THE PROTECTIVE EFFECTS AND RELATED MECHANISMS OF NATURAL POLYSACCHARIDES ON PROBIOTIC BACTERIA AGAINST ANTIBIOTIC DAMAGE

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The Protective Effects and Related Mechanisms of Natural Polysaccharides on Probiotic Bacteria against Antibiotic Damage

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CERTIFICATE OF ORIGINALITY

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Mao Yuheng

4th July, 2019

Abstract

Antibiotics are regarded as a class of the most successful drugs in history for their efficacy in the treatment of infectious diseases and having saved billions of lives. In recent decades, however, excessive use of antibiotics in poultry farming and aquafarming processes has imposed a health threat worldwide owing to the development of antimicrobial resistance as well as the many side effects of antibiotics on human health. The excess and unabsorbed antibiotics in the upper gut may enter the large intestine and disrupt the gut microbial balance by inhibiting the beneficial bacteria, increasing the colonization of resistant microbes and pathogenic organisms. The gut microbiota plays an important role in human health and an imbalanced gut microbiota is linked to a wide range of local and systemic disorders in the human body. This research project aimed to assess the protective effects and possible mechanisms of exopolysaccharides (EPS) produced by a medicinal fungus, Cs-HK1, against the inhibitive effects of antibiotics on pure cultures of several bifidobacterial species and on human fecal microflora *in vitro* fermentation. The antibiotics used in this study were selected from those that are widely used in therapy and animal husbandry, including enrofloxacin, penicillin, ampicillin, tetracycline and streptomycin. EPS fractions with different molecular weights (MW) were assessed together with some well-known prebiotic carbohydrates such as inulin, galactooligosaccharide (GOS) and konjac glucomannan (KGM), a high MW polysaccharide originated from the plant tuber of *Amorphohallus konjac*.

In pure bifidobacterial cultures, the high-MW Cs-HK1 EPS (~2.252×10^8 Da) and KGM (~8.8×10^8 Da) showed the most significant protective effect on most of the bifidobacterial species against antibiotic inhibition, as shown by the drastic increase in the minimal inhibitory and bactericidal concentration (MIC and MBC) dramatically. In general, the protective effect was positively correlated to the MW of polysaccharide fractions and also varied with the

bacterial species. Cs-HK1 EPS and KGM significantly increased the tolerance of bifidobacteria to the common antibiotics, suggesting their potential use as protection reagent for human gut bacteria, especially under the condition of intended or unexpected antibiotic exposure.

To understand the mechanisms of EPS and KGM's protective effect, we assessed the possible contribution by the nutritional effects and physical interactions. EPS, KGM and inulin were barely utilized as a carbon source for the bifidobacterial growth in the pure cultures. On the other hand, electron microscopic observation showed that the Cs-HK1 EPS formed a viscous layer around the bacterial cell, which could resist the access by antibiotics. Furthermore, the polysaccharide layer surrounding the bacteria cell also promoted the aggregation of bacterial cells to form biofilms, and EPS also enhanced the bifidobacterial adhesion to Caco-2 cell monolayer. Additionally, the EPS showed moderate ability to adsorb some of the antibiotics. These results suggested that EPS and other high MW polysaccharides protected bifidobacteria mainly through physical interactions.

Human fecal fermentation was performed to evaluate the effects of EPS on the complex and diverse microbial community of intestinal microflora. EPS fractions with different MW ranges were tested in this system. While barely utilized by individual bifidobacterial species in pure cultures, all the EPS fractions were consumed by more than 75% during 24-48 h of the *in vitro* fecal fermentation. As a consequence, there was a significant increase in the production of short chain fatty acids (SCFAs) including acetic, propionic and butyric acid. The consumption rates and production levels of SCFAs varied slightly with the different EPS fractions. EPS also influenced the composition and diversity of fecal microflora, increasing the relative abundance of *Firmicutes* but suppressing that of *Proteobacteria*, which may be beneficial to human health. This result demonstrated the fermentability of EPS by gut microbiota and proposed the potential of Cs-HK1 EPS as prebiotic in the food industry.

Similar to that in the pure bacterial cultures, Cs-HK1 EPS and KGM also protected the bacteria against antibiotic damage during *in vitro* fecal fermentation. Particularly, the Cs-HK1 EPS significantly increased the viability of major groups of gut bacteria compared with the well-known prebiotics, inulin and GOS. The presence of EPS in the fecal culture was able to maintain the operational taxonomic unit (OTU) of fecal microflora at the level of the control. The fermentative metabolism of EPS also increased the relative abundance of *Clostridium*. In contrast, inulin and GOS induced a significant decrease of the OTU number. The beneficial effects of EPS on the gut microbial diversity and microbial distribution in the presence of ampicillin further validated the protective effects of Cs-HK1 EPS on gut bacteria against antibiotic damage.

In summary, the following are the major findings from this research project:

(1) The high MW EPS produced by the medicinal fungus Cs-HK1 had protective effects against antibiotic inhibition of bifidobacteria in liquid cultures but could not be well utilized as a carbon source for bacterial growth.

(2) The protective effect of EPS on bifidobacteria was mainly attributed to physical interactions, the formation of a viscous layer surrounding the bacterial cell that increased the resistance to antibiotics.

(3) Cs-HK1 EPS also showed promising prebiotic function during *in vitro* human fecal fermentation, being well fermented by the fecal microflora to produce SCFAs and also beneficial for the stability and diversity of microbial populations.

These findings lay a theoretical foundation for further development and application of Cs-HK1 EPS as new prebiotic nutraceutical and functional food products for improving gut health.

List of publications

Journal papers

1. Song, A.-X., **Mao, Y.-H.**, Siu, K.-C. & Wu, J.-Y. (2019). Protective effects of exopolysaccharides of a medicinal fungus on probiotic bacteria during cold storage and simulated gastrointestinal condition. *International Journal of Biological Macromolecules*, 133, 957-963.
2. **Mao, Y.-H.**, Song A.-X., Wang, Z.-M., Yao, Z.-P. & Wu, J.-Y. (2018). Protection of Bifidobacterial cells against antibiotics by a high molecular weight exopolysaccharide of a medicinal fungus Cs-HK1 through physical interactions. *International Journal of Biological Macromolecules*, 119, 312-319.
3. **Mao, Y.-H.**, Song A.-X., Yao, Z.-P. & Wu, J.-Y. (2018). Protective effects of natural and partially degraded konjac glucomannan on Bifidobacteria against antibiotic damage. *Carbohydrate Polymers*, 181, 368-375.
4. Song, A.-X., **Mao, Y.-H.**, Siu, K.-C. & Wu, J.-Y. (2018). Bifidogenic effects of Cordyceps sinensis fungal exopolysaccharide and konjac glucomannan after ultrasound and acid degradation. *International Journal of Biological Macromolecules*, 111, 587-594.

Manuscripts submitted/in preparation:

1. Mao, Y.-H., Song A.-X., Li, L.-Q., Yao, Z.-P. & Wu, J.-Y. Evaluation of the prebiotic effects of exopolysaccharide fractions from a medicinal fungus by in vitro fermentation of human fecal microflora. Submitted to Journal of Functional Foods

2. Mao, Y.-H., Song A.-X., Yao, Z.-P. & Wu, J.-Y. Application of in vitro colonic batch fermentation to study the protective effects of Cs-HK1 EPS on human gut microbiota against the damage of antibiotics. Manuscript in preparation.

Conference papers and posters (International)

1. 3rd Microbiome R&D and Business Collaboration Congress: Asia. Hong Kong.
2. Mao, Y.-H., Song A.-X., Wang, Z.-M., Yao, Z.-P. & Wu, J.-Y. (2018). The protective effect of Cs-HK1 EPS on human gut probiotic bacteria. (4th) International Conference on Gut Microbiota and Health. Shanghai, China.
3. 4th Microbiome R&D and Business Collaboration Congress: Asia. Hong Kong.
4. Mao, Y.-H., Song A.-X., Wang, Z.-M., Yao, Z.-P. & Wu, J.-Y. (2019). The protective effects of Cs-HK1 exopolysaccharides on gut bacteria against antibiotic damage. Microbiome: Chemical Mechanisms and Biological Consequences (C3). Montreal, Canada.

Conference presentations and posters (Local)

1. Mao, Y.-H., Song A.-X., Yao, Z.-P. & Wu, J.-Y. (2017) The protective effects of Konjac glucomannan against antibiotic damage of probiotic gut bacteria. The 24th Symposium of Chemistry Postgraduate Research in Hong Kong.

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List of abbreviations and symbols

EPS	Exopolysaccharides
PS	Polysaccharides
KGM	Konjac glucomannan
GOS	Galactooligosaccharides
US	Ultrasound
MW	Molecular weight
HPGPC	High performance gel permeation chromatograph
SEM	Scanning electron microscopy
CLSM	Confocal laser scanning microscopy
AFM	Atomic force microscope
SCFA	Short chain fatty acid
UPLC	Ultra-performance liquid chromatography
MS	Mass spectrometry
GC	Gas chromatography
FID	Flame ionization detector

Chapter 1 Introduction

The human intestinal microflora or gut microbiota is a diverse microbial community which is composed mainly of bacteria. Among the numerous bacterial phyla that have been detected in the human gut, *Firmicutes* and *Bacteroidetes* are two of the dominant ones, and *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* are also commonly found as the minor constituents of microbiota. It is generally believed that the gut microbial composition and diversity have a close association with the host's physiology and pathology. Diet is the most probable factor affecting the microbial composition and balance. Non-digestible dietary fibers and prebiotics including complex polysaccharides and oligosaccharides can reach the large intestine and be fermented by the gut bacteria (Rios-Covian et al., 2016). The selective consumption of the non-digestible carbohydrates by different microbial species directly affects their proliferation and relative abundance in the gut microbiota. On the other hand, the fermentative metabolism of carbohydrates in the gut yields short chain fatty acids (SCFAs) as the end products, including acetic acid, propionic acid and butyric acid, which have important and beneficial functions both locally and systemically (Pluznick et al., 2013).

Antibiotics had been regarded as the most successful drugs for a long time since the discovery of penicillin during the World War II because of their high efficacy for treatment of infectious diseases and for saving billions of lives (Modi, Collins, & Relman, 2014a). With the rapid advancement and expansion of poultry farming and aquaculture in the postwar years, antibiotics have been increasingly used as feed additives for the prevention of diseases in animals and for the promotion of animal growth (Blaser, 2016; L. Zhao, Dong, & Wang, 2010). However, the excessive use of antibiotics in the poultry and aquafarming processes in recent decades has posed a health threat worldwide owing to the development of antimicrobial

resistance as well as the many side effects of antibiotics on human health. Some of the unabsorbed antibiotics in the upper gut that enter the large intestine may disrupt the gut microbial balance by inhibiting the beneficial bacteria, increasing the colonization of resistant microbes and pathogenic organisms. The imbalanced gut microbiota may consequently lead to a number of diseases, such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and even other chronic metabolic diseases (Keeney, Yurist-Doutsch, Arrieta, & Finlay, 2014a). *Bifidobacteria*, which represent an important group of beneficial probiotic bacteria in human gut microbiota, were found to suffer a significant loss after antibiotic treatment (Dethlefsen, Huse, Sogin, & Relman, 2008). Effective strategies for counteracting the side-effects of antibiotics are urgently needed.

Natural polysaccharides extracted from edible plants, fungi, macro alga and other sources have been widely explored as functional food ingredients because of their notable health benefits, such as their antitumor and immunomodulation (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Yan, Wang, Li, & Wu, 2011), and antioxidant properties (I. C. Ferreira et al., 2015; Q.-L. Huang, K.-C. Siu, W.-Q. Wang, Y.-C. Cheung, & J.-Y. Wu, 2013; Yue, Ye, Zhou, Sun, & Lin, 2013). As many of the bioactive natural polysaccharides are non-starch and non-digestible, one of their primary sites of action may be in the large intestine on the gut bacteria (Ramberg, Nelson, & Sinnott, 2010; Singdevsachan et al., 2016). Besides the nutritional and biological functions, natural polysaccharides can affect the gut bacteria with their physicochemical properties, such as the thickening and gelling effects in aqueous media. The exopolysaccharides or extracellular polymeric substances (EPS) of lactic acid bacteria and other microorganisms may have a protective function for bacteria, such as resistance to antimicrobials with the formation of a biofilm barrier to the diffusion and uptake of antimicrobials (Mah & O'Toole, 2001). Edible mushrooms or fungi which are rich in bioactive

polysaccharides have been recognized as the potential source of prebiotics (Aida, Shuhaimi, Yazid, & Maaruf, 2009). Mushroom polysaccharides also enhanced the survival rate of probiotic bacteria in yogurts during cold storage and improved tolerance in simulated gastric and bile juices (Chou, Sheih, & Fang, 2013). Nonetheless, only a few studies have been made on the protective effects of natural polysaccharides on probiotic gut bacteria against antibiotics.

Cordyceps sinensis (Berk.) Sacc., generally known as Dong-Chong-Xia-Cao in Chinese, is a valuable and rare medical fungus with a wide range of health promoting effects such as antioxidant, anticancer, immunomodulation, antifatigue, hypoglycemic and hypolipidemic properties (X. Chen, Ding, Wang, Siu, & Wu, 2014; Leung, Zhao, Ho, & Wu, 2009; Wu, Leung, Wang, & Xu, 2014; Zhou, Gong, Su, Lin, & Tang, 2009). Moreover, *C. sinensis* fungus can have protective effects on the liver, kidney and cardiovascular system in human body (Zhou et al., 2009). Due to the scarcity of natural Cordyceps, the increasing demand of this traditional Chinese medicine can only be fulfilled by a more economic method, mycelial fermentation (Wu et al., 2014; Yan et al., 2011). Our lab has established a mycelial culture of Cs-HK1, which is an anamorphic fungal species isolated from a natural Cordyceps fruiting body and can produce significant amount of exopolysaccharides (EPS) in liquid culture (J.-K. Yan et al., 2009).

Konjac glucomannan (KGM), is a well-known polysaccharide isolated from the tuber of plant *Amorphophallus konjac,* which is widely used as a gelling and thickening agent in liquid foods and also as an edible film coating of food and pharmaceutical products (Herranz, Borderias, Solas, & Tovar, 2012; X. Xu, Li, Kennedy, Xie, & Huang, 2007). Recently, KGM has been increasingly used as dietary fiber in functional foods for improving gut health, lowering blood sugar and cholesterol and the risk of type II diabetes and obesity (Behera &

Ray, 2016; Tester & Al-Ghazzewi, 2013, 2016; Y.-Q. Zhang, Xie, & Gan, 2005). Native and enzyme-hydrolyzed KGM products have been evaluated as prebiotic substrates for the growth of lactobacilli and bifidobacteria (Al-Ghazzewi, Khanna, Tester, & Piggott, 2007; Al-Ghazzewi & Tester, 2012; J. Yang et al., 2017) and other probiotic bacteria of human or animal gut microbiota (Connolly, Lovegrove, & Tuohy, 2010; Harmayani, Aprilia, & Marsono, 2014).

To the best of our knowledge, however, no previous studies have assessed the protective effects of *C. sinensis* EPS and KGM on gut bacterial, especially bifidobacteria or any other probiotic bacteria against antibiotics. This research project was to evaluate the protective effects of EPS produced by Cs-HK1 mycelial fermentation, KGM and their degraded products on gut bacteria against the inhibition of antibiotics in pure culture and in *in vitro* fecal fermentation and to investigate the possible mechanisms.

Chapter 2 Objectives and significance

2.1 Objectives

This research project aims to evaluate the protective effects of natural and partially hydrolysed EPS produced by *C. sinensis* fermentation and KGM on *Bifidobacteria* against the inhibition of antibiotics and to investigate the possible mechanisms of the protection. Particularly, we will carry out the experimental studies as follows:

1) To assess the protective effects of EPS and KGM fractions with different molecular weights (MW) on five important bifidobacterial species against selected representative antibiotics used in medicine and farming.

2) To investigate the mechanisms of the protective effects through the assessment of biofilm formation of *Bifidobacteria*, the absorption of antibiotics to the polysaccharides, the observation of the physical interactions between EPS and *Bifidobacteria* by microscopic imaging techniques, and measurement of bacterial adhesion on caco-2 cell monolayer.

3) To evaluate the potential prebiotic function of Cs-HK1 EPS fractions in different MW ranges in vitro fecal fermentation.

4) To evaluate the protective effects of EPS and KGM on counteracting the disruption of antibiotics in *in vitro* fecal fermentation and investigate the mechanisms of the protective effect.

2.2 Significance

EPS from *Cordyceps sinensis* and KGM are two well-known polysaccharides with many demonstrated bioactivities. The present study is the first to study the protective effects of EPS and KGM on bifidobacteria against antibiotic damage. Additionally, the possible mechanisms for the protection effect were hypothesized and preliminarily demonstrated to be the adsorption of antibiotics by PS and the improvement of biofilm formation.

EPS from *Cordyceps sinensis* had many bioactivities, however, this is the first study that evaluated the prebiotic potential of the *Cordyceps sinensis* EPS in in vitro fecal fermentation. Moreover, the protective effects of EPS and KGM on counteracting the side effects of antibiotics were assessed in in vitro fecal fermentation. All the results may be useful for further study on the application of natural polysaccharides (Cs-HK1 EPS and KGM) on protecting the gut microbiota balance, and the enhancement of this protective effects.

Chapter 3 Literature Review

Natural polysaccharides extracted from plants and other sources have various bioactivities. As many of the bioactive natural polysaccharides are non-starch and non-digestible, one of their primary sites of action may be in the large intestine on the gut bacteria (Ramberg et al., 2010; Singdevsachan et al., 2016). Besides the nutritional and biological functions, natural polysaccharides can affect the gut bacteria with their physicochemical properties such as the thickening and gelling effects in aqueous media. The exopolysaccharides or extracellular polymeric substances (EPS) of lactic acid bacteria and other microorganisms may have a protective function for the bacteria, such as the resistance to antimicrobials with the formation of a biofilm barrier to the diffusion and uptake of antimicrobials (Mah & O'Toole, 2001). Mushroom polysaccharides enhanced the survival rate of probiotic bacteria in yogurts during cold storage and improved the tolerance in simulated gastric and bile juices (Chou et al., 2013). In this chapter, the properties and bioactivities of two natural polysaccharides were reviewed, and the relationship between polysaccharides and gut microbiota were also discussed.

3.1 Polysaccharides from *Cordyceps sinensis* and konjac glucomannan

3.1.1 Polysaccharides from *Cordyceps sinensis*

Cordyceps sinensis, which is well-known as “Dong-Chong-Xia-Cao” in Chinese and “Tochukaso” in Japanese, is a precious Chinese medicine that composed by a fungal fruiting body and caterpillar larva. *C. sinensis* is found on the Qinghai Tibetan plateau (above 2000 m) and grows in a special mode. In late autumn, the fungus infects the ghost moth larvae and the larvae keeps alive for a period before the growing fungus occupied its body and finally dead

and frozen in soil in winter (Figure 3-1). This herbal medicine was documented to be beneficial to lung and kidney more than 300 years ago in Ben-Cao-Cong-Xin (Yan, Wang, & Wu, 2014).

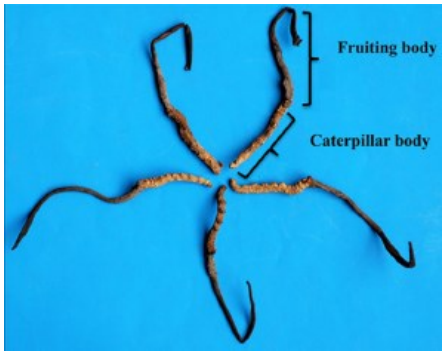

Figure 3-1 Wild *Cordyceps sinensis* emerging out of the head of a head caterpillar (Shashidhar, Giridhar, Sankar, & Manohar, 2013).

Many studies found that *C. sinensis* provides beneficial health effects on immune, respiratory, cardiovascular, circulatory systems. It can enhance immune functions and possess antitumor, anti-aging, anti-fatigue, and antioxidant functions. These beneficial effects may attribute to the chemical components, such as polysaccharides, nucleosides, amino acids, alkaloids, and some inorganic elements. As wild *C. sinensis* is very scarce in nature that cannot satisfy the increasing needs and extremely expensive that few people can afford it, mycelial fermentation of cordyceps fungal species became a valuable and feasible mean to produce enough fungus and active compounds at present (X. Chen, Wang, Nie, & Marcone, 2013; Nie, Cuia, Xie, Phillips, & Phillips, 2013).

The polysaccharides (PS) in *C. sinensis* represent a major class of component provides the beneficial health effects. Although there are many inconsistent or controversial results on the biological functions of PS from C. sinensis, many evidences showed that PS from C. sinensis have potential effects on immune regulation (Moradali et al., 2007), anti-fatigue (Wu et al., 2014), anti-oxidation (X. Chen, Ding, et al., 2014; Leung, Zhang, & Wu, 2006), and anti-

tumor (Leung et al., 2006). The PS extracted from fungal mycelium are known as intracellular PS (IPS) and those from the fermentation liquid medium as exopolysaccharides (EPS). EPS is usually isolated from C. sinensis liquid culture medium by ethanol precipitation. In fact, this crude EPS is a complex of polysaccharides and protein. So far, it is not clear about the association between polysaccharides and protein and how the fractions with different MW and chemical components contribute to biological activities (Q.-L. Huang, K. C. Siu, W.-Q. Wang, Y. C. Cheung, & J.-Y. Wu, 2013).

3.1.2 Konjac glucomannan

Konjac glucomannan (KGM) is a soluble dietary fiber which can provide satiety, reduce and delay the absorption of glucose, inhibit the synthesis of fatty acid. KGM helps to stabilize blood sugar and decrease blood fat and liver fat without toxic side effects. KGM has many physical and chemical properties, including the high viscosity, the excellent ability of water absorption and expansion. Because the processing technology of konjac is limited, the concentration of KGM in konjac food is generally low (D. Yang et al., 2017).

KGM is composed of glucose and mannose at the ratio 1.0-1.6. There are some branch points on the C-3 position of mannose residues. However, some more recent researches showed that there are also some branch points on the C-6 position with branch degree of 8%. The ratio of glucose residues to mannose residues is approximately 2 (Ni et al., 2016). KGM contains many acetyl groups (about per 19 sugar residues with an acetyl group) that provide a certain water solubility of KGM. Under the condition with the presence of alkali, the gel can be formed by heating, because alkali can remove the acetyl group. The absorption peak of the acetyl group in the infrared absorption spectrum was 1730 cm-1 and so was KGM film, and this absorption peak disappeared after alkali treatment (H. Zhang et al., 2001). The result was shown in Figure 3-2.

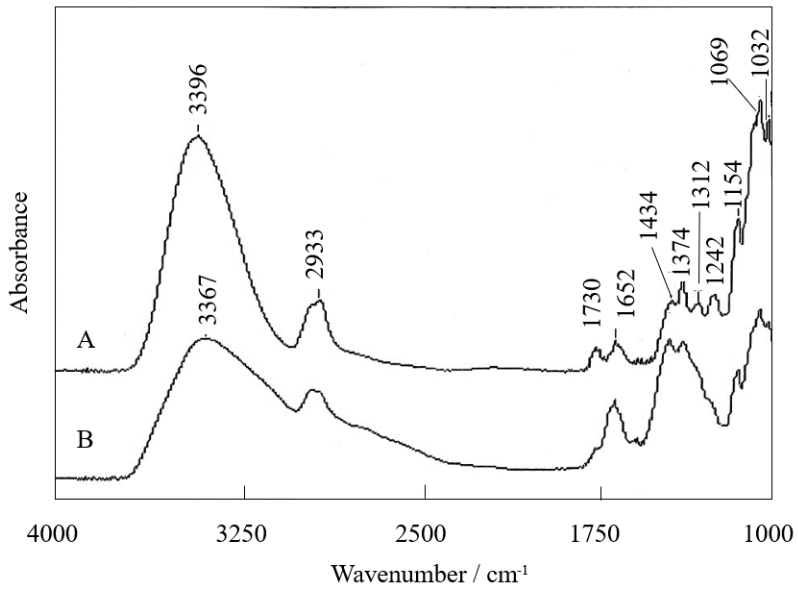

Figure 3-2 Absorbance FTIR spectra of LM-4 with the assignment of the main bands. A: untreated; B: treated with Na_2CO_3 (H. Zhang et al., 2001).

Under room temperature, gel permeation chromatography (GPC) is applied to determine the MW of each KGM component. Non-degraded KGM solution is gel with high viscosity, so it is more challengeable to determine the MW of original KGM by GPC. Some MWs of Non-degraded KGM components are higher than 1.0×10^6 Da, while no components lower than 1.0×10^4 Da. To the water-soluble KGM, the MW distribution range has the similar width (Clegg, Phillips, & Wiiliams, 1990) and nearly cover the whole range of 4.0×10^4 Da to 2.0×10^7 Da. Because lack of standard polysaccharide with WM higher than 1.0×10^6 Da, the exclusion limit of GPC column is 5.0×10^7 Da. Therefore, it is hard to conclude the MW for the separated components at early time.

As an important dietary fiber, KGM has certain therapeutic effects in the early stage of diabetes. Vuksan et al. (Vuksan et al., 1999) studied the effects of KGM as a supplementary food on the treatment of type-2 diabetes among patients with multiple risk factors of coronary heart disease (CHD), including hypertension, hyperlipidemia and hyperglycemia. The results showed that KGM can relief three dangerous factors, which indicated the possibility of decreasing CHD risk. Meanwhile, KGM can decrease the drug doses, which lead to lower cost

and higher acceptability of treatment. A further study also showed that KGM reduced the corresponding risk factors such as hypertension and high blood lipids, and improve insulin resistance by regulating the absorption rate of nutrients in the small intestine (Vuksan et al., 2000). They continued to investigate the potential of KGM for the treatment of type-2 diabetes and found some evidences to support such a conclusion (Vuksan et al., 2001).

Cairella et al. (Cairella & Marchini, 1995) showed that low calories diet with glucomannan is more effective than pure low-calorie diet in improving human weight, blood sugar, total serum cholesterol, hunger and fullness. A large number of clinical trials have been carried out to study the effect of dietary fiber KGM on obesity in children or adults (Vido, Facchin, Antonello, Gobber, & Rigon, 1993; Walsh, Yaghoubian, & Behforooz, 1984). The results showed that after a period of time, the average weight significantly decreased after intaking KGM with high purity. More importantly, no significant side effects were found in all subjects. Additionally, a study also showed that konjac food have very good effect on preventing and treating hyperglycemia (Huang et al., 1990).

Additionally, KGM also showed good effect on treating constipation, which is a common disease in western countries. A study investigated the effectiveness and acceptability of glucomannan among 93 patients with constipation (Passaretti et al., 1991) and found that glucomannan can be used as an ideal treatment for patients with constipation for the effectiveness and long-term effectiveness.

3.2 Intestinal microbiota

3.2.1 Composition and function of gut microbiota

There is a wide variety of intestinal microbes and they play a lot of functions in our intestine. It is generally believed that the gastrointestinal tract is a system consisting mainly of two (Firmicutes and Bacteroides), but it should be noted that at least 10 various phyla have an

important contribution to their function (Marchesi et al., 2016). When we look at the gut microbiota, we generally focus on bacteria; only a few papers have studied viral components (or virions) and micro-eukaryotes (including protozoa and fungi). It can be seen from the relatively large cohorts (for example, more than 100 individuals) that the ratio of *Firmicutes*: *Bacteroidetes* is different in all individuals. At present, we are still unclear about the importance of the proportional changes (severe or small changes) of these two phyla to health. Studies have shown that the reduction of a single species belonging to the *Firmicutes* phylum (eg, *Faecalibacterium prausnitzii*) is associated with IBD (Sokol et al., 2009). However, in another scientific literature, it has been reported that this species is unrelated to IBD (Gerasimidis et al., 2014; Rajilic-Stojanovic & de Vos, 2014).We know that the gut microbiota is critical to the normal functioning and development of the host, but it is not sure which are the key species and whether the function of the microbiota is more important than the composition of the population. But this view is too simple. In some cases, differences in strains within a species may be differences between pathogens/pathogens and probiotics: for example, *E. coli* is associated with IBD and colorectal cancer (CRC)7, but an *E. coli* strain is used as probiotic.

In fact, most of the bacteria in the gut microbiota belong to five phyla. There are approximately 160 species in the colon of everyone, but few species are shared between unrelated individuals (Rajilic-Stojanovic & de Vos, 2014). However, everyone's gastrointestinal tract shows similar functions. This observation leads to conclude that function is more important than the identity of the species itself. However, differences in gut microbiota may also be important as these may result in differences in functional effectiveness. For example, although the ability to synthesize short-chain fatty acids (SCFAs) is found in the gut of all people, their amounts can vary (Louis, Young, Holtrop, & Flint, 2010).

Until now, it is still difficult to measure microbial diversity, but the precise molecular techniques have confirmed that there are about 500 species of bacteria existing in the gut environment. In the whole intestinal environment, the main microbial genera have been identified but with limit information. *Bifidobacterium* and *Lactobacillus* are regarded as good bacteria in gut microbes, and their proliferation marks the health level.

Fermentation of carbohydrates that escape from the digestion of gastric or duodenal digestive enzymes is a key activity of the human gut microbiota (Marchesi et al., 2016). The release of the nutrients in the large intestine maintains the overall function of the intestines and the absorption of micro-nutrients, such as the vitamin B and vitamin K. Intestinal microbes play a very important role in the host health. Non-digestible carbohydrates are fermented in the large intestine to organic acids (such as SCFAs), finally promote the proliferation of intestinal epithelial cells, while kill pathogenic microorganisms (Rios-Covian et al., 2016).

3.2.2 Study models

***In vitro* models.** So far, several *in vitro* models have been recognized, including the mimic of physical and chemical properties of the different parts of the gastrointestinal tract. Generally, temperature, pH value, oxidation reduction potential and running time are controlled. The movements of the gastrointestinal can be simulated by stirring. Some other models were used to mimic the excretion and absorption of gastrointestinal. In vitro model allows to control the conditions in the laboratory to simulate human intestinal micro ecosystem, while maintaining the diversity of the intestinal microbial community and test the fermentation characteristics of a series of substrate. More and more experiments show that these models are effective in the developing process of probiotics and prebiotics. One of the most commonly used is the five-stage in vitro model of human gastrointestinal developed by Gent University, Belgium , which was also known as the Simulator of the Human Intestinal Microbial

Ecosystem (SHIME®) (De Boever, Deplancke, & Verstraete, 2000). This model is shown in Figure 3-3.

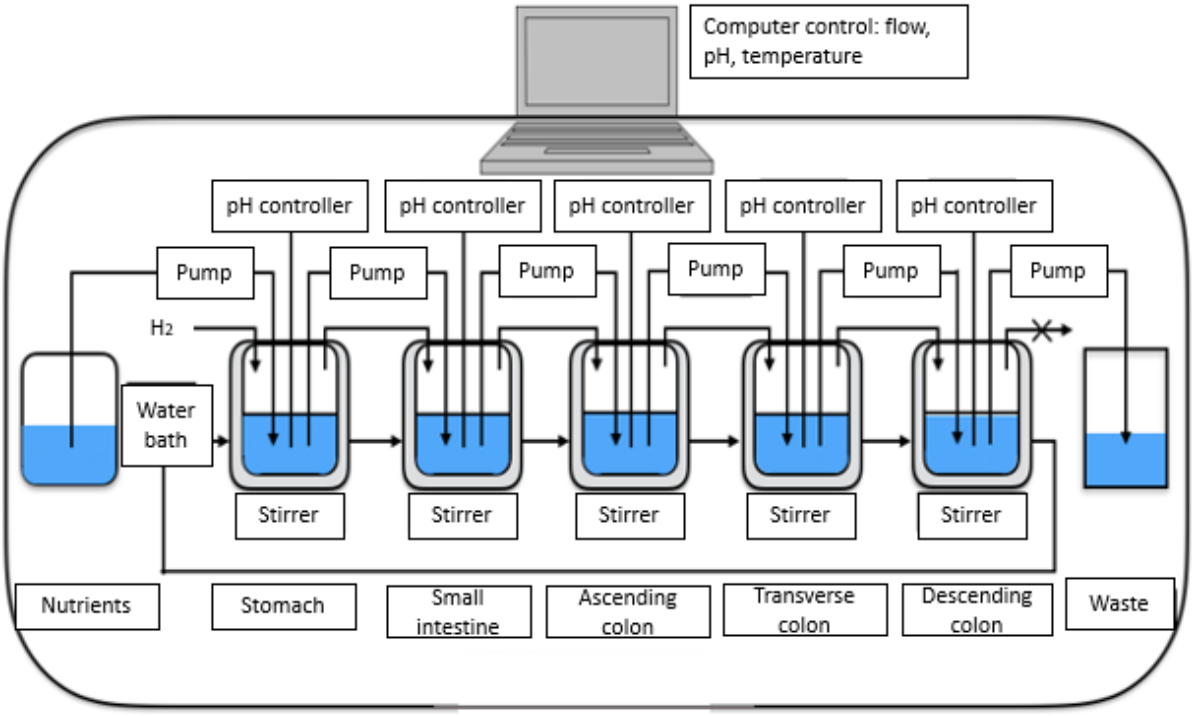

Figure 3-3 Schematic representation of the SHIME® (De Boever et al., 2000).

