polyphenols in high-risk individuals of liver cancer: modulation of urinary excretion of green tea polyphenols and 8-hydroxydeoxyguanosine. *Carcinogenesis*, **27**, 262–268.
- Manach, C., Williamson, G., Morand, C., Scalbert, A., and Rémésy, C. (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.*, **81**, 230S–242S.
- Martin, F.-P.J., Wang, Y., Sprenger, N., Yap, I.K.S., Lundstedt, T., Lek, P., Rezzi, S., Ramadan, Z., van Bladeren, P., Fay, L.B., *et al.* (2008) Probiotic modulation of symbiotic gut microbial–host metabolic interactions in a humanized microbiome mouse model. *Mol. Syst. Biol.*, **4**.
- McNulty, N.P., Yatsunenko, T., Hsiao, A., Faith, J.J., Muegge, B.D., Goodman, A.L., Henrissat, B., Oozeer, R., Cools-Portier, S., Gobert, G., *et al.* (2011) The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. *Sci. Transl. Med.*, **3**, 106ra106–106ra106.
- Nielsen, H.B., Almeida, M., Juncker, A.S., Rasmussen, S., Li, J., Sunagawa, S., Plichta, D.R., Gautier, L., Pedersen, A.G., Le Chatelier, E., *et al.* (2014) Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. *Nat. Biotechnol.*, **32**, 822–828.
- Nocker, A., Burr, M., and Camper, A.K. (2007) Genotypic microbial community profiling: a critical technical review. *Microb. Ecol.*, **54**, 276–289.
- Oliver, T.H., Heard, M.S., Isaac, N.J.B., Roy, D.B., Procter, D., Eigenbrod, F., Freckleton, R., Hector, A., Orme, C.D.L., Petchey, O.L., *et al.* (2015) Biodiversity and resilience of ecosystem functions. *Trends Ecol. Evol.*, **30**, 673–684.
- Qian, G., Xue, K., Tang, L., Wang, F., Song, X., Chyu, M.-C., Pence, B.C., Shen, C.-L., and Wang, J.-S. (2012) Mitigation of Oxidative Damage by Green Tea Polyphenols and Tai Chi Exercise in Postmenopausal Women with Osteopenia. *PLOS ONE*, **7**, e48090.
- Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W., Guan, Y., Shen, D., *et al.* (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*, **490**, 55–60.
- Scalbert, A., Manach, C., Morand, C., Rémésy, C., and Jiménez, L. (2005) Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.*, **45**, 287–306.

- Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., and Huttenhower, C. (2011) Metagenomic biomarker discovery and explanation. *Genome Biol*, **12**, R60.
- Shen, C.-L., Chyu, M.-C., and Wang, J.-S. (2013) Tea and bone health: steps forward in translational nutrition. *Am. J. Clin. Nutr.*, **98**, 1694S–1699S.
- Sinha, R., Abnet, C.C., White, O., Knight, R., and Huttenhower, C. (2015) The microbiome quality control project: baseline study design and future directions. *Genome Biol.*, **16**, 276.
- Sohail, M.U., Hume, M.E., Byrd, J.A., Nisbet, D.J., Shabbir, M.Z., Ijaz, A., and Rehman, H. (2015) Molecular analysis of the caecal and tracheal microbiome of heat-stressed broilers supplemented with prebiotic and probiotic. *Avian Pathol.*, **44**, 67–74.
- Stalmach, A., Mullen, W., Steiling, H., Williamson, G., Lean, M.E.J., and Crozier, A. (2010) Absorption, metabolism, and excretion of green tea flavan-3-ols in humans with an ileostomy. *Mol. Nutr. Food Res.*, **54**, 323–334.
- Tang, L., Tang, M., Xu, L., Luo, H., Huang, T., Yu, J., Zhang, L., Gao, W., Cox, S.B., and Wang, J.-S. (2008) Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention. *Carcinogenesis*, **29**, 411–417.
- The Human Microbiome Project Consortium (2012) A framework for human microbiome research. *Nature*, **486**, 215–221.
- Thomas, F., Hehemann, J.-H., Rebuffet, E., Czjzek, M., and Michel, G. (2011) Environmental and gut bacteroidetes: the food connection. *Front. Microbiol.*, **2**.
- Tilg, H. (2010) Obesity, metabolic syndrome, and microbiota: multiple interactions. *J. Clin. Gastroenterol.*, **44**, S16–S18.
- Tims, S., Derom, C., Jonkers, D.M., Vlietinck, R., Saris, W.H., Kleerebezem, M., de Vos, W.M., and Zoetendal, E.G. (2013) Microbiota conservation and BMI signatures in adult monozygotic twins. *ISME J.*, **7**, 707–717.
- Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., *et al.* (2009) A core gut microbiome in obese and lean twins. *Nature*, **457**, 480–484.
- Uronis, J.M., Arthur, J.C., Keku, T., Fodor, A., Carroll, I.M., Cruz, M.L., Appleyard, C.B., and Jobin, C. (2011) Gut microbial diversity is reduced by the probiotic VSL#3 and correlates with decreased TNBS-induced colitis: *Inflamm. Bowel Dis.*, **17**, 289–297.

- Van Amelsvoort, J., Van Het Hof, K., Mathot, J., Mulder, T., Wiersma, A., and Tijburg, L. (2001) Plasma concentrations of individual tea catechins after a single oral dose in humans. *Xenobiotica*, **31**, 891–901.
- Verdam, F.J., Fuentes, S., de Jonge, C., Zoetendal, E.G., Erbil, R., Greve, J.W., Buurman, W.A., de Vos, W.M., and Rensen, S.S. (2013) Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. *Obesity*, **21**, E607–E615.
- Visioli, F., Lastra, C.A.D.L., Andres-Lacueva, C., Aviram, M., Calhau, C., Cassano, A., D'Archivio, M., Faria, A., Favé, G., Fogliano, V., *et al.* (2011) Polyphenols and Human Health: A Prospectus. *Crit. Rev. Food Sci. Nutr.*, **51**, 524–546.
- Wang, J., Tang, L., Glenn, T.C., and Wang, J.-S. (2016) Aflatoxin B₁ induced compositional changes in gut microbial communities of male F344 rats. *Toxicol. Sci.*, **150**, 54–63.
- Wang, J., Tang, L., and Wang, J.-S. (2015) Biomarkers of dietary polyphenols in cancer studies: current evidence and beyond. *Oxid. Med. Cell. Longev.*, **2015**, 1–14.
- Yang, C.S., Chen, L., Lee, M.-J., Balentine, D., Kuo, M.C., and Schantz, S.P. (1998) Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. *Cancer Epidemiol. Prev. Biomark.*, **7**, 351–354.

CHAPTER 6

CONCLUSIONS

Ample evidence has demonstrated that commensal microbiota can affect the host response to xenobiotics exposure by influencing the absorption and metabolism of the xenobiotics (Nicholson *et al.*, 2005; Leser and Mølbak, 2009). It is less known that how those xenobiotics impact the commensal microbiota which would then modulate the host physiology and/or ultimately health (Figure 6-1). This dissertation is starting to fill this gap by evaluating the toxic or health impacts of xenobiotics on the gut microbiome of rat models, using aflatoxin B₁ and green tea polyphenols as model xenobiotic agents. Using 16S rRNA gene sequencing analysis and whole genome shotgun sequencing based metagenomic analysis, the compositional and functional profile of the gut microbiome have been evaluated in response to the repeated doses of model xenobiotics agents. By using multiple doses of xenobiotics, the dose-response relationship between xenobiotic exposures and the compositional and functional changes of the gut microbiome could be established. Given these results and in reflection of the specific goals of this dissertation, we conclude that:

- (I) AFB₁ induced a dose-dependent modification of the gut microbial community structure of F344 rats. The modification was likely due to the suppression of rare species and enrichment of the resistant species in the microbial community. Some beneficial bacteria, such as lactic acid bacteria, have been suppressed.

- (II) GTP induced dose-dependent modification of gut microbiome. Treatment of 3 months was sufficient to modify the gut microbiome, which was persistent through 6 months. A decrease of community biodiversity and a directional change of the gut microbiota structure was observed consistently across sampling times.
- (III) GTP treatment consistently enriched *Bacteroidetes*, *Oscillospira*, depleted *Peptostreptococcaceae*, in a dose-dependent manner. Among tens of gene orthologs modified at both sampling times, four gene orthologs have been consistently modified, among which, NADH Flavin oxidoreductase/NADH oxidase is related to energy metabolism. The modification of gut microbiome might be ascribed to the various beneficial effects of GTP.
- (IV) The library preparation procedure and data analysis protocol are sufficient and valid to elicit the responses of the gut microbiome to AFB1 and GTP exposure.

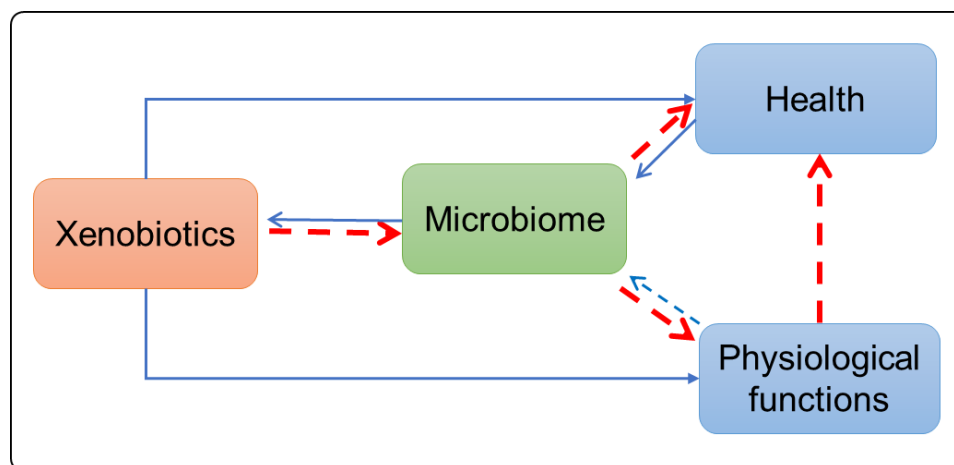


Figure 6-1 Central diagram for studying the xenobiotic interactions with the gut microbiome

References

- Leser, T.D. and Mølbak, L. (2009) Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environ. Microbiol.*, **11**, 2194–2206.

Nicholson, J.K., Holmes, E., and Wilson, I.D. (2005) Opinion: Gut microorganisms, mammalian metabolism and personalized health care. *Nat. Rev. Microbiol.*, **3**, 431–438.

CHAPTER 7

FUTURE DIRECTIONS

The overall objective of studying the interaction between xenobiotics and the commensal microbiome is to evaluate whether xenobiotics could impact the host health by modulating its microbiota (Figure 7-1). This dissertation is a starting step for this endeavor which still has a long way to go. To fully achieve this objective, future work could be continued in the following directions:

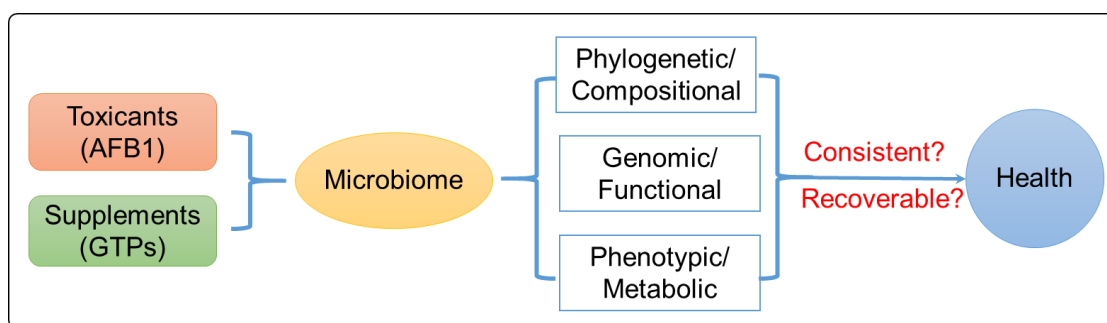


Figure 7-1 Research strategy for evaluating the interactions between xenobiotic agents and the microbiome and the overall health impacts.

- (I) From the perspective of xenobiotics impacts on gut microbiota, this dissertation only examined the compositional and functional genome profile of the gut microbiota. However, the changes at these levels might not necessarily be translated into a change in the function of the microbial community due to functional redundancy and horizontal gene transfer. Consequently, transcriptomics, proteomics, and metabolomics analysis, which could capture the phenotypic changes of the microbiota, would need to be implemented to advance current understanding.

(II) In addition, this dissertation, as well as the majority of research studying the xenobiotic impacts on the microbiome at the current time, adopted high-throughput sequencing technologies and generated a considerable amount of data regarding changes of the sequences and their inferred biological features (e.g. gene orthologs, proteins, metabolites). However, at present, valid inferences that could be drawn from these findings are still quite preliminary, if not over-interpreted. The main reason is that current high-throughput sequencing methods are still not cost-effective enough to obtain a complete and accurate profile of all relevant changes in the microbiome. The information obtained for genes and enzymes at present usually is not sufficient to construct a whole biological pathway. Another reason is that current bioinformatic tools vary widely in their accuracy of linking the sequence data to their biological features. Overall, the current microbiome research is still in the hypotheses-generating stage.

As for this dissertation, several very interesting results have been discovered. For example, lactic acid bacteria were suppressed by AFB₁ and there appeared to be a plateau in the response of the gut microbiome to AFB₁ in that the microbiome in medium and high dosing groups were structurally quite similar (see Figure 4-2 and Figure 4-3); several beneficial bacteria were enriched, one possible pathogen was suppressed, and one microbial gene related to energy metabolism was enriched in GTP treated rats. These findings are intriguing, suggesting that further studies using methods would be necessary appropriate for confirmation and mechanisms exploration (hypotheses validation process).

- (III) To evaluate whether the changes of the microbiome in response to xenobiotics would lead to a change in health status, further studies should be conducted to check if the changes in microbiome are consistent across time, which we touched upon a little bit in the GTP project, and if the changes in the microbiome would revert after withdrawing the xenobiotics.
- (IV) In this dissertation, two model xenobiotics have been tested. During this process, another mycotoxin, fumonisin B₁ was also briefly studied, which did not show any effects on the gut microbiome (data not shown). Consequently, more types of xenobiotics that might be interacting with the commensal microbiota should be tested to elicit the potential common modes of action.