PREBIOTIC FUNCTIONS AND MECHANISMS OF NATURAL POLYSACCHARIDES ON DIFFERENT BACTERIAL SPECIES

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Prebiotic functions and mechanisms of natural polysaccharides on different bacterial species

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20 August 2019

Abstract

Natural polysaccharides (PS) from various sources have been increasingly recognized as potential pharmaceutical and nutraceutical materials due to their notable bioactivities and health benefits. The natural PS with complex structures and high molecular weights (MWs) usually have a strong resistance to digestion in the human gastrointestinal system and can reach the large intestine. In other words, these PS may perform prebiotic functions on the gut bacteria, resulting in various health benefits to the human host. Therefore, the various health benefits of natural and bioactive PS may be connected to their prebiotic functions. The aim of this project was to investigate the potential prebiotic functions of three natural PS from fungal and plant sources in pure cultures of probiotic bacteria.

In the first part of this study, the bifidogenic effects of an exopolysaccharide (EPS) from a medicinal fungus (*Cordyceps sinensis*) and two well-known food PS, konjac glucomannan (KGM) and arabinoxylan (AX), with different MW ranges were evaluated in liquid cultures of *Bifidobacterium*. The preliminary results showed that the native EPS and KGM could not be well utilized as a carbon source by the bifidobacteria for growth due probably to their high MW and complex structures. Therefore, the native EPS and KGM were partially degraded with high intensity ultrasound (US) to increase the water solubility and lower the viscosity, and to much lower MW by acid hydrolysis with trifluoroacetic acid (TFA). Only the acid-degraded fractions (EPS-AH and KGM-AH) were able to significantly ($p < 0.05$) increase the growth of all five bifidobacterial

species compared to the control without any carbon source, but the effects were much less than that of glucose or galacto-oligosaccharide (GOS). The US-degraded high MW fractions, EPS-US and KGM-US, could slightly support the growth of some bacterial species. All EPS fractions increased the acetic acid production of most bacterial species. Very interestingly, the high MW EPS-US and KGM-US fractions significantly enhanced the cell viability, giving rise to much higher CFU counts than the cultures with glucose or the prebiotic reference GOS as the carbon source, indicating a strong protective effect for the survival of bifidobacteria. These results suggested that EPS and KGM could be used as prebiotic fibres for a healthy gut microbiota.

Further study was carried out to evaluate the protective effects of high MW EPS fractions on the probiotic bacteria including *Lactobacillus* and *Bifidobacterium*. The EPS at 5 g/L significantly increased the survival rate of the probiotic bacteria during cold storage (4 °C) and in simulated gastric acid, reducing the death rate of different bacterial species by 50% to 70%. The protective effect of EPS was weaker when the concentration was decreased to 3 g/L or when the MW of EPS was reduced by partial degradation with power US. EPS also showed significant protective effect on the four bacterial species in bile juice. Compared with EPS, two commercial prebiotic fibers including inulin and GOS showed much less or no significant protective effect on probiotic bacteria in these conditions. In addition, the EPS had a total dietary fiber content about 70%, which was close to its total carbohydrate content; it was resistant to artificial gastric acid (pH2) with no more than 4% hydrolysis in 6 hours. The results

have demonstrated the potential value of Cs-HK1 EPS as a novel prebiotic fiber for the formulation of synbiotic products with probiotic bacteria.

Arabinoxylan (AX) is an important dietary fiber which is abundant in many cereal grains such as rye, wheat, barley, oats and rice. In addition to its multiple health benefits, AX has shown prebiotic function, stimulating the growth of beneficial bacteria such as *Bifidobacterium* in the gut. However, the mechanism for utilization of AX by the bifidobacteria is still unclear due to the substrate specificity of AX hydrolases and the complex structures of native AX as well as the different metabolism processes in different bifidobacterial species. In the preliminary experiments, it was interesting to find that AX could only be consumed effectively by *B. longum* (CICC6186) but barely used by another four species of bifidobacteria. Further study was performed to investigate the enzymatic metabolism of wheat AX by *B. longum*. Based on the enzyme activities and consumption rates of AX and its low MW hydrolysates, a strategy was proposed involving extracellular cleavage of xylose backbone and intracellular degradation of both backbone and arabinose substitution. Another interesting finding was that the *B. longum* had a preference on the AX with a single substitution and a relatively higher degree of polymerization. This suggested that the polymer chain structure of AX played a key role in the uptake and metabolism of AX by the bifidobacterial cell. Genomic analysis showed the lack of the well-known β-xylosidase in the bifidobacterial strain, suggesting the existence of a novel or unknown enzyme or metabolic pathway. According to these results, a hypothetical model was proposed to describe the pathway for uptake and metabolism of AX by the *B. longum* cell.

In summary, following are the chief findings from this project:

(1) In pure cultures of bifidobacteria, the high MW EPS produced by the Cs-HK1 fungus or the high MW KGM could not be well utilized by the bacteria as a carbon source for growth, even after acid hydrolysis to much lower MW fractions.

(2) The high MW PS especially the EPS produced by the Cs-HK1 fungus could provide protective effects on bifidobacteria and lactobacilli in harsh conditions.

(3) Arabinoxylan (AX), a well-known dietary fiber from plant foods, could be selectively utilized by certain bifidobacterial species for growth, e.g. *B. longum* in this study, and the utilization and bacterial growth was dependant strongly on the AX chain structure.

These findings are new and useful for understanding the probiotic functions of natural PS and the relationships to their structure and properties and for development and application of natural PS as prebiotics in food and pharmaceutical industry.

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. **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.
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5. Li, J., Li, B., Geng, P., **Song, A.-X**., & Wu, J.-Y. (2017). Ultrasonic degradation kinetics and rheological profiles of a food polysaccharide (konjac glucomannan) in water. *Food hydrocolloids, 70*, 14-19.

Paper in preparation

Song, A.-X., Mao, Y.-H., Siu, K.-C., & Wu, J.-Y. Mechanistic insight into wheat arabinoxylan untilisation by *Bifidobacterium longum*.

Conference presentations and posters

1. **Song, A.-X.**, Mao, Y.-H., Siu, K.-C., & Wu, J.-Y. (2019). Bifidogenic and protective effects of exopolysaccharide from *Cordyceps sinensis* fungus. Microbiome: Chemical Mechanisms and Biological Consequences. Montreal, Canada.
2. Wu, J.-Y., Yin, J.-Y., **Song, A.-X.**, Mao, Y.-H., & Siu, K.-C. (2018). Partial degradation of large polysaccharides with power ultrasound for improving water solubility and bioactivity. The 14th International Hydrocolloids Conference. Nanchang, China.
3. Wu, J.-Y., Mao, Y.-H. & **Song, A.-X.** (2018). Exopolysaccharide produced by the *Cordyceps sinensis* fungus Cs-HK1: potential health benefits through the gut microbiota. 2018 年全国糖生物学会议. Shanghai, China.
4. Wu, J.-Y., Mao, Y.-H., **Song, A.-X.**, & Li, L.-Q. (2018). Exopolysaccharide from *Cordyceps sinensis* Cs-HK1 mycelial fermentation: Potential prebiotic and anti-inflammatory activities. The 6th International Symposium on Edible & Medicinal Plant Resources and the Bioactive Ingredients Nanjing, China.
5. **Song, A.-X.**, Mao, Y.-H., Wu, J.-Y. (2018). Molecular properties and Prebiotic Functions of Polysaccharides Produced by *Cordyceps sinensis* Fungus Cs-HK1.

Functional Food Center's 23rd International Conference. California, USA.

6. **Song, A.-X.**, Mao, Y.-H., Siu, K.-C., & Wu, J.-Y (2017). Physiochemical properties and prebiotic functions of two important plant and fungal polysaccharides. 3th Microbiome R&D and Business Collabration Congress: Asia. Hong Kong.
7. Wu, J.-Y., Geng, P., **Song, A.-X** (2017). Invited talk: Partial degradation of high-molecular natural polysaccharides with power ultrasound for improving functional properties. The 17th Congress of Asian-Pacific Confederation of Chemical Engineering. Hong Kong.
8. **Song, A.-X.**, SIU Ka Chai, WU Jian Yong (2016). Physiochemical properties and dietary fiber functions of two important plant and fungal polysaccharides. The 23rd Symposium on Chemistry Postgraduate Research in Hong Kong.