Animal models. These models can be used to study the whole-body reaction and host reaction caused by the whole intestinal microorganism, and the disorder state of the gastrointestinal system can be simulated by modeling (Butel, 2003; Cummings, Macfarlane, & Macfarlane, 2003; Hambly, Rumney, Fletcher, Rijken, & Rowland, 1997). Because the reactions between immune response and probiotics and prebiotics are often specific, it is ideal to evaluate the biological effects and prebiotic effects by using the animals have the similar microbial community with human beings (Edwards et al., 2003).

Tissue models. Tissue of the normal animal and pathological biopsy can be used to study the intestinal niche and intestinal microbial surface connection (Schultsz, Van, Ten, Tytgat, & Dankert, 1999). Many in vitro and animal experiments used fecal microorganisms as an initial

inoculum. Though fecal microbes are a good representative of the intestinal microbes, but they are not exactly the same as the epithelial adhesion bacteria (Macfarlane, Macfarlane, & Gibson, 1998). The development of molecular tools has greatly increased the possibility to study microorganisms from living tissues. Tissue culture is another method used to describe the characteristics of adhesion of probiotics and prebiotics. Though tissue culture has been recognized, but still with some limitations, such as that the model is derived from cancer cell lines, many probiotics are anaerobic bacteria while tissue culture is aerobic. In addition, when the supply with probiotics or prebiotics, tissue culture may appear immune response. And tissue culture needs to be optimized to reflect the true state of the intestinal system, especially under the pathological conditions.

Human trials. Well-designed human trials can be applied to evaluate the effect of probiotics and prebiotics. Under ideal conditions, the experiment must include a double-blind, randomized, placebo-controlled design, and a multi-center comparative studies are also preferred. As prebiotics, placebo selection method is not for certain now. Some studies use non-degradable polysaccharides (starch or MALT) or fully digested sugars (glucose or lactose) (Beards, Tuohy, & Gibson, 2010). Finally, recording food intake and intestinal characteristics during the test can provide more valuable information.

3.2.3 Impact of antibiotics on gut microbiota

It is undeniable that the discovery and application of antibiotics has made remarkable contributions to human health. However, the human uptake of antibiotics, especially at excessive doses, can induce several side effects, including improper drug compatibility, disordered metabolism and adsorption of nutrients, increasing colonization of resistant microbes and changed sensitivity to infections. One of the most common side effects was antibiotic-associated diarrhea (AAD), which sometimes can be fatal. This serious disease was

demonstrated to be connected to the altered microbial flora in the gut, which commonly known as gut microbiota. Recently, with advanced genetic methodology the study of the microbiota change became possible and accurate. This section will discuss the major side effects of antibiotics on gut microbiota.

(1) Alteration of microbiota composition

The study of the alteration of different classes and families in the human gastrointestinal tract (GIT) by clinically related antibiotics has just at a very early stage. The findings of several studies examining the microorganism populations influenced by the antibiotics were summarized in the Table 3-1. But Keeney K.M. (Keeney et al., 2014a) stated that the methods used by those studies were still preliminary, and more economic and comprehensive methods were expected to apply in future studies as the quick development of the microbiota composition characterizing techniques. When those advanced methods are available, the investigation of the changed metabolites will help us to understand more specific mechanisms.

In the earlier stage, the culture-based studies only examined the crude alterations in the organism composition in oral cavity, GIT and vagina in patients who were administrated with various antimicrobial agents (Nord, 1993). However, only general changes of large classes of microorganisms could be revealed in the microbiota analysis. Moreover, those studies failed to discover the effects of antimicrobials on normal gut microbiota of healthy human. For example, in some culture-based studies, ciprofloxacin (a broad-spectrum antibiotic usually used to treat different bacterial infections) was found to reduce the number of enterobacteria in feces, but in a study used denaturing gradient gel electrophoresis (DGGE), it had little influence on the whole composition of the GIT microbiota (Donskey et al., 2003). However, in later studies used 16S rRNA sequencing technique, some significant changes in the microbiota in the patients treated with ciprofloxacin were observed (Dethlefsen et al., 2008; Dethlefsen &

Relman, 2011). Nearly 30% of the taxa in the gut were influenced by a single treatment, however the microbiota mostly reversed within 4 weeks after the treatment (Dethlefsen & Relman, 2011). The other study revealed that some taxa were never regained (Dethlefsen et al., 2008). In addition, repeated treatment by ciprofloxacin induced more significant changes in both long term and short term (Dethlefsen & Relman, 2011).

Table 3-1 Antibiotic treatment alters the gut microbiota.

Antibiotic treatment	N	Method of analysis	General findings
Ciprofloxacin, 500 mg, 2 times a day, 7 days;	1	DGGE coupled with specific band sequencing	Treatment with ciprofloxacin resulted in minor changes (73% similarity to original state); clindamycin led to extreme change (18% similarity to original state, 11%% similarity to post-ciprofloxacin state) (Donskey et al., 2003)
Amoxicillin (875 mg) and clavalanic acid (125 mg), 2 times a day, 10 days	1	16S rRNA cone library screen	Changes to all major bacterial groups (*Bacteroides* spp., *Clostridium* cluster IV, *Clostridium* cluster XIVa, *Bifidobacterium* spp.) (Young & Schmidt, 2004) Overgrowth of Enterobacteriaceae by day 4; general reversal of the community by day 24, except for Bifidobacterium spp., which did not return
Ciprofloxacin, 500 mg, 2 times a day, 5 days	3	16S rRNA tag pyrosequencing	30% of the taxa in the gut were influenced by the treatment; the microbiota mostly regenerated within 4 weeks after treatment (Dethlefsen et al., 2008)
Ciprofloxacin, two 5-day treatments 6 months apart	3	16S rRNA tag pyrosequencing	25-50% of taxa wiped out by treatment; mostly regenerated post-treatment (Dethlefsen & Relman, 2011)

Indeed, whether the microbiota recovers to the same as the original state after the treatment is a crucial issue. But there are not many such studies on the long-term effects of antibiotic usage in human beings, and the results analyzed by high- and low-resolution analysis techniques are different (Table 5). In a DGGE-based study, 5 out of the 6 patients regained the original gut microbiota 60 days after a short course of treatment with various antibiotics, while in a study used 16S rRNA, the gut microbiota of the patients with a short course of treatment with clindamycin in fecal samples was found to have extreme long-term alterations (Jernberg, Lofmark, Edlund, & Jansson, 2007).

Interestingly, no diarrhea, the most common side effect, was reported among the patients, which implied that the shifted microbiota still had basic normal function. Actually, this finding is consistent with another research result of HMPC that there were many different microbiota compositions having the same basic function. However, in some cases the alteration of gut microbiota can cause serious outcomes, such like fatal AAD or other intestinal diseases. A study on a healthy man who was administrated with amoxicillin showed that AAD might appeared when all of clostridium cluster XIVa were wiped out (Young & Schmidt, 2004). Because those classes produce butyrate which is crucial to maintain the healthy flora.

(2) Increase of susceptibility to pathogenic organisms

In healthy human gut, the microbiota is resistant to the colonization of “bad” bacteria or at least control them in limited amount. Researchers found that the increase of susceptibility to pathogenic organisms in the host may also related to the use of antibiotics. Additionally, different antibiotics caused different effects, and microbiota composition is the crucial issue to the susceptibility or prevention to certain subsequent diseases. The evidences and findings of three common pathogens are discussed here.

Clostridium difficile was firstly isolated from healthy infants by Hall and O'Toole in 1935 (Hall & Duffett, 1935). Because it is very difficult to isolate and culture manually, it was named like this. *C. difficile* can excrete toxin which causes damages to intestinal epithelium that leads to colitis and even death in animals. In humans, it can cause AAD or more serious form disease, such as pseudomembranous colitis (Kelly, Pothoulakis, & LaMont, 1994). Although the total incidence of *C. difficile*-associated diarrhea (CDAD) is low, up to 20% patients in epidemic conditions in hospital setting can develop into CDAD. Some more recent studies showed that a new hyper-virulent strain was contributing to the increasing incidence of CDAD (Warny et al., 2005). There are still many uncertain aspects for CDAD. But presently it can be proved that *C. difficile* naturally exists in the gut microbiota in some healthy individuals. However, different surveyed populations were observed to contain various percentages of healthy carriers. For example, some researchers reported 1% carriers in population while some others showed one fifth of individuals carry the pathogen (McFarland, Mulligan, Kwok, & Stamm, 1989). It is also uncertain that whether the patients themselves are carriers before getting CDAD or they infect *C. difficile* after the gut microbiota dysbiosis. Some studies indicated that not the indigenous overgrowth, but later acquisition from outside caused CDAD. Furthermore, the researchers also suggest that the colonization resistance to *C. difficile* is related to some forms of adaptive immunity (Shim, Johnson, Samore, Bliss, & Gerding, 1998). This suggestion was demonstrated in some murine experiments. In a mice model of CDAD, vancomycin was used to treat the infected mice. The results showed that in fact the initial colonization of *C. difficile* might help the host to resist the progress of CDAD by the adaptive immunity. Therefore, it is clearer that the initial colonization of *C. difficile* is not the causative factor for CDAD, but the GIT dysbiosis (usually caused by antibiotic treatment) is. Actually, the *C. difficile* infection model can only be established in germ-free animals or massive antibiotic treated animals (X. Chen et al., 2008; Chung et al., 2012).

Salmonellosis typhimurium susceptibility can also be increased by antibiotics. This pathogen can cause fever, diarrhea and abdominal cramps. Although most patients can recover within several days after infection, many weak populations including young people and elderly people who with weak immune function might be more susceptible to the pathogen. In addition, those patients with antibiotic treatment are also more sensitive than those without antibiotic treatment. One early study in 1964 suggested that even a single dosage of streptomycin can enlarge the susceptibility to salmonellae by more than 100,000 times. other early studies also showed that many antibiotics can aggravate the situation of a patient with salmonellosis infection (Bornside & Cohn, 1965). A more recent study discovered that streptomycin and vancomycin caused certain changes to GIT microbiota which make them more sensitive to S. Typhimurium, and this correlation is dose-dependent (Sekirov et al., 2008). Subsequently, researchers investigated the microbiota changes on a certain type of mice that were less sensitive to S. Typhimurium and observed the outcomes of different diseases and pathology. They found that only streptomycin or vancomycin can cause enterocolitis, while metronidazole had no such effects. Ferreira found that *Bacteroidetes* and *Porphyromonadaceae* were related to the colitis resistance (R. B. Ferreira et al., 2011). Beyond this, another study proved that antibiotic can also lead to intestinal metabolism change as well as the microbiota alteration. For example, streptomycin was found to reduce steroid and eicosanoid hormones which are usually increased after the infection of S. Typhimurium (R. B. Ferreira et al., 2011). The studies on the metabolite alterations will reveal the behind mechanism of the influence of the changed GIT microbiota and are expected to be conducted more.

Citrobacter rodentium is a common pathogen naturally colonized in mice, which is usually used as a model for attaching and effacing pathogens (pathogenic *E. coli*). A high dosage of streptomycin can induce the colonization resistance breaking and finally results in ten to fifty times of *C. rodentium* colonization (Bergstrom et al., 2010). Another antibiotic

metronidazole can increase the attachment of the *C. rodentium* to intestinal epithelium and exacerbate the severity of colitis induced by *C. rodentium*. The increase of the pathogen was corresponding to the reduction of *Porphyromonadaceae* and an increase of lactobacilli. In addition, Muc2, an important component of mucous layer, was also under-expressed which leads to the thinner mucin layer (Wlodarska et al., 2011).

(3) Other diseases caused by microbiota dysbiosis

GIT dysbiosis can be induced by antibiotic-associated treatment, environmental infections or other genetic factors, is demonstrated to connect with various diseases, including IBD, type 1 diabetes, obesity, rheumatoid arthritis, asthma and atopy. Some studies were summarized in Table 3-2. Two most obvious characteristics of the microbial dysbiosis are the significant decrease of bacterial diversity and the change of the ratio of *Firmicutes* to *Bacteroides*, two phyla composed most bacterial members in human intestine. All the epigenetic, environmental and genetic factors contribute to the development of above diseases, especially during the early stages. Because certain bacterial members of microbiota are constantly interacted with the host's immune system (Chung et al., 2012; Rakoff-Nahoum, Paglino, Eslami-Varzaneh, Edberg, & Medzhitov, 2004).

Table 3-2 Dysbiosis of GIT microbiota in the patients with inflammatory bowel disease, type 1 diabetes, obesity, rheumatoid arthritis, atopy and asthma.

Evidence of dysbiosis	Identified microorganisms
IBD, CD and UC	
1. GIT microbiota in patients with CD and UC are disturbed (Frank et al., 2007). 2. Certain species of bacteria are connected to diseases in genetically susceptible models (Sydora, Tavernini, Doyle, & Fedorak, 2005).	1. Higher recurrence risk associated with overall reduction in *Faecalibacterium prausnitzii* and *Firmicutes.* 2. 46 clostridial strains prevent colitis in mice. 3. Numbers of *Enterococcus faecalis* and *Enterobacteria* increased in CD patients. 4. *Bifidobacterium* strains decreased in CD patients. 5. *Bacteroides fragilis* prevents colitis in animals.
Type 1 diabetes	
1. Both the ratio of *Firmicutes* : Bacteroidetes and the bacterial diversity reduced in T1D children (Giongo et al., 2011). 2. Gut microbiota changed prior to the disease (Brugman et al., 2006). 3. Sulfatrim prevented the virus induced T1D rats (Hara et al., 2012)	1. Number of *Bacteroides* increased in T1D patients. 2. *Clostridiales* order decreased. 3. Lower levels of *Bifidobacterium*, *Bacteroides* and *Lactobacillus* in BBDP rats.
Obesity	
1. The ratio of *Firmicutes*:*Bacteroidetes* increased in both obese individuals and mice (Ley et al., 2005). 2. Microbiota of obese mice induced obesity to normal mice (Turnbaugh, Backhed, Fulton, & Gordon, 2008). 3. Antibiotics including penicillin, macrolides, glycopeptide and tetracycline caused weight gain in animals (Keeney et al., 2014a).	1. Lower level of *Bacteroidetes* in obese population. 2. Higher level of *Metbanobrevibacter smitbii.* 3. Higher level of *Lactobacillus.* 4. Higher level of *Faecalibacterium prausnitzii* in obese children

Evidence of dysbiosis	Identified microorganisms
4. Antibiotic administration before 6 months of age related to the weight gain in 1-3 years of age in children (Turta & Rautava, 2016). 5. Vancomycin induced weight gain in patients with endocarditis (Thuny et al., 2010). 6. Azithromycin induced weight gain in patients (6-18 years of age) treated for cystic fibrosis (Saiman et al., 2003). 7. Clarithromycin caused weight gain in adult patients treated for *Helicobacter pylori* infection (Lane et al., 2011).	
Rheumatoid arthritis	
1. Reduced GIT bacterial diversity was observed (Scher et al., 2013). 2. Oral microbiota changed (Martinez-Martinez et al., 2009). 3. Exposure of beta-glucans induced RA-like disease in SPF (Ruutu et al., 2012).	1. Higher level of *Prevotella* spp. in recently diagnosed patients. 2. Reduced *Clostridia*, *Lacbnospiraceae* and *Bacteroides.* 3. Reduced number of bifidobacteria and *Bacteroides fragilis.* 4. Higher level of *Bacteroides* spp. and *Porphyromonas gingivalis.*
Asthma and atopy	
1. Differences in GIT microbiota between healthy children and children with atopy (Bjorksten, Sepp, Julge, Voor, & Mikelsaar, 2001). 2. Vancomycin worsened asthma in mice (Russell et al., 2012). 3. A meta-analysis (23 studies included) suggested 20% increased risk of asthma for infants born via caesarean (Thavagnanam, Fleming, Bromley, Shields, & Cardwell, 2008)	1. Reduced *Helicobacter pylori* and *Mycobacterium vaccae* in mice with airway disease. 2. Fewer members of *Bacteroides* spp. and *Bifidobacterium* in infants born via cesarean

For those extra-intestinal diseases including atopy and asthma, it is still unclear how the certain bacteria affect the immune function via the immune response. Some studies in animals provided some crucial clues about this mechanism. As showed in Figure 3-4, some studies also suggested a possible opportunity window during the microbiota shifts, in which some particular time this microbiota shifts are critical for the immune function change and disease progress (Russell et al., 2012). Additionally, some obesity studies also implied that antibiotics are causative agent for the development of obesity. The relation between antibiotics and weight gain had been well know and applied in livestock culturing.

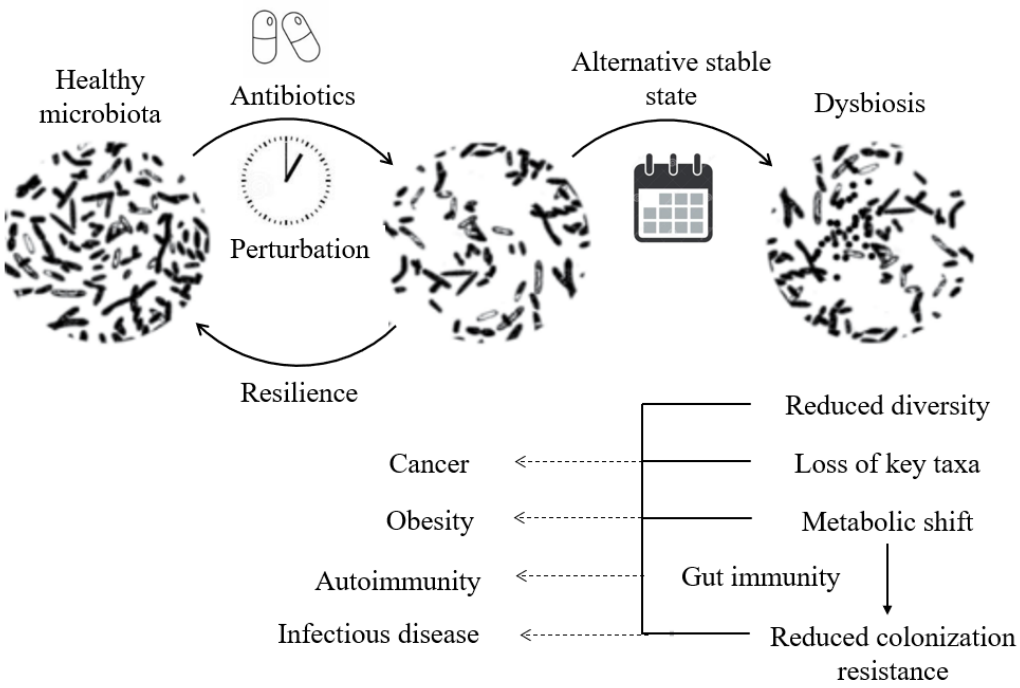

Figure 3-4 The effects of antibiotics on human microbiota and subsequent disease (Keeney et al., 2014a).

3.3 Effects of polysaccharides on gut microbiota

3.3.1 Nutritional functions

Compared with probiotics which have hundreds of years of research history, the research of prebiotics as functional foods is developing quickly just recently (T. S. Chen & Chen, 1989). The prebiotic concept originated from the observation of oligosaccharides, which are fermented selectively by Bifidobacterium. They can induce intestinal flora itself to improve human health without uptake live microorganism (X. Wang & Gibson, 1993). The latest definition of prebiotics is the food component that cannot be digested by upper gastrointestinal system, but will reach the large intestine, and fermented by beneficial microorganisms in the digestive tract (Roberfroid, Van Loo, & Gibson, 1998). Most prebiotics are oligosaccharides and polysaccharides with low or moderate MW, because high MW carbohydrates (such as dietary fiber) cannot be selective fermentation (Crittenden et al., 2001). So far, the mechanism of selective fermentation is not very clear, such as the relation between MW and selectivity. In some cases, when the polysaccharides were degraded into lower MW molecule, the selectivity increased (Olano-Martin, Gibson, & Rastell, 2002). Most of the research are about inulin, fructo-oligosaccharide (FOS) and lactulose etc., because these components are easy to be obtained.

However, in recent years many studies have shown that some high-MW polysaccharides can play a similar role of the prebiotic effect. In a study, 34 bifidobacterial strains from nine species, of which most are derived from human intestine, are used to test whether they are capable to utilize arabinogalactan from different resources, including larch wood, potato, pectic and so on. The results showed larch arabinogalactan cannot stimulate the growth of any strain, while just 11 of the 34 strains tested were able to grow well on potato-derived pectic arabinogalactan. These data indicated that certain human-derived bifidobacteria can use arabinogalactan derived from potato, therefore, this sugar may be a selective growth substrate for certain strains (Motherway, Fitzgerald, & van Sinderen, 2011). Another study was conducted in vivo and in vitro to investigate the possible prebiotic effect of an aqueous extract

of Anoectochilus formosanus (SAEAF) and a non-digestible polysaccharide (AFP) separated from SAEAF. The average MW of AFP was 29 kDa. After four weeks of oral feeding to rats, a series of index including cecum pH, calcium absorption and fecal bifidobacteria amount are determined. The result showed that all those parameters increased, that indicated SAEAF exhibited prebiotic effects.

Furthermore, with a bioactivity-guided separation strategy, AFP was proven to be a bifidogenic component in vitro fermentation by fecal strains and in vivo treatment to mice. The RT-PCR analysis of *Bifidobacterium* presented that AFP was able to increase the expression of certain transporter relating to nutrient uptake. Therefore, AFP was demonstrated to be a prebiotic and were beneficial to improve gut microbiota (Motherway et al., 2011). The similar study researched the polysaccharide from the seeds of *Plantago asiatica L.*. After oral administration to mice for 30 days with a dosage of 0.4 g/kg body weight, researchers found that the apparent absorption of lipid decreased significantly, so did the total triglyceride, cholesterol and several other parameters. In addition, the colon bacterial diversity was higher than the control group. *Bacteroides sp., Eubacterium sp.*, butyrate-producing bacteria *Butyrivibrio sp.*, and probiotics *B. bifidum*, *L. fermentum*, and *L. reuteri* were all increased after polysaccharide intake. These indicated that polysaccharide from *Plantago asiatica L.* has prebiotic effect on colon microbiota (Hu et al., 2014).

Unlike probiotics, prebiotics can completely reach the colon but without the concern of survival rate. This property provides many advantages of prebiotics. In addition, their processing characteristics, such as thickening and sweetness, can make them more suitable for industrial production. Prebiotics have a longer shelf life than probiotics, and they can be added to more kinds of food, such as baby food, weaning food, cereal, candy, cakes, beverages, dairy products and dietary supplements. However, there are also some side effects in the application. Human tests showed that, because prebiotics cannot be digested in upper digestive tract, the

volume of food ingredients and fermentation product increased (Bouhnik et al., 1996), which may leads to higher frequency of defecation and weight. High dose intake of prebiotics (20g/d), such as inulin and lactulose, may cause diarrhea reaction (Bouhnik, Vahedi, Achour, Attar, Salfati, Pochart, & Rambaud, 1999). Therefore, the key of probiotics product development is avoid increasing the number of aerogen. The recommended dosage of commonly used prebiotics was defined. For FOSs, the clinical dosage is 3-20 g/d for adults, 0.4-3.0 g/d for infants (Bouhnik, Vahedi, Achour, Attar, Salfati, Pochart, Marteau, et al., 1999; Moro et al., 2002).

The current probiotics are allowed by the generally recognized safety standards (GRAS). The natural prebiotics and the prebiotics exist in the ordinary foods that have been used for a long time and have been assessed as risk free food so far. But whether they have a long inoculation effect is not clear if terminating the intake of prebiotics. In many feeding experiments, taking prebiotics can significantly change the colonic microbiota of animals, but this reaction gradually stopped as the interrupt of treatment. Therefore, long period of daily intake of prebiotics to achieve the best effect of long-term maintenance is necessary. However, few studies have reported that prebiotics have a long-term effect.

3.3.2 Protective effects

The human intake of antibiotics directly for medical treatment of microbial infections and indirectly from antibiotic contaminated foods has become a public health threat. Besides the spread of antibiotic resistance, another adverse effect of uptake of antibiotics on humans is the disruption of the ecological balance of the gut microbiota. Gut microbiota plays an important role in human health and links with gut disorders and many other diseases. Antibiotics in the gut can cause damage of the beneficial probiotic bacteria. Because some studies suggested that natural polysaccharides originated from edible plants and fungi may

have prebiotic activity, it is possible that they also have protective effect on the prebiotic against antibiotics.

There are some studies on the protective effect of natural polysaccharides extracted from plants, cereals or mushrooms on probiotic strains supplemented in functional foods. Chou reported that polysaccharides extracted from stipe or base of three mushrooms (*Lentinula erodes* stipe, *Flammulina velutipes* base, and *Pleurotus eryngii* base) can increase the survival rate of three probiotic strains in the storage under low temperature at only 0.1% to 0.5% addition. Those polysaccharides can provide synergistic effects with yogurt culture extract to maintain the probiotic strains beyond 10^7 CFU/mL (the concentration required to provide probiotic effects) during cold storage. The similar results were reported by Rosburg, who found that the addition of oat beta-glucan in yogurt can provide the protective effects on two bifidobacteria (B. breve and B. longum) in yogurt when under low-temperature stress. Both the survival rate and the time maintained above functional level of two probiotic strains in the groups with polysaccharide addition are better than the control group (Rosburg & White, 2010).

To provide certain probiotic effect, one critical requirement for probiotic strains is resistance to gastrointestinal acid and digestive juice. Using polysaccharides to enhance the variability of probiotic strains have been studied for a few years. Chou reported that three mushroom polysaccharides extracted from culture waste provide significant protective effects for three probiotic strains in mimic gastrointestinal environment (Chou et al., 2013). Michida also reported that the viability of Lactobacillus plantarum, another strain usually be used as probiotic strain in functional food in very recent years, can be enhanced by cereal extracts under modulated gastrointestinal conditions (Figure 3-5 and 3-6). Interestingly, the author pointed out that using cereal fiber to immobilize the strain can enhance its tolerance (Figure 3-7) to bile and the addition of cereal extracts can provide further protection to bile juice (Michida et al., 2006). This may due to the lower activity of the strains, the less sensitivity to the adverse

environment. Considering this, some polysaccharides with high viscosity that can immobilize in certain level may exhibit protective effects against some unfavourable conditions, such as the antibiotics.

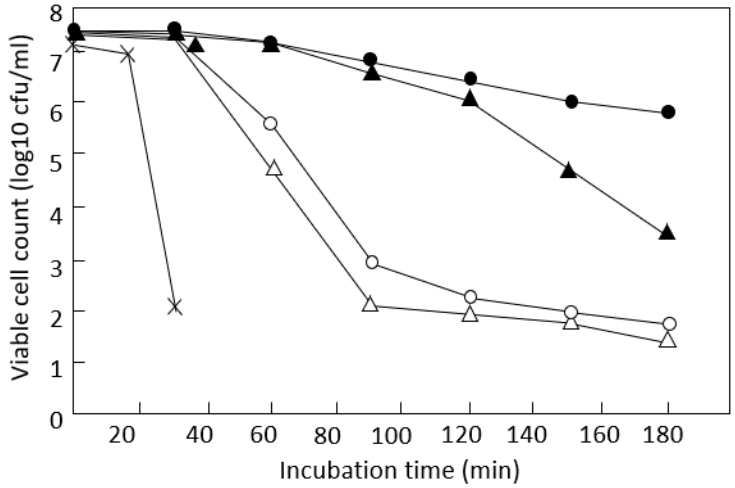

Figure 3-5 Viability of free *Lactobacillus plantarum* cultured in MRS during 180 min incubation in the presence of simulated gastric juice with 1% (circle) or 2% (filled circle) malt and 1% (△) or 2% (filled triangle) barley extract and without cereal extract (×) (Michida et al., 2006).

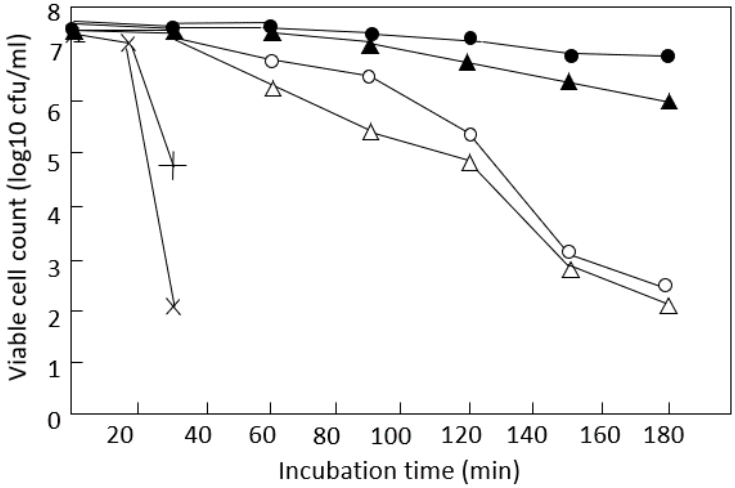

Figure 3-6 Viability of *L. plantarum* immobilized within malt and barley fiber during 180 min incubation in the presence of simulated gastric juice with and without cereal extracts. Cells in malt (×) and barley (+) fiber without cereal extracts; cells in malt fiber with 1% (circle) or 2% (filled circle) malt extract; cells in barley fiber with 1% (△) or 2% (filled triangle) barley extract (Michida et al., 2006).

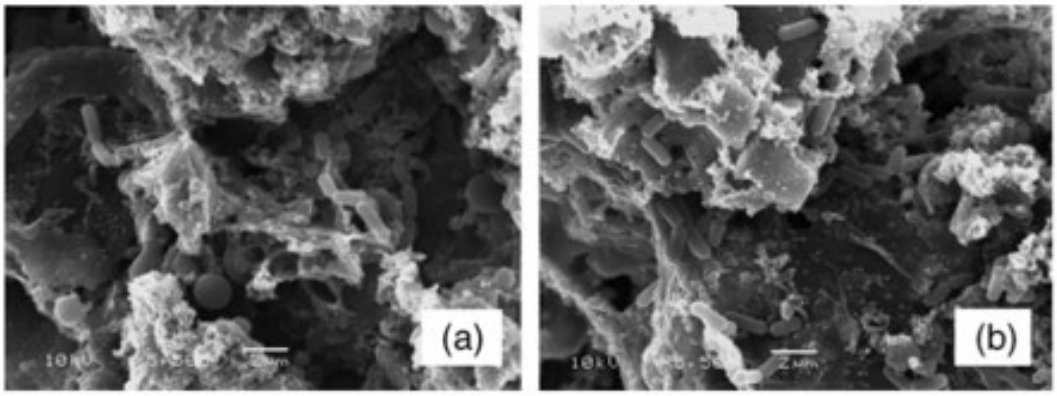

Figure 3-7 Scanning electron micrographs (5500×) of *L. plantarum* immobilized within (a) malt and (b) barley fiber (Michida et al., 2006).

Another study conducted by Parkar showed that monoK pectin, the most effective pectin extracted from kiwifruit, can enhance the adhesion ability of Lactobacillus rhamnosus to Caco-2 (intestinal epithelial cells) more significantly than inulin, the conventional functional polysaccharides. In addition, monoK pectin and inulin can also improve the adhesion ability of Bifidobacterium bifidum. Another type of conventional polysaccharides, guar gum had no such effect on any strain. The author concluded that kiwifruit fiber may be a potentially natural functional ingredient which can be used to deliver probiotics into the target position in body (Parkar et al., 2010).

3.4 Possible solutions for GIT microbiota against antibiotics

The most effective measure for preventing the GIT microbiota from the damage of antibiotics is minimizing the use. However, this is not always possible for antibiotics are most effective for the treatment of serious infectious diseases and we may ingest antibiotics unknowingly from foods. Antibiotics have been used as the feed supplement for decades to increase the weight gain and prevent animal diseases. Although there are regulations and laws restricting the residue maximum limit, some studies showed that there were still counting cases with high residues in the animal products. Therefore, some protective or preventive methods

should be considered to prevent the GIT microbiota destroyed by the therapeutic or non-therapeutic antibiotics. Probiotics and prebiotic products are used in functional foods frequently and are believed to provide health benefits. Some more recent studies also suggested that probiotic strains can provide protective effects to the host by toxic adhesion. Natural polysaccharides are also discussed in this chapter for their potentially prebiotic effect and some other physiological functions related to antibiotic-associated situations.

3.4.1 Probiotic products

Probiotics are generally defined as living organisms that can improve the intestinal microbial balance in host. There are some commonly used probiotics and also some debatable strains, including *Streptococcus faecalis*, *Escherichia coli* and yeast (*Saccharomyces boulardii* and *Saccharomyces cerevisiae*). As living organisms, except for the enterococcus and yeast, the use of probiotics has been confirmed. Probiotic strains traditionally used for the production of fermented milk have been recognized as safety strains (GRAS). People realized that fewer and fewer species can be used as probiotics, so they should be more careful to examine the potential risks in their applications. In theory, these risks are due to the growth of the probiotic bacteria and endogenous intestinal microorganisms might cause the excessive growth. Scientists found that *Bifidobacterium* and Lactobacillus are not closely linked to the endogenous intestinal microbes that are known to people (Jacobsen et al., 1999). The transmission of the *Bifidobacterium* and the *Lactobacillus* is quite rare. Few translocations of *Lactobacillus* caused by drug complications. From worldwide safety reports, researchers found that the death risk caused by *Lactobacillus* and *Bifidobacterium* is negligible (Borriello et al., 2003).

Some studies have shown that the supplements with probiotic strains can resolute the discomfort caused by therapeutic antibiotics. In 1994, Orrhage conducted a clinical double-

blind trial lasted for 21 days on 30 healthy individuals to examine the possibly protective effects of *Bifidobacterium longum* and *Lactobacillus acidophilus* on gut microbiota during the 7-times administration of clindamycin (Orrhage & Nord, 1994). Three groups were settled, with group 1 received a complex supplement of *B. longum* and *L. acidophilus* meanwhile, group 2 received a single strain of *B. longum*, and group 3 received a placebo as control. The results turned out that all groups experienced a reduction of anaerobic microorganisms, but the reduction of *Bacteroides* in group 1 was significantly less than in group 3. No detectable bifidobacteria was observed in group 3 on day 7. Additionally, less individuals in group 3 reported gastrointestinal discomfort comparing to other two groups. Interestingly, the difference of the reduction of total *Bacteroides* in group 2 and group 3 had no statistically significant, which might implicate that the composition of the probiotics more complex, the more possibility they work.

3.4.2 Oligosaccharides and polysaccharides

Compared with probiotics which have hundreds of years of research history, the research of prebiotics as functional foods is developing quickly just recently (T. S. Chen & Chen, 1989). The concept of prebiotic originated from the observation of oligosaccharides that fermented selectively by Bifidobacterium. They can induce intestinal flora to improve human health without live microorganism uptake (X. Wang & Gibson, 1993). The latest definition of prebiotics is the food component which cannot been digested by upper gastrointestinal system, but will be fermented by beneficial microorganisms in the digestive tract after arriving the large intestine (Roberfroid et al., 1998). Most prebiotics are carbohydrates with low or moderate molecular weight, because carbohydrates with high molecular weight (such as dietary fiber) cannot be selectively fermented (Crittenden et al., 2001). So far, the mechanism of selective fermentation is still not very clear, such as the relation between MW and selectivity. In some cases, when the polysaccharides were degraded into molecule with lower molecular weight,

the selectivity increased (Olano-Martin et al., 2002). Most of the studies are upon inulin, fructooligosaccharide (FOS) and lactulose etc., because these components are easy to obtain massively.

Only a few literatures reported the protective effects of prebiotic products and more such studies are expected in the future. Laura P. Johnson studied whether dietary supplements - another method for regulating intestinal composition and function - can be used to counteract the side effects of antibiotics (Johnson et al., 2015). They used gentamycin and ampicillin to disrupt intestinal bacterial flora in in vitro anaerobic cultures. The dietary supplements include pectin (a non-fermentable fiber), inulin (a common prebiotic that promotes the growth of beneficial bacteria), or neither. The results showed that, in most cases, the side effects of antibiotics eliminate the beneficial effects of dietary supplements. However, in some therapeutic combinations, particularly ampicillin and inulin, dietary supplements alleviated the side effects of antibiotics, suggesting that dietary supplements can be used to alleviate adverse effects of antibiotics.

In another study, Beatriz Míguez used oligofructose (FOS) and xylooligosaccharide (XOS) as substrates for in vitro elder fecal fermentation to assess their effects on alleviating the changes of gut microbiota and metabolic activity caused by different doses of three antibiotics (AB) (Miguez, Gomez, Parajo, & Alonso, 2018). The results showed that the type and dose of antibiotics hardly affected the total bacterial count and microbiota composition after 24 hours of fermentation. However, in the presence of ABs, the relative percentage of *Lactobacillus* (from 11.4% to 3.2% in the presence of XOS1) and butyrate production were reduced, while the population of *Bacteroides* was significantly increase (from 27.5% to 55.7%) with the presence of XOS1. FOS counteracted the negative effects of antibiotics by increasing the yield of butyrate and the number of Lactobacillus, while keeping the number of *Bacteroides*

almost constant and reducing Clostridium. XOS2 (mainly DP = 2-4) also increased the percentage of *Bifidobacterium* and the production capacity of butyrate and acetate.

Chapter 4 General materials and methods

This chapter only covers the common materials and methods applied in most parts of the research project. Other materials and methods will be described in the chapter where they are first used.

4.1 Cs-HK1 mycelial fermentation and exopolysaccharides (EPS) production

4.1.1 Cs-HK1 mycelial fermentation

Cs-HK1 is a fungus species which was isolated from a wild *Cordyceps sinensis* fruiting body by Wu's group and has been maintained in mycelial culture as reported previously [16]. As described previously, the liquid medium for Cs-HK1 mycelial fermentation consisted of 40 g/L glucose, 5 g/L peptone, 1 g/L KH2PO4, 0.5 g/L MgSO4·7H2O and 10 g/L yeast extract. The liquid fermentation was carried out in 1 L Erlenmeyer flasks each filled with 200mL of the liquid medium at 20 °C in a shaking incubator at 200 rpm for 7 days. The mycelial fermentation liquid was then centrifuged at 12,000 rpm (21,612 ×g) for 15 min and the supernatant medium was collected for recovery of EPS.

4.1.2 Production of EPS fractions

The exopolysaccharide (EPS) was isolated from the Cs-HK1 liquid fermentation medium by ethanol precipitation (with 80% v/v ethanol). Another two EPS fractions by two-step ethanol precipitation, firstly using 40% (v/v) ethanol to attain EPS-H with a much higher MW and secondly using 80% (v/v) ethanol to obtain EPS-L with a much lower MW than the EPS attained by one step precipitation using 80% v/v ethanol. All the EPS precipitates were recovered from the liquid by centrifugation at 12,000 rpm for 15 min, re-dissolved in a small amount of deionized (DI) water and freeze-dried as the final EPS fractions for experiments.

4.1.3 Ultrasonic treatment of EPS

The EPS-US fraction with a lower MW and high water-solubility was prepared by irradiation of the liquid fermentation medium with high-intensity ultrasound (US) before ethanol precipitation. Power US was generated with a VCX 750 processor with a fixed frequency of 20 kHz and a maximum output power of 750W (Sonics and Materials Inc., Newton, USA). the US power was fixed at 80% amplitude and the treatment period was 30 min. Finally, the EPS-US solution was freeze-dried for later experiments. The production process of different EPS fractions is shown in Figure 4-1.

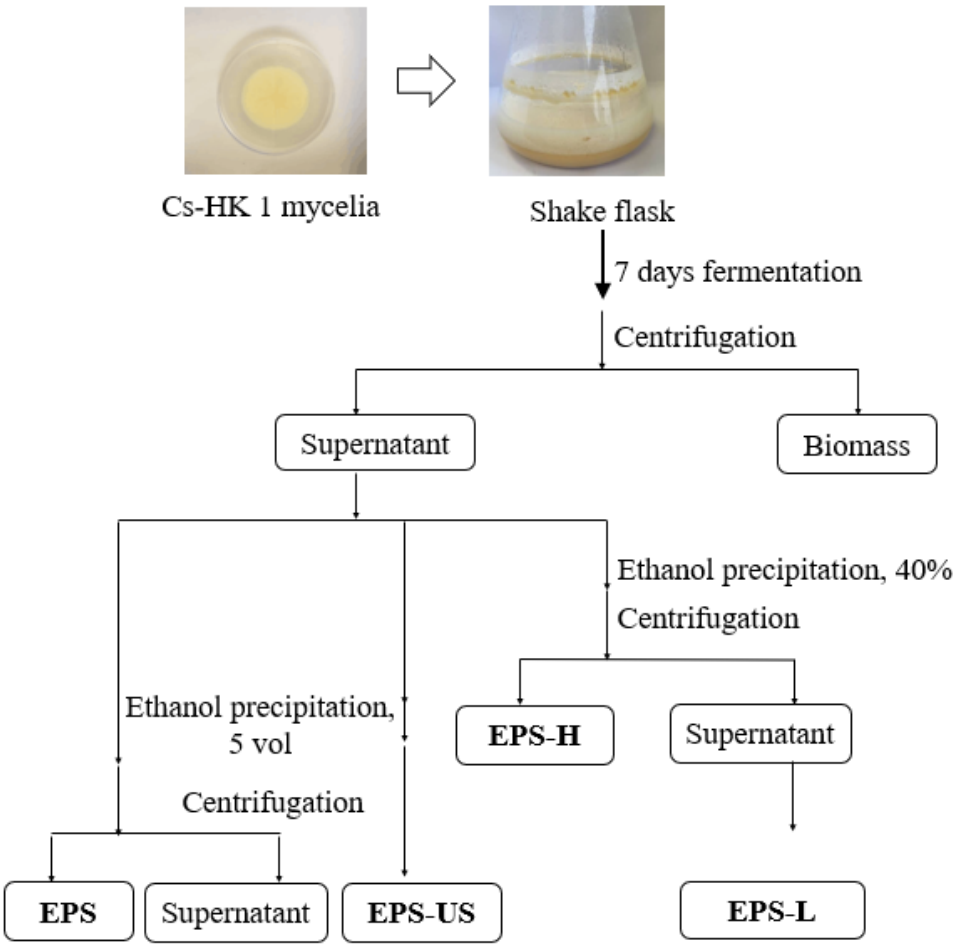

Figure 4-1 Preparation of different EPS fractions.

4.2 Physiochemical analysis of poly- and oligo-saccharides

4.2.1 Molecular weight analysis

Molecular weight (MW) of poly- and oligo-saccharide samples was measured by a high-pressure gel permeation chromatography (HPGPC) instrument equipped with a Waters 1515 isocratic pump and a 2414 refractive index detector (Waters Co, Milford, MA, USA) as described previously (Qi-Lin Huang et al., 2013). A series of three columns was used including Waters Ultrahydrogel 120, 250 and 2000 (7.8 × 300 mm) and the column temperature was 50 °C. The mobile phase was Milli-Q water at a flow rate of 0.6 ml/min. All samples were dissolved in distilled water (0.2 mg/ml for KGM, EPS, EPS-H, 1 mg/ml for KGM-US and EPS-US, and 3 mg/ml for KGM-AH, EPS-L, inulin and GOS) and centrifuged at 6000 rpm for 15 min. The supernatant was collected and filtered through 0.45 μM membrane before the injection. Dextran MW standards 1, 5, 12, 25, 50, 80, 270, 410 and 670 kDa were used to obtain the calibration curve.

4.2.2 Intrinsic viscosity

The intrinsic viscosity of poly- and oligo-saccharides and degraded products was determined as described previously (J. K. Yan et al., 2009). The poly- and oligo-saccharide samples were dissolved with distilled water overnight under constant stirring. The solution was diluted with water in series and filtered through a Watman No. 1 paper and the viscosity was measured with an Ubbelohde viscometer (0.5-0.6 mm capillary diameter) at 25 ± 0.1°C. The intrinsic viscosity [η] was derived from the following equations,

$$\eta_{sp}=(\eta_{sample}-\eta_{ref})/\eta_{ref}=(t_{sample}-t_{ref})/t_{ref} \quad \text{(Eq.1)}$$

$$\eta_{red}=\eta_{sp}/C=[\eta]+k'[\eta]^2C \quad \text{(Eq.2)}$$

where η_{sp} is the specific viscosity and η_{red} the reduced viscosity and η_{ref} the viscosity of reference (distilled water).

Chapter 5 Protective effects of natural and partially degraded konjac glucomannan on Bifidobacteria against antibiotic damage

5.1 Introduction

Konjac glucomannan (KGM) isolated from the tuber of plant *Amorphophallus konjac C*. Koch is commonly used as a gelling and thickening agent in liquid foods and also as an edible film coating of food and pharmaceutical products (Herranz et al., 2012; X. Xu et al., 2007). Recently, KGM has been increasingly used as a dietary fiber in functional foods for improving gut health, lowering blood sugar and cholesterol, the risk of type II diabetes and obesity (Behera & Ray, 2016; Tester & Al-Ghazzewi, 2013, 2016; Y.-Q. Zhang et al., 2005). Native and enzyme-hydrolysed KGM products have been evaluated as prebiotic substrates for the growth of lactobacilli and bifidobacteria (Al-Ghazzewi et al., 2007; Al-Ghazzewi & Tester, 2012; J. Yang et al., 2017) and other probiotic bacteria of human or animal gut microbiota (Connolly et al., 2010; Harmayani et al., 2014). To the best of our knowledge, however, no previous studies have assessed the protective effects of KGM on bifidobacteria or any other probiotic bacteria against antibiotics.

Penicillin, enrofloxacin, tetracycline and streptomycin are among the most widely used antibiotics in veterinary medicine and animal feed (Schwarz, 2001; Sumano, Gutierrez, & Zamora, 2003; Voldrich, 1965). Their antimicrobial actions are based on different mechanisms. Penicillin breaks the bacterial cell walls indirectly by targeting on the peptidoglycans of bacteria, and is more effective against the Gram positive bacteria (Winstanley & Hastings, 1989). Enrofloxacin, an approved veterinary medicine by the USFDA, kills bacteria by targeting on the DNA gyrase (Trouchon & Lefebvre, 2016). Tetracycline and streptomycin

mainly prevent bacterial protein synthesis by inhibiting the combination of aminoacyl tRNA with bacterial ribosome (Chopra & Roberts, 2001; Igarashi, Ishitsuka, & Kaji, 1969).

This study was to evaluate the protective effects of natural and partially hydrolysed KGM on *Bifidobacteria* against the inhibition of antibiotics and to investigate the possible mechanisms. The natural KGM was first treated by high-intensity ultrasound (US) to attain partially degraded KGM with relatively high molecular weights. The US-degraded KGM was further degraded to much lower molecular weight with trifluoroacetic acid (TFA). The potential protective effects of various KGM fractions were assessed on five important bifidobacterial species against four representative antibiotics used in medicine and farming, penicillin, tetracycline, enrofloxacin and streptomycin. Two well-known prebiotic carbohydrates, inulin and galactooligosaccharide (GOS) were used as references and tested together with KGM fractions. The possible formation of biofilms on solid surfaces and the absorption of antibiotics to KGM were analysed.

5.2 Material and methods

5.2.1 Bacterial strains and culture conditions

Five strains of *Bifidobacterium* were used in the present study (Table 5-1), which were generously donated by Biostime Ltd. The bacterial strains were stored in 15% (v/v) glycerol tubes at -80 °C. The bacteria were cultured in Reinforced Clostridium Medium (RCM) (Guangdong Huankai Bio-Technology Co., Ltd., Guangzhou, China). The RCM medium was composed of 5 g/l glucose, 10 g/l beef extract, 10 g/l peptone, 3 g/l yeast extract, 1 g/l soluble starch, 0.5 g/l cysteine HCl, 5 g/l sodium chloride, 3 g/l sodium acetate and 0.5 g/l agar for RCM broth or 15 g/l for RCM agar with a final pH of 6.8 ± 0.2 (unadjusted). The culture media were sterilized at 121 °C for 20 min. Prior to the culture experiments, the bacterial strains taken from the storage were cultured on RCM agar solid medium for 48 h. A single colony spot was

picked out from the solid culture and inoculated into 5 ml of RCM broth liquid medium in a 10 ml centrifuge tube, followed by shaking incubation at 200 rpm for 24 h. The final bacterial suspension was inoculated at 1% (v/v) into the RCM broth under the same conditions as for the culture experiments. The closure of the centrifuge tube was punctured to ascertain anaerobic atmosphere in the ullage of the tube. All the bacterial cultures were maintained at 37 ^{o}C under anaerobic condition in air-tight jars with anaerobic gas generating sachets (AnaeroGen TM, Thermo Scientific Oxoid, USA) or (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) (Sabine A Tanner et al., 2014).

Table 5-1 Five strains of *Bifidobacterium* used in this study.

Microorganism	Strain code	Origin
B. adolescentis	CICC[a] 6070	Intestine of adult
B.bifidum	CICC 6071	Infant feces
B. breve	CICC 6079	Intestine of infant
B.infantis	CICC 6069	Intestine of infant
B.longum	CICC 6186	Intestine of adult

[a] CICC: China Center of Industrial Culture Collection (Beijing, China)

5.2.2 Preparation of ultrasound- and acid-degraded KGM

Konjac glucomannan (KGM) was provided by Hubei Konson Konjac Gum Co., Ltd. (Ezhou, Hubei, China). KGM was dissolved in distilled water at 10 g/l and 150 ml of the KGM solution was added to a centrifugal bottle for ultrasonic degradation. Ultrasonic degradation of KGM was carried out as described previously (Li, Li, Geng, Song, & Wu, 2017) with a VCX 750 processor (Sonics and Materials Inc., Newton, USA) with a fixed frequency of 20 kHz and at a maximum output power of 750 W. A probe horn with a tip diameter of 13 mm was used and the sample was irradiated at a fixed power level of 80% amplitude for 30 min, yielding US-degraded fraction KGM-US. The KGM-US (0.15 g) was treated with 60 ml of 2 M

trifluoroacetic acid (TFA) at 70 °C for 4 h, yielding acid-hydrolyzed KGM fraction KGM-AH. After the US and acid treatment, the KGM solutions were evaporated to dryness with a rotary evaporator under vacuum at 40 °C and then washed by methanol. Finally, the degraded KGM samples were re-dissolved in 10 ml DI water and freeze dried and stored in a desiccator at room temperature before use.

5.2.3 Preparation of poly- and oligo-saccharide solutions for bacterial cultures

For investigation of the effects of KGM and its degraded products on the antibiotic-treated bifidobacteria, the KGM samples were added to the bacterial medium RCM at three different concentrations (0.5, 2 and 5 g/l). KGM and other poly- and oligo-saccharides were all dissolved in distilled water at the desired final concentrations by stirring for overnight, and then 38 g/l of RCM powder was mixed with each sample solution. The RCM medium containing the KGM and other poly- and oligo-saccharides was sterilized by autoclaving at 121°C for 20 min.

Putative prebiotic carbohydrate fibers were tested as references for comparison with the KGM fractions including galactooligosaccharides (GOS) and inulin. GOS with purity of 80% was obtained from New Francisco Biotechnology Co., Ltd. (Yunfu, China) and inulin (from dahlia tubers, DP ≈ 36) from Sigma (St. Louis, MO, USA). The solutions were prepared in the same way as for KGM.

According to the guidelines from the Institute of Medicine, American Heart Association and Chinese Nutrition Society, the recommended intake of dietary fiber for an adult is 25 g to 38 g/day or 14 g/1,000 kcal/day while the mean intake was slightly more than 15 g/day (King, Mainous, & Lambourne, 2012). If an adult takes 10 g of carbohydrate fibers per day as dietary fiber supplement, the concentration is about 5 g/l in a total intestinal volume of 2 l (Parkar et

al., 2010). Therefore, the concentration of 5 g/l was chosen in the experiments for evaluating the protective effects of KGM and other carbohydrate fibers.

5.2.4 Preparation of antibiotic solutions

Four of the most common antibiotics used in human and animal care were chosen for this study. Enrofloxacin and streptomycin sulfate are two antibiotics commonly applied in livestock husbandry and fishery, while penicillin G and tetracycline hydrochloride are widely used in human and animal medicine. The four were all purchased from Guangzhou XiangBo Bio-Technology Co., Ltd. (Guangdong, China). Antibiotic solutions were freshly prepared in the culture medium at a concentration of 2.5 mg/ml.

5.2.5 Determination of minimum inhibitory and bactericidal concentrations

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were used to represent the sensitivity of bifidobacteria to antibiotics. MIC was defined as the lowest concentration required for complete inhibition of the bacterial growth (Karakoc & Gerceker, 2001), and MBC as the lowest concentration for killing 99.9% of the initial inoculum (Standards, 1991). MIC and MBC were determined by microtiter plate assays (Cleusix, Lacroix, Vollenweider, Duboux, & Le Blay, 2007). The antibiotics were dissolved in distilled water at 2.048 mg/ml. A serial two-fold dilution of the antibiotic solution was prepared, and the diluted solution was transferred at 100 µl aliquots into a 96-well polystyrene microtiter plate (SPL Lifesciences Inc., Pocheon, Korea) containing 100 µl of RCM broth per well. The bifidobacteria were cultured to mid-log phase (16-18 h) in RCM broth as described above. The optical density (OD) of bacterial suspension was adjusted to 0.1 with fresh RCM broth using a Ledetect microtiter plate reader (Labexim, Lengau, Austria) at 600 nm (Mota-Meira, LaPointe, Lacroix, & Lavoie, 2000). Then the standardized bacterial suspension was

inoculated at 100 µl into each well, the microtiter plates were incubated anaerobically at 37°C for 48 h and the OD_{600} was recorded. A control inoculated with the tested culture in RCM and a blank containing only RCM were included on each microtiter plate. The first well with OD value equal to the control was taken as MIC. For the MBC assay, 20 µl was withdrawn from the first well showing no visible growth on the RCM agar and the lowest concentration with no colony appearing on the RCM agar was taken as MBC (Cleusix et al., 2007). The microtiter plate assay was performed in four replicates for each antibiotic–bacterium combination and the median MIC or MBC values were recorded as the result.

5.2.6 Test of KGM on bifidobacterial growth

For examination of their effects on the growth of bifidobacteria, KGM, degraded KGM and prebiotic references were added to the RCM medium at 5 g/l final concentration, and then subjected to serial 2-fold dilution from 5 to 0.0782 g/l. The liquid medium was dispended into a 96-well microtiter plate at 200 µl per well, followed by inoculation of the *Bifidobacteria* (4×10^5 colony forming units in total volume) and incubation for 48 h at 37 °C in anaerobic atmosphere. RCM inoculated with bacteria was included as the control and RCM with KGM and prebiotic but no bacteria as the blank. The bacterial concentration was determined by measurement of OD at 600 nm and the treatment effect was represented by $(OD_{test} - OD_{blank})/OD_{control}\times100\%$.

5.2.7 Detection of biofilm formation of *Bifidobacteria*

The formation of biofilm as a possible mechanism for the protective effect of KGM against antibiotic damage was detected by modified methods from literature (Stepanovic, Vukovic, Dakic, Savic, & Svabic-Vlahovic, 2000) on bacterial adhesion to surfaces in culture tubes and microplates. In the tube test, a bacterial strain cultured on RCM agar plates was

inoculated into glass tubes (13×100 mm) filled with 2.6ml of RCM broth containing 5 g/l KGM or KGM-US. The broth was mixed by pipetting gently and repeatedly and 0.6 ml of the broth was removed from each tube for the microtiter plate test. The tubes containing RCM supplemented with or without KGM and KGM-US (5 g/l) were included in the test as negative control. After incubation anaerobically at 37 °C for 48 h, the liquid was removed from the tubes with a pipette, followed by addition of 2 ml of 0.25% safranin solution into each tube for staining. After removal of the liquid with a pipette, the tubes were placed upside down at room temperature overnight. The amount of bacterial adhered on the inner tube wall was compared by visible observation and recorded as absent (0), weak (+), moderate (++), or strong (+++).

In the microtiter plate test, 200 µl of bacterial suspension from above tube test was filled in each of three wells of a 96-well microtiter plate (for suspension culture) and the wells filled with 200 µl of RCM but no polysaccharides were included as the control. The covered plates were incubated anaerobically at 37°C for 48 h. After removal of the liquid content, each well was washed three times with 250 µl of sterilized physiological saline. The plates were shaken vigorously to remove all planktonic bacteria. Then 200 µl of 99% methanol was added into each well to fix the attached bacteria. After 15 min, the plates were emptied and dried with a hair drier. Then, each well was stained with 200 µl of 2% crystal violet solution for 5 min and rinsed off the excess stain with running tap water. After drying the plates with a hair drier, 160 µl of 33% (v/v) glacial acetic acid was added to each well to re-dissolve the dye bound to the adherent bacteria. Finally, OD was recorded with an automated microtiter plate reader at 570 nm. Based on the OD values of bacterial films, the results of the microtiter plate test were classified into four categories, non-adherent ($OD \leq OD_C$), weakly adherent ($OD_C < OD \leq 2 \times OD_C$), moderately adherent ($2 \times OD_C < OD \leq 4 \times OD_C$) and strongly adherent ($OD > 4 \times OD_C$), where OD_C is the cut-off OD equal to three times of standard deviation (SD) over the mean OD of the negative control.

5.2.8 Determination of antibiotic adsorption to KGM

The antibiotic adsorption of KGM and degraded products was determined by a self-devised method. The KGM samples were dissolved at 5 g/l with Milli-Q water under constant stirring overnight. Each of the antibiotics was dissolved completely at 1 mg/ml to the KGM solution with vigorous agitation. The solution (7 ml) was transferred into a dialysis tubing (MWCO 3.5 kDa, Spectrum Laboratories, USA) and placed into a beaker, which contained 28 ml Millie-Q water agitated constantly with a magnetic stirrer at room temperature. After dialysis for 24 h, antibiotic concentration in the dialyzing water was analyzed by high performance liquid chromatography (HPLC). The HPLC system consisted of an Agilent 1100 series equipped with a UV-VIS detector and an auto-sampling equipment (Agilent 1200 Series) and a C_{18} analytical column (250 mm×4.6 mm×5 μm, Alltech, USA).

Penicillin analysis was according to a reported method (Benito-Peña, Partal-Rodera, León-González, & Moreno-Bondi, 2006) with minor modifications. The standard solution was prepared by dissolving 122.8 mg penicillin G standard (Sigma, St. Louis, Mo, USA) in 0.9% NaCl solution in a 10 ml volumetric flask and was serial diluted with 0.9% NaCl solution. The HPLC mobile phase consisted of 0.02 mol/l NaH_2PO_4 (38%) and Methanol (62%) (pH of NaH_2PO_4 solution adjusted to 3.1 with phosphoric acid), flowing at 1.0 ml/min. The sample injection volume was 10 μl, column temperature 25°C and UV detection at 242 nm.

Tetracycline analysis was performed based on a reported procedure (Shariati, Yamini, & Esrafili, 2009) with modifications. Tetracycline standard (Sigma, St. Louis, Mo, USA) was dissolved in Millie-Q water (100 mg in 10 ml) in a volumetric flask and the solution was diluted in a series. The HPLC mobile phase consisted of 0.02 mol/l NaH_2PO_4 : Methanol (47% : 53%), flowing at 1.2 ml/min. The sample injection volume was 10 μl, column temperature 25 °C and US detection at 270 nm.

5.2.9 Statistical analysis

MIC and MBC assays were conducted with four replicates. *Kruskal-Wallis* test and *Nemenyi* test were used for the MIC and MBC data analysis and the median was taken for the results. Other experiments were performed in triplicate and the results were averaged. Student's *t* test was applied for the comparison of OD values and antibiotic concentrations. The data analysis was performed using SPSS 23.0 program.

5.3 Results and discussion

5.3.1 Intrinsic viscosity and MW distribution of KGM and degraded products

Figure 5-1 and Table 5-2 present the intrinsic viscosity and MW distribution results of KGM and partially degraded KGM as well as GOS and inulin used in the experiments (GPC profiles for MW in supplemental data). The intrinsic viscosity of KGM was significantly lower after the US treatment, and the major MW peaks showed a general shift from high to low MW and the percentage (relative peak area) of high MW components decreased. The acid-hydrolyzed KGM product KGM-AH was relatively homogenous with a single low MW peak at 1369 Da.

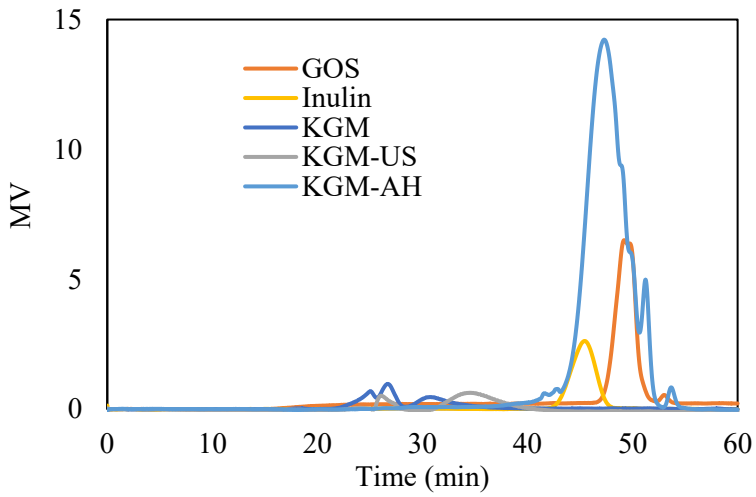

Figure 5-1 GPC profiles (molecular weight distributions) of KGM and degraded products.

Table 5-2 The intrinsic viscosity and molecular weight of GOS, inulin, KGM and partially degraded KGM (GPC profiles in Supplemental data).

Sample	Intrinsic viscosity (dL/g)	MW (Da)	% Area
KGM	1.2457	1.679×10^{8}	29.62
		7.066×10^{7}	33.95
KGM-US	0.5392	1.169×10^{8}	22.46
		1.301×10^{6}	77.54
KGM-AH	ND	1369	98.89
GOS	ND	530	97.91
Inulin	ND	3463	~100

5.3.2 Effects of KGM and degraded products on sensitivity of bifidobacteria to antibiotics

Table 5-3 shows the results of MIC and MBC tests of antibiotics on five bifidobacterial strains cultivated in the RCM culture medium supplemented with various poly- and oligosaccharides. The MIC and MBC values of a given antibiotic varied with the bacterial strains. Except for a few cases, GOS and inulin (at 5 g/l) had very small effects on the MIC and MBC values of four antibiotics compared with those of the control. For most bifidobacterial strains in the control, penicillin was the most potent with the lowest MIC values (all $\leq$ 1 μg/ml) and MBC values (all $\leq$ 16 μg/ml). The MIC value of penicillin was increased most dramatically by KGM-US at 5 g/l to > 512 μg/ml for all five bifidobacterial strains. On the other hand, the native KGM significantly increased the MBC value of penicillin for all strains.

The MIC value of enrofloxacin varied in a much wider range than penicillin from 1 to 128 μg/ml with the bifidobacterial strains. In comparison, *B. bifidum* and *B. breve* were less sensitive to enrofloxacin with higher MIC values (64-128 μg/ml) than other strains. KGM-US at 5 g/l increased the MIC of *B. adolescentis* (64-128 μg/ml versus 1-2 μg/ml for the control) and *B. bifidum* (512 μg/ml versus 64-128 μg/ml for the control).

The MIC values of tetracycline for the bifidobacterial strains varied from below 1 μg/ml to 32 μg/ml. *B. adolescentis* was the most sensitive to both enrofloxacin and tetracycline with the lowest MIC values of 1-2 μg/ml compared with those for other bacterial strains. KGM (at 5 g/l) decreased the MIC but increased the MBC significantly to ≥ 512 μg/ml for most of the bifidobacterial strains.

All five bifidobacterial strains were relatively resistant to streptomycin with high MIC and MBC values. The phenomenon is consistent with that in a previous study (Kheadr, Bernoussi, Lacroix, & Fliss, 2004). Streptomycin inhibited the bifidobacteria at high concentrations from 16 to >512 μg/ml. *B. infantis* was most resistant to streptomycin with high MIC and MBC values > 512 μg/ml. Nevertheless, KGM and KGM-US (5 g/l) increased the MIC and MBC values for other four strains by 2-16 folds in most cases.

Tables 5-3 Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) in μg/ml of antibiotics on five strains of *Bifidobacterium* in RCM supplemented with native, and degraded KGM, GOS and insulin (all at 5 g/L if not specified otherwise)

OS/PS (5 g/l or specified)	Enrofloxacin		Penicllin		Tetracycline		Streptomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. adolescentis								
Control (none)	1-2	2	<1	<1	<1	32-128	256	256-512
GOS	2	2	<1	<1	1	8	128	128
Inulin	2	1-2	<1	<1	1	8	128	128
KGM	<1	>512	<1	>512	<1	>512	>512	>512
KGM-US	64-128	>512	>512	>512	1-2	>512	>512	>512
KGM-AH	16	ND	<1	ND	<1	ND	256-512	ND
KGM-US (0.5 g/l)	1	16	<1	1	<1	128	128	256
KGM-US (2 g/l)	1	64	4-8	32	<1	64	128	>512
B. bifidum								
Control (none)	64-128	32	<1	8	8	128	128	128
GOS	128-256	512	<1	1	16-32	32	8	16

OS/PS (5 g/l or specified)	Enrofloxacin		Penicllin		Tetracycline		Streptomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Inulin	128-256	512	<1	1	32	64	32	16-32
KGM	8	>512	<1	>512	<1	>512	16	512
KGM-US	512	>512	>512	>512	2	128	256-512	>512
KGM-AH	256	ND	<1	ND	8	ND	128	ND
KGM-US (0.5 g/l)	32	32-64	1	1-4	2	128	16	32
KGM-US (2 g/l)	128	32-64	4	32	1	64	32	64
B. breve								
Control (none)	64	512	1	8	8	4-16	16	16-32
GOS	16-32	128	<1	4	1	4	16	16-32
Inulin	16	128	<1	16	1	4	32-64	32-64
KGM	<1	256	1	512	<1	512	2	64
KGM-US	64	>512	>512	>512	64-128	64-128	128-256	>512
KGM-AH	64	ND	2	ND	8	ND	16-32	ND
KGM-US (0.5 g/l)	8	32	≤ 1	8	<1	8	8	16
KGM-US (2 g/l)	2-8	32-64	1-2	64	2	16	16	32
B. infantis								
Control (none)	4-8	32	<1	8-16	32	16	>512	>512
GOS	8	8-16	<1	<1	32	64	>512	>512
Inulin	4	8-16	<1	<1	16-32	64	>512	>512
KGM	4-8	256	<1	512	8	512	>512	>512
KGM-US	8	>512	>512	>512	8	32-64	>512	>512
KGM-AH	32	ND	<1	ND	16	ND	>512	ND
KGM-US (0.5 g/l)	8-32	64	<1	2-4	8	32	>512	>512
KGM-US (2 g/l)	8-32	64	4-16	32	8	32	>512	>512
B. longum								
Control (none)	4	4-16	<1	8-16	1-2	32-128	64	32-512
GOS	8	8-16	<1	2-4	2-4	8	32	32
Inulin	8	8	<1	2-4	4	8	128	256-512
KGM	4	>512	<1	>512	<1	>512	>512	>512
KGM-US	4	>512	>512	>512	2	16	>512	>512
KGM-AH	8	ND	<1	ND	1-2	ND	128	ND
KGM-US (0.5 g/l)	8	64-128	<1	8	1-2	64	512	>512
KGM-US (2 g/l)	4	32-64	4	16-32	1-2	64	512	>512

Note: Each data point is the median of four replicates. ND: Not determined.

Table 5-4 shows the relative protective effects of native and partially degraded KGM by comparison of the MIC and MBC values in Table 3. KGM-US was the most effective, followed by the native KGM, in protecting the bifidobacteria against the inhibition of penicillin and streptomycin. KGM-US at 5 g/l increased both the MIC and MBC of penicillin for all five bacterial strains by more than 64-fold, while KGM at 5 g/l increased the MBC of penicillin by more than 8-fold for *B. infantis* and by more than 64-fold for other four strains. Except for *B. infantis*, KGM or KGM-US also increased the MIC and MBC of streptomycin by 7-fold. However, KGM and KGM-US showed little effect on the MIC value of enrofloxacin and tetracycline but significant effect for enhancing the MBC of the two antibiotics for most bifidobacterial strains.

Table 5-4 The protective effects of native, and degraded KGM, GOS and insulin (all at 5 g/L if not specified otherwise) bifidobacteria against antibiotic inhibition based on results of MIC and MBC assays presented in Table 3.

OS/PS (5 g/l or specified)	Enrofloxacin		Penicllin		Tetracycline		Streptomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. adolescentis								
KGM	-	+++	-	+++	-	++	+	+
KGM-US	++	+++	+++	+++	+	++	+	+
KGM-AH	++	ND	-	ND	-	ND	+	ND
KGM-US (0.5 g/l)	-	+	-	+	-	-	-	-
KGM-US (2 g/l)	-	++	+	++	-	-	-	+
B. bifidum								
KGM	-	++	-	+++	-	+	-	+
KGM-US	+	++	+++	+++	-	-	+	+
KGM-AH	+	ND	-	ND	-	ND	+	ND
KGM-US (0.5 g/l)	-	+	+	-	-	-	-	-
KGM-US (2 g/l)	-	+	+	+	-	-	-	-
B. breve								
KGM	-	+	-	+++	-	+++	-	+
KGM-US	-	+	+++	+++	++	++	++	++
KGM-AH	-	ND	+	ND	-	ND	+	ND
B. infantis								
KGM	-	++	-	++	-	++	-	-
KGM-US	-	++	+++	+++	-	+	-	-
KGM-AH	+	ND	-	ND	-	ND	-	ND
B. longum								
KGM	-	+++	-	+++	-	++	++	+
KGM-US	-	+++	+++	+++	-	-	++	+
KGM-AH	+	ND	-	ND	-	ND	+	ND

Note: -: no positive effect; +: value ≤ 8 times of control; ++: 8 < value ≤ 64 times of control; +++: value > 64 times of control; ND: not determined.

Since KGM-US showed the most consistent and notable protective effects, it was also tested at two lower concentrations, 0.5 and 2 g/l for *B. adolescentis* and *B. bifidum*. The MIC and MBC values usually increased with the concentration increase from 0.5 to 5 g/l, indicating a dose-dependent effect.

5.3.3 Effects of KGM, GOS and inulin on bifidobacterial growth

As shown in Fig. 5-2, the effects of KGM, GOS and inulin on the bifidobacterial growth varied with the bacterial strains. The prebiotic reference GOS improved the growth of four bacterial strains, *B. adolescentis* (Fig. 5-2A), *B. breve* (Fig. 5-2C), *B. infantis* (Fig. 5-2D) and *B. longum* (Fig. 1E) at relative high concentrations. Inulin had a marginal effect, either positive or negative, on most bacteria strains; KGM showed a slightly negative effect on most bacterial strains. The acid hydrolysed KGM (KGM-AH) only improved the growth of *B. bifidum* and *B. breve* (Fig. 5-2B-C). All the poly- and oligo-saccharides had little effect on *B. infantis* and *B. longum* (Fig. 5-2D-E). In summary, KGM and its degraded products had very small influence on the growth of most bifidobacterial strains. Although inulin is widely recognized as a prebiotic carbohydrate rich of fructooligosaccharides (FOS), it did not support the bifidobacterial growth. Similarly, in a previous study, only eight out of the 55 *Bifidobacterium* strains could utilize inulin as carbon source for growth (Rossi et al., 2005). Yang et al. (J. Yang et al., 2017) also reported that original KGM could not or only slightly stimulate the growth of *Lactobacilli* and *Bifidobacteria*.

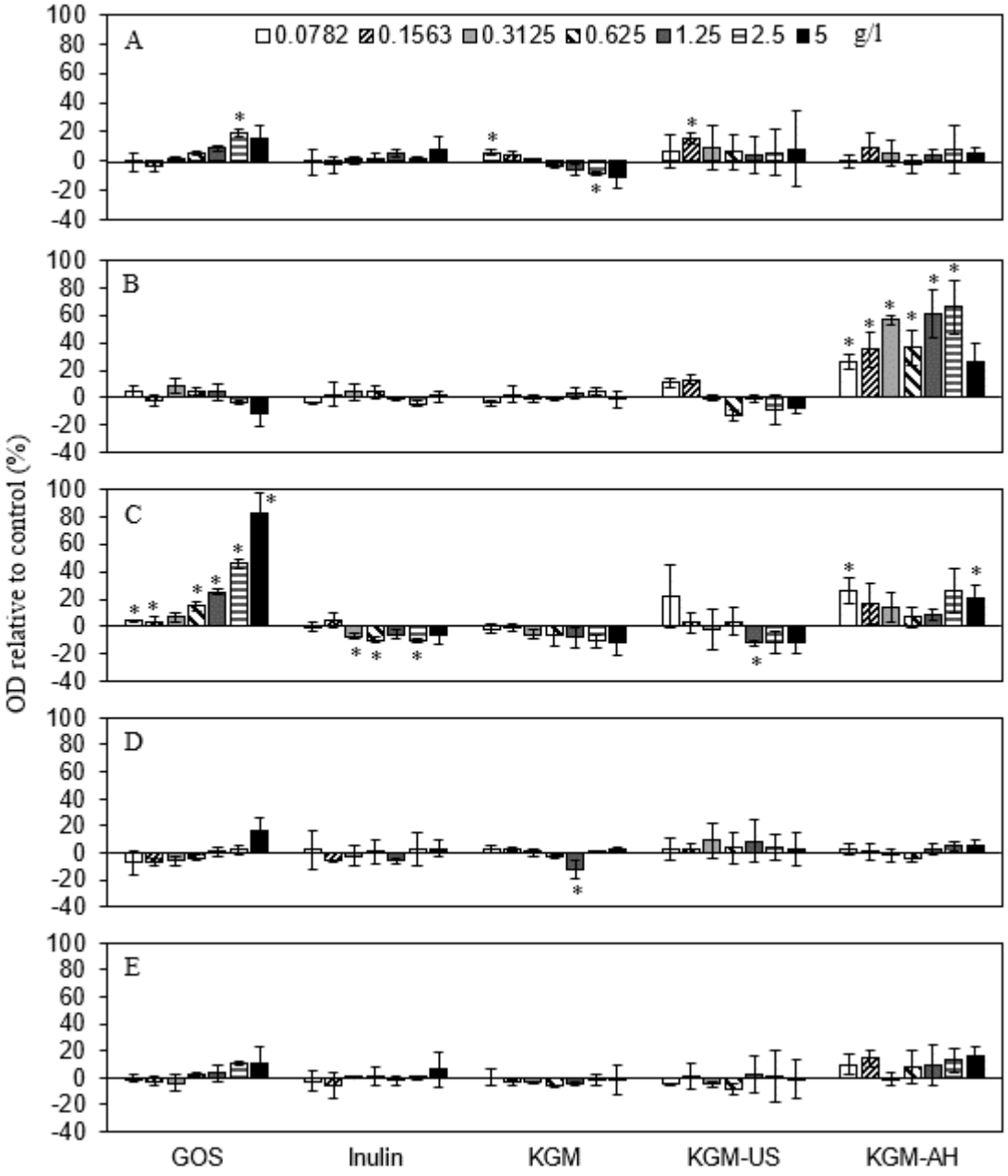

Figure 5-2 Effects of GOS, inulin, native KGM and partially degraded KGM on growth of five bifidobacterial strains, *B.adolescentis* (A), *B. bifidum* (B), *B. breve* (C), *B. infantis* (D) and *B. longum* (E). (Inoculum 4×10^5 colony forming unites (cfu) in 200 µl; incubation 48 h. Error bars for SD (n =3); *: significant difference ($p < 0.05$) compared with the control).

5.3.4 Formation of biofilm in presence of KGM and KGM-US

A possible mechanism for the protection of KGM or KGM-US against antibiotic inhibition is the formation of a viscous layer surrounding the bacterial cell by the high MW

polysaccharide, which acts as a barrier to the antibiotic molecules. On the other hand, the viscous layer can also block or slowdown the transfer of nutrients to the bacterial cell. As reported previously (Fernandes et al., 2012a), the inhibitory effect of chitooligosaccharides (COS) at a relatively high concentration of 10 g/l on probiotic bacteria including *Lactobacilli* and *Bifidobacteria* was attributed to the resistance to nutrient transport created by the COS surrounding or covering the bacteria cell. Moreover, the viscous layer surrounding the bacterial cell can enhance cell adhesion and formation of biofilms on solid surfaces. Bacterial cells in biofilm are also more resistant to antibiotics (Stewart & William, 2001). KGM was chosen for the biofilm test because of its high viscosity while KGM-US was chosen because of its most significant protection for the bifidobacteria. As shown in Table 5-5, KGM and KGM-US only increased the adherence of *B. infantis* to the inner surface of glass tube and had no effect on other four strains. However, the tube test was not so reliable for quantifying the biofilms for several reasons (Christensen et al., 1985). The microtiter-plate test showed more positive results with strong or moderate adherence of bacteria to the polystyrene surface. Both KGM and KGM-US increased the biofilm formation for *B. adolescentis*, *B. infantis* and *B. longum* by one degree (from "++" to "+++"), though KGM caused a slight reduction of biofilm formation for *B. bifidum*.

Table 5-5 Adhesion ability of five strains of *Bifidobacteria* by tube and microtiterplate tests.

Test	*B. adolescentis*	*B. bifidum*	*B. breve*	*B. infantis*	*B. longum*
Tube test					
Control (none)	-	+	-	-	-
KGM (5 g/l)	-	+	-	+	-
KGM-US (5 g/l)	-	+	-	+	-
Microtiter-Plate test					
Control (none)	++	+++	++	++	++
KGM (5 g/l)	+++	++	+++	+++	+++
KGM-US (5 g/l)	+++	+++	++	+++	+++

Note: -: not adherent, +: slightly adherent, ++ moderately adherent, and +++ strongly adherent, compared to the negative control. Cultured for 2 d at 37 °C.

The result of microtiter-plate test may be more relevant to this study because the protective effects were conducted in polystyrene micro-titer plates. The improved biofilm formation of most bacterial strains with the addition of 5 g/l KGM and KGM-US was quite consistent with the protective effect of KGM and KGM-US against antibiotics. The different results from glass tube and microtiter-plate tests indicated that the material property influences the adherent ability of the bacteria. To mimic the large intestine environment for microbiota, some researchers have applied the intestinal epithelial cell model established by colonic carcinoma (Caco-2) cells to assess the adherence of bacteria in intestine (Parkar et al., 2010).

5.3.5 Adsorption of antibiotics by KGM and KGM-US

Another possible mechanism for the protective effect of KGM and degraded products against antibiotic inhibition of the bifidobacteria is the adsorption of the antibiotics to the polysaccharides, thus decreasing the free antibiotic concentration in the culture medium (Fig. 5-3). Because penicillin was the most affected and tetracycline the least affected among the four antibiotics by KGM and KGM-US based on the MIC and MBC assays, the two antibiotics were chosen for this test via the dialysis experiment. Considering the water binding capacity of polysaccharide, theoretically, the final concentration of antibiotics in the water outside of the dialysis tubing should be at least 0.2 mg/ml if polysaccharide adsorbs no antibiotics. Penicillin and tetracycline were proven to traverse the membrane freely because there was no significant difference between the concentration of control group and the theoretical concentration (0.2 mg/ml) for penicillin or tetracycline by the *t* test ($p > 0.05$).

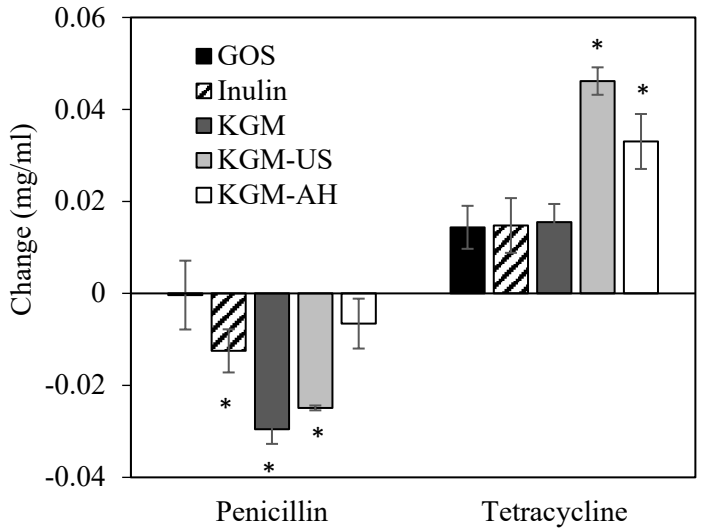

Figure 5-3 The change of antibiotic concentration outside the dialysis tubing (initially containing 5 g/l of poly- or oligo-polysaccharide and 1 mg/ml of antibiotics; dialysis against water at 20 °C for 24 h). (Error bars for SD, n =3; *: significant difference at $p < 0.05$ compared with control).

As shown in Fig. 5-3, the concentrations of penicillin in KGM, KGM-US and inulin groups were significantly lower than in the control, which confirmed the adsorption of penicillin by these PS molecules. KGM showed the highest adsorption capacity with about 0.03 mg/ml. In contrast, the concentrations of tetracycline in all test groups were higher than in the control, especially significant in the KGM and degraded KGM groups. The higher concentration of tetracycline observed may be attributed to the water absorbability of KGM and degraded products. Moreover, the sharply different absorption ability of KGM for penicillin and tetracycline may be attributed to the different molecular properties of the two antibiotics, especially the polarity as penicillin is very polar and soluble in water while tetracycline is less polar and less soluble in water (Chlou, Malcolm, Brinton, & Klle, 1986; Soren, 2003).

The results of adsorption experiments are in general agreement with the finding from the above that KGM and KGM-US had a significant protective effect on the bifidobacteria against penicillin but little effect against tetracycline. However, a quantitative correlation could not be

found between the adsorption concentrations and the changes in MIC or MBC due probably to the simplistic adsorption experimental system and the tedious MIC and MBC assay procedure. Therefore, the protective effect of KGM and KGM-US against some of the antibiotics can be partially attributed to the adsorption of these antibiotics to the polysaccharides thereby decreasing the concentration of free antibiotic in the system.

5.4 Summary

The present study has revealed the protective effect of KGM, especially the US-degraded KGM on bifidobacteria against some common antibiotics including penicillin and streptomycin. Partially degraded KGM by high intensity ultrasound was more effective than the native KGM and the low molecular weight, acid-hydrolysed KGM. Two prebiotic standards GOS and inulin showed no significant protective effect. The protective effect of KGM on the bifidobacteria may be attributed to the adsorption of antibiotics and the formation of a viscous layer surrounding the bacteria by the polysaccharides. However, the present study has only detected the protective effect of KGM in the pure cultures of a few bifidobacteria. As the protective effect varied with the bacterial strains, further investigation should be carried out in mixed cultures of gut microflora to evaluate the potential application in human gut microbiota.

Chapter 6 Protection of Bifidobacterial cells against antibiotics by Cs-HK1 exopolysaccharide through physical interactions

6.1 Introduction

Antibiotics are widely used in clinical therapy for human infectious diseases and also in poultry farming and aquaculture for decades (Modi, Collins, & Relman, 2014b). However, the human gut microbiota is very sensitive to antibiotics and other food contaminants. Antibiotics may cause disruption of the healthy balance of gut microflora (Keeney, Yurist-Doutsch, Arrieta, & Finlay, 2014b). The imbalanced gut microbiota may be implicated in a number of diseases, such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and other chronic metabolic diseases (Keeney et al., 2014b). On the other hand, dietary carbohydrate fibres are beneficial for growth and colonization of Firmicutes in the intestine (De Filippo et al., 2010).

In the former study from our group, a high-molecular weight (MW) (7.066×10^7-1.679×10^8 Da) food polysaccharide, konjac glucomannan (KGM) has been shown to offer significant protective effect on the survival of *Bifidobacteria* against specific antibiotics, while the acid-degraded low-MW fraction of KGM lost the protective effect (Y.-H. Mao, A.-X. Song, Z.-P. Yao, & J.-Y. Wu, 2018). In another study, the higher-MW fractions of KGM and EPS from Cs-HK1 mycelial fermentation showed more significant protective effect on Bifidobacteria in liquid culture (Song, Mao, Siu, & Wu, 2018). According to these studies, it is hypothesised that the Cs-HK1 EPS can have a MW-dependant protective effect on Bifidobacteria against antibiotic inhibition through physical interaction with the bacterial cells.

This work was to investigate the protective effect of EPS from Cs-HK1 mycelial liquid fermentation and its partially degraded products against the inhibition of antibiotics with

respect to the relative MW and the physical mechanisms. The enhanced biofilm formation as a possible mechanism for the protective effect was directly observed by scanning electronic microscopy (SEM) and atomic force microscopy (AFM), and confocal laser scanning microscopy (CLSM). Moreover, the Caco-2 cell monolayer was applied as a model to detect the effect of EPS on bifidobacterial adhesion on the intestinal mucosa (Mah & O'Toole, 2001).

6.2 Material and methods

6.2.1 Chemicals and biologicals

Reinforced Clostridium Medium (RCM) and four antibiotics (enrofloxacin, streptomycin, penicillin and tetracycline) were purchased from Guangdong Huankai Bio-Technology Co., Ltd. (Guangzhou, China). Five strains of Bifidobacteria were attained originally from China Center of Industrial Culture Collection (CICC, Beijing, China). Cs-HK1 fungus for the production of exopolysaccharide (EPS) was originally isolated from the fruiting body of a wild *Cordyceps sinensis* and preserved at China General Microbiological Culture Collection Center (Reg. No. 6004).

Reagents for the confocal laser scanning microscope (CLSM), phosphate buffer solution (PBS) glutaraldehyde solution, and propidium iodide (PI) were from Shanghai Yuanye Bio-Technology Co., Ltd., (Shanghai, China), and fluorescein isothiocyanate conjugated concanavalin A (FITC-ConA) solution from Sigma (USA). Caco-2 cell line (human colonic adenocarcinoma cell) was purchased from American Type Culture Collection (ATCC) (Perez, Minnaard, Disalvo, & De Antoni, 1998). The culture medium and supplements, Dulbecco's modified Eagle Medium (DMEM) from Gibco™, Invitrogen, fetal bovine serum was attained from Biosera (France), 1% penicillin-streptomycin from Sigma (USA).

6.2.2 Bifidobacterial culture conditions

Five strains of Bifidobacteria were used in this study including *B. adolescentis* (CICC6067), *B. bifidum* (CICC6071), *B. breve* (CICC6079), *B. infantis* (CICC6069) and *B. longum* (CICC6186). The bacterial cultures were maintained in Reinforced Clostridium Medium (RCM), which was composed of beef extract (10 g/l), peptone (3 g/l), yeast extract (3 g/l), soluble starch (1 g/l), glucose (5 g/l), cysteine HCl (0.5 g/l), sodium chloride (5 g/l), sodium acetate (3 g/l), agar (0.5 g/l for broth, 15 g/l for agar plate). Experiments were carried out in bacterial suspension cultures at 37°C for 24 h under anaerobic condition in airtight jars with anaerobic sachets (S. A. Tanner et al., 2014). Details of the storage and culture conditions have been described previously (Y.-H. Mao et al., 2018; Song et al., 2018).

6.2.3 Test of EPS on bifidobacterial growth

The EPS fractions and inulin as a prebiotic reference were dissolved in DI water at the desired final concentrations with vigorous stirring for overnight. After the addition of 38 g/l RCM powder into each sample solution, the mixture solution was sterilized at 121 °C for 20 min. The EPS fractions were added to RCM at a final concentration of 5 g/l, and then 2-fold diluted into a series of concentration from 5 to 0.0782 g/l. The liquid medium was dispensed into a 96-well microtiter plate and each well was inoculated with 4×10^5 colony forming units (CFUs) of the Bifidobacteria (pre-cultured for 24 hours as described in 2.1) to a total liquid volume 200 µl per well. The microtiter plate was incubated at 37 °C under anaerobic condition for 48 h. RCM inoculated with bacteria was included as the control and RCM with EPS but no bacteria as the blank. The effect of EPS fractions on the bifidobacterial growth was represented by $(OD_{test} - OD_{blank})/OD_{control} \times 100\%$, where the optical density (OD) was measured at 600 nm.

6.2.4 Treatment of bifidobacteria with selected antibiotics and the EPS fractions

Four antibiotics that are commonly applied to livestock animals and fishery (enrofloxacin

and streptomycin) and human medicine (penicillin and tetracycline) were chosen for this study. Antibiotic solutions were prepared freshly in culture medium at an initial concentration 2.048 mg/mL and used within 24 h. Two assays were applied to evaluate the sensitivity/tolerance of bifidobacteria to antibiotics, minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC), which were performed in microtiter plates as described previously (Y.-H. Mao et al., 2018). In brief, each antibiotic solution was subject to serial two-fold dilution and the diluted antibiotic solution was transferred at 100 µl aliquots into a 96-well microtiter plate containing 100 µl of fresh bacterial culture medium in each well. The optical density at 600 nm of bifidobacterial cell suspension in the mid-log growth stage was adjusted to 0.1 with fresh RCM medium and the EPS tested, and 100 µl of the standardized bacterial suspension was inoculated into each plate well and incubated for 48 h. Antibiotic-free RCM medium was included as a control and bacterium-free RCM medium as a blank for each plate test. For the MBC assay, 20 µl of the bacterial suspension from each plate well was inoculated on RCM agar and the lowest concentration of a tested antibiotic with no visible growth after 48 h incubation was taken as the MBC value. All plate assays were performed in four replicates and the median MIC and MBC values were recorded for each antibiotic–bacterium combination.

6.2.5 Microscopic examination of bacteria and EPS interactions

Microscopic experiments were performed for direct observation of the microstructures of the bifidobacteria and EPS fractions and their interactions. Three microscopy techniques, scanning electron microscope (SEM), atomic force microscope (AFM) and confocal laser scanning microscope (CLSM) were employed to capture the fiber structures of EPS fractions, the cell surface and biofilm formation of bifidobacteria. Since the effects of EPS fractions on the different bifidobacterial strains showed a similar trend, only one of the bacterial strains, *B.*

adolescentis, was taken for the microscopic assessment.

SEM was performed with a JSM 6701F SEM (JEOL Ltd., Tokyo, Japan) to examine the surface and morphological structures of *B. adolescentis* and EPS fibers. The *B. adolescentis* was cultured in RCM for 24 h as described in 2.1. Based on preliminary experiments, 400 µl of the bacterial suspension was washed by sterilized saline and then centrifuged twice. The bacterial pellet was re-suspended in 2 ml of 1 g/l of EPS solution and was immediately frozen in liquid nitrogen, and then freeze-dried for SEM imaging.

AFM imaging was performed on a Bruker Nanoscope-8 device (Bruke, Billerica, Massachusetts, USA) equipped with a J-type scanner to examine EPS surrounding the bacterial cells. Forty microliters of the bacterial suspension in liquid medium containing the EPS fractions (1 and 5 g/L) was loaded onto a clean glass slide and then air-dried. The loaded glass slide was gently rinsed with DI water to remove salt crystals and air dried again for the AFM analysis (Fernandes et al., 2012b). The AFM was performed in tapping mode in air, using silicon cantilevers with a resonant frequency about 150 kHz (MikroMasch, Tallinn, Estonia). For each EPS sample, three independently prepared specimens were analyzed, and several areas were captured, but only the characteristic images are presented in the results.

CLSM was performed with a Leica DMi8 Confocal Microscope (Leica Microsystems GmbH, Wetzlar, Germany), to visualize the biofilms formed by the bifidobacteria in the presence of EPS in the culture medium. Glass cover slides were cleaned and autoclaved, and then suspended in the RCM broth with EPS fractions in petri dishes and RCM without EPS as a control. The *B. adolescentis* bacterial suspension from above liquid culture was inoculated at 1% (v/v) into the petri dishes and incubated for 48 h with gentle shaking at 30 rpm. The glass covers were carefully and aseptically removed, and treated as the follows. One side of the slide was scrubbed with isopropyl alcohol cotton swab, rinsed with phosphate buffer solution (PBS) three times, fixed with 100 µl of 2.5% (w/v) glutaraldehyde solution at 4°C for 1.5 h. After

rinsing with PBS twice, the slide was stained with 100 µl of 100 µg/ml fluorescein isothiocyanate conjugated concanavalin A (FITC-ConA) solution for 30 min at 4°C in dark (Liu et al., 2015). After PBS rinsing, it was stained with 100 µl of 50 µg/ml propidium iodide (PI) solution for 15 min at 4°C in dark, and then rinsed with PBS and dried at 20°C in dark (Mongkolrob R., Taweechaisupapong S., & S., 2015). It was finally treated with the antifade solution and sealed with nail polish, and ready for CLSM. The FITC-ConA combined with EPS exhibited green fluorescence under the laser at 488 nm, while the PI combined with the DNA of bacterial cell exhibited red fluorescence under the laser at 543 nm. The bacteria exhibited orange fluorescence when the two images overlapped.

6.2.6 Test of bacterial adhesion on Caco-2 monolayer

Caco-2 cell monolayer is commonly used as an in vitro model for intestinal epithelium to screen for adhesive strains (Tuomola & Salminen, 1998). The Caco-2 cell culture was maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 4.5 g/l glucose, 1% non-essential amino acids, 20% inactivated (30 min, 56°C) fecal bovine serum and 1% penicillin-streptomycin. The Caco-2 cells were seeded at 4.5 ×103 cells per well in a 96-well culture plates or 5 ×104 cells per well in 24-well plates and incubated at 37 °C with 5% CO2 in the atmosphere. The medium was replenished every 48 h and the cells maintained for 14 d to obtain a differentiated intestinal epithelial cell monolayer for the assay (Delie & Rubas, 1997). For bacterial adhesion assays, MEM without antibiotics was used in the last two rounds of medium replacement.

At 1 hour after the last medium replacement, each of the EPS fractions was added in triplicate to the microplate wells at a final 1 or 5 g/l final concentration. After 1 h incubation, *B. adolescentis* suspension (10 µl) was added to all the wells, maintaining a multiplicity of infection ratio of 100 bacteria to one Caco-2 cell. Prior to the test, the *B. adolescentis* bacterial

suspension had been centrifuged at 1000 g for 10 min, washed three times with phosphate buffered saline (PBS), and then diluted with DMEM to 5 $\times 10^9$ colony forming units (cfu)/ml before the following assy. After incubation for 2 h under 5% CO2 at 37 °C, the Caco-2 cells were washed thrice with pre-warmed PBS to remove loosely adherent bacteria and lysed for 10 min with 1% Triton X- 100. Total viable bacteria adherent to or internalized by the cells as well as the initial number of bacteria added were quantified by serial dilution and plating on RCM agar. Control cells were treated with the medium alone. The percentage change in the number of viable adherent bacteria was represented by the average cfu in the treated cells/average cfu in the untreated control × 100.

6.2.7 Determination of Caco-2 cell viability

Caco-2 cells grown in the 96-well plates for 14 d (see Section 2.8) were used for this assay. Fresh DMEM was added 1 h before the assay. The carbohydrates, EPS and inulin, were then added to the wells at final concentrations of 5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0783 mg/ml in triplicate. After incubation for 2 h under 5% CO2 at 37 °C, all the wells were aspirated and refilled with 100 μl DMEM and 10 μl of MTT reagent. After incubation for 4 h, all wells were aspirated and added with 100 μl of DMSO reagent followed by gentle shake for 10 min. Finally, the culture plate was read at 570 nm by a microplate reader.

6.2.8 Statistical analysis

The microplate assays were performed in four replicates and all other experiments performed in triplicate and the results were averaged. Kruskal-Wallis test and Nemenyi test were applied in the analysis of MIC and MBC data. Student's *t* test was performed to compare the statistical significance of treatment effects on the bacterial adhesion.

6.3 Results and discussion

6.3.1 Molecular properties of EPS

Table 6-1 shows the molecular weight (MW) data of EPS, EPS-US and EPS-L attained from HPGPC analysis. Compared with the native EPS, the EPS-US attained after 30 min US treatment of the native had overall shift of the average MW to a lower range with the major MW fraction decreased from 2.252×10^8 to 2.699×10^7 Da. The MW reduction caused by the US treatment was similar to that reported previously (X. Chen, Siu, Cheung, & Wu, 2014). The EPS-L had an even lower MW distribution with a major MW fraction (58%) at 3.346×10^6.

Table 6-1 The intrinsic viscosity and molecular weight of different EPS fractions.

Sample	MW (Da)	% Area
EPS	2.252×10^8	85.8
	1.858×10^5	4.61
EPS-US	2.699×10^7	85.1
	1.492×10^4	3.49
EPS-L	5.784×10^7	26.9
	3.346×10^6	58.9

According to composition analysis, the total carbohydrate content was 71% for the native EPS, 70% for EPS-US, and 28% for EPS-L and the total protein content was 6.97% for EPS, 18.4% for EPS-US, and 40% for EPS-L. The native EPS consisted of three monosaccharide constituents, mannose, glucose and galactose at a molar ratio of 1.73:1.81:1.

6.3.2 Effect of EPS on bifidobacterial growth

As shown in Fig. 6-1, the native EPS and EPS-US at 1.25 g/L or a higher concentration suppressed the growth of most bifidobacteria strains while EPS-L with relatively low MW had a slightly positive effect on some of the bacterial strains but negative effect on others. Among

the three EPS fractions, the native EPS with the highest MW caused most notable inhibition on the growth of the five bacteria strains, especially at higher concentrations, and EPS-L with the lowest MW caused slight or not growth inhibition. As the major difference among the three EPS fractions was the MW, the inhibitory effect became more severe as the MW was higher. A plausible explanation for the inhibitory effect of high MW EPS fractions on the bacterial growth is the resistance to nutrient transport to the bacteria cell created by the highly viscous EPS in the liquid medium. The increased resistance to molecular transport by the high MW EPS may also contribute to the protective effect on the bacteria against the harmful chemicals such as the antibiotics as shown below (Mah & O'Toole, 2001).

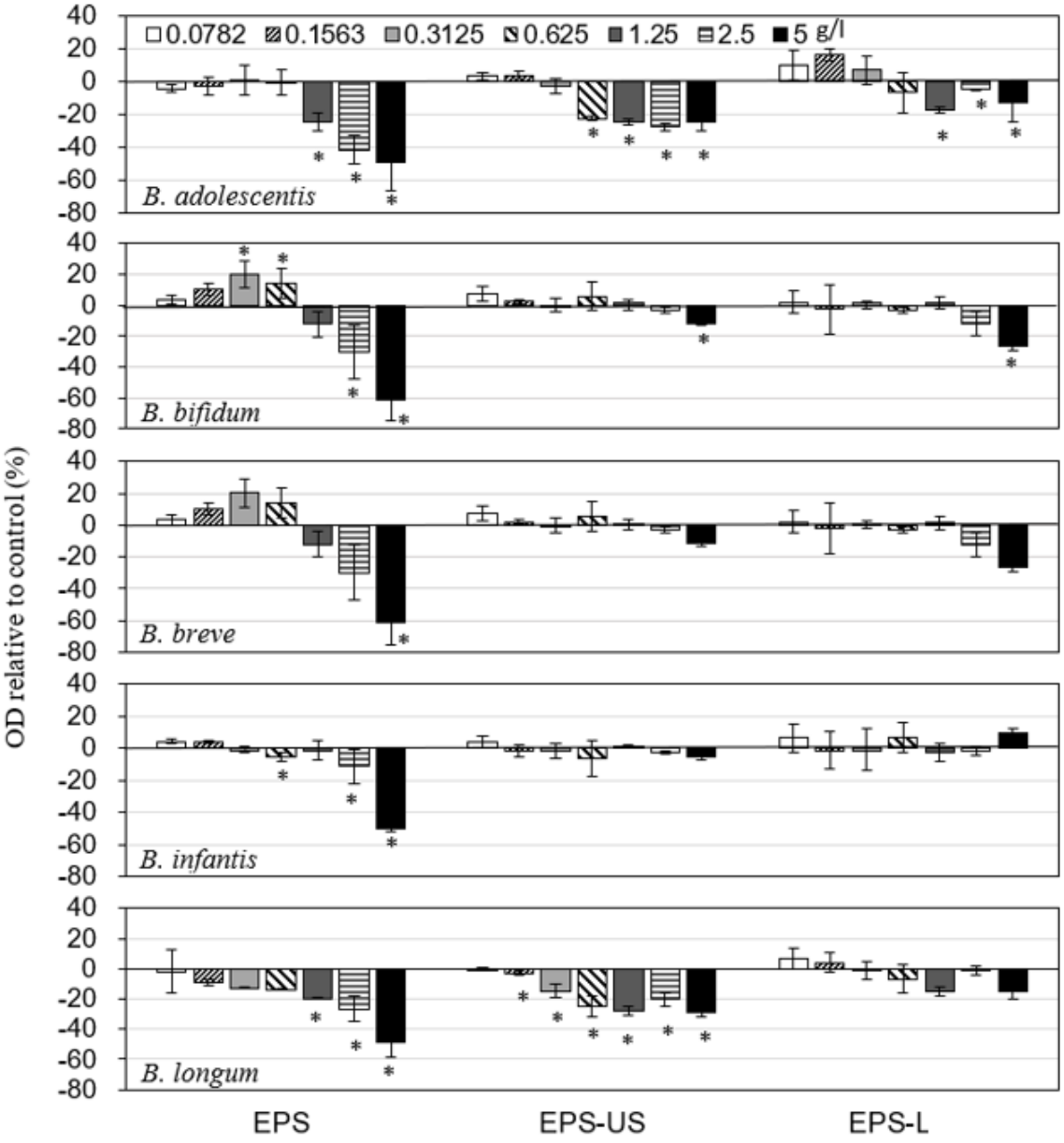

Figure 6-1 Effects of different EPS fractions on proliferation of five strains of *Bifidobacteria*. Inoculum concentration: 4×10^5 colony forming unites (cfu) in 200 µl; incubation 48 h. Error bars for standard deviations (SD, n =3); *: significant difference ($p < 0.05$) compared with the control.

6.3.3 Protective effects of EPS against antibiotic inhibition of bifidobacteria

Table 6-2 presents the MIC and MBC values of four antibiotics on the five bifidobacterial strains cultured with or without various EPS fractions. In the control cultures, penicillin was the most potent (with lowest MIC and MBC values) to inhibit the five strains of bifidobacteria

compared with the other three antibiotics. On the other hand, most of the bifidobacteria were more resistant or tolerant to streptomycin than the three antibiotics with lower MIC and MBC values ranged from 16 to >512 μg/ml. Additionally, the MIC and MBC values of enrofloxacin and tetracycline were widely varied with the different bacterial strains. The prebiotic reference inulin added to the bacterial medium (at 5 g/L) caused little or no change in the MIC or MBC values of antibiotics as compared with the control.

Table 6-2 MIC and MBC (μg/ml) of antibiotics against five strains of *Bifidobacterium* in RCM supplemented with different carbohydrates (Control in TCM medium).

OS/PS (5 g/l or specified)	Enrofloxacin		Penicllin		Tetracycline		Streptomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. adolescentis								
Control (none)	1-2	2	<1	<1	<1	32-128	256	256-512
EPS (1 g/l)	8	64	<1	4	2	16	256	512
EPS	32	>512	>512	>512	2	>512	256-512	>512
EPS-US (1 g/l)	1	32	<1	1	<1	32	128	256
EPS-US	>512	>512	>512	>512	>512	>512	>512	>512
EPS-L (1 g/l)	8	32	<1	1	4	16	256	256
EPS-L	128	128	<1	4	32	32	256	256
B. bifidum								
Control (none)	64-128	32	<1	8	8	128	128	128
EPS (1 g/l)	512	512	1	8	16	128	128	256
EPS	>512	>512	>512	>512	>512	>512	>512	>512
EPS-US (1 g/l)	128	64	<1	2-4	1-2	64	16-64	32
EPS-US	>512	>512	>512	>512	64	64	>512	>512
EPS-L (1 g/l)	256	128-256	2	8	8	128	128	64-128
EPS-L	64	128	2	8	16	128	128	128
B. breve								
Control (none)	64	512	1	8	8	4-16	16	16-32
EPS (1 g/l)	256	>512	4	32	128	128	512	>512
EPS	64-128	>512	>512	>512	>512	>512	64	>512
EPS-US (1 g/l)	8	32	<1	8-16	<1	16	16	32
EPS-US	>512	>512	>512	>512	256	256	>512	>512
EPS-L (1 g/l)	256	256-512	8	32	128	128	32	32
EPS-L	128-256	256-512	16	32-64	512	256-512	256	256-512
B. infantis								
Control (none)	4-8	32	<1	8-16	32	16	>512	>512
EPS (1 g/l)	64	64	<1	16	16	32	>512	>512
EPS	>512	>512	>512	>512	>512	>512	>512	>512
EPS-US (1 g/l)	32	64	<1	8	8	32	>512	>512
EPS-US	256	256-512	>512	>512	64	64	>512	>512
EPS-L (1 g/l)	64	128	<1	16	32	32	>512	>512
EPS-L	64	128	<1	16	32	64	>512	>512
B. longum								
Control (none)	4	4-16	<1	8-16	1-2	32-128	64	32-512
EPS (1 g/l)	16	16	<1	32	2	32	256	256
EPS	>512	>512	>512	>512	512	>512	128-256	>512
EPS-US (1 g/l)	4	16-32	<1	4-8	1	32	128	>512
EPS-US	64-512	64-512	>512	>512	64	64	>512	>512
EPS-L (1 g/l)	8	8	8	4-8	2	32-64	64	64-128
EPS-L	16	64	4-8	16	2	16-32	128	128

Both EPS and EPS-US fractions at 5 g/L significantly increased the MIC and MBC values to over 512 μg/ml of four antibiotics on most of the bacterial strains. The relative significance of the effects between EPS and EPS-US varied with the different antibiotics and bacterial species.

With a lower concentration (1 g/L) of EPS and EPS-US, the MIC and MBC values of four antibiotics were increased less significantly than with 5 g/l in most cases. The lower MW EPS fraction EPS-L at both concentrations also increased the MIC and MBC values of antibiotics on most bacterial species, but less significantly than EPS and EPS-US.

The MIC and MBC results indicate that the high-MW EPS fractions had a significant, concentration-dependent protective effect against antibiotic inhibition of the bifidobacteria, and the lower MW EPS-L had a less significant protective effect. The results suggest that a relatively high MW is an important factor for the protective effect of EPS fractions on the bacteria. In this regard, the lack of protective effect by the prebiotic inulin was due probably to the low MW. Similarly, konjac glucomannan (KGM) and US-degraded KGM with high MW showed a significant protective effect on bifidobacteria against antibiotics but the low-MW acid-hydrolyzed KGM had a significantly lower or no protective effect (Mao et al., 2017). In general, EPS and EPS-US showed more stable protective effect than KGM on all four antibiotics due probably to the relatively high MW and different polymer structure of EPS compared with KGM.

In our previous study (Y.-H. Mao et al., 2018), three possible mechanisms have been proposed for the protective effects of KGM on the bifidobacteria, the physical adsorption of antibiotic molecules, the formation of a viscous layer surrounding the bacteria, and the increased formation of biofilms by the bacteria by the polysaccharides. However, the EPS fractions showed no significant adsorption to any of the four antibiotics (data not shown) and the other two mechanisms were investigated in the following experiments.

6.3.4 Microstructures and interactions of bacterial cells and EPS

Fig. 6-2 shows the SEM images of *B. adolescentis* bacteria and EPS fractions. The native EPS and US-treated EPS-US appeared as a relatively smooth and planner sheet (Fig. 6-2 a; Fig. 6-2 b), the lower EPS fraction EPS-L as an irregular and distorted sheet (Fig. 6-2 c), and inulin appeared as aggregates. Many bacteria were attached on the sheet surface of EPS and EPS-US, and much fewer were found on the surface of EPS-L and inulin.

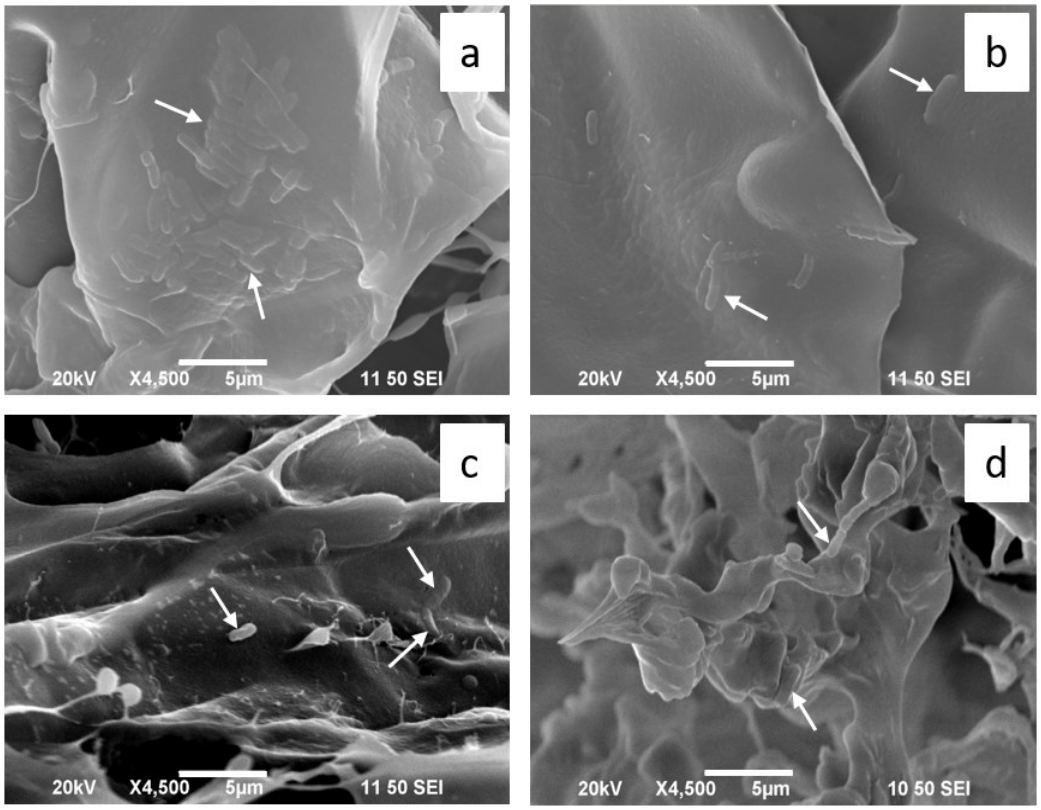

Figure 6-2 SEM images of B. adolescentis in different EPS fractions: (a) EPS; (b) EPS-US; (c) EPS-L; (d) Inulin. Arrows indicating the bacterial cells.

Fig. 6-3 shows the AFM images of *B. adolescentis* samples in absence or presence of the EPS fractions. In the absence of any EPS (Fig. 6-3a), few or no *B. adolescentis* cells were attached to the glass slide. In the presence of different EPS fractions (Fig. 6-3b-g), the bacteria cells had similar size and morphology, but their surface roughness varied, increasing in the

order EPS>EPS-US>EPS-L, and with the concentration. The increased cell surface roughness with the high-MW EPS suggests the presence of a thin coat or layer with the viscous EPS on the bacterial cell. This viscous EPS layer surrounding the bacterial cell may contribute to the protective effect against the antibiotics and also to the inhibitory effect on the bacterial growth as seen above (Fig. 6-3), by creating a barrier to the antibiotic and nutrient transport. Similarly, in previous studies the inhibitory effect of chitoligosaccharides at a high concentration (10 g/l) on *Bacillus cereus* growth was attributed to the formation of a film layer on the bacterial cell surface, although no direct observation or experimental evidence was provided (Fernandes et al., 2012b; Fernandes, Eaton, Gomes, Pintado, & Malcata, 2009).

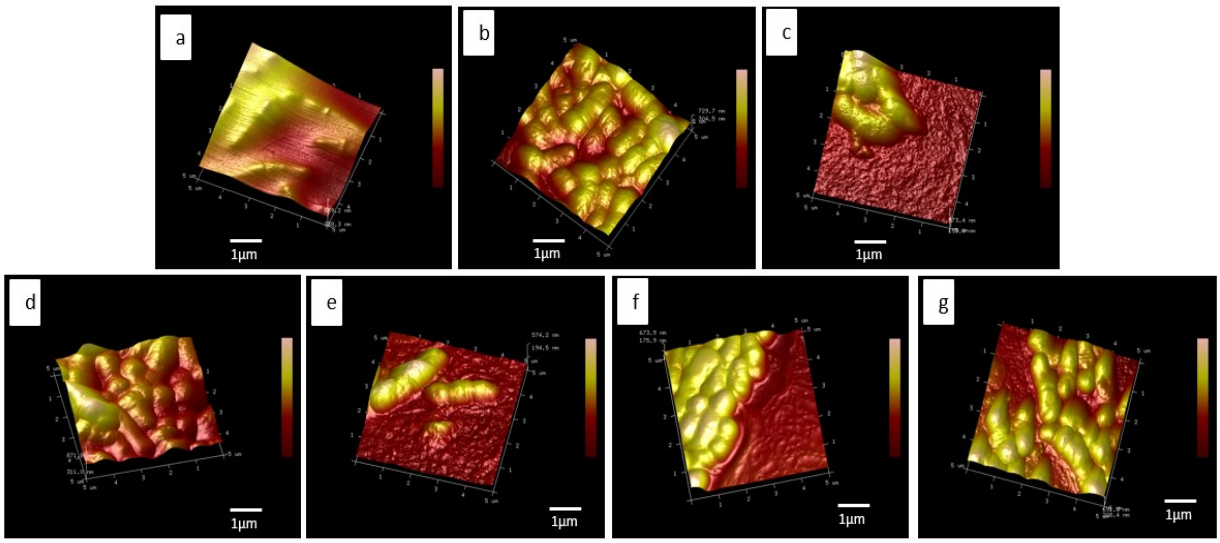

Figure 6-3 AFM images (tapping mode) of the effect of different EPS fractions upon *B. adolescentis* morphology: (a) Control with no EPS; (b) EPS 1 g/l; (c) EPS 5 g/l; (d) EPS-US 1 g/l; (e) EPS-US 5 g/l; (f) EPS-L 1 g/l; (g) EPS-L 5 g/l.

6.3.5 Biofilm formation of B. adolescentis in presence of EPS

Fig. 6-4 shows the CLSM images of bifidobacteria on glass slides stained with the fluoresce marker. In the control sample without any EPS (Fig. 4a), few biofilm spots (green spots) were present on the glass slide. In the samples with 1 g/l of EPS (Fig. 6-4a) or EPS-US

(Fig. 6-4c), some small pieces of biofilms appeared. With 5 g/L EPS (Fig. 6-4b) or EPS-US (Fig. 6-4d), many small pieces of biofilms were formed. No biofilms appeared in the sample with EPS-L at 1 g/L or 5 g/L (Fig. 6-4f-Fig. 6-4g). Therefore, EPS and EPS-US promoted the formation of biofilms by the bifidobacterial cells in a dose dependant manner. The effects of various EPS fractions on biofilm formation were consistent or correlated with their protective effects against antibiotic inhibition as shown above (Table 6-2). The results suggest that the protective effect of EPS and EPS-US on the bifidobacteria was attributable to the promotion of biofilm formation. The similar relationship has been suggested for the protective effect of KGM on the bifodobacteria (Y.-H. Mao et al., 2018). Formation of biofilms by bacterial cells can usually increase their resistance to antimicrobial agents (Mah & OToole, 2001).

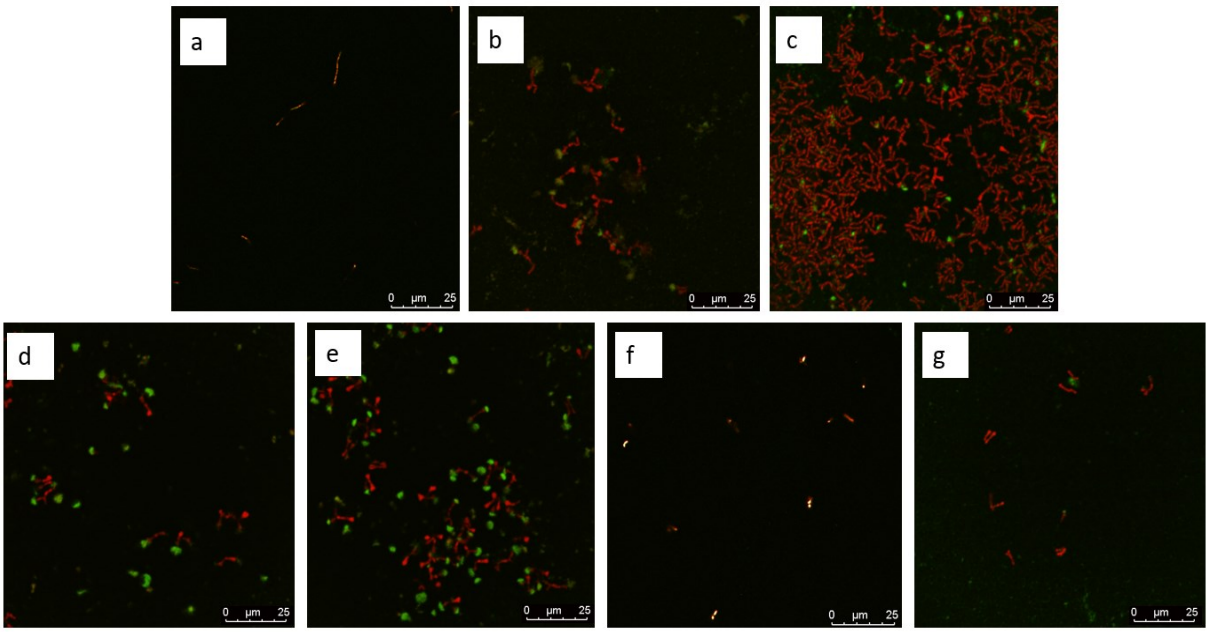

Figure 6-4 Confocal laser scanning microscopy (CLSM) images of B. adolescentis biofilms on glass slides in RCM with existence of different polysaccharides. Inoculation with 1% (v/v) bifidobacterial suspension. Cultured for 2 days at 37 °C, 30 rpm. (a) Control with no EPS (b) EPS 1 g/l, (c) EPS 5 g/l, (d) EPS-US 1 g/l, (e) EPS-US 5 g/l, (f) EPS-L 1 g/l, (g) EPS-L 5 g/l. Bacteria were stained by PI and emitted red light, while biofilms were stained by FITC-ConA and emitted green light. The overlap parts showed orange.

A previous study has shown that biofilm formation of bifidobacteria was enhanced under bile stress due probably to the increase in the surface hydrophobicity of bacterial cell (Ambalam P., Kondepudi K. K., Nilsson I., Wadstrom T., & A., 2014). In *E. coli*, RpoS protein (σ^S) as a chief factor for response to general stresses was produced at higher concentration when the bacterial growth was terminated due to starvation or the lack of major nutrients such as carbon, nitrogen, phosphorus and amino acids (S.-X. Wang et al., 2012). It has been found that RpoS plays a crucial role in the biofilm formation by *E. coli* (Corona-Izquierdo & Membrillo-Hernandez, 2002). These previous research findings suggest that biofilm formation by bacteria cells is enhanced in response to environment stress and nutrient limitation. In the present study, the high-MW EPS and EPS-US probably retarded the transfer of the nutrients to the bifidobacterial cell leading to slower growth rate. In addition to nutrient limitation, the increase in the liquid viscosity or formation of a viscous layer surrounding the bacterial cell may also promote the aggregation of bacterial cells and formation biofilm.

6.3.6 Effect of EPS on adhesion of B. adolescentis to Caco-2 monolayer

As shown in Fig. 6-5, both EPS and EPS-US increased the adhesion of bifidobacteria to Caco-2 cell monolayer, especially at the higher concentration of 5 g/L, by about 35% compared to the control. The prebiotic reference inulin at 5 g/l also increased the adhesion by 15.6%. However, EPS-L had no statistically significant effect on the adhesion. The increase in bacterial adhesion to the Caco-2 monolayer has a positive correlation with the molecular weight and concentration of polysaccharides. Additionally, most of the Caco-2 cells remained alive with the three EPS fractions. The result suggests that the high-MW EPS fractions have the ability to bifidobacteria to intestinal epithelium. Similarly, a previous study has shown based on molecular techniques that the EPS produced by a well-known probiotic, *B. animal subsp.* Lactis influence bacterial surface properties so as to increase the adhesion to the intestinal epithelial

cell HT29 monolayer (Castro-Bravo, Hidalgo-Cantabrana, Rodriguez-Carvajal, Ruas-Madiedo, & Margolles, 2017). The enhanced adhesion of probiotic bacteria to the intestinal epithelium may facilitate their colonization in the intestine.

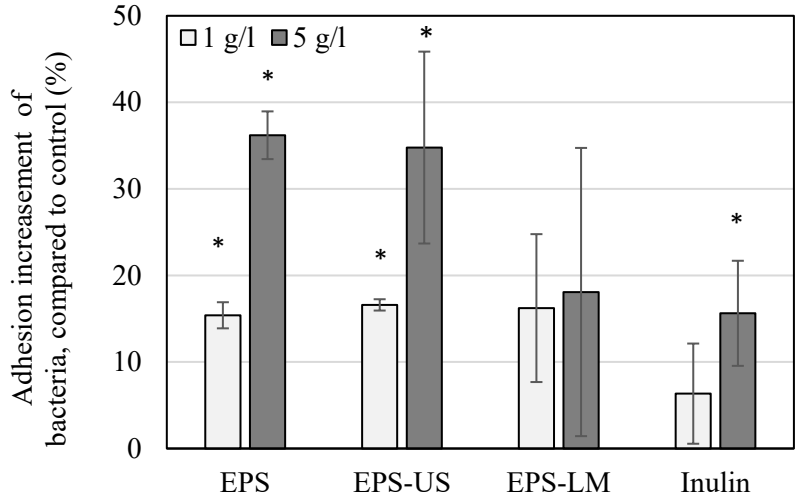

Figure 6-5 Effect of inulin and different Cs-HK1 EPS on adhesion and survival of *B. adolescentis* to Caco-2 cells. The cells were pre-treated with EPS for one hour and then incubated with bacteria for two hours. The number of adherent bacteria was counted by the plate count method. The values are the mean of triplicates ± SD. The control is untreated Caco-2 monolayer incubated with bacteria, and the adhesion increase of bacteria = (average log10(cfu in treated cells)/average log10 (cfu in untreated control) - 1) × 100. The asterisks denote the significance of $p < 0.05$, in comparison with the control.

6.4 Summary

The high-MW EPS produced by the Cs-HK1 fungus exhibited a significant protective effect on several strains of bifidobacterial against four commonly used antibiotics. The protective effect of different EPS fractions was generally more significant with a higher MW and higher concentration, which proved the initial hypothesis of MW-dependent protection. Microscopic observation suggests the formation of a viscous coat on the bacterial cell to by the high MW EPS. This viscous coat not only retarded the access of bacteria cell to the antibiotics but also to useful nutrients, leading to slower growth rate. The physical and biological effects

of EPS on the bacterial cells also enhanced the formation of biofilm and adhesion to epithelia cell layer by the bifidobacteria. Overall, the results suggest that Cs-HK1 EPS was beneficial to the bifidobacteria by protecting against the antibiotics and by promoting their adhesion to and colonization in the intestine epithelium. Our preliminary experiments have shown that the Cs-HK1 EPS are resistant to degradation by simulated gastric acid and digestive enzymes, and not significantly protective for *E. coli* or *Staphylococcus aureus* against the antibiotics (unpublished results). Further work is needed to verify the potential benefits of EPS to the gut microbiota in animal experiments and to investigate the mechanism for the EPS effects on the bifidobacterial cells such as the expression of RpoS protein during stress conditions and biofilm formation.

Chapter 7 Effects of Cs-HK1 exopolysaccharides on fecal microflora during *in vitro* fermentation

7.1 Introduction

The human intestine is inhabited by trillions of microbes. This complex ecological community, so called "gut microbiota" has a profound influence on human health and nutritional status through multiple mechanisms. For most adults, Firmicutes and Bacteroidetes are two dominative phyla in microbiota, whereas *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* are other generally found but minor constituents. A great many of evidences showed that microbial composition and metabolites are intimately related to host physiology and pathology. The indigestible food ingredients escaped from the digestive juice and enzymes in the upper digestive tracts are fermented by microbiomes in large intestine (Rios-Covian et al., 2016). The end products of fermentable complex carbohydrate in intestine are short chain fatty acids (SCFAs) (Macfarlane & Macfarlane, 2012). The most abundant SCFA in colon are acetic acid, propionic acid and butyric acid, which account for more than 95% in total of the SCFA content. Butyric acid is the major fuel for enterocytes and supplies approximately 60-70% of the needed energy of colon cells (Ardawi & Newsholme, 1985), while acetic acid and propionic acid have some other physiological functions, including blood pressure modulation (Pluznick et al., 2013).

Cordyceps sinensis is a rare medical fungus widely used to promote health for many activities including antitumor (Yoshida et al., 1989), antioxidant (L. Wang et al., 2011), antifatigue (Nakamura, Shinozuka, & Yoshikawa, 2015) and other bioactivies (Zhou et al., 2009). Because natural *C. sinensis* is rare, mycelial fermentation is usually applied for producing biomass and polysaccharides of *C. sinensis* to meet the increasing demand (Wu et

al., 2014). However, few references were found on the impact of polysaccharides originated from the *C. sinensis* species on human gut microbiota. Our previous study showed that the Cs-HK1 EPS or ultrasound-treated EPS were limitedly utilized in pure cultures of single bifidobacterial strains (Song et al., 2018). However, many non-digestible carbohydrates cannot be utilized in pure culture because of lacking complex enzyme system, while can be utilized with cross-feeding, which is an integral component of the complex microbial ecosystem in the human colon (Rios-Covian, Gueimonde, Duncan, Flint, & de los Reyes-Gavilan, 2015). Therefore, the fermentability of EPS fractions is needed to be evaluated before studying the protective effects and the mixture fermentation system is also needed to further study the prebiotic potential of the EPS fractions.

Therefore, this study applied in vitro fecal batch fermentation model to evaluate the impact of Cs-HK1 EPS with different molecular weight on the human fecal microbiota. The microbiota composition of human fecal microbiota was examined by 16S rRNA gene analysis, while the culturable bacteria was enumerated by selective agar plates. The utilization of polysaccharides and SCFA production was measured during the fermentation.

7.2 Material and methods

7.2.1 Fecal sample preparation

Fecal material was donated by three healthy volunteers (two females, one male; age 26-30 years). Donors were free of known gastrointestinal diseases and metabolic disorders and had not received any antibiotic treatment, probiotic or prebiotic supplementation for at least 3 months prior to experimentation. Fecal samples were collected in sterile tubes and immediately stored in anaerobic jars containing anaerobic sachets (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan), then used within 30 minutes of collection. Fecal samples were diluted in ten times the volume of pre-reduced sterile phosphate-buffer saline (PBS) and homogenized for 2

minutes and centrifuged at 500 rpm for 5 min to remove large particles. Resulting fecal suspension were used as the initial inoculation in the batch culture.

7.2.2 In vitro batch cultures

The basal medium consisted of peptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), dipotassium phosphate (0.04 g/l), monopotassium phosphate (0.04 g/l), sodium bicarbonate (2 g/l), magnesium sulfate heptahydrate (0.01 g/l), calcium chloride hexahydrate (0.01 g/l), Tween-80 (2 ml/l), hemin (50 mg/l), phylloquinone (vitamin K1, 10 ml/l), L-cysteine (0.5 g/l), bile salts (0.5 g/l), resazurin (1 mg/l) and distilled water (Johnson et al., 2015). The control methylcellulose (Sigma, Saint Louis, USA) and EPS fractions were added (5 mg/ml) to the respective fermentation tubes with vigorous stirring for overnight. The pH was adjusted to 6.8 and then autoclaved. Ten-milliliter sterile tubes were filled with 5.4 ml of sterilized enriched medium. Each tube was inoculated with 0.6 ml fresh prepared fecal slurry (1:10, w/w). Batch culture were conducted for 24 h, and samples of collected at 0, 5, 10, 24, 48 h for counting of bacterial populations, 16S rRNA gene sequencing, determination of carbohydrate utilization profiles and SCFA production during fermentation.

7.2.3 Enumeration of bacteria by selective agar plates

Plate counting of total anaerobic bacteria, total aerobic bacteria was carried by BHI agar, *Clostridia, Bacillus coli, Staphylococcus, Enterococcus, Lactobacillus* and *Bifidobacterium* were counted by Tryptose Sulfite Cysloserine (TSC), MacConkey agar, Mannistol Salt Agar (MSA) Enterococcus agar, MRS agar, Bifidobacterium agar (Haibo Ltd. Co., Qingdao, China), respectively. The fecal slurry was diluted with PBS sequentially from 10^{-1} to 10^{-12} according to preliminary results, then 100 μl was spread onto agar plate. After incubation in anaerobic or aerobic atmosphere at 37 °C for 48 h, the number of colonies on the plate was counted.

7.2.4 DNA extraction, 16S rRNA gene sequencing and microbial composition analysis

The fermentation samples were centrifuged at 12000 rpm for 5 min. The residues were used for gene analysis and 1.4 ml of the supernatants for monosaccharide determination and 0.2 ml for SCFA analysis. DNA extraction of the sample was carried out using Tiangen stool DNA extraction kit (Tiangen, Beijing, China), according to the instruction provided by manufacturer. The microorganism DNA concentration was determined by NanoDrop 2000 (Thermo Fisher, Massachusetts, USA) to be 6-100 ng/μl, total initiation mass ≥ 30 ng. The 16S rRNA gene sequencing was conducted by BGI (Shenzhen, China). Database used for species annotation is Greengene (default): V201305[8]; RDP: Release11_5,20160930.

7.2.5 Quantification of monosaccharides by LC-MS

D-Arabinose, D-fructose, D-galactose, D-glucose, L-rhamnose monohydrate, D-mannose, D-glucose 13C1, trifluoroacetic acid (TFA), HPLC grade acetonitrile (ACN), and methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA) were used in Monosaccharide quantification. Individual stock solutions of each monosaccharide and internal standard (IS) D-glucose $13C_1$ were prepared at a concentration of 10 mg mL^{-1} with Milli-Q water. Working standard solutions were prepared by appropriate dilution of the stock solutions with ACN/H2O (50:50 v/v). External calibration standard solutions were prepared at concentrations from 0.5 to 50 μg/mL. Each external standard solution contained IS at a concentration of 1 μg/ml. All solutions were stored at 4 °C.

To investigate the monosaccharide composition of different EPS fractions, five milligrams of different EPS fractions were dissolved in 1.4 ml of water for overnight; to investigate the monosaccharides existed in fermentation samples, 1.4 ml of supernatant in 2.5 was collected. Then 0.6 ml TFA was added to achieve final acid hydrolysis condition (4 M

TFA). The solutions were transferred into hydrolysis tubes and were hydrolysed at 100 °C for 8 h. Excessive TFA was removed by vacuum evaporation and the residue was reconstituted with 2 mL of water. Further clean-up was accomplished by SPE. The SPE cartridge was preconditioned by 3 ml of methanol followed by 3 ml of ultrapure water. The sample solution was loaded onto the cartridge and the eluent was directly collected. The eluate was evaporated to dryness under vacuum and then reconstituted with 2 mL (or greater if response was too high) of ACN/ H_2O (50:50 v/v) for LC/MS/MS analysis. An amount of 0.01 mg of IS was added to the reconstituted solution. For evaluation of matrix effects, the vacuum-dried residue was reconstituted to 2 mL with ACN/H_2O (80:20 v/v) and was used to prepare matrix-containing standard solution. The treated samples were diluted to proper concentration by ACN/H_2O (80:20 v/v) before injection.

Liquid chromatographic separation was achieved using a normal phase method on an ACQUITY UPLC® BEH amide column (2.1mm×100mm, 1.7 μm particles; Waters) at a flow rate of 0.3 ml/min. Mobile phase A and B are water and acetonitrile, respectively (Honeywell, Burdick and Jackson, MS Grade). Monosaccharide elution was accomplished primarily by a linear gradient over 30 min from 95 to 60 % (v/v) mobile phase B, followed by a column wash and re-equilibration. Column temperature was maintained at 20 °C; autosampler plate temperature control was set at 4 °C. An Agilent 6460 HPLC system (Santa Clara, CA) was coupled in-line with an electrospray ionisation triple quadrupole mass spectrometer (Foster City, CA) equipped with an AJS electrospray ionisation source. Ions were detected using a multiple-reaction monitoring (MRM) method in negative polarity with a dwell time of 200 ms. Source conditions were as follows: gas temperature = 300 °C, gas flow = 8 l/min, nebulizer = 45 psi, sheath gas temperature = 320 °C, sheath gas flow = 11 l/min, capillary positive voltage = 3500 V (positive) and 3000 V (negative), nozzle voltage = 500 V. Data acquisition was performed using MassHunter Quantitative Analysis software.

7.2.6 Short chain fatty acid analysis

The short chain fatty acids (SCFAs) generated during the fermentation were analysed by gas chromatograph combined with a flame ionization detector (GC-FID) described in previous paper (Song et al., 2018). Briefly, the 0.2 ml of supernatant in 2.5 was diluted by 0.8 ml of milli-Q water and the pH value was adjusted to 2-3 with 1 M HCl. An internal standard (2-ethylbutyric acid) was added at 1 nM final concentration before injection into the GC system. An Agilent 7980B GC system accompanied with a flame ionization detector (FID) (Agilent Technologies Inc., USA) and a fused silica capillary column (dimension 30 × 0.32 mm) (DB-FFAP 123-3232, Agilent Technologies Inc.) was used in the determination. Nitrogen gas was applied as the mobile phase with a flow rate of 0.6 ml/min. The initial oven temperature 80 °C was maintained for 2 min, then raised gradually to 180 °C at 6 °C/min and maintained for 4min. The injection volume is 1 μl with injection temperature 200 °C. The detector temperature was controlled at 220 °C. Six SCFA standards (Aladdin®, Shanghai, China) were used for identification and quantification including acetic acid, propionic acid, n-butyric acid, i-butyric acid, n-valeric acid, and i-valeric acid. The n-butyric and i-butyric acid were combined in the analysis.

7.2.7 Statistical analysis

The one-way ANOVA was used to compare the monosaccharide composition of EPS fractions after fecal fermentation. The two-way ANOVA with Tukey's hoc tests was used for the comparison of cfu among different groups. Kruskal-Wallis test was used to compare the OTU difference between MC and experimental groups. SPSS 23.0 was applied for statistical analysis. The paired *t*-test was used to compare different taxonomic compositions between experimental and control groups.

7.3 Results and discussion

7.3.1 Monosaccharide composition of different EPS fractions

The molar ratio value of released monosaccharides in different EPS fractions was determined after hydrolyzation. As shown in Table 7-1, all four EPS fractions are mainly composed by mannose, glucose, galactose and fructose with different molar ratios. For EPS and EU, the molar ratio of four monosaccharides is similar (1.00:2.57:0.97:0.03 for EPS and 1.00:3.32:1.16:0.03 for EU). EHMW and ELMW were different with EPS or EU. A higher ratio of glucose was found in EHMW (1.00:6.83:1.04:0.07). In contrast, ELMW was composed by a lower ratio of glucose and higher ratio of galactose (1.00:0.34:2.02:0.01).

Table 7-1 Properties and composition of EPS fractions.

	Avg MW (Da)	[η], dl/g	Mannose	Fructose	Glucose	Galactose
EPS	1.93×10^8	6.03	1.00*	0.03	2.57	0.97
EPS-US	2.30×10^7	4.65	1.00	0.03	3.32	1.16
EPS-H	2.57×10^8	12.47	1.00	0.07	6.83	1.04
EPS-L	1.76×10^7	ND	1.00	0.01	0.34	2.02

* All standard variations < 5%

ND: not detected

As the Cs-HK1 EPS was resistant to hydrolysis by digestive enzymes and simulated gastric acid (Song, Mao, Siu, Tai, & Wu, 2019), the undigested EPS fractions were used in the following experiments.

7.3.2 Substrate consumption

From the Fig. 7-1, compared with the total monosaccharide contents at 0 h, MC was not utilized during the fermentation, since the total hydrolysed glucose in MC medium maintained

unchanged compared with the concentration at time 0 h. In contrast, four EPS fractions can be utilized well by gut flora with total monosaccharides concentration at 48 h ranged from 3.45% to 23.38% (time 0 h normalized to 100%). The fermentation of EPS, EU, EPS-L were started after 10 h of inoculation and utilized at a similar rate during 10 to 24 h in the fermentation course and no more fermentation during 24 to 48 h. In contrast, the total monosaccharides in EPS-H medium decreased steady from 10 to 48 h, and 60% of total carbohydrates remained at 24 h of fermentation, which indicated that EPS-H is a slower-fermented carbohydrate compared with other three EPS fractions. Although EPS-H was more resistant to the utilization, the concentration of total monosaccharides decreased to 5.07% compared the time 0 h. The concentration of different monosaccharides all decreased proportionally during the fermentation, which indicates that gut microbiome did not have an obvious preference on the degraded monosaccharides.

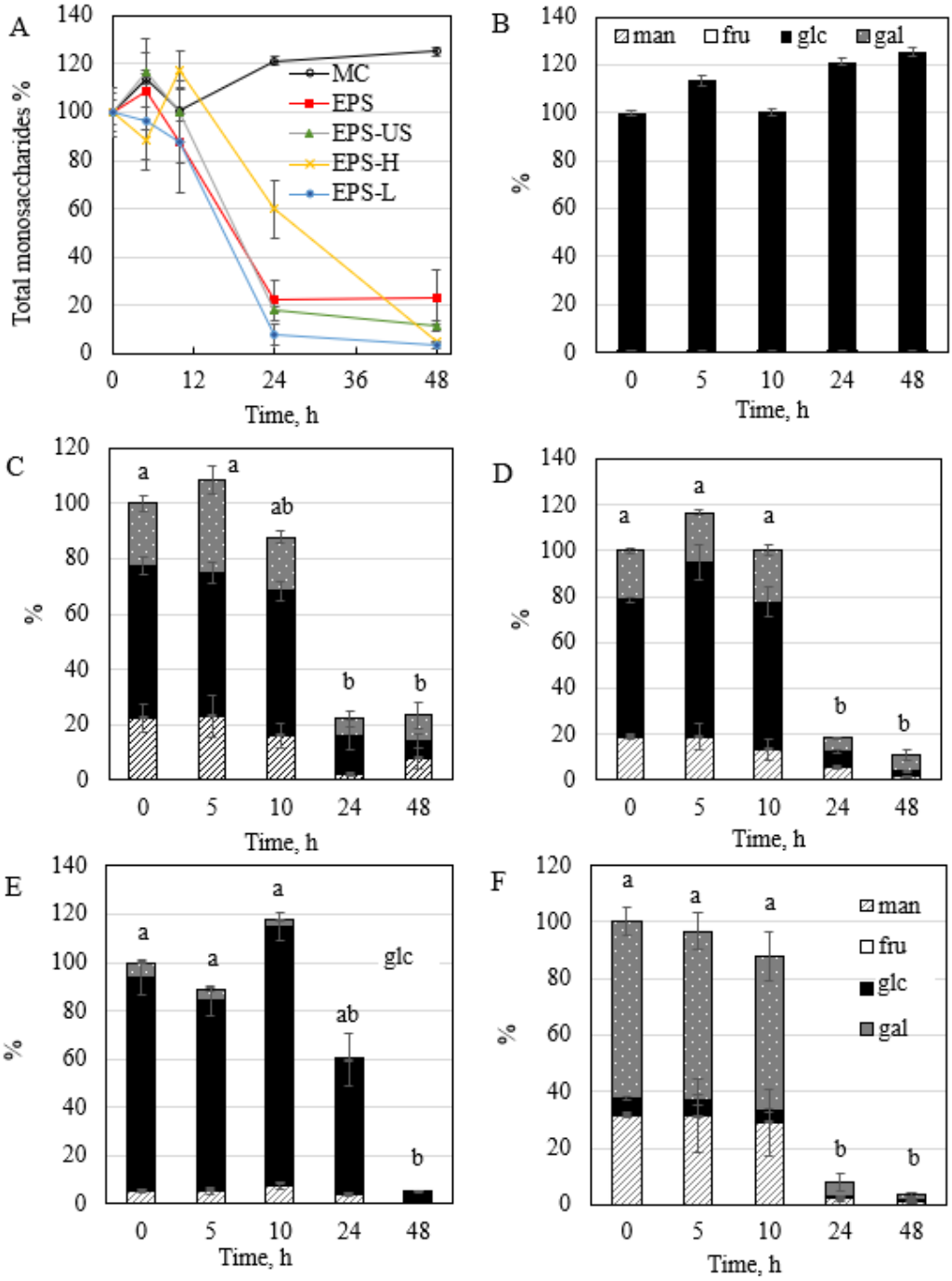

Figure 7-1. The changes of total monosaccharide concentration (A) and monosaccharide constitute (B-F: MC, EPS, EPS-US, EPS-H and EPS-L) of different EPS during fecal fermentation (3 donors). Total monosaccharides at 0 h was normalized to 100%. Significant differences ($p < 0.05$) of total monosaccharides (in %) between different time points with the same substrate are indicated with letters. One-way ANOVA with LSD hoc tests were used for the statistical analysis.

Table 7-2 shows the molar ratios of monosaccharides in the EPS fractions before and after 48 h of fecal fermentation. For the EPS-H and EPS-US fractions, the molar ratio of glucose decreased more dramatically, while that of galactose increased significantly over the fermentation period. This suggests that the majority of fecal bacterial community preferred using glucose as the major carbon source than galactose or mannose. In contrast, for the EPS-H and EPS-L fractions, galactose was consumed more during the fermentation. It is possible that the EPS-H and EPS-L was taken up as a whole and consumed by the fecal bacteria. It has been suggested that polysaccharides are mostly transported into and metabolized within probiotic bacterial cells (Pokusaeva, Fitzgerald, & van Sinderen, 2011; J. Zhao & Cheung, 2013). It has been suggested that the metabolism of polysaccharides in the gut microbiota is dependent on their molecular properties (Koropatkin, Cameron, & Martens, 2012).

Table 7-2 The change of monosaccharide composition (molar ratio) of EPS fractions over 48 hours of fecal fermentation (total molar ratio 100%).

	Time (h)	mannose	fructose	glucose	galactose
MC	0	ND [a]	0.30 ± 0.02 c [b]	99.70 ± 1.03 a	ND
	5	ND	0.67 ± 0.03 a	99.33 ± 2.19 a	ND
	10	ND	0.52 ± 0.02 b	99.48 ± 1.27 a	ND
	24	ND	0.50 ± 0.02 b	99.50 ± 1.78 a	ND
	48	ND	0.56 ± 0.02 ab	99.44 ± 1.87 a	ND
EPS	0	22.23 ± 5.00 b	0.59 ± 0.10 b	54.53 ± 3.00 ab	22.65 ± 3.00 b
	5	21.06 ± 7.43 ab	0.57 ± 0.09 b	47.28 ± 4.00 b	31.09 ± 5.07 ab
	10	18.26 ± 4.19 b	0.49 ± 0.11 b	59.21 ± 3.45 a	22.04 ± 2.16 b
	24	19.79 ± 0.60 b	0.13 ± 0.01 c	63.79 ± 5.37 a	26.29 ± 2.81 b
	48	34.56 ± 3.77 a	1.57 ± 0.19 a	24.84 ± 2.57 c	39.04 ± 4.82 a
EPS-US	0	18.86 ± 1.00 b	0.61 ± 0.05 a	59.20 ± 1.00 a	21.34 ± 1.00 c
	5	16.06 ± 5.76 bc	0.66 ± 0.10 a	64.74 ± 7.45 a	18.54 ± 1.18 c
	10	13.09 ± 4.35 bc	0.53 ± 0.12 a	63.93 ± 6.70 a	22.45 ± 2.45 c
	24	29.91 ± 0.57 a	0.37 ± 0.02 b	36.98 ± 0.89 b	32.74 ± 0.60 b
	48	13.03 ± 0.22 c	0.16 ± 0.01 c	23.94 ± 0.29 c	62.87 ± 2.30 a
EPS-H	0	5.32 ± 0.90 a	0.61 ± 0.10 a	87.95 ± 7.00 a	6.12 ± 0.50 a
	5	5.99 ± 1.57 a	0.67 ± 0.09 a	88.16 ± 6.48 a	5.19 ± 0.84 a
	10	6.65 ± 1.40 a	0.61 ± 0.11 a	90.53 ± 5.87 a	2.21 ± 0.50 b
	24	6.44 ± 0.99 a	0.61 ± 0.11 a	92.73 ± 10.92 a	0.22 ± 0.05 c
	48	6.88 ± 0.12 a	ND b	93.12 ± 0.26 a	ND d
EPS-L	0	31.71 ± 1.00 a	0.34 ± 0.10 a	5.46 ± 0.50 c	62.48 ± 5.00 a
	5	32.36 ± 12.90 a	0.34 ± 0.12 a	5.78 ± 1.79 c	61.51 ± 6.60 a
	10	32.93 ± 11.92 a	0.34 ± 0.09 a	4.61 ± 0.70 c	62.12 ± 8.89 a
	24	26.40 ± 0.95 b	0.27 ± 0.02 a	11.71 ± 0.15 b	61.62 ± 3.17 a
	48	36.82 ± 0.89 a	ND b	16.61 ± 0.03 a	46.57 ± 0.68 b

[a] ND: not detected

[b] Different letters mean significantly ($p < 0.05$) from each other in the same column with the same substrate fermentation.

7.3.3 The pH and Short chain fatty acid production in fecal batch cultures

Analysis of pH value change and SCFA production at different time points during the fermentation course confirmed that the fermentation of EPS, EU, EHMW and ELMW accelerated after 10 h of inoculation (Fig. 7-2&7-3). For EPS, EU and ELMW, the pH maintained > 6 during the first 10 h, followed by a decrease to pH < 5 at 24 h, and maintained unchanged or a slight further decrease at 48 h. For EHMW, the pH maintained 5.45 at 24 h and the decrease of pH from 24 to 48 h is larger than that from 10 to 24 h (Fig. 7-2). It is noteworthy that the pH drops in all EPS groups at 48 h (Δ pH ranged from 2.2 to 3.04) were larger as compared to a pH drop to 5.85 at 48 h (Δ pH 1.42) observed for MC group.

The pH decreased as a result of SCFA production. In line with pH change, the greatest increase in the concentration of total SCFAs was generally observed from 10 to 24 h, followed by a further slighter increase from 24 to 48 h (Fig. 7-3). The concentrations of acetic acid, propionic acid and butyric acid produced by four EPS fractions at 24 or 48 h were significantly higher than MC at the same time points (except for butyric acid production of EU and ELMW at 48 h). For four EPS fractions, the most predominant SCFA produced was acetic acid, then the propionic acid and butyric acid. For all EPS fractions, the total SCFAs at 48 h are generally in line with pH change that no significance was found among different EPS groups except EU. It should be noted that EPS with higher MW seemed to produce more butyric acids and the concentration of butyric acid produced by EHMW fermentation (8.67 mM) was approximately one time higher than other three EPS groups (3.38 to 4.58 mM). This outstanding performance of EHMW on butyric acid production may due to its structure and composition containing high concentration of glucose that may be β-glucan, which has an ability to increase butyric acid formation in intestine (Hamer et al., 2008; Zielke et al., 2017).

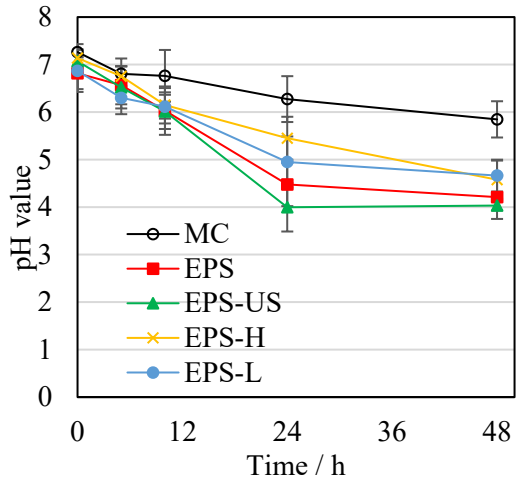

Figure 7-2 The change of pH value after inoculation (0 h) and after 5, 10, 24 and 48 h of fermentation. Error bar indicate SD (n=3).

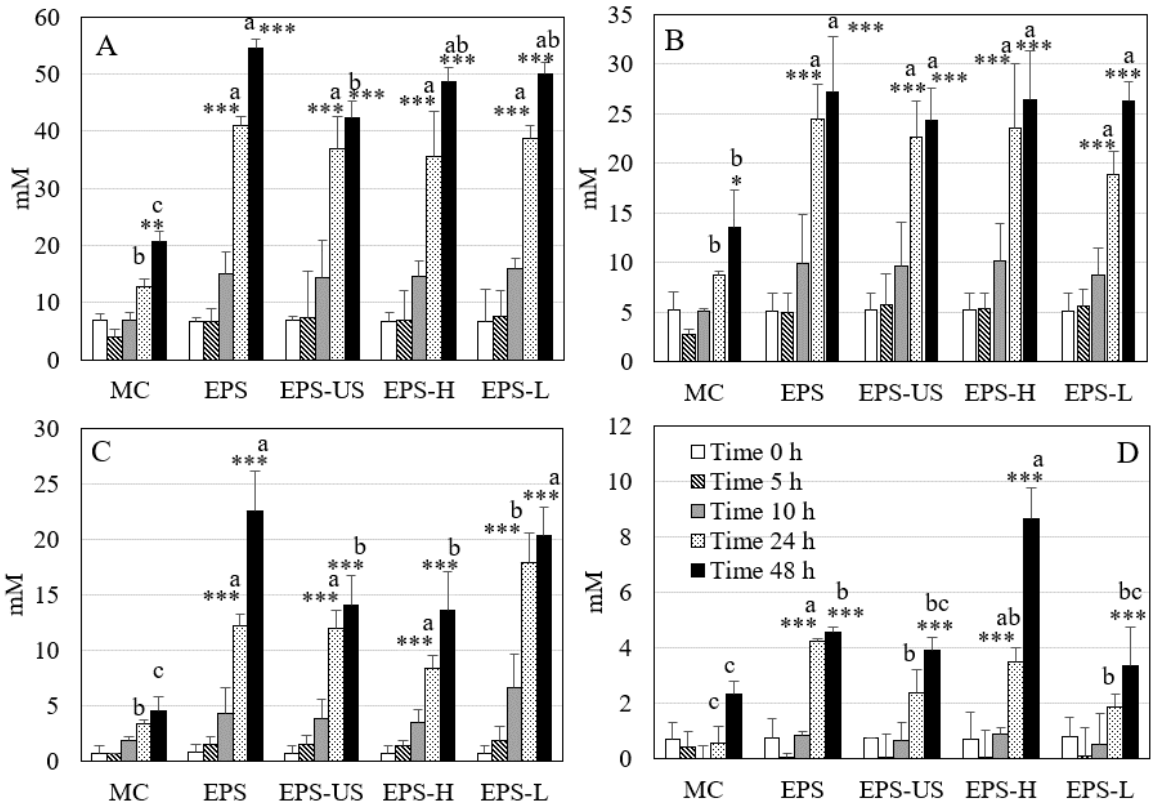

Figure 7-3 Total SCFAs (A), acetic acid (B), propionic acid (C) and butyric acid (D) concentrations (mM) obtained in batch cultures after inoculation (0 h) and after 5, 10, 24 and 48 h of fermentation of different EPS fractions. Error bar indicates SD (n=3). Significant differences ($p<0.05$) between substrate at the same time point are indicated with letters. Asterisks indicate significantly different compared to 0 h within the same substrate *$p<0.05$; **$p<0.01$; ***$p<0.001$. Two-way MANOVA with Tukey's hoc tests were used for the statistical analysis.

7.3.4 Enumeration of specific bacterial population

Although recent advances in technology enable the evaluation of microbiota more efficiently, enumeration by traditional cultivation on selective agar plates can assess the viability of bacteria while gene analysis cannot. As shown in Fig. 7-4, fermentation time affected the bacterial cfu more than the substrate did. The enumeration roughly increased in all experimental and control groups as fermentation time extended. No significant change of the different groups of bacterial cells were found after 24 or 48 h of fermentation of different EPS fractions compared with MC. However, it is notable that EPS significantly increased the cfu of Bifidobacterium, Clostridium, total anaerobic and aerobic bacteria, at 10 h compared with MC. But this significance disappeared since there was a slightly decrease of cfu at 24 or 48 h. In our previous study, we found that adding EPS with higher MW (EPS and EPS-H) can protect bifidobacteria from the damage of antibiotic through forming a slim layer outside the bacteria (Y.-H. Mao et al., 2018). Maybe this protection mechanisms explains the increase of cfu at the beginning of the fermentation, however, as layers formed by EPS fractions began to be utilized by bacteria, this protection disappeared. In general, four EPS fractions showed no significant effects in increasing the cfu of different bacteria after enough fermentation time as long as normal transit time in colon. From our results, we found that EPS have double effects on microbiota that protective effects before and nutritious function after being utilized. On the other side, since most bacteria cannot be cultured by selective agar plates (Zhu, Wang, & Li, 2010), gene analysis is also necessary to investigate the change of microbial composition.

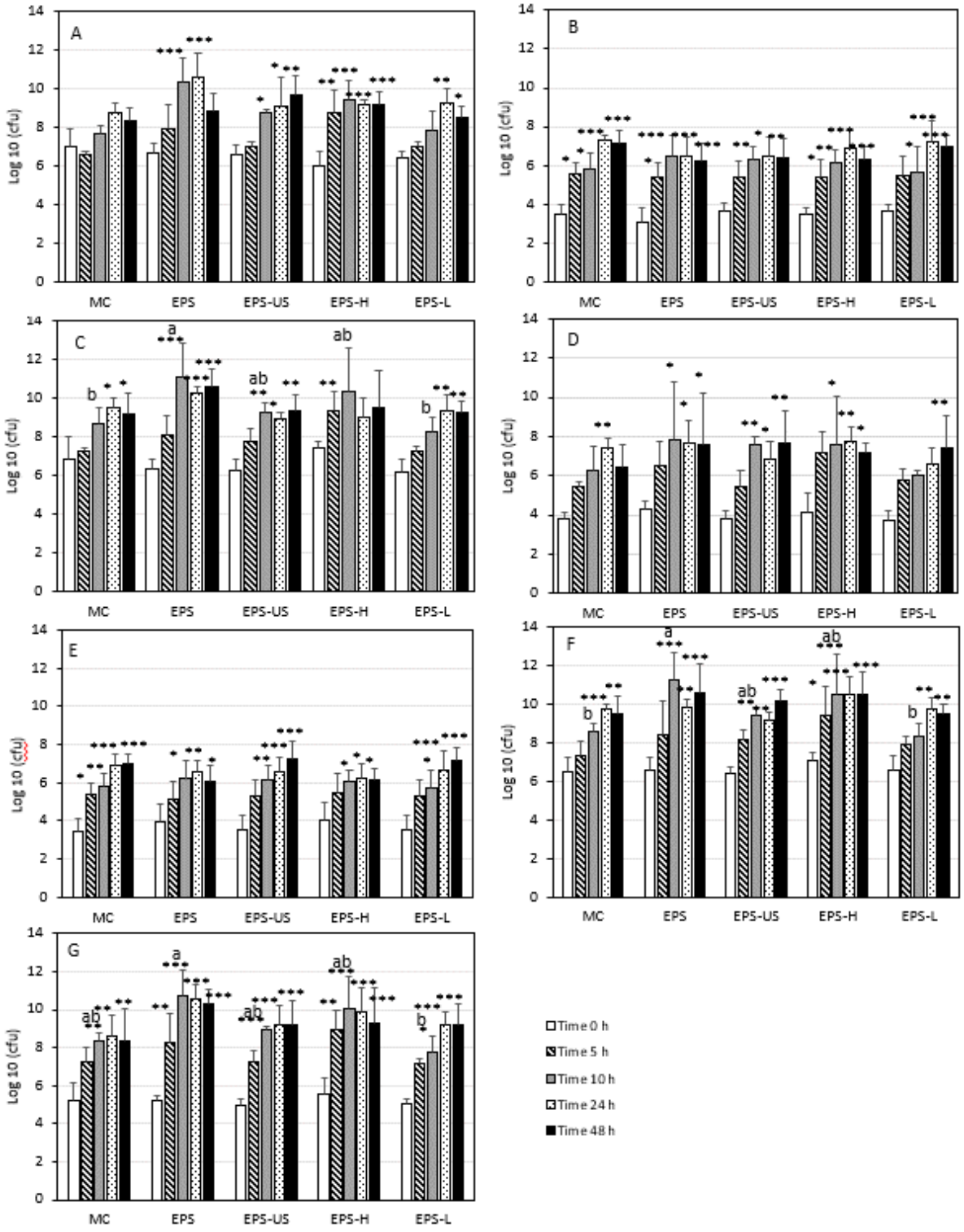

Figure 7-4 Log colony form unit (cfu) of different groups of bacteria at 0, 5, 10, 24 and 48 h of fermentation with different EPS fractions by selective agar plate. Error bar indicates SD (n=3). Significant differences between substrate at the same time point are indicated with letters. Asterisks indicate significantly different compared to 0 h within the same substrate *P<0.05; **p<0.01; ***p<0.001. Two-way MANOVA with Tukey's hoc tests were used for the statistical analysis.

7.3.5 Impact of EPS on diversity of fecal microflora

There was no shift in the microbial diversity observed in the fecal microflora with MC or any of the EPS fractions (Kruskal-Wallis test, *p*-value = 0.556) (Fig. 7-5). In contrast, the fecal materials from different donors affected the results of in vitro fecal fermentation (Kruskal-Wallis test, *p*-value = 0.022) (Fig. 7-6), which may contribute to the reduced richness in D3 incubations (Dunn's test, *p*-value < 0.1). Although the diversity or richness of bacterial populations is generally regarded as an indicator for the healthy balance and resilience of gut microbiota (Le Chatelier et al., 2013b), some previous studies have observed that the high microbiome richness indices were associated with gastrointestinal disorders including prolonged transit times (Roager et al., 2016), hard stools (Vandeputte et al., 2016), and increased proteolytic fermentation (Macfarlane, Cummings, Macfarlane, & Gibson, 1989). This signifies a complex relationship between community richness and gut health.

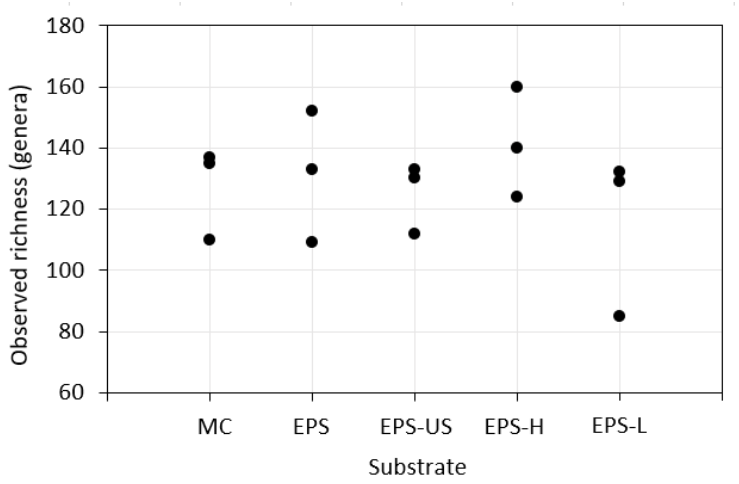

Figure 7-5 After 24 hours of incubation with the substrates, no significant substrate-specific differences in observed richness were found (Kruskal-Wallis test, *p*-value = 0.556).

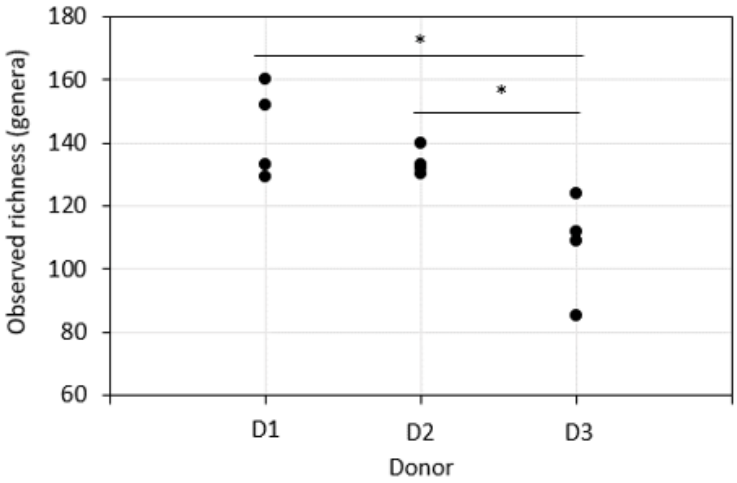

Figure 7-6 Donor-specific differences in observed richness were still present after 24 hours of incubation with the substrates (Kruskal-Wallis test, p-value = 0.022; each data point represent richness observed after a single 24 hours EPS fraction incubation). These were attributed to lowest richness in D3 incubations (Dunn's test). Dunn's adj p-value < 0.1.

7.3.6 Effect of EPS on composition of fecal microflora

To identify substrate specific responsive genera, the taxa relative abundances after 24 h of EPS fermentations were compared with the outcome of MC incubations in matching donor fecal slurries at different levels (Fig. 7-7). On phylum level, Cs-HK1 EPS fractions were found to result in increased Firmicutes relative abundances (paired *t*-test, EPS, p=0.059; EPS-US, p=0.058; EPS-H, p=0.042; EPS-L, p=0.006) with an expense of Proteobacteria relative abundances(EPS, p=0.031; EPS-US, p=0.13; EPS-H, p=0.013; EPS-L, p=0.014) (Table 7-3). On family level, EPS fractions decreased the relative abundance of *Desulfovibrionaceae* (EPS, p=0.043; EPS-US, p=0.034; EPS-H, p=0.045; EPS-L, p=0.033) and *Enterobacteriaceae* (EPS, p=0.029; EPS-US, p=0.15; EPS-H, p=0.008; EPS-L, p=0.017). On genus level, EPS fractions were found to induce specific changes in slurry microbiota compositions. The genera *Escherichia* belonging to the family *Enterobacteriaceae* (EPS, p = 0.018; EPS-H, p = 0.015; EPS-L, p = 0.012), *Bilophila* belonging to the family *Desulfovibrionaceae* (EPS-US, p = 0.048; EPS-L, p = 0.049), *Parabacteroides* (EPS-US, p = 0.026) and *Phascolarctobacterium* (EPS-

US, p = 0.029) were underrepresented following different EPS fraction fermentations (Table 7-4). In contrast, the only significant increase (p = 0.029) was found on EPS-L in *Phascolarctobacterium* relative abundance. Unexpectedly, all EPS fractions (p > 0.05) had no significant influence on increasing *Bifidobacterium* relative abundances although the SCFA production increased significantly. In general, the addition of Cs-HK1 EPS fractions to fecal slurries resulted in the expense of the *Proteobacteria*. Of all EPS fractions under investigation, EPS-US and EPS-L displayed the broadest impact range on microbiota composition that induced significant changes on three genus relative abundances.

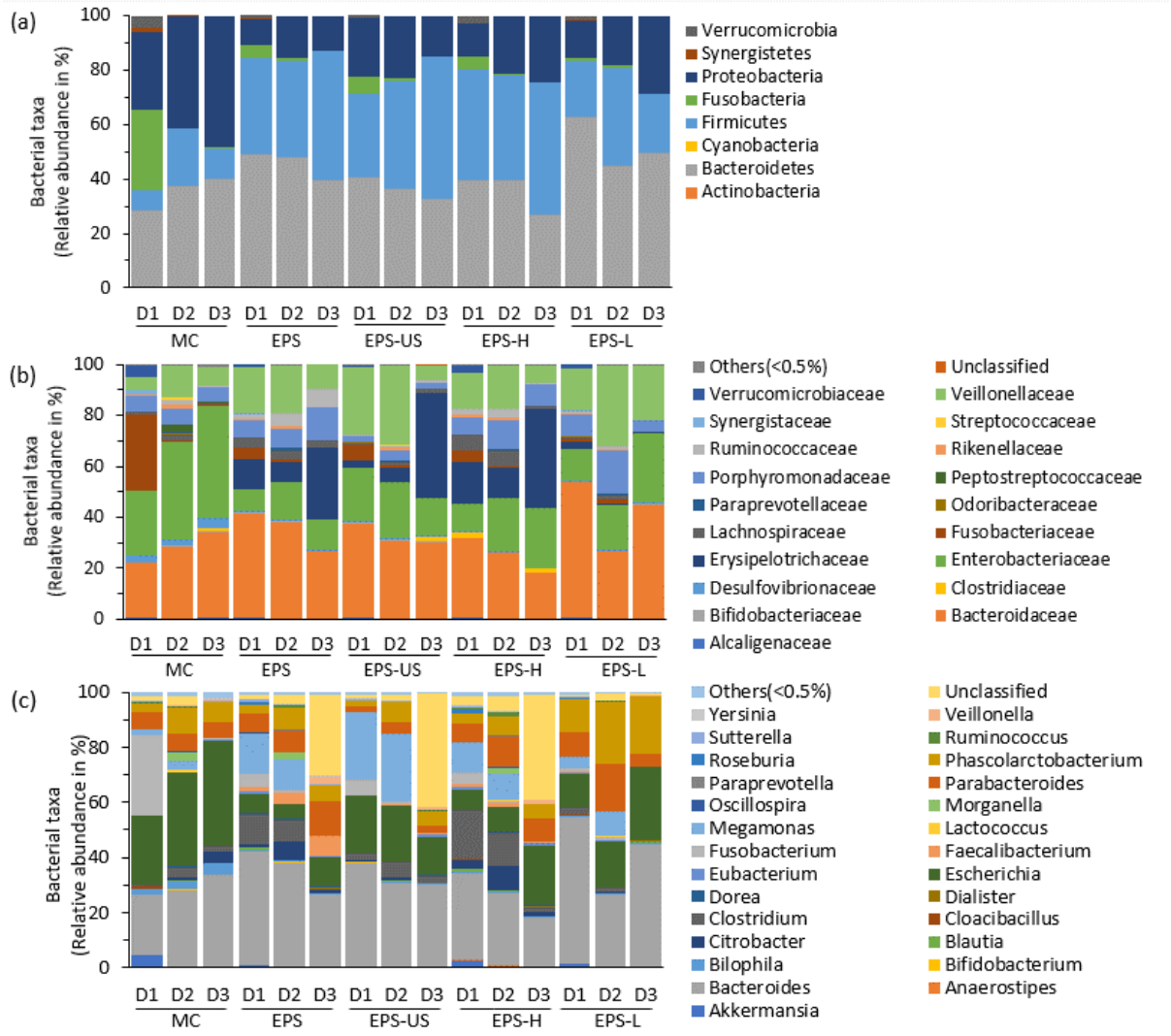

Figure 7-7 The taxonomic composition distribution at the phylum (a), family (b) and genus level (c).

The compositional changes of the gut microbial community are associated with many diseases. Although the mechanisms driving this dysbiosis are incompletely unveiled, the most robust and consistent ecological pattern observed during intestine dysbiosis is an expansion of bacteria that belong to the phylum *Proteobacteria* (Yael Litvak, Mariana X Byndloss, and, & umler, 2017). As the largest and most diverse phylum of bacteria, *Proteobacteria* were associated with many diseases (Mukhopadhya, Hansen, El-Omar, & Hold, 2012). In Proteobacteria, a great number of evidences showed that *E. coli* is associated with gastrointestinal disease, particular in inflammatory disorders such as Crohn's disease and ulcerative colitis (Arnott et al., 2004; Mow et al., 2004; Tabaqchali, O'Donoghue, & Bettelheim, 1978). Therefore, the significant reduction of *Proteobacteria* (particularly *E. coli*) ratio with inoculation of EPS fractions found in this study may indicate a beneficial effect on gut microbiota.

Unexpectedly, no significant changes ($p > 0.05$) on the relative abundance of *Bifidobacterium* with the addition of EPS fractions (Table 7-5). This may closely relate to the low relative abundance of this genera originally in fecal materials provided by three donors (0.24%, 0.26% and 1.01%, respectively). Therefore, EPS fractions failed to effectively enhance the relative abundance of *Bifidobacterium* because of the competitive inhibition from other abundant genera could utilize polysaccharides (Shi, Odt, & Weimer, 1997). However, the relative abundances of butyrate producers *Eubacterium* (EPS, p=0.32; EPS-US, p=0.307; EPS-H, p=0.152; EPS-L, p=0.354), *Faecalibacterium* (EPS, p=0.136; EPS-US, p=0.098; EPS-H, p=0.118; EPS-L, p=0.17) and *Roseburia* (EPS, p=0.425; EPS-US, p=0.474; EPS-H, p=0.419; EPS-L, p=0.448) (Table 7-5) all increased in each donor - EPS fraction (especially EPS-H) combination although without statistical significance because of great variations, which is consistent with the results of SCFA production. Nevertheless, the effects of substrate on gut microbiota should be considered with combination with metabolisms and functions instead of

microbial composition alone (Flint, Duncan, Scott, & Louis, 2015). To minimize the variation among donors, the enterotype classification may also be considered in future study (Arumugam et al., 2011; Tremaroli & Backhed, 2012).

Table 7-3 Paired *t*-test (*p* values) of the relative abundance on phylum level between samples inoculated with methylcellulose and different EPS fractions

	EPS	EPS-US	EPS-H	EPS-L
Acidobacteria	-	-	-	-
Actinobacteria	0.181	0.643	0.12	0.709
Bacteroidetes	0.231	0.849	0.999	0.187
Cyanobacteria	0.407	0.999	0.291	0.662
Firmicutes	0.059	0.058	**0.042**	**0.006**
Fusobacteria	0.433	0.428	0.42	0.434
Proteobacteria	**0.031**	0.13	**0.013**	**0.014**
Synergistetes	-	-	-	-
Verrucomicrobia	-	-	-	-
Firmicutes/Bacteroidetes	0.148	0.102	0.112	0.071
Firmicutes/Proteobacteria	**0.037**	0.114	0.061	0.059
Firmicutes/Actinobacteria	0.053	0.313	0.06	0.147

Table 7-4 Paired *t*-test (*p* values) of the relative abundance on family level between samples inoculated with methylcellulose and different EPS fractions.

	EPS	EPS-US	EPS-H	EPS-L
Alcaligenaceae	0.289	0.231	0.569	0.213
Bacteroidaceae	0.45	0.488	0.731	0.298
Bifidobacteriaceae	0.169	0.522	0.102	0.794
Clostridiaceae	0.535	0.56	0.44	0.377
Desulfovibrionaceae	0.043	0.034	0.045	0.033
Enterobacteriaceae	0.029	0.15	0.008	0.017
Erysipelotrichaceae	0.128	0.317	0.115	0.296
Fusobacteriaceae	0.433	0.428	0.42	0.434
Lachnospiraceae	0.078	0.775	0.129	0.039
Odoribacteraceae	0.571	0.396	0.591	0.357
Paraprevotellaceae	0.423	0.423	0.423	0.423
Peptostreptococcaceae	0.393	0.395	0.393	0.392
Porphyromonadaceae	0.281	0.026	0.167	0.381
Rikenellaceae	0.853	0.565	0.708	0.535
Ruminococcaceae	0.135	0.781	0.136	0.368
Streptococcaceae	0.33	0.336	0.32	0.293
Synergistaceae	0.422	0.422	0.422	0.421
Veillonellaceae	0.168	0.211	0.269	0.023
Verrucomicrobiaceae	0.423	0.423	0.423	0.423
Unclassified	0.612	0.479	0.148	0.485
Others (<0.5%)	0.272	0.637	0.705	0.156

Table 7-5 Paired *t*-test (p values) of the relative abundance on genus level between samples inoculated with methylcellulose and different EPS fractions.

	EPS	EPS	EPS-US	EPS-H
Akkermansia	0.423	0.423	0.423	0.423
Anaerostipes	0.363	0.314	0.217	0.979
Bacteroides	0.45	0.488	0.731	0.298
Bifidobacterium	0.169	0.522	0.102	0.794
Bilophila	0.062	0.048	0.059	0.049
Blautia	0.703	0.524	0.612	0.463
Citrobacter	0.675	0.451	0.468	0.329
Cloacibacillus	0.422	0.422	0.422	0.421
Clostridium	0.325	0.075	0.233	0.765
Dialister	0.479	0.402	0.552	0.589
Dorea	0.434	0.928	0.271	0.381
Escherichia	0.018	0.138	0.015	0.012
Eubacterium	0.32	0.307	0.152	0.354
Faecalibacterium	0.136	0.098	0.118	0.17
Fusobacterium	0.431	0.427	0.419	0.432
Lactococcus	0.421	0.423	0.421	0.425
Megamonas	0.209	0.184	0.193	0.265
Morganella	0.421	0.422	0.424	0.422
Oscillospira	0.848	0.534	0.804	0.455
Parabacteroides	0.281	0.026	0.167	0.381
Parabacteroides	0.281	0.026	0.167	0.381
Paraprevotella	0.423	0.423	0.423	0.423
Phascolarctobacterium	0.19	0.029	0.257	0.014
Roseburia	0.425	0.474	0.419	0.448
Ruminococcus	0.422	0.309	0.277	0.106
Sutterella	0.275	0.221	0.551	0.214
Veillonella	0.439	0.526	0.461	0.667
Yersinia	0.952	0.529	0.51	0.293
Unclassified	0.415	0.438	0.374	0.144
Others (<0.5%)	0.01	0.041	0.351	0.003

7.4 Summary

In summary, despite the heterogeneous composition of the gut microbiome between donors and the limitations of in vitro batch fecal fermentation model, all the Cs-HK1 EPS fractions manifested beneficial changes in SCFAs and key microbiota. Three main SCFA productions were improved in all EPS groups, while EHMW remarkedly increased butyric acid production. Although no statistically significant changes were found on "beneficial" genera, the reduced relative abundance of genus *Escherichia* belonging to phylum *Proteobacteria* also indicates the possibility to achieve a healthier gut ecosystem by Cs-HK1 EPS. EPS with higher molecular weight showed protective effects on some groups of bacteria before they began to be utilized. Further animal studies with these Cs-HK1 EPS are required to confirm the potential beneficial effects.

Chapter 8 Effects of Cs-HK1 EPS on human fecal microflora exposed to antibiotics during in vitro fermentation

8.1 Introduction

Non-digestible carbohydrates (fibers) are the main source of nutrition for colonic bacteria (Flint, Scott, Duncan, Louis, & Forano, 2012). Bacterial fermented fibers produce short-chain fatty acids (SCFAs) (Wong, de Souza, Kendall, Emam, & Jenkins, 2006), which mainly include acetic acid, propionic acid and butyric acid (Cummings et al., 1979). SCFAs provide a host of benefits for the host, including providing an energy source for the intestine and initiating the hormone pathway (Sleeth, Thompson, Ford, Zac-Varghese, & Frost, 2010). Natural polysaccharides extracted from edible plants, fungi and seaweed are of interest as functional food ingredients and are beneficial to human health. Although many studies have studied the prebiotic effects of plant or fungal polysaccharides using an in vitro fermentation system that mimics the human gut (Gu et al., 2018; Monteagudo-Mera et al., 2018), only a few studies have investigated the possibility of prebiotics reducing antibiotic-induced intestinal microbiota disorder (Johnson et al., 2015 ; Ladirat et al., 2014). In addition, the results in the literature are inconsistent, and most of them focus on traditional prebiotics, such as galactooligosaccharides (GOS), fructooligosaccharides (FOS), xylooligosaccharides (XOS) (Miguez et al., 2018)and inulin, but only few studies on large molecular weight complex polysaccharides. Cordyceps sinensis is a rare medicinal fungus widely used to promote many physiological activities (Nakamura et al., 2015; L. Wang et al., 2011; Yoshida et al., 1989; Zhou et al., 2009), and artificial culture is now commonly used to meet the growing needs. Konjac glucomannan (KGM) isolated from the tubers of Flaorphophallus konjac C. Koch is commonly used as a gelling and thickening agent in liquid foods and as edible film coating for foods and

pharmaceuticals. Our previous studies have shown that exopolysaccharides (EPS) (Mao, Song, Wang, Yao, & Wu, 2018) and KGM (Y.-H. Mao et al., 2018) extracted from the fermentation liquid of Cordyceps sinensis Cs-HK1 mycelium can protect bifidobacteria from antibiotic damage. However, pure culture of single strains lacks consideration of the interaction between complex taxa in the human gut.

This study aimed to explore whether dietary supplementation can be used to reduce the adverse effects of antibiotic treatment on gut microbiota diversity and functioning by using in vitro fecal fermentation. If so, this might help to devise refined interventions combining antibiotic and prebiotic use. We measured the interactive effects of antibiotics and dietary supplements on faecal microbial composition and SCFA production in anaerobic batch cultures. Ampicillin was applied in this study because it is a widely used broad-spectrum antibiotic. We monitored bacterial composition by Illumina-sequencing of the 16S rRNA gene. We also tracked counts of several typical groups of bacteria over 48 h using selective agar culture. The substrate utilization and SCFA production were determined using mass spectrometry (MS) and gas chromatography (GC).

8.2 Material and methods

8.2.1 Fecal slurry preparation

Fecal material was donated by three healthy volunteers (two females, one male; aged 26-30). Donors have no known gastrointestinal or metabolic disorders and have not received any antibiotic treatment, probiotics or prebiotic supplements for at least 3 months prior to the experiment. Fecal samples were collected in sterile tubes and immediately stored in an anaerobic tank containing an anaerobic sachet (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) and then used within 30 minutes after collection. Fecal samples were diluted by10 times the volume of pre-reduced sterile phosphate buffered saline (PBS) and homogenized for 2

minutes and centrifuged at 500 rpm for 5 minutes to remove large particles. The resulting stool suspension was used as an initial inoculation in batch culture.

8.2.2 Substrates and antibiotics

According to our previous studies (Song et al., 2018), the Cs-HK1 mycelium was fermented in a shake flask at 20 ° C for 6 days, and the exopolysaccharide (EPS, 2.25×10^8 Da) was separated from the Cs-HK1 liquid fermentation medium by ethanol precipitation. Konjac glucomannan (KGM) was provided by Hubei Kangsen Konjac Gum Co., Ltd. (Ezhou, Hubei, China). In the study, galactooligogalactose (GOS) with a purity of 80% from New Francisco Biotechnology Co., Ltd. and inulin (from Dahlia tubers, DP≈36) purchased from Sigma (St. Louis, MO, USA) were used as traditional prebiotic references. Non-fermentable methylcellulose (MC) (Sigma, Saint Louis, USA) was used as a blank control. Ampicillin from Yuanye Co. Ltd. (Shanghai, China) was added to the fermentation tube by the experimental dosage (1 mg / 6 ml) reported in a previous study (Johnson et al., 2015) (Johnson et al., 2015).

8.2.3 *In vitro* batch cultures

One liter of the basal medium contained peptone water 2 g, yeast extract 2 g, NaCl 0.1 g, dipotassium phosphate 0.04 g, monopotassium phosphate 0.04 g, sodium bicarbonate 2 g, magnesium sulfate heptahydrate 0.01 g, calcium chloride hexahydrate 0.01 g, Tween-80 2 ml, hemin 50 mg, phylloquinone (vitamin K1, 10 ml), L-cysteine 0.5 g, bile salts 0.5 g, resazurin 1 mg and distilled water (Johnson et al., 2015). The control methylcellulose (Sigma, Saint Louis, USA), EPS, KGM, inulin and GOS were added at a concentration of 5 mg/ml to the respective fermentation tubes with vigorous stirring overnight. The pH was adjusted to 6.8 and then the medium was autoclaved. A 10 ml sterile tube was filled with 5.4 ml of sterilized enriched medium. Each tube was inoculated with 0.6 ml of freshly prepared fecal slurry (1:10,

w/w). Batch cultures were conducted for 48h, and samples were collected at 0, 5, 10, 24, 48 hours for counting bacterial populations, 16S rRNA gene sequencing, determination of carbohydrate utilization and SCFA production were carried out during the fermentation course.

8.2.4 Enumeration of bacteria

Plate counting of total anaerobic bacteria and total aerobic bacteria was carried out using BHI agar, *Clostridia, Bacillus coli, Staphylococcus, Enterococcus, Lactobacillus* and *Bifidobacterium* were counted by Tryptose Sulfite Cysloserine (TSC), MacConkey agar, Mannistol Salt Agar (MSA) Enterococcus agar, MRS agar, Bifidobacterium agar (Haibo Ltd. Co., Qingdao, China), respectively. According to the preliminary results, the fecal slurry was diluted by 10^{-1} to 10^{-12} with sterilized PBS sequentially, and then 100 μl was spread on an agar plate. After 48 hours of incubation at 37 °C in an anaerobic or aerobic atmosphere, the number of colonies on the plates was counted.

8.2.5 DNA extraction, 16S rRNA gene sequencing and microbial composition analysis

The fermentation samples was centrifuged at 12000 rpm for 5 min. The residue was used for genetic analysis, and 1.4 ml of the supernatant was used for monosaccharide determination, and 0.2 ml was used for SCFA analysis. DNA extraction of the samples was performed using a Tiangen stool DNA extraction kit (Tiangen, Beijing, China) according to the instructions provided by the manufacturer. The microbial DNA concentration was determined by NanoDrop 2000 (Thermo Fisher, Massachusetts, USA) to be 6-100 ng/μl, and the total initiation mass ≥ 30 ng. The 16S rRNA gene sequencing was performed by BGI (Shenzhen, China). The database used for species annotation is Greengene (default): V201305[8]; RDP: Release11_5,20160930.

8.2.6 Monosaccharide quantification by LC-MS

Analytical standards (D-Arabinose, D-fructose, D-galactose, D-glucose, L-rhamnose monohydrate, D-mannose, D-glucose $13C_1$), trifluoroacetic acid (TFA), HPLC grade acetonitrile (ACN), and methanol for monosaccharide quantification were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual stock solutions of each external and internal standard were prepared using Milli-Q water at a concentration of 10 mg mL^{-1}. Working standard solutions were prepared by appropriately diluting the stock solutions with ACN/H2O (50:50 v/v). External calibration standard solutions were prepared at concentrations from 0.5 to 50 μg/mL. Each external standard solution contained IS at a concentration of 1 μg/ml. All solutions were stored at 4 °C.

An amount of 1.4 ml supernatant in 8.2.5 was collected and 0.6 ml TFA was added to achieve the final acid hydrolysis condition (4 M trifluoroacetic acid). The solution was transferred to a hydrolysis tube and hydrolysed at 100 °C for 8 hours. Excessive TFA was removed by vacuum evaporation and the residue was re-dissolved with 2 ml of water. Further purification was accomplished with SPE. The SPE column was activated with 3 ml of methanol followed by 3 ml of ultrapure water. The sample solution was loaded onto the column and the eluent was collected directly. The eluate was evaporated to dryness under vacuum and then reconstituted with 2 ml (or greater, if response was too high) of ACN/ H_2O (50:50 v/v) for LC/MS/MS analysis. An amount of 0.01 mg of IS was added to the reconstituted solution. For evaluation of matrix effects, the vacuum-dried residue was reconstituted to 2 ml with ACN/H_2O (80:20 v/v) and was used to prepare matrix-containing standard solution. The treated samples were diluted to the appropriate concentration by ACN/H_2O (80:20 v/v) prior to injection.

Liquid chromatography was carried out using a normal phase method on an ACQUITY UPLC® BEH amide column (2.1mm×100mm, 1.7 μm particles; Waters) at a flow rate of 0.3

ml/min. Mobile phases A and B were water and acetonitrile (Honeywell, Burdick and Jackson, MS Grade), respectively. Monosaccharide elution was accomplished primarily by a linear gradient from 95 to 60 % (v/v) mobile phase B in 30 min followed by column washing and rebalancing. The column temperature was maintained at 20 °C; autosampler plate temperature control was set at 4 °C. An Agilent 6460 HPLC system (Santa Clara, CA) was coupled in-line with an electrospray ionisation triple quadrupole mass spectrometer (Foster City, CA) equipped with an AJS electrospray ionisation source. Ions were detected using a multiple-reaction monitoring (MRM) method in negative polarity with a dwell time of 200 ms. The source conditions were as follows: gas temperature = 300 °C, gas flow = 8 l/min, nebulizer = 45 psi, sheath gas temperature = 320 °C, sheath gas flow = 11 l/min, capillary positive voltage = 3500 V (positive) and 3000 V (negative), nozzle voltage = 500 V. Data acquisition was performed using MassHunter Quantitative Analysis software.

8.2.7. Short chain fatty acid analysis

The short chain fatty acids (SCFAs) produced during fermentation were analysed by gas chromatography in conjunction with a flame ionization detector (GC-FID) as described in a previous paper (Song et al., 2018). In brief, the 0.2 ml of supernatant in 8.2.5 was diluted with 0.8 ml of milli-Q water and the pH was adjusted to 2-3 with 1 M HCl. The internal standard (2-ethylbutyric acid) was added at a final concentration of 1 nM prior to injection into the GC system. Detection was performed using an Agilent 7980B GC system equipped with a flame ionization detector (FID) (Agilent Technologies Inc., USA) and a fused silica capillary column (dimension 30 × 0.32 mm) (DB-FFAP 123-3232, Agilent Technologies Inc.). Nitrogen gas was used as the mobile phase at a flow rate of 0.6 ml/min. The initial oven temperature was maintained at 80 °C for 2 min and then gradually raised to 180 °C at 6 °C/min and maintained for 4min. The injection volume was 1 µl and the injection temperature was 200 °C. The detector

temperature was controlled at 220 °C. Six SCFA standards (Aladdin®, Shanghai, China) were used for identification and quantification, including acetic acid, propionic acid, n-butyric acid, i-butyric acid, n-valeric acid, and i-valeric acid. In the analysis, n-butyric and i-butyric acid were combined as butyric acid.

8.2.8 Statistical analysis

Paired *t*-test was used for the comparison of cfu, SCFA, different taxonomic compositions between experimental and control groups. Non-parametric statistical analysis (Wilcoxon paired test) was used to compare the OTU difference between MC and experimental groups. SPSS 23.0 was applied for statistical analysis.

8.3 Results and discussion

8.3.1 The impact of ampicillin and EPS on the viability of bacteria

Fig. 8-1 shows that ampicillin significantly decreased the cfu of different groups of gut bacteria by ~10^2 (*Clostridium*) to ~10^5 times (*Enterococcus*) compared with the blank (only contained basal medium), except total anaerobic bacteria (decreased with no significance). This also reflected the property of ampicillin that can better kill enterococcus, even those antibiotic-resistant strains (Magdesian, 2017). EPS showed outstanding effect on enhancing cfu of all groups of bacteria (by ~10 to ~10^4 times), a results that was consistent with our previous study results that EPS would maintain the viability of bifidobacteria under harsh conditions including cold storage and simulated gastrointestinal conditions (Song, 2019). The most notable was that compared with ampicillin group, adding EPS along with ampicillin significantly increased the viabilities of nearly all groups of bacteria (except for *Lactobacillus*) to be comparable with or even higher than blank, which further indicated the protective effects of EPS on gut microbiome against ampicillin. In our previous study, EPS was found to enhance the tolerance

of five strains of *Bifidobacterium* to antibiotics by forming a slim layer outside the bacterial cells. Considering the thickening and nutritious effect of EPS, non-nutritious MC was used as control, and the protective effects of EPS on the bacterial variability were also compared with those of other commercial polysaccharides or oligosaccharides during the whole fermentation course.

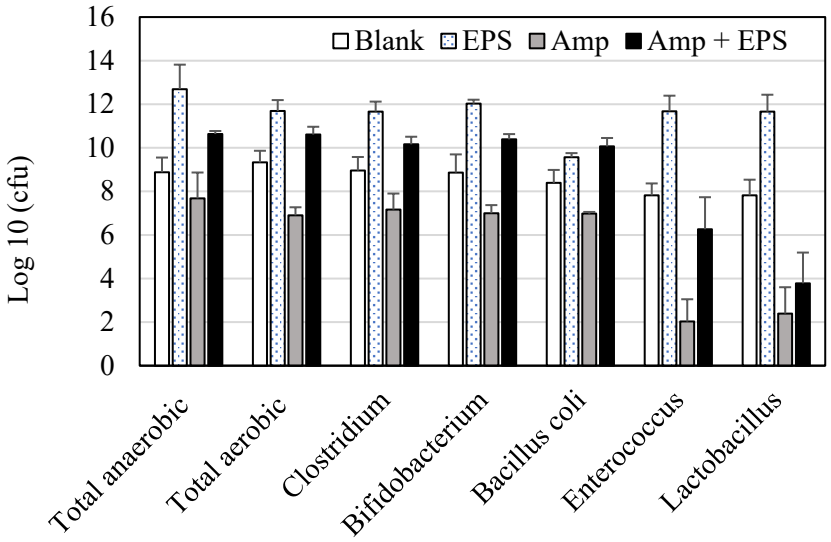

Figure 8-1 The log (cfu) of different groups of bacteria after 24-hour fecal fermentation under different conditions. Blank: only basal medium, EPS: 5 g/l, Ampicillin: 1 mg/6 ml. Error bar indicates SD (n=3).

8.3.2 EPS enhanced the bacterial viability in the presence of ampicillin

As shown in Table 8-1, EPS had a much wider influence on the viability of different groups bacteria at any time points during the whole fermentation course, though with no statistical significance for a few groups of bacteria (total anaerobic bacteria and Clostridium) at 48 h because of the large variation among donors. Especially during 5 to 24 h, the bacterial viability of four or five groups of bacteria was significantly ($p < 0.05$) increased by EPS compared with MC. In contrast, KGM barely changed the bacterial viability during former 24 h, while inulin and GOS changed the bacterial viability of a few groups at an earlier time

compared with MC. It was notable that inulin and GOS decreased the total anaerobic bacteria and *Bifidobacteria* after 5 h of fermentation, which was not consistent with some studies that concluded supplementation of prebiotics alleviated the damage on *Bifidobacteria* caused by antibiotics (Johnson et al., 2015). This difference might be due to the individual difference and some environmental conditions.

After 24 h of fermentation (the normal intestinal transit time), in the presence of ampicillin, EPS significantly increased the viable bacteria of five groups ($p < 0.05$). On the contrary, GOS decreased the live bacteria of several groups, especially *Enterococcus* ($p < 0.05$), in the presence of ampicillin. Additionally, inulin and KGM showed no significant effect on changing the number of live bacteria compared with MC. This finding was consistent with our previous minimum inhibitory (MIC) and bactericidal concentration (MBC) test that pointed out with supplementation of inulin or GOS, sometimes MIC or MBC tended to decrease rather than to increase (Yu-Heng Mao, Ang-Xin Song, Zhao-Mei Wang, Zhong-Ping Yao, & Wu, 2018). This might be due to the fact that the prebiotic accelerates the bacterial grow rate, which made the bacteria more sensitive to antibiotics (Stewart & William, 2001). Therefore, unlike under the antibiotic-free condition, the fast-fermented oligosaccharides or relative short-chain polysaccharides may perform as a double-edged sword in the presence of antibiotic. On one hand, fast-fermented carbohydrates would provide convenient nutrients for bacteria to grow faster, however, on the other hand, the fast growth weakened the tolerance of bacteria to antibiotics.

Table 8-1 The log(cfu) changes [a] of different groups of bacteria induced by different OS/PS after 0, 5, 10, 24 and 48 h fecal fermentation in the presence of ampicillin (compared with methylcellulose).

	EPS	KGM	Inu	GOS
0 h				
Total anaerobic bacteria	-0.03 ± 0.21	0.04 ± 0.12	0.07 ± 0.15	-0.10 ± 0.26
Total aerobic bacteria	-0.07 ± 0.15	-0.13 ± 0.29	0.10 ± 0.26	0.07 ± 0.06
Clostridium	-0.07 ± 0.15	-0.03 ± 0.21	-0.07 ± 0.32	-0.03 ± 0.40
Bifidobacterium	-0.03 ± 0.12	0.04 ± 0.21	0.03 ± 0.38	-0.03 ± 0.38
Bacillus coli	0.03 ± 0.12	-0.47 ± 0.55	-0.20 ± 0.85	-0.01 ± 1.14
Lactobacillus	0.20 ± 0.08 *	0.20 ± 0.01	0.35 ± 0.05 **	-0.01 ± 0.19
Enterococcus	0.13 ± 0.06 *	0.06 ± 0.14	0.06 ± 0.31	0.16 ± 0.31
5 h				
Total anaerobic bacteria	2.10 ± 0.31 **	-1.88 ± 1.45	-1.12 ± 0.37 **	-2.00 ± 1.14 *
Total aerobic bacteria	1.92 ± 0.38 **	-1.26 ± 0.83	-0.50 ± 0.80	-0.97 ± 1.17
Clostridium	2.46 ± 0.52 **	-0.95 ± 0.84	-0.80 ± 1.00	-1.49 ± 0.53 **
Bifidobacterium	1.73 ± 0.66 **	-1.52 ± 1.25	-1.15 ± 0.63 *	-1.83 ± 0.47 **
Bacillus coli	1.50 ± 0.45 **	-1.50 ± 1.15	-0.68 ± 0.86	-1.66 ± 0.90 *
Lactobacillus	0.71 ± 0.22 **	0.01 ± 0.39	0.01 ± 0.70	0.06 ± 0.91
Enterococcus	0.68 ± 0.53	-0.47 ± 0.55	-0.70 ± 0.49	-1.30 ± 0.90
10 h				
Total anaerobic bacteria	1.73 ± 0.45 **	-1.85 ± 0.44 **	-1.11 ± 0.72	-0.71 ± 1.54
Total aerobic bacteria	2.31 ± 0.39 **	-0.48 ± 1.76	-0.39 ± 1.24	0.99 ± 0.72
Clostridium	2.55 ± 0.56 **	-0.49 ± 0.70	-1.14 ± 1.28	-0.72 ± 1.57
Bifidobacterium	1.87 ± 0.81 *	-1.24 ± 1.70	-1.31 ± 1.10	-1.02 ± 1.38
Bacillus coli	2.29 ± 0.54 **	-1.33 ± 1.30	-1.78 ± 0.95 *	-0.04 ± 1.40
Lactobacillus	0.27 ± 0.37	0.16 ± 0.29	-0.16 ± 0.42	-0.28 ± 0.62
Enterococcus	-0.19 ± 0.36	-0.57 ± 1.00	-1.05 ± 0.73	-1.73 ± 0.98 *
24 h				
Total anaerobic bacteria	1.69 ± 0.48 **	-0.40 ± 1.47	-0.35 ± 0.77	0.30 ± 1.49
Total aerobic bacteria	1.75 ± 0.63 **	-0.12 ± 1.43	-0.42 ± 0.58	0.12 ± 1.83
Clostridium	1.35 ± 0.41 **	0.17 ± 0.82	-0.97 ± 1.67	-0.83 ± 1.2
Bifidobacterium	1.95 ± 0.28 **	0.10 ± 1.22	0.38 ± 0.31	0.37 ± 1.03
Bacillus coli	1.93 ± 0.59 **	-0.28 ± 1.25	-0.56 ± 1.57	-0.51 ± 0.9
Lactobacillus	0.98 ± 0.04 **	0.11 ± 0.77	0.09 ± 0.77	-0.43 ± 1.26
Enterococcus	0.28 ± 0.88	-0.39 ± 0.54	-0.14 ± 0.89	-1.58 ± 0.3**

	EPS	KGM	Inu	GOS
48 h				
Total anaerobic bacteria	4.11 ± 2.72	-0.31 ± 1.36	1.68 ± 0.86 *	-0.53 ± 0.44
Total aerobic bacteria	2.27 ± 0.36 **	0.60 ± 0.99	0.76 ± 0.59	-0.40 ±1.48
Clostridium	1.43 ± 1.04	-1.97 ± 1.13 *	-1.18 ± 1.70	-1.30 ± 1.60
Bifidobacterium	2.90 ± 1.41 *	0.70 ± 0.10 **	1.66 ± 0.48 **	-0.77 ± 0.41 *
Bacillus coli	2.15 ± 0.82 **	-4.70 ± 5.71	-0.63 ± 0.89	-1.43 ± 1.44
Lactobacillus	0.52 ± 1.07	0.35 ± 1.10	-0.69 ± 1.47	-0.50 ± 1.78
Enterococcus	0.25 ± 1.14	0.56 ± 1.00	-0.54 ± 2.32	-1.12 ± 3.08

a Values were mean ± SD of three donors.

* means p-value < 0.1, ** means p-value < 0.05, by paired t-test.

In some previous studies, both EPS and KGM were shown to protect the bifidobacterial strains against antibiotics in pure culture (Y. H. Mao, A. X. Song, Z. M. Wang, et al., 2018; Y. H. Mao, A. X. Song, Z. P. Yao, & J. Y. Wu, 2018). But in this study, KGM caused no remarkable changes on Bifidobacteria until after 48 h of fermentation. Furthermore, the slight increase on log (cfu) (Δ log(cfu) 0.70) might only due to the cross-feeding by microbiota (nutritious effect). The disappearance of the protective effect of KGM might be related to its property change in different solutions. The viscosity of KGM solution in Reinforced clostridium medium (RCM) used for pure culture was higher than that in fecal fermentation basal medium used in this study. It is suggested that viscosity affects antibiotic susceptibility not only by reducing diffusion, but also by altering the phenotype of the planktonic bacteria more resistant to antibiotics, as seen in biofilms (Kostenko, Ceri, & Martinuzzi, 2007).

8.3.3 Fecal fermentation impact microbiota composition

Generally, 24 h fermentation of MC resulted in no significant changes of genus richness when compared to donor material (paired t-test, p = 0.146; Table 8-2). However, the relative abundance of *Proteobacteria* was increased by the in vitro fermentation condition (paired t-

test, p = 0.013) (Table 8-3). The changes in taxa abundances included the increased relative abundance of *Escherichia* (paired *t*-test, p = 0.043) and reduced proportions of *Blautia* (p = 0.03), *Butyricicoccus* (p = 0.087), *Roseburia* (p = 0.083) and *Streptococcus* (p = 0.066) (Table 8-4). MC incubations were set up with fecal material from each volunteer. Given its limited fermentability by colon bacteria (Mudgil and Barak, 2013), in these control cellulose fermentations, the effect of experimental conditions was expected to dominate over the impact of substrate fermentation. Hence, they allowed assessing the impact of the experimental set-up on the fecal microbiota composition. In summary, experimental conditions were shown to induce proportional blooming of *Proteobacteria*, but there were no changes on bacterial richness. The explosion of *Proteobacteria* under in vitro fermentation condition was also reported in another study (Kevin D'hoe & Sara Vieira-Silva, 2018).

Table 8-2 Paired-T test (p values) of the OTU between samples inoculated with methylcellulose and initial feces, different antibiotics and MC groups.

	Feces after fermentation compared to Initial	Ampicillin compared to no antibiotic
OTU	0.146	0.162

Table 8-3 Paired-T test (p values) of the relative abundance of major phylum between samples inoculated with methylcellulose and initial feces, different antibiotics and MC groups.

	Feces after fermentation compared to Initial	Ampicillin compared to no antibiotic
Actinobacteria	1	0.349
Bacteroidetes	0.095	0.146
Firmicutes	0.559	0.525
Proteobacteria	**0.013**	**0.049**

8.3.4 Ampicillin impact microbiota composition

Ampicillin specifically impacted on taxonomic composition, although the addition of ampicillin at a concentration of 1 mg/ 6 ml brought no significant changes on operational taxonomic unit (OTU) (paired *t*-test, $p = 0.162$) (Table 8-2). Ampicillin induced a further increase of relative abundance of *Proteobacteria* (paired *t*-test, $p = 0.049$) (Table 8-3) beyond the experimental condition did. Changes in taxa abundances included increase of *Lachnospira* ($p = 0.05$) and decrease of *Butyricicoccus* ($p = 0.046$) (Table 8-4). This result varied from another study (Johnson et al., 2015), in which the same experimental concentration of ampicillin (1 mg / 6 ml) reduced the absolute abundance of *Bacteroides* and *Bifidobacteria*. There might be many factors attributed to this difference, including the individual differences (European vs. Asian), the different measure techniques (FISH vs. 16S rRNA gene sequencing) and the extensive environmental and animal antibiotic-resistance in China in recent years (Su et al., 2018; J. Xu et al., 2015).

Table 8-4 Paired-T test (*p* values) of the relative abundance on genus level among samples inoculated with methylcellulose and initial feces, different antibiotics and MC groups.

	Feces after fermentation compared to Initial	Ampicillin compared to no antibiotic
Alistipes	-	0.15
Bacteroides	0.159	0.416
Bifidobacterium	0.454	0.362
Bilophila	0.593	0.467
Blautia	**0.03**	0.114
Butyricicossus	0.087	**0.046**
Butyricimonas	-	0.328
Clostridium	0.426	0.452
Collinsella	0.905	0.409
Coprobacillus	-	0.423
Coprococcus	0.37	0.298
Dialister	0.298	0.503
Dorea	-	0.139
Enterococcus	0.175	0.174
Escherichia	**0.043**	0.794
Eubacterium	-	0.304
Faecalibacterium	0.127	0.375
Fusobacterium	0.184	0.184
Gemmiger	0.221	0.361
Lachnospira	0.149	0.05
Lactobacillus	0.681	0.389
Lactonifactor	-	0.415
Odoribacter	-	0.311
Oscillospira	0.182	0.27
Parabacteroides	0.33	0.212
Phascolarctobacterium	-	0.388
Plesiomonas	-	0.423
Paraprevotella	0.423	0.423
Peptoniphilus	0.422	0.422
Phascolarctobacterium	0.483	0.419
Prevotella	0.423	0.413
Roseburia	0.083	0.426
Ruminococcus	0.131	0.136
Snodgrassella	-	-
Streptococcus	0.066	0.205
Sutterella	0.211	0.232
Veillonella	0.409	0.402
Unclassified	0.192	0.87
Others (<0.5%)	0.054	0.656

8.3.5 EPS and KGM maintained the richness of gut microbiota

Fig. 8-2 showed the difference in OTU between MC and four other experimental substrates. Surprisingly, it was found that inulin (Wilcoxon paired test, $p = 0.015$) and GOS ($p = 0.030$) induced a significant decrease of OTU, while EPS ($p = 0.222$) and KGM ($p = 0.431$) maintained the OTU at the level of the control with perturbance of ampicillin compared with MC (Table 8-5). As we know, fecal microbiome richness has been put forward as a readout of colonic microbiota stability or resilience (Vieira-Silva et al., 2016), with reduced estimates thought to be indicative of ecosystem dysbiosis (Le Chatelier et al., 2013a; Qin et al., 2012).

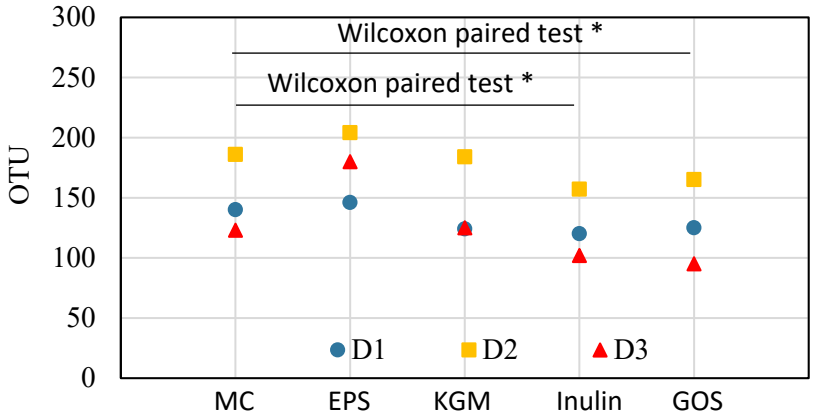

Figure 8-2 OTU of different groups

Table 8-5 Wilcoxon paired test (p values) of the OTU among samples inoculated with methylcellulose and different EPS fractions in the presence of three antibiotics.

	EPS	KGM	Inulin	GOS
p value	0.109	0.414	**0.015**	**0.030**

8.3.6 EPS specifically improved gut microbiota

Fig. 8-3 showed the bacterial relative abundance at the phylum and genus levels. At the phylum level (Fig. 3a), *Bacteroidetes* relative abundance was increased by EPS (paired t-test, $p = 0.03$) and KGM (paired t-test, $p = 0.01$) fermentation (Table 8-6). Some animal and human

studies showed that obese groups had higher ratio of Firmicutes/Bacteroidetes (the ratio of F/B) (L. Zhao, 2013), and antibiotic usage may lead to obesity (Ray, 2012). Therefore, the ratio of F/B was also compared between different experimental groups and the MC group, but no significant differences were found (Table 8-7).

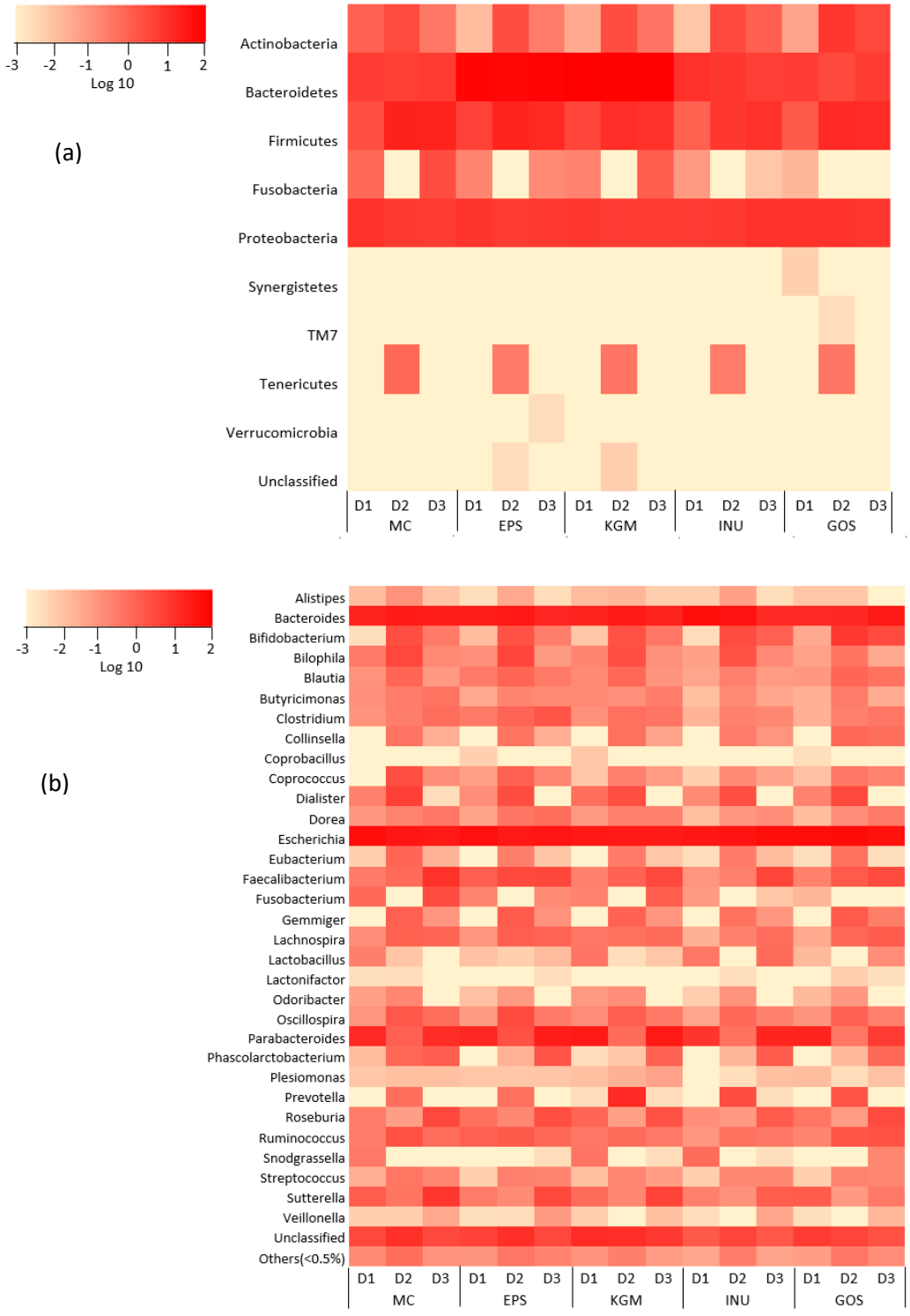

Figure 8-3 Log-scaled relative abundance heat maps of the phylum (a) and genus level (b).

To identify substrate-specific responsive genera in the presence of ampicillin, the study compared the taxa relative abundances after 24 h of experimental substrate fermentations with the outcome of MC incubations in matching donor fecal slurries (Table 8-7). At the genus level (Figure 3b), EPS fermentation resulted in increased *Clostridium* relative abundance (paired *t*-test, $p = 0.02$), while GOS microbiome profiles were characterized by lower proportion of *Dorea* (paired *t*-test, $p = 0.01$) (Table 8-7). *Clostridium* cluster IVa bacteria, encompassing several colon butyrate producers. *Dorea*, on the other hand, was reported to be positively linked with valerate concentrations (Kevin D'hoe & Sara Vieira-Silva, 2018) – although the taxon is known as a major gas producer (Rajili´c-Stojanovi´c and de Vos, 2014). However, no significant changes induced by KGM or inulin on any genus relative abundance were observed. Their neutral effect on fecal microbiome composition was consistent with the results of bacterial viability.

Table 8-6 Paired-T test (*p* values) of the relative abundance on major phylum among samples inoculated with methylcellulose and different EPS fractions in the presence of three antibiotics.

	EPS	KGM	Inulin	GOS
Actinobacteria	0.37	0.55	0.36	0.19
Bacteroidetes	**0.03**	**0.01**	0.37	0.27
Firmicutes	0.63	0.25	0.16	0.15
Proteobacteria	0.68	0.3	0.93	0.16
Firmicutes / Bacteroidetes	0.4	0.2	0.2	0.74
Firmicutes / Proteobacteria	0.56	0.27	0.18	0.18
Firmicutes / Actinobacteria	0.46	0.31	0.27	0.3

Table 8-7 Paired-T test (*p* values) of the relative abundance at the genus level among samples inoculated with methylcellulose and different EPS fractions in the presence of three antibiotics.

	EPS	KGM	Inulin	GOS
Alistipes	0.29	0.41	0.27	0.34
Bacteroides	0.93	0.57	0.45	0.5
Bifidobacterium	0.38	0.51	0.26	0.2
Bilophila	0.7	0.34	0.33	0.36
Blautia	0.13	0.91	0.36	0.47
Butyricimonas	0.15	0.3	0.16	0.32
Clostridium	**0.02**	0.85	0.26	0.17
Collinsella	0.42	0.28	0.68	0.2
Coprobacillus	0.42	0.42	-	0.42
Coprococcus	0.47	0.41	0.41	0.45
Dialister	0.39	0.5	0.4	0.41
Dorea	0.25	0.49	0.12	**0.01**
Escherichia	0.8	0.27	0.89	0.2
Eubacterium	0.41	0.4	0.41	0.39
Faecalibacterium	0.79	0.44	0.36	0.49
Fusobacterium	0.32	0.26	0.3	0.3
Gemmiger	0.4	0.41	0.42	0.24
Lachnospira	0.47	0.49	0.2	0.87
Lactobacillus	0.45	0.42	0.35	0.76
Lactonifactor	0.68	0.18	0.38	0.67
Odoribacter	0.26	0.61	0.22	0.26
Oscillospira	0.56	0.29	0.24	0.23
Parabacteroides	0.34	0.2	0.87	0.76
Phascolarctobacterium	0.95	0.27	0.49	0.19
Plesiomonas	0.17	0.22	0.15	0.74
Prevotella	0.42	0.42	0.42	0.42
Roseburia	0.66	0.72	0.38	0.67
Ruminococcus	0.77	0.37	0.28	0.61
Snodgrassella	0.43	0.4	0.42	0.75
Streptococcus	0.45	0.18	0.38	0.4
Sutterella	0.29	0.31	0.32	0.41
Veillonella	0.52	0.31	0.86	0.2
Unclassified	0.21	0.19	0.17	0.58
Others (<0.5%)	0.71	0.27	0.15	0.33

In general, the addition of inulin and GOS in the presence of ampicillin induced OTU loss and GOS resulted in a decrease of valerate producer *Dorea*. On the opposite, EPS supplementation proportionally stimulated the growth of a possible butyrate producer *Clostridium*. This result was consistent with bacterial enumeration result. It might further indicate that supplementation of traditional prebiotic substrates might adversely influence the stability and some beneficial taxa by increasing the sensitivity to antibiotics.

8.3.7 Substrate consumption

As shown in Figure 8-4A, compared with the total monosaccharide contents at 0 h (normalized to 100%), MC was not utilized during the whole fermentation process, since the total hydrolysed monosaccharides in the medium at different time points remained unchanged compared with the concentration at time 0 h. In contrast, four experimental substrates could be utilized well by gut flora with total monosaccharides concentration at 48 h ranging from 3.43% (GOS) to 18.18% (KGM). As a fast-fermentable substrate, GOS began to be consumed from 10 h, while the significant consumption of the other three polysaccharides was found after 24 h. Although EPS (Yu-Heng Mao et al., 2018), KGM (Yin, Ma, Siu, & Wu, 2019) and inulin (Sulieman et al.) had different monosaccharide profiles, their utilization rates were similar.

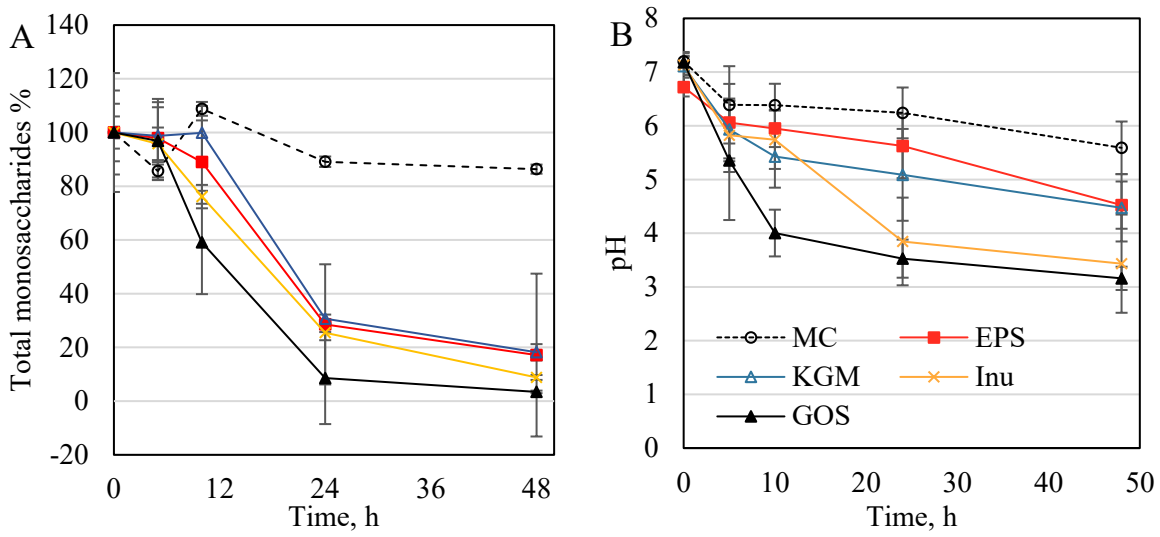

Figure 8-4 Changes in total monosaccharide concentration of different substrates (A) and pH value (B) during fecal fermentation (3 donors). Ampicillin: 1 mg/6 ml; PS / OS: 5 g/l. Total monosaccharides at 0 h were normalized to 100%. Error bar indicates SD (n=3).

8.3.8 Changes in pH value

Figure 8-4B showed the changes in pH value at different time points during the fermentation course, which confirmed that the fermentation of EPS, KGM and inulin accelerated after 10 h, while the fermentation of GOS began earlier (from 5 h). For EPS and KGM, the pH maintained > 5.5 during the first 10 h, followed by a steady decrease to pH < 5 at 48 h. For inulin, the pH sharply decreased from 10 to 24 h (Δ pH 1.89), then slowly decreased to 3.43 at 48 h. The pH changes were consistent with the different substrate consumption trends. A dramatic pH decrease (Δ pH = 3.18) during first 10 h was observed for GOS, followed by a steady decrease from 10 to 48 h, which also reflected the fast fermentation property of GOS.

8.3.9 SCFA production

Table 8-8 shows that compared with MC, GOS increased SCFA production from an earlier time point of fermentation (before 10 h), while EPS, KGM and inulin significantly increased the production of SCFAs the most after fermenting for a relative longer time (24 or 48 h), which was consistent with the substrate consumption trend and pH changes. At the end

of fermentation (48 h), EPS, KGM and inulin showed better ability in increasing propionic acid production than GOS, and EPS and KGM produced higher concentration of butyric acid than inulin (even with no statistical significance). This result was consistent with the gene analysis, that EPS fermentation induced a higher relative abundance of *Clostridium* which included the butyrate producer *Clostridium* cluster IVa bacteria.

At 24 h (the normal colon transit time), KGM and inulin significantly increased the production of acetic acid, propionic acid and total SCFA compared with MC. It was notable that EPS and GOS also increased total SCFA production by 11.11 mM and 19.61 mM, respectively, but with no statistical significance because of the large variation among different donors. With the presence of ampicillin, the utilization and metabolites of substrate were different from the condition without antibiotic, since each donor had different response to antibiotic. For EPS, the substrate concentration decreased but no significant increase of SCFA production was found. This might be due to the compositional and functional changes of gut microbiota, which might induce some other organic acid (including pyruvic acid, lactic acid, succinic acid and so on) (Rios-Covian et al., 2016) production during the fermentation.

Table 8-8 Changes in SCFA concentrations (mM) [a] after 5, 10, 24 and 48 h of fermentation of different polysaccharides and oligosaccharide (5 g/l) compared with MC, in the presence of ampicillin (1 mg/6 ml) in batch cultures.

	EPS	KGM	Inulin	GOS
5 h				
Acetic acid	0.76 ± 0.46	3.63 ± 1.34 *	1.31 ± 1.09	1.92 ± 0.51 *
Propionic acid	0.51 ± 0.73	0.79 ± 0.95	0.35 ± 0.51	0.34 ± 0.67
Butyric acid	0.47 ± 0.42	0.40 ± 0.35	ND	ND
Total	1.74 ± 1.56	4.81 ± 2.04	1.66 ± 0.61 *	2.25 ± 0.29 *
10 h				
Acetic acid	2.74 ± 3.16	5.91 ± 3.82	3.97 ± 2.99	8.85 ± 0.82 *
Propionic acid	0.73 ± 0.82	1.38 ± 1.21	0.33 ± 0.79	0.40 ± 0.78
Butyric acid	0.68 ± 0.59	0.70 ± 0.61	ND	ND
Total	4.15 ± 4.33	7.99 ± 5.50	4.29 ± 3.75	9.25 ± 1.42 *
24 h				
Acetic acid	4.71 ± 3.49	8.24 ± 2.42 *	7.33 ± 1.22 *	13.75 ± 3.01 *
Propionic acid	5.47 ± 4.65	4.90 ± 0.66 *	2.52 ± 1.82 *	2.41 ± 3.28
Butyric acid	0.94 ± 0.85	0.91 ± 0.85	ND	3.45 ± 5.98
Total	11.1 ± 8.31	14.05 ± 3.03 *	9.86 ± 2.05 *	19.6 ± 11.0
48 h				
Acetic acid	8.36 ± 8.46	10.8 ± 6.68	13,0 ± 8.24	14.3 ± 3.98 *
Propionic acid	11.1 ± 1.65 *	9.92 ± 2.42 *	8.72 ± 4.65	0.96 ± 2.71
Butyric acid	2.63 ± 2.86	3.13 ± 3.20	ND	0.32 ± 0.56
Total	22.1 ± 12.5	23.9 ± 9.40 *	21.7 ± 7.23 *	15.6 ± 6.15 *

[a] Values were mean ± SD of three donors.

* means p-value < 0.05, by paired t-test.

Additionally, it is interesting to note that the standard deviation of each SCFA produced by EPS and KGM was generally larger than inulin and GOS at the same time point, which indicated that the ability to utilize more complex carbohydrate varied more among different individuals, especially under the condition of antibiotic disturbance. This maybe because a more complex enzyme system is needed during EPS and KGM utilization due to their complex molecular structure. In the presence of antibiotic, for some donors, the critical species that

participate in the carbohydrate utilization are inhibited, which may finally induce the changes in utilization pathway.

8.4 Summary

The impacts of different polysaccharides and oligosaccharides on fecal microbiota with perturbance of ampicillin were very different. We surprisingly found that traditional prebiotic inulin and GOS induced OTU loss and less production of propionic acid and butyric acid compared with EPS and KGM. In contrast, EPS maintained or increased the viability of several major groups of bacteria and enhanced the production of propionic acid and butyric acid compared with most other substrates. This study suggests that more consideration should be given to providing a proper direction for prebiotic supplementation when aiming at relieving or counteract antibiotic influence. Additionally, *in vivo* studies are needed to confirm the findings of this study.

Chapter 9 General conclusions and future work

9.1 General conclusions

This research project has accomplished an experimental study on the protective effects of two natural polysaccharides (PS), exopolysaccharides (EPS) from Cs-HK1 mycelial fermentation and konjac glucomannan (KGM), on gut bacteria, and the related mechanisms for the protection. The chief findings from this project are summarized below.

1. Partially degraded KGM by high intensity ultrasound was more effective than the native KGM and acid hydrolysed low MW KGM on bifidobacteria against some common antibiotics including penicillin and streptomycin. Two prebiotic standards GOS and inulin showed no significant protective effect. The protective effect of KGM on the bifidobacteria may be attributed to the adsorption of antibiotics and the formation of a viscous layer surrounding the bacteria by the polysaccharides.

2. The high-MW EPS produced by the Cs-HK1 fungus exhibited a significant protective effect on several strains of bifidobacterial against four commonly used antibiotics. The protective effect of different EPS fractions was generally more significant with a higher MW and higher concentration, which proved the initial hypothesis of MW-dependent protection. Microscopic observation suggested the formation of a viscous coat on the bacterial cell by the high MW EPS. This viscous coat not only retarded the access of bacteria cell to the antibiotics but also to useful nutrients, leading to a slower growth rate. The physical and biological effects of EPS on the bacterial cells also enhanced the formation of biofilm and adhesion to epithelia cell layer by the bifidobacteria. Overall, the results suggested that Cs-

HK1 EPS was beneficial to the bifidobacteria by protecting against the antibiotics and by promoting their adhesion to and colonization in the intestine epithelium.

3. The EPS fractions with different MWs and compositions derived from the EPS were all well utilized to produce SCFAs during fecal fermentation. Despite the heterogeneous composition of the fecal microflora among different donors and the limitations of the in vitro fermentation model, the fecal fermentation experiments were simple and effective for evaluation of various EPS fractions for their effects on the diversity and composition of fecal microflora. The significant decrease in the relative abundance of *Proteobacteria* suggested the beneficial effect of EPS toward a healthier gut microbial ecosystem.

4. EPS was effective to avoid the harmful effect of antibiotics such as ampicillin, maintaining the viability of several major groups of bacteria and enhancing the production of propionic acid and butyric acid. In contrast, traditional prebiotic inulin and GOS induced OTU loss and less production of propionic acid and butyric acid compared with EPS and KGM. EPS fermentation also resulted in increased possible butyric acid producer *Clostridium* relative abundance. This study suggested that the more complex non-digestible carbohydrates are more effective to relieve or counteract antibiotic influence on gut microbiome.

9.2 Future studies

The results from this project provide a foundation and useful references for further research, development, and application of natural PS. Specifically the following areas are suggested for future work.

1. There is a need to verify the potential benefits of EPS to the gut microbiota in animal experiments and to investigate the mechanism for the EPS effects on the bifidobacterial cells such as the expression of RpoS protein during stress conditions and biofilm formation.

2. Further studies through animal experiments are warranted to assess and verify the prebiotic function and related health benefits of EPS.

3. More *in vivo* studies are needed to further understand the protective effects of EPS because of the limitations of the *in vitro* fermentation.

4. Animal and human studies are needed to prove the beneficial functions of EPS and other natural polysaccharides for protection of the adverse effects of antibiotics on gut health.

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