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List of abbreviations

AX	Arabinoxylan
AXOS	Arabinoxylan oligosaccharide
CFU	Colony forming unit
EPS	Exopolysaccharide
GOS	Galactooligosaccharide
HPGPC	High performance gel permeation chromatograph
HPLC	High performance liquid chromatograph
IPS	Intracellular polysaccharide
KGM	Konjac glucomannan
MW	Molecular weight
NMR	Nuclear magnetic resonance
OD	Optical density
PMP	1-phenyl-3-methyl-5-pyrazolone
PS	Polysaccharides
SEM	Scanning electron microscope
TFA	Trifluoroacetic acid
US	Ultrasound
XOS	Xylooligosaccharide

Chapter 1 Introduction

Polysaccharides (PS) are biopolymers of homo or hetero monosaccharides with degrees of polymerization (DP) greater than 10. Usually natural PS have very high molecular weight (MW) from thousands to over millions. The structures of natural PS are complex and variable not only with the source but also with the extraction and purification processes (Nie & Xie, 2011). In recent decades, natural PS have attracted increasing attention from scientific community and commercial industry due to their significant bioactivities, such as immunomodulation, anti-tumor, anti-inflammatory and anti-oxidant effects (Yu, Shen, Song, & Xie, 2018; Zong, Cao, & Wang, 2012).

For several reasons such as improved living standards, ageing population and skyrocketing medical cost, there has been a constant increase worldwide in the development and application of functional foods for promotion and preservation of health. Prebiotics represent a class of the most popular functional foods, in connection with the important role of gut microbiota in human health and disease. The term 'prebiotic' was first introduced by Gibson (1995), referring to a non-digestible food component that promotes the growth and/or activities of good bacteria in colon, thus beneficially affecting the health of host. Consumption of prebiotic is believed to have positive effects on human health by modulating the immune system, decreasing the risk of intestinal infections and promoting the general well-being (Roberfroid et al., 2010; Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013). These beneficial effects on health are mainly attributed to their positive influences on the healthy balance in the

structure and composition of gut microbiota. Numerous studies have reported the anti-tumor and anti-cancer effects after ingestion of foods containing prebiotics (Roberfroid et al., 2010; Al-Sheraji et al., 2013). These reports have clearly shown the changes of composition in gut microbiota, especially the increasing concentration in bifidobacteria (Scott, Martin, Duncan, & Flint, 2014). Saccharolytic genera like *Bifidobacterium* are considered potentially health-promoting bacteria which play a key role in improving health status as well as reducing disease risk (Cummings et al., 2004; Gibson & Roberfroid, 1995).

With the increasing concerns on food safety, consumers nowadays tend to purchase prebiotic products originally from natural sources. Natural PS with no toxicity and a high resistance to human gastrointestinal conditions provide a rich source of novel prebiotics (Singdevsachan et al., 2016; Yoo, Kim, & Paek, 2012). PS are abundant in the nature and found in nearly all living organisms, such as animals, plants, fungi, bacteria and yeasts (Xie et al., 2016). The use of fungi and plants as food or pharmacological products has a long history all over the world, such as Traditional Chinese Medicine (TCM). Recently, the prebiotic functions of PS from fungi and plants have also attracted wide interests due to the low cost of source materials and the convenience of production process. However, the potential of fungi and plants PS as valuable prebiotic sources remain to be widely explored. In this project three natural PS were investigated on their potential prebiotic functions, one from a medicinal fungus and two from plant foods.

Cordyceps sinensis (syn. *Ophiocordyceps sinensis*) known as Dong Chong Xia Cao in Chinese is a valuable medicinal fungus which has been used in TCM for hundreds of years (Zhang, Li, Wang, Li, & Liu, 2012). *C. sinensis* has been widely used as functional food for various health promotion functions such as improving of physical performance, reducing exhaustion and anti-aging effect (Kumar et al., 2011; Li, Li, Li, Dou, & Gao, 2010). It has also been applied as a medicinal herb for treatment of many diseases like chronic kidney disease, cardiovascular disease and hyperlipidemia (Guo et al., 2010; Yan et al., 2013; Zhang et al., 2014). PS are considered as the major bioactive components in *C. sinensis* with immunomodulatory, antitumor and some other medicinal properties (Yan, Wang, & Wu, 2014). Cs-HK1 is a *Cordyceps* species isolated from wild *C. sinensis* in our group and the liquid fermentation of Cs-HK1 has been well established to produce mycelial biomass and exopolysaccharides (EPSs) (Yan et al., 2014). Although the pharmaceutical application of *C. sinensis* has been intensively studied, there is little or no information on the prebiotic activity of PS originated from this fungi.

Konjac glucomannan (KGM) is a well-known dietary PS extracted from the corns of *Amorphophallus konja*c plant. Japan and China are the two major countries for production, marketing and consumption of *A. konjac* (Chua, Baldwin, Hocking, & Chan, 2010). KGM has been used in the food industry as a thickening and gelling agent for its high viscosity and multi-physical properties in solution. Recently, KGM has been increasingly used as a major source of dietary fiber for reducing the risk of obesity, diabetes and cholesterol (Chen, Cheng, Wu, Liu, & Liu, 2008; Salas-Salvadó et al.,

2008; Tester & Al-Ghazzewi, 2016) and been permitted as a food additive in Europe (Al-Ghazzewi, Khanna, Tester, & Piggott, 2007). The health-promotion effects of KGM may be associated with its function as a prebiotic material. Previous studies have shown the promotion effect of KGM oligosaccharides on the growth of bifidobacteria and lactobacilli. However, the potential use of KGM as prebiotic still needs further investigation.

Arabinoxylan (AX) is an important dietary fiber with multiple health benefits, which is mainly found in most cereal grains (Broekaert et al., 2011). Arabinoxylan-oligosaccharide (AXOS) and xylooligosaccharide (XOS) derived from enzymatic hydrolysis of AX have proven prebiotic functions based on ample evidence. They are resistant to gastric acid and digestive enzymes, and can be effectively fermented in gut microbiota and selectively stimulate the growth of beneficial gut flora (Courtin, Swennen, Verjans, & Delcour, 2009; Geraylou et al., 2012; Neyrinck et al., 2011; Sanchez et al., 2009). Although the prebiotic effect of AX has been widely accepted by the scientific community, the mechanism of AX metabolism by intestinal bacteria is still not fully understood. In addition, most of these reports have shown that the native AX was not so fermentable as AXOS and XOS in pure cultures of bifidobacteria (Crittenden et al., 2002; Van Laere, Hartemink, Bosveld, Schols, & Voragen, 2000).

This research project was aimed to study the beneficial effects of EPS, KGM and AX on probiotic bacteria, particularly bifidobacteria in pure cultures. These three PS are representative of dietary and bioactive PS of high MW from difference sources. The study in pure bacterial cultures is a simple and convenient approach for evaluation of

the effects of complex natural PS with respect to their molecular properties and for investigation of the underlying mechanisms. The approach is also meaningful for application because the addition of natural PS to probiotic bacterial cultures is an industrial strategy for the formulation of synbioitc products. Moreover, the mechanisms for the effects of PS on the probiotic bacteria and their metabolism by the bifidobacteria were investigated.

Chapter 2 Objectives and significance

2.1 Objectives

This research project was aimed to investigate the potential prebiotic application of three natural polysaccharides (PS), exopolysaccharides (EPS) from Cs-HK 1 mycelial fermentation, konjac glucomannan (KGM) and arabinoxylan (AX), and to evaluate the protection of EPS on probiotic bacteria from different harsh conditions as well as the metabolic mechanism of *Bifidobacterium longum* feeding on AX. These will be achieved through the following studies:

1) Fractionation of EPS and KGM with different molecular weights (MWs) after ultrasound (US) degradation and acid hydrolysis and analysis of the physiochemical properties of native and degraded EPS and KGM, including chemical component, monosaccharide composition and MW distribution.
2) Evaluation of the prebiotic effects of EPS and KGM fractions in pure cultures of commercial bifidobacterial species and quantification of the short chain fatty acids and pH change during bacterial fermentation.
3) Evaluation of the protective effect of EPS on bifidobacteria and lactobacilli in harsh conditions, including cold storage and simulated gastrointestinal conditions.
4) Investigation of the protective mechanisms of EPS on probiotic bacteria based on H_2O_2 accumulation during cold storage and microscopic observation of the interactions between EPS and bacteria.

5) Examination of the fermentability of AX and its low MW factions in pure cultures of different bifidobacterial species and screening of the bacterial species (*B. longum*) which could efficiently utilize AX as a single carbon source for growth.
6) Investigation of the uptake and consumption mechanisms of AX in *B. longum* cell by cellular and molecular approaches.

2.2 Significance

With the increasing awareness of healthy living and well-being, more and more consumers have chosen to use functional foods which are natural products containing bioactive ingredients. Natural polysaccharides (PS) with diverse molecular structures and a wide range of bioactivities and potential health benefits provide a rich source of potential prebiotics. Although many PS isolated from plants and fungi have been reported for their applications as health food or pharmaceutical products, the potential use as prebiotics of these PS still needs further investigation.

Cordyceps sinensis is a valuable medicinal fungus in traditional Chinese medicine and PS are major bioactive constituents of *C. sinensis*. However, there is nearly no available information about the prebiotic function of *C. sinensis* PS. This project will provide new evidence and scientific foundation for the potential application of EPS from *C. sinensis* fungi Cs-HK1 as prebiotics. Meanwhile, the research will find the relationships between MW of PS (KGM and EPS) and their prebiotic functions. In addition, the investigation of the degradation process of AX by *B. longum* will be useful

to reveal the mechanisms adopted by the gut bifidobacterial population for the utilization of AX. All these results will lay the foundation for further development of natural PS as prebiotics and also be useful for further study on the dynamic effects that different non-digestible carbohydrates have on the intestinal microbiota population.

Chapter 3 Literature review

Polysaccharides (PS) isolated from natural sources have attracted much attention due to their pharmacotherapy using for a long time. Various bioactivities of these bio-macromolecules have been widely reported such as immune-regulation, anti-cancer, anti-tumor and anti-oxidant activities. Recently, increasing attention has been paid on these natural polymers for their potential prebiotic functions. A lot of bioactive PS such as konjac glucomamman (KGM) and arabinoxylan (AX) are considered to perform their physiological activities through the interaction with gut microbiota. In this chapter, three PS from different sources are reviewed including their occurrences, structures and bioactivities. Gut flora and probiotics are also briefly introduced to give a basic information on the whole project.

3.1 *Cordyceps sinensis*

3.1.1 History and development of *C. sinensis*

Cordyceps sinensis, Dong Chong Xia Cao (winter worm, summer grass), is a valuable medicinal fungus and used as traditional Chinese medicine (TCM). The phylogenetic classification in 2017 announced this fungi into phylum *Ascomycota* class *Sordariomycetes* order *Hypocreales* family *Ophiocordycipitaceae* genus *Ophiocordyceps* (Shrestha, Sung, & Sung, 2017) and changed the name to *Ophiocordyceps sinensis*. The medicinal properties of *Cordyceps* have been recognised and used since long time ago. As early as Tang Dynasty, ‘Yue Wang Yao Zhen’

(Somaratsa, AD710) recorded the treatment for lung disease of *C. sinensis* (Zhu, Halpern, & Jones, 1998b). Later in a medicinal document 'Zang Ben Cao' (AD780), the function of improving lung and kidney was described. The detailed description of *C. sinensis* was first documented in 'Ben Cao Cong Xin' (New compilation of Materia Medica) in 1775 during the Qing Dynasty.

The modern study of *C. sinensis* started in 1950s. Researchers from the Institute of Zoology, Chinese Academy of Sciences first confirmed the host insect species *Hepialus armoricans* for cordyceps in 1973 and also proved the description 'winter worm, summer grass' by investigating the larva-fungi complexes in Qinghai and Sichuan provinces (Chen, Tang, & Mao, 1973). Since then the study on the host insect diversity of cordyceps has attracted increasing attention from Chinese scientists and several new genera were recorded (Wang & Yao, 2011). Meanwhile, the influence of environmental condition for the growth of cordyceps has been investigated (Zhang, YU, Wu, & Liu, 2011) and artificial growth has also been explored (Yu, 2004).

C. sinensis has received western attention within recent 40 years after several world records were established by Chinese runners in 1993 (Zhu et al., 1998b). The coach contributed their physical performance and endurance to a cordyceps-containing diet and he thought it could provide special nutrients. Since then numerous studies have investigated the bioactive components from *C. sinensis*, like cordycepin, adenosine, PS, ergosterol, mannitol and amino acid (Chen, Wang, Nie, & Marcone, 2013; Nakamura, Shinozuka, & Yoshikawa, 2015; Tsai, Lin, & Tsai, 2010). Cordycepin is considered as the index component for cordyceps in Chinese Pharmacopoeia. The health benefits of

this fungi such as antitumor, anti-cancer and immunomodulatory activities has also been explored (Khan, Tania, Zhang, & Chen, 2010; Wu et al., 2014).

3.1.2 Habitat and biology of *C. sinensis*

C. sinensis mainly distributes in the high altitude area (3500-5000 m above sea level) of the Tibet Plateau in China, including Qinghai, Tibet, Sichuan, Yunnan and Gansu province. It may also be found in some countries around Himalaya Mountains like Nepal, Bhutan and India (Zhu et al., 1998b). *C. sinensis* is an insert larva-fungal fruit body complex (Figure 3-1). The fungi are parasitic to a larva of moth which belongs to the family *Heplialidae/Thitarodes* (Chu, Wang, & Han, 2004). The parasitic fungal spores can infect some of the host larvae and release fungal mycelium in every summer or autumn. The fungi grow and obtain nutrients inside the larva until the whole larva become a sclerotium, which is called 'winter worm'. By early summer of the following year, the fruit body of fungi grows from the sclerotium and protrudes from the ground and this is called 'summer grass' (Pegler, Yao, & Li, 1994; Yao, 2004; Zhu et al., 1998b).

Insect host of *C. sinensis* belongs to the family *Heplialidae/Thitarodes*, primarily *Heplialus armoricanus*. Until now, there are seven genera and 82 species or subspecies found belonging to *Heplialidae* (Chu et al., 2004). However, due to the lack of study on determining whether they are the hosts of the fungi or the relationship between the insects and the fungi, the total number of insect host of *C. sinensis* is still unclear (Wang & Yao, 2011). The fungal mycelium of *C. sinensis* is principally *Paecilomyces hepiali*

(Yue, Feng, Liu, & Bao, 1995). Because the natural *C. sinensis* is rare and expensive, isolation of the fermentable fungal mycelia is of great importance for both scientific exploration and manufactory application. Scientists in The Institute of Material Media in Chinese Academy of Medical Science successfully isolated the Cs-4 from wild cordyceps in 1982. In the following years, several fungal species were isolated from wild cordyceps and some of them were manufactured through artificial cultivation, including *Cephalosporium sinensis, Hirsutella sinensis, Mortierella hepiali* Chen lu *sp. nov*., *Paecilomyces sinensis, Scytalidium hepiali* G. L. Li *sp. nov*., and *Tolypocladimn sinensis* C.I. *sp. nov* (Yin & Tang, 1995).

Figure 3-1 Natural *Cordyceps sinensis*

3.1.3 Cultivation and mycelial fermentation of *C. sinensis*

C. sinensis from natural source is rare and expensive. Therefore, artificial cultivation has been applied for the production of this valued fungi. There are two ways for the cultivation of *C. sinensis*. One is to mimic the natural process by using cordyceps fungi to infect the insect larva and then forming the fruit body. Sichuan Institute of

Traditional Chinese Medicine successfully get the artificial cordyceps by infecting the *Hepialus gonggaensis* moth using *Hirsutella sinensis* (Yin & Tang, 1995). Because of the time consumption and high cost of the whole process, this *in vivo* cultivation cannot be widely applied in manufactory.

The other is mycelial fermentation in liquid medium, which is a reliable process for mass production of the *C. sinensis*. In general, fungal mycelia are inoculated into a nutrient medium which is contained in a culture vessel with constant agitation and temperature. After a selected period when the major nutrients in the medium are used out and the mycelia step into the stationary phase, fungal mycelia can be collected by centrifugation or filtration, followed by drying to get the crude products. Many studies have reported the optical culture condition for the production of mycelial biomass and exopolysaccharides (EPS) (Hsieh, Tsai, Hsu, Chang, & Lo, 2005; H. Kim & Yun, 2005; Y.-S. Liu & Wu, 2012). The liquid medium commonly contain glucose or sucrose as carbon source, yeast extract, peptone or corn steep as nitrogen source and inorganic salts such as $MgSO_4$, KH_2PO_4 and K_2HPO_4, and some of the surfactants like Tween are also added to improve the mycelial morphology. Usually the culture period is 4-7 days and the pH is 4.0-7.0 for different species (Yan et al., 2014). Mycelial fermentation is a preferred method for its low-cost and well-controlled property as well as its production of bioactive compounds with constant quality and quantity.

3.1.4 Bioactive constituents

Seven classes of chemical compositions are found in *C. sinensis* both from natural sources and artificial fermentation, including proteins (peptide, amino acids and polyamines), saccharides (sugar derivatives), sterols, nucleoside compounds, organic acids, vitamins and inorganics (Zhu et al., 1998b). The bioactive mechanisms of these compounds are incomplete understood.

PS are the major pharmacologically active constituents of *C. sinensis*. Cordycepic acid initially considered to be one of the major bioactive compounds in *C. sinensis* is actually d-mannitol which also belongs to the sugar derivative (Chen & Chu, 1996). There are two kinds of PS produced by *C. sinensis*, intracellular polysaccharide (IPS) and extracellular polysaccharide, also called exopolysaccharide (EPS). IPS can be extracted from mycelial biomass by pure water, acidic or alkaline buffer under heating. EPS is produced by the mycelial cells into the fermentation broth and can be obtained by methanol precipitation. Usually the PS from *C. sinensis* are polysaccharide-protein (PSP) complexes with mannose, glucose and galactose as the major monosaccharide components (Yan et al., 2014). The molar ratio of these monosaccharides differs in PS from different Cordyceps species. Table 3-1 shows some purified PS from mycelial fruit body or culture broth of two Cordyceps fungal species. It shows that even the PS are originally from the same strain, they can be various in their molecular weights and linkage types based on the extraction process.

Nucleosides are considered as the key bioactive components in *C. sinensis* (Li, Li, Dong, & Tsim, 2001). Several nucleosides and their derivatives have been isolated from cordyceps including adenine, adenosine, cordycepin (3’-deoxyadenosine), cordycepin

triphosphate, deoxyuridines, 2'-3'-dideoxyadenosine, guanidine, deoxyguanidine, hydroxyethyladenosine, hypoxanthine, inosine, thymidine, uracil and uridine (Li et al., 2004; Shashidhar, Giridhar, Sankar, & Manohar, 2013; Zhu et al., 1998b). The contents of these nucleosides and their analogues are various among natural and cultural cordyceps. Figure 3-2 shows some structures of these nucleoside compounds. Cordycepin firstly extracted from *C. militaris* by Cunningham et al (1950) is recommended by Chinese Pharmacopoeia as the index component for quality control of cordyceps fungal products.

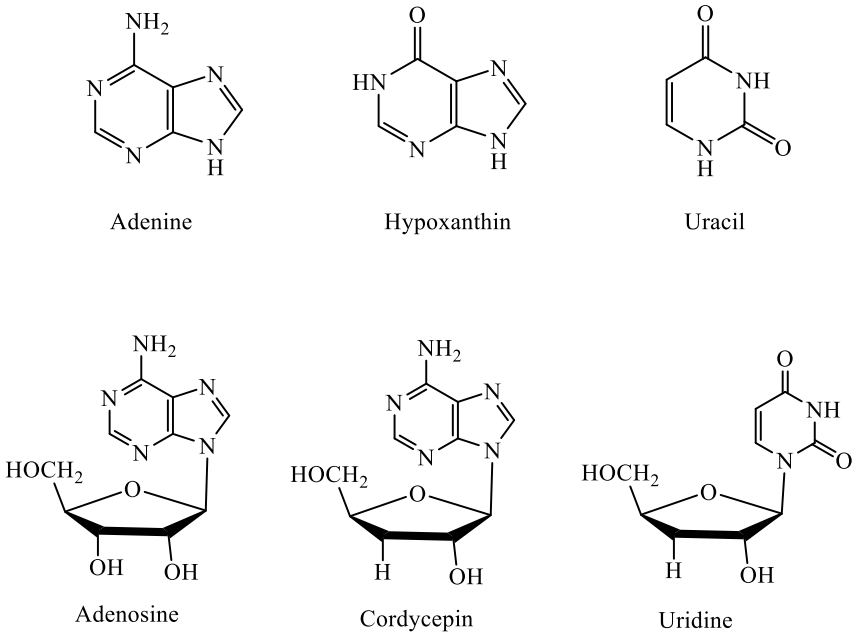

Figure 3-2 Structures of nucleoside compounds from natural and cultural *C. sinensis*.

Table 3-1 Polysaccharides from *Cordyceps* fungi

Species	PS type	Extract methods	Monomer	MW	Major linkage	Reference
Cephalosporium sinense Chen	IPS	Boiling water	β-Glu	13.6 kDa	(1→3)-β-d-glucosyl residues carrying a single (1→4)-β-d-glucosyl residue	Wu, Sun, and Pan (2005)
		0.05 M phosphate buffer	α-Glu	184 kDa	(1→4)-D-glucosyl residues carrying a single (1→6)-D-glucosyl residue.	Wu, Sun, and Pan (2006)
		0.05 M acetate buffer	Glu:Man = 9:1	7.7 kDa	(1→3)- and (1→4)-α-D-glucan backbone with α-D-(1→6)-Man*p* side chain	Wu, Hu, Pan, Zhou, and Zhou (2007)
		Hot water after purification by polar and non-polar solvents sequentially	Glc:Man:Ara:Gal = 8:90:1:1	8.3 kDa	-	Wu, Sun, Qin, Pan, and Sun (2006)
Tolypocladium sinensis	EPS	Ethanol precipitation and fractionation by ion-exchange chromatography	Glu:Man:Gal = 15.2:3.6:1	40 kDa	(1→6)-α-D-glucose and (1→6)-α-D-mannose residues as backbone with β-D-galactose residue as side chain	Yan, Li, Wang, and Wu (2010)
	EPS	Precipitation and rough fractionation by ethanol, further fractionation by ion-exchange chromatography	Man:Gal = 1.7:1	50 kDa	(1→2)-α-D-Man*p* backbone with Gal*p* branches	Chen, Ding, Wang, Siu, and Wu (2014)
	IPS	Hot water	α-Glu	1180 kDa	(1→4)-linked α-D-Glc*p* backbone with (1→6)-α-D-Glc*p* side chains	Yan, Wang, Li, and Wu (2011)
	IPS	1.25 M NaOH and 0.04% $NaBH_4$ aqueous solution	α-Glu	1150 kDa	(1→4)-linked α-D-Glc*p* backbone without branches	Yan et al. (2011)

Proteins, peptide and all essential amino acids have been found in cordyceps. The protein content in fungal fruit body is highest (30.4%), followed by dead larvae (29.9%) and fermented mycelia (14.8%) (Shashidhar et al., 2013). Aspartic acid, arginine and glutamic acid are found to have the highest contents in larvae-fruit body by amino acid profile (Hsu, Shiao, Hsieh, & Chang, 2002). Some cyclic dipeptides related to anti-tumor and antibiotic activities also exist in cordyceps such as cyclo-(Leu-Pro), cyclo-(Phe-Pro), cyclo-(Val-Pro) and cyclo-(Ala-Leu) (Rhee, 2004).

Cordyceps also contains some other compounds like sterol (ergosterol, ergosterol peroxides, campasterol, daucosterol and β-sitosterol), fatty acids (saturated and unsaturated), vitamins (vitamins B_1, B_2, B_{12}, E and K) and inorganic elements (Al, Ca, Cr, Cu, Ga, Fe, K, Mg, Mn, Na, Ni, Zn).

3.1.5 Bioactivity of polysaccharides from *C. sinensis*

Immunoregulation activity of PS from *C. sinensis* has been widely reported. PS from different preparation processes can promote proliferation and differentiation of phagocyte, induce phagocytosis and stimulate macrophage activities on mononuclear phagocyte system (Chen, Zhang, Shen, & Wang, 2010; Chen, Shiao, Lee, & Wang, 1997; Kuo, Chang, Cheng, & Wu, 2007). The cellular immunological regulation of the PS is contributed to the stimulation on T suppressor cell of T lymphocyte subset (Guan, Hu, & Hou, 1992). Because of the immune regulation without any influence on hemopoietic system and lymphocytotoxicity, PS from both natural cordyceps and its fermentation products have been applied in clinic treatment for immunocompromised and immunological disease.

C. sinensis has been traditionally used as supplements in many functional food for its anti-aging function which is mainly contributed to the anti-oxidation effect of

cordyceps PS. Li et al (2001) investigated the anti-oxidation activities of several *C. sinensis* from different sources. Results showed that all the species had very strong anti-oxidant effects, and the natural cordyceps and the cultured mycelia possessed the activities at the same level. They also found that when PS were removed from the fungal mycelia, the anti-oxidant activity lost. It suggested the PS played a key role in exhibiting the anti-oxidation effect. PS isolated from both cordyeps mycelia and fermentation medium latterly have been confirmed to have anti-oxidant activity (Chen et al., 2006; Leung, Zhao, Ho, & Wu, 2009; Li et al., 2003).

Although anti-cancer or anti-tumor function of cordyceps is considered to result mainly from cordycepin, cordyceps PS may also have this function due to their mediation on cellular immunity and scavenging of free radical (Zhu, Halpern, & Jones, 1998a). PS cordlan isolated from *C. militaris* could induce phenotypic maturation of dendritic cells which were directly related to the success of cancer immunotherapy (Hyung Sook Kim et al., 2010). Animal test also showed the water extracts of *C. militaris* mainly containing PS significantly shrunk tumors in NCI-460 cell-transplanted mice and prolonged their lifespan (Park, Kim, Lee, Yoo, & Cho, 2009). An in vitro study on B16F10 melanoma cells found that a purified PS from *C. sinensis* mycelia inhibited matrix metalloproteinases expressions, thus suppressing the migration of cancer cells (Jayakumar et al., 2014). All these suggest the potential use of cordyceps PS as candidates for cancer treatment.

The effects of *C.sinensis* PS on blood glucose metabolism have also been evaluated by many research groups. An *in vivo* study using diabetic mice model caused by streptozotocin (STZ) and epinephrine showed that a purified PS isolated from *C. sinensis* cultured mycelia significantly decreased the blood glucose level in normal and hyperglycemic mice (Kiho, Ookubo, Usui, Ukai, & HiranoI, 1999). Similarly, Li et al

(2006) applied *C. sinensis* PS CSP-1 with different concentration on normal and diabetic mice. Their results showed that CSP-1 significantly reduced the blood glucose level in both normal and diabetic mice and the effect was dosage dependent.

Some other bioactivities of *C. sinensis* PS have been reported including anti-inflammation, anti-fatigue and promotion on respiratory and cardiovascular function (Zhu et al., 1998a). Although the mechanisms of these functions are incompletely understood, the application of *C. sinensis* in functional food and clinic treatment still attracts much attention from consumers and patients.

3.1.6 Commercial *Cordyceps* products

Since 1982 when the species Cs-4 was successfully isolated from wild *C. sinensis* by Chinese scientists from Institute of Materia Media in Chinese Academy of Medical Sciences, the investigation on Cs-4 fermentation products has swept over China. Both fundamental studies including chemical composition, bioactive function, safety and animal test, and clinic trials have been carried out in China. The studies on industrial fermentation process of Cs-4 leaded to a commercial product named JinShuiBao capsule which has been approved by the National New Drug Review and Approval Committee of the Chinese Ministry of Public Health.

Besides Cs-4, there are several Cordyceps species isolated from natural sources and some of them have also been commercially produced by mycelial fermentation (Zhu et al., 1998b). For example, *Cephalosporium sinesis* (NingXinBao) was isolated by the QingHai Institute of Liverstock and Veterinary Sciences, *Scydalium* sp. (832) by the Navy Institute of Medicine, and *Paecilomyces sinensis* (Cn80-2) by FuJian QingLiu Country Hospital and Institute of Microbiology, Chinese Academy of Sciences.

3.2 Konjac glucomannan

3.2.1 *Amorphophallus* and *Amorphophallus konjac*

Genus *Amorphophallus* which belongs to family *Araceae* is a large group containing more than 200 tuberous herbaceous plants. It is natively distributed in tropical and subtropical zones from West Africa to Polynesia (Sedayu, Eurlings, Gravendeel, & Hetterscheid, 2010). According to Hetterscheid et al (1996), there are 170 species of this genus and they have rather high specificities in regional endemism. Only four species are found to have a fair distribution range (*A. paeoniifolius*, *A. muelleri*, *A. konjac* and *A. abyssinicus*). The morphology and bionomics of *Amorphophallus* vary greatly from species to species in size, flora organ, bulbil and habit (P.-Y. Liu, 2004). *A. titanium* has the largest inflorescence with ~2.5 m length while the spadices of *A. eichleri* is only 20 cm (Figure 3-3). Some of the *Amorphophallus* species have rough rhizome tissues which cannot form edible corms and some have toxicity in their corms. Therefore, most of the 170 species are inesculent and there are only 20 used as food or medical products (Liu, 2004). Table 3-2 shows the name and distributions of these edible species.

A. konjac is the most widely used one among all the 170 species due to its nutritive value and health benefit function. It has a fair geographical range from the Himalayas to Thailand and Myanmar and also has an extensive distribution in China (Liu, 2004). It has been an important cultivated species in China, Japan and South East Asia for a long time. The corms of this plant species have more than 50% of glucomannan and certain contents of starch, cellulose, hemicellulose, amino acid and inorganic salts. It has an over 2000-year history in China been used as food and Traditional Chinese Medicine (TCM) (Chua et al., 2010). An ancient Chinese pharmacopoeia, ‘Ben Cao Gang Mu’, recorded several processing methods of *A. konjac* for its medicinal

application (Xu et al., 2001). According to 'National Compilation of Chinese Herbs' in 1950s, extracts from the corms of *A. konjac* were used for phthisis, hernia, burns and cough and its leaves had extraordinary efficacy on the treatment of insect bites.

(a) (b) (c)

Figure 3-3 Flora of (a) *A. titanium* and (b) *A. eichleri*, and (c) corms of *A. konjac*

3.2.2 Konjac glucomannan

Glucomannan is a component of the cell well in many microorganisms and also exists in some plants like lily, iris, aloe and green bean (Kato, Yamaguchi, Mutoh, & Ueno, 1976; Liu, 2004). Until now, *Amorphophallus* are the only genus who can produce glucomannan in a large scale among all these plants. Purified glucomannan extracted from the corms of *A. konjac* is called konjac glucomannan (KGM) and considered as the major bioactive component of *A. konjac*. KGM is a PS with D-glucose and D-mannose residues linked by β-1,4 glycosidic bond (Zhang & Yang, 2014). Branches randomly take place at mannosyl and glucosyl residues (Maeda, Shimahara, & Sugiyama, 1980). The molar ratio of glucose and mannose is 1.6:1 and this value differs in various konjac breeds. There are acetyl groups occurring every 9-19 sugar

units on the main chain, which contributes to the high solubility of KGM (Chua et al., 2010).

Table 3-2 Edible *Amorphophallus* species

Species	Distribution
A. albus P. Y. Liu et J. F. Chen	Chinese endemic species in Sichuan and Yunnan province
A. aphyllus Hutch	Senegal, Sudan
A. bulbifer Br.	India, Java
A. corrugatus N. E. Brown	China, Thailand, Myanmar
A. dracotioides N. E. Br	From Madagascar to Polynesia
A. harmandii Engl. Et Grhrm	Central Africa, the Ivory Coast
A. kachinensis Engl. et Gehrm	China, Myanmar, Thailand, Laos
A. kiusianus Makino	North China, Taiwan, South Japan
A. konjac K. Koch	China, Japan, Indonesia, Philippines
A. krausei Engl	China, North Thailand, Myanmar
A. nanus H. Li et C. L. Long	Chinese endemic species in Yunnan province
A. odoratus Hett. et H. Li	Chinese endemic species in Hong Kong
A. oncophyllus Prain et Hook. f.	Indo-China Peninsula, Southeast Asia
A. paeoniifolius (Dennst.) Nicolson	India, Cambodia, Vietnam
A. prainii Hook. f.	Thailand, West Malaysia, East Kalimantan
A. titanium (Becc.) Becc. ex Arcang.	Myanmar, Sumatra
A. tonkinesis Engl. et Gehrm	China, Vietnam
A. variabilis Blume	Philippines, Java, Malaysia
A. yuloensis H. Li	Chinese endemic species in Yunnan province
A. yunnanensis Engl	China, Thailand, Laos, Vietnam

Until now, the studies on KGM have involved the extraction methods, structure analysis, physicochemical properties, medicinal and health functions, and chemical modification. KGM has very complex structures so that even it has been used worldwide for over several centuries, the investigations mainly focus on the primary structure and there are few reports about its advanced structure. Besides, the

controversies still exist on the primary structure of KGM such as the molar ratio of the composed monosaccharide, the length and position of the branches and the degree of acetylation.

Due to the physicochemical property and physiological function, KGM has been extensively applied in the field of food, medicine, and chemical engineering (Zhang, Xie, & Gan, 2005). The addition of KGM as supplement in some food products can improve their sensory properties, texture and storage stability (Lin & Huang, 2003; Zhou et al., 2013). KGM is also used for the treatment of obesity, hyperglycemia and hypercholesterolemia (Chua et al., 2010). The great gel-forming and film-forming ability as well as biological degradability make KGM a novel material for drug delivery and blend film. In addition, the application of KGM in cosmetics has been studied in Japan based on its water adsorption and emulsification (Zhang et al., 2005).

3.2.3 Bioactivity and prebiotic function of konjac glucomannan

KGM as well as some non-starch PS is thought to have immuno-regulation function. The mechanism of the function are suggested to perform through two different strategies. First KGM can directly stimulate the immune cells and promote the production of cytokines, thus inducing the immune response. An *in vitro* study showed that the degraded KGM by enzymatic hydrolysis significantly activated the human promyceloccytic leukemia and monoblast leukeia cells (Ohya, Ihara, Murata, Sugitou, & Ouchi, 1994). This might be contributed to the high contents of mannose and glucose units in KGM which were considered as the pathogen associate molecular patterns molecules and could be recognised by the mannose or β-glucan receptors on the cells (Wang, Liu, Li, Wang, & Wang, 2015). Lv et al (2006) cultivated macrophages with saccharides and found KGM had the best affinity to macrophage mannose receptors.

Onishi et al (2007) also found that KGM had a positive control of Th1/Th2 cytokine production and increased plasma immunoglobulin E (IgE) in mice. Secondly, the regulation of KGM on gut microbiota based on its prebiotic function results partially in the immuno-modulation effect. Although the exact mechanism of this strategy is incompletely established, it has been considered to involve the physical contact between lactic acid bacteria and intestinal immune cells, and also the production of short chain fatty acid from the fermentation of non-digestible fibers by gut microbiota.

As mentioned above, KGM has prebiotic function and this effect has been investigated by *in vitro* and *in vivo* models. Addition of KGM in ultrahigh-temperature processed milk was reported to increase the number of lactobacilli in the milk (Al-Ghazzewi, Khanna, Tester, & Piggott, 2007). Besides, the bacterial colony forming units (CFU) from KGM-containing milk was significantly higher than another group which was supplemented by inulin. An animal test by Chen et al (2005) investigated the modulation of KGM and its acid-hydrolysed products on gut microbiota. Their results showed both KGM and the hydrolysed KGM significantly increased the count of bifidobacteria and anaerobic bacteria in mice faeces. In addition, the number of faecal *Clostridium perfringens* was greatly decreased by the native and hydrolysed KGM. Elamir et al (2008) got a similar result that the daily supplementation with acid-hydrolysed KGM promoted the growth of colonic lactobacilli and anaerobic bacteria but decreased faecal *Escherichia coli* and *C. perfringens* counts in mice. The prebiotic effect of KGM has also been investigated in clinic study with both healthy and constipated adults by Chen et al (2006; 2008). They got the similar results with the findings in animal test that the KGM had the ability to increase the stool weight and

faecal counts of lactobacilli and bifidobacteria at the same time reduced the number of clostridium.

There are many other pharmacological functions of KGM such as anti-obesity, laxative, anti-inflammatory and anti-tumor activity and many of these properties are thought to be related to its prebiotic effect. Although the therapeutic mechanism of KGM still needs further studies, the application of this natural PS in clinic treatment is of great potential.

3.3 Arabinoxylan

3.3.1 Occurrence and structure of arabinoxylan

Arabinoxylan (AX) is one of the major components of plant cell walls (Broekaert et al., 2011). AX together with cellulose, arabinogalactan, glucan and lignin constitutes the majority of dietary fiber in cereal grains. 60-70% of the totally carbohydrate content in most cereal cell walls is contributed to AX (Grootaert et al., 2007). This complex PS can also occur in rice (40%, w/w) and barley (20%, w/w) (Fincher, 1986). AX basically consists of xylan backbone (β-1,4 linked D-xylopyranoside units) with L-arabinofuranose substitutions attached by α-1,2 and/or α-1,3 glycosidic bond (Figure 3-4). Xylose residues sometimes are substituted with acetyl groups or glucuronic acids while arabinose residues can be esterified by *p*-coumaric or ferulic acids (Rivière et al., 2014).

The structure of AX differs greatly based on different sources. A/X ratio referring to the degree of substitution of arabinose is of great importance on the physicochemical properties of AX. AX originally from rice and sorghum has a relatively higher A/X ratio while lower substituted AX is found in barley, wheat and rye. Clear variances are

also existed in AX from different organs in the same plant. The seed coat and aleurone of wheat have very low A/X ratio (0.1-0.4) and this value in endosperm and bran tissues (0.5-0.7) is relatively higher. Wheat pericarp were reported to have a highest content of double substituted AX (Maes & Delcour, 2002). The molecular weight of cereal AX varies greatly depending on the extraction and purification process. Water extracted AX was reported to have molecular weight range from 65,000 to 66,000 by sedimentation, and 70,000 to 1,000,000 by gel filtration. High performance gel permeation chromatography was also established to determine the molecular weight range of 219,000-255,000 for AX from wheat and 519,000-770,000 from rye (Izydorczyk & Biliaderis, 1995).

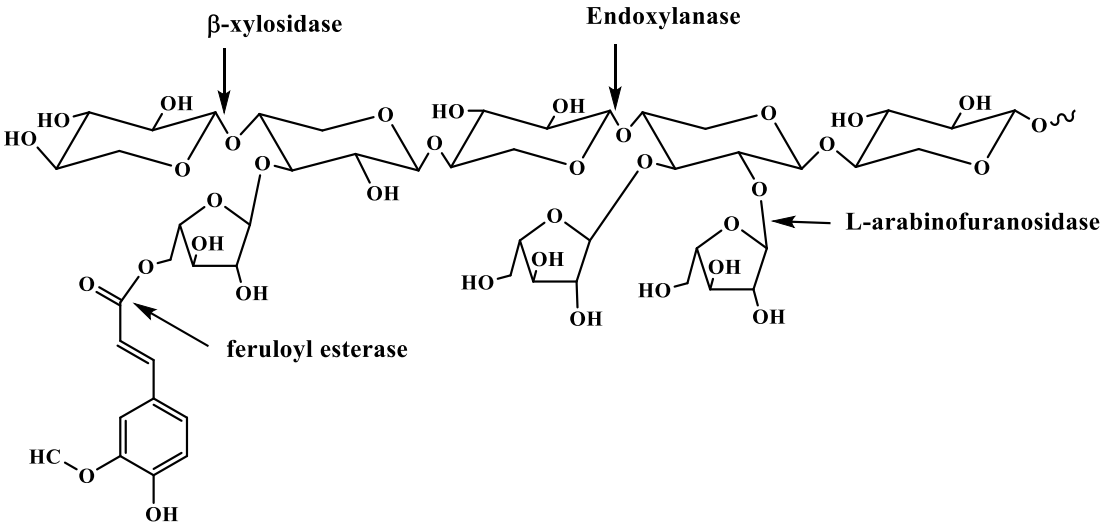

Figure 3-4 Chemical structure of AX and the enzymatic site of different enzymes.

Only a small portion of cereal AXs is extractable with water from cell wall materials. The low capability of water extraction on AX might be contributed to the different substitution patterns and the physical entanglements as well as the covalent bond formations between or within the molecules.

3.3.2 Arabinoxylan oligosaccharide and enzymatic degradation of AX

Recent attention has been paid to the nutrient properties of AX and its enzymatically degraded products, arabinoxylan-oligosaccharide (AXOS) and xylooligosaccharide (XOS). These carbohydrates are considered to be candidate supplements in daily and functional food due to their prebiotic properties. The degradation of AX may generate AXOS and XOS at the same time. Therefore, (A)XOS is usually used to indicate both AXOS and XOS. (A)XOS can be present and purified from AX-rich materials, or produced in food industry by acidic/enzymatic hydrolysis of AX, or generated by microbial degradation of AX in human colon (Broekaert et al., 2011).

No matter the enzymatic degradation of AX occurs *in vivo* or *in vitro*, there are three basic enzymes involved in the cleavage process, endo-1,4-β-xylanase, β-xylosidase and α-L-arabinofuranosidase (Figure 3-4). Arabinofuranosidase hydrolyzes arabinose branches from the backbone. Endoxylanase cleaves the backbone at the endo position but its activity is frequently blocked by arabinose side chains (Lagaert, Pollet, Courtin, & Volckaert, 2014). Therefore arabinofuranosidase and endoxylanase usually act in synergy to generated unbranched XOS. β-xylosidase is needed to complete the degradation by attacking the non-reducing ends of backbone to remove xylose residues. Some other enzymes are also needed for the debranching process such as ferulic acid esterase, α-glucuronidase and acetylesterase (Sørensen, Meyer, & Pedersen, 2003).

3.3.3 Metabolism of AX

Lots of investigations have reported the potential prebiotic properties of AX and (A)XOS. The resistance of (A)XOS to gastric acid and mammalian enzymes are studied by the *in vitro* studies. Sanchez et al. (2009) mimicked the stomach and small intestinal

condition to test the degradation of AXOS and found that this carbohydrate could not be hydrolysed to its component monosaccharides. In addition, (A)XOS was reported to have much stronger resistance to gastric condition compared to a commercial prebiotics frucooligosaccharide (Courtin, Broekaert, et al., 2009). Until now, there is no evidence showing that human has the enzymes to hydrolyze AX, AXOS and XOS.

Utilization of AX and (A)XOS by gut microbiota has been investigated for a long time. The fermentation of these carbohydrates by gut flora yields energy for the bacterial growth meanwhile the end products like short chain fatty acid (SCFA) can be absorbed by the host to perform the health benefits (Mendis, Leclerc, & Simsek, 2016). The fermentability of AX from different origins by gut bacteria varies greatly. AX from maize, rice and wheat showed different capability of SCFA production by human faecal microbiota (Rose, Patterson, & Hamaker, 2009). The highest SCFA concentration was observed in the maize AX-treated group and the researchers contributed this to the less branched structure of maize AX. However, a similar *in vitro* study using human feceal fermentation suggested that the fermentation rate was free from molecular weight and degree of substitution of AX (Rumpagaporn et al., 2015). They showed that it was the complexity of branches governing the fermentation rate of AX and the simpler the branches were, the faster the fermentation rate was. All these indicate that the fine stricture of AX is the main factor that affects its fermentation.

The mechanism of microbial degradation of AX in human gut has also been widely investigated. *Bacteroides* is the only genus who possesses the most expanded gene collection involved in the degradation of XOS and its derivatives (Zhang et al., 2014). There exist expanded PS utilization loci (PULs) within bacteroides that encode the utilization pathway of AX and some other non-starch PS. The metabolic systems of these non-starch PS in bacteroides are similar to its starch utilization system (Sus) and

therefore has been termed Sus-liked system (Koropatkin, Cameron, & Martens, 2012). A recent identification of xylan-induced gene cluster in bacteroidetes is named the xylan utilization system (Xus) which also acts in the same way as Sus (Dodd, Mackie, & Cann, 2011). In the predicted model of the Xus cluster (Figure 3-5), a set of binding proteins on the surface of bacterial outer membrane would bind extracellular xylan. Then the PS is degraded into oligosaccharides by the endo-xylanase which is also located on the bacterial surface, followed by importation in periplasm by transporters across the outer membrane. In the periplasm, these fragments are further cleaved to monomers and then transported into the cell by the inner membrane monosaccharide importers. Although some bacteria belong to *Bifidobacterium* and *Lactobacillus* are reported to have the capability of using (A)XOS, the metabolic mechanisms are still unclear. According to the results from Riviere et al (2014), the degradation process of AX in bifidobacteria was species dependent. Further study should be performed to reveal the secret in this field.

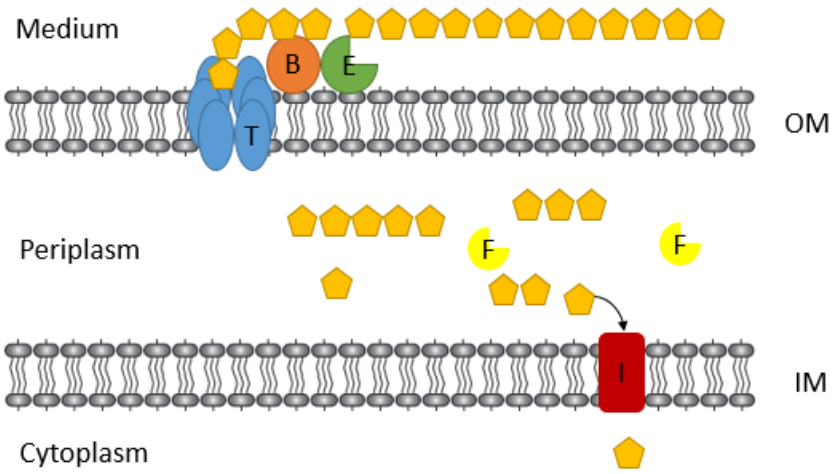

Figure 3-5 Predicted model of the Xus. E, endoxylanase; B, binding protein; T, transporter; F, free endoxylanase or xylosidase; I, importer.

3.4 Gut flora and probiotics

3.4.1 Gut flora

Intestinal micro-ecology is an important part of the human micro-ecosystem. There are large diversity and amount of bacteria colonizing in human gastrointestinal tract (GIT). The collective genome of these bacteria cells, also called the microbiome, makes them highly adapted to the complex and volatile environment. Human gut as a natural habitat for a large bacterial community contains 300-500 different species of symbiotic bacteria (Simon & Gorbach, 1984), whose number is nearly the same as the number of cells in human body. The intestinal microflora is a positive health asset that crucially influences the normal structural and functional development of human physiology and pathology. In general, intestinal bacteria can be divided into three type according to the physiological effect on host: beneficial, pathogenic and conditional pathogenic bacteria. The structure and composition of gut flora in adult human are dependent on many factors, including genetic background, daily diet, age, physiological state, life-style and microbiota interactions. A recent culture-independent study on the gastrointestinal tract microbiota composition in adults, which applied sequence analysis of amplified microbial ribosomal RNA-encoding genes, demonstrated that most of the GIT microbiota are members of five bacterial phyla, *Firmicutes* (79.4%), *Bacteroids* (16.9%), *Actinobacteria* (1%), *Proteobacteria* (0.1%) and *Verrucomicrobia* (0.1%), and of seven genera, *Bacteroids*, *Bifidobacterium*, *Coprabacillus*, *Eubacterium*, *Faecalibacterium*, *Roseburia* and *Ruminococcus* (Tap et al., 2009). The microorganisms in human gut are predominantly anaerobic bacteria whose number is 100-100 times more than the total number of aerobic and facultative anaerobic bacteria (Simon & Gorbach, 1984).

3.4.2 Physiological functions of gut flora

Based on the pathological and physiological impact of these microorganisms on host, gut microbial community can be considered as a virtual organ to (1) supply energy and absorbable products by fermentation of non-digestible dietary fibers and anaerobic metabolism of proteins and peptides, (2) contribute to the development of a health, mature and competent immune system, and (3) prevent pathogen or exogenous microbes from colonization and invasion by competing the attachment sites on intestinal epithelial cells.

Fermentation of non-digestible dietary fibers and supply energy and absorbable products for the host are the major metabolic function of microbial community (Guarner & Malagelada, 2003). A major source of energy in colon is provided by the fermentation of non-digestible carbohydrates including large PS, some oligosaccharides and alcohols (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987; Cummings, Beatty, Kingman, Bingham, & Englyst, 1996). Anaerobic metabolism of carbohydrates and proteins can generate short chain fatty acids (SCFAs) which are important substrates for host health. SCFAs are easily absorbed by human colon and can promote the absorption of calcium, magnesium and iron (Miyazawa, Iwabuchi, & Yoshida, 1996; Roberfroid, Bornet, Bouley, & Cummings, 1995). In addition, gene diversity endows gut flora with the distinct biochemical pathways from host to produce enzymes and nutrients which cannot be synthesized by host themselves. Some metabolites are regulated by host while some are controlled by the synergy between host and microflora, for example, primary bile acids are produced by the metabolism of cholesterol in liver via classical or alternative pathway, and then the primary bile acids enter the colon in which secondary bile acids was generated solely by the metabolic effect of intestinal bacteria. Besides, colonic bacteria also play an

important role in the synthesis of vitamin (Conly, Stein, Worobetz, & Rutledge-Harding, 1994; Hill, 1997).

The mucosal immune system in intestine is the largest immune system for mammals and the intestinal mucosa is the main interface between the external environment and the immune system. The gut bacteria play a necessary role in the development of a competent and health immune system. Gut-associated lymphatic tissue (GALT) which is composed of lymphoid nodule, antibody secretory cell and lymphocytes of the intestinal mucosal epithelium is an important part in mucosal immune system. GALT and enterocyte are the frontline soldiers of immune defense, engaging invading virus and pathogens. Animal tests reported that the low density of lymphoid cells, small specialized follicle structure and low immunoglobulins in blood circulation were observed in germ-free animals (Butler, Sun, Weber, Navarro, & Francis, 2000; Falk, Hooper, Midtvedt, & Gordon, 1998; Tannock, 2001).In rats and mice, proliferation of intraepithelial lymphocytes (Helgeland, Vaage, Rolstad, Midtvedt, & Brandtzaeg, 1996; Umesaki, Setoyama, Matsumoto, & Okada, 1993), cell producing rate of germinal centers (Cebra, Periwal, Lee, Lee, & Shroff, 1998) and concentration of immunoglobulin arose rapidly immediately after exposure to the luminal microorganisms. These investigations indicated the composition of gut-associated lymphoid tissue was greatly affected by the gut flora.

Resident bacteria are highly relevant in prevention from colonization and invasion of pathogens or exogenous microbes by competing the attachment sites on intestinal epithelial cells (Bernet, Brassart, Neeser, & Servin, 1994) (Figure 3-6) and nutrient availability in ecological niches (Hooper, Xu, Falk, Midtvedt, & Gordon, 1999). Germ-free host is more easily infected by potential pathogen (Baba, Nagaishi, Fukata, & Arakawa, 1991).When host is treated by antibiotics, the equilibrium between resident

bacteria which are very stable in the microbial population under normal condition may be broken down, resulting in susceptibility to infection. Use of antibiotics in normal mice can allow overgrowth of toxigenic *Clostridium difficile* which is associated with pseudomembranous colitis (Waaij, 1989). *In vitro* study also suggested the production of a specific antimicrobial substance called bacteriocins by resident bacteria to inhibit the growth of the competitors (Brook, 1999). Liévin et al. (2000) separated 14 bifidobacteria species from infants and found two of them could secrete a specific substance to inhibit the invasion of *Salmonella typhimurium* SL 1344 on human Caco-2 cells. In addition, after colonization of these two bacteria in germ-free mice, they could survive and suppress the infection of *Salmonella typhimurium* C5.

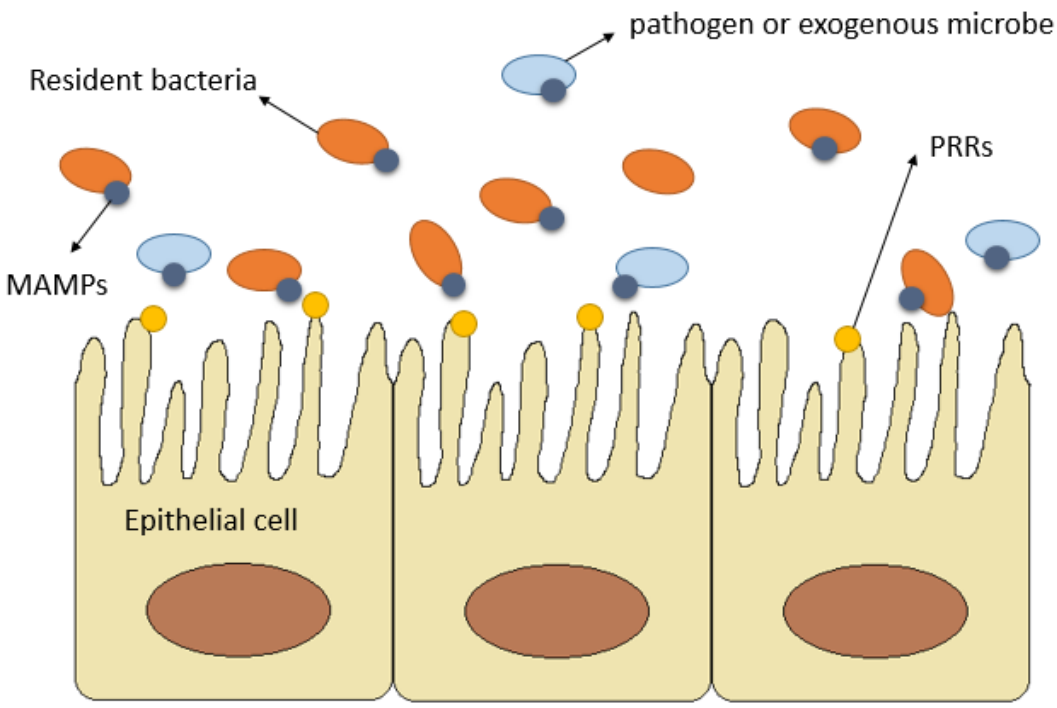

Figure 3-6 Host pattern recognition receptors (PRRs) on epithelial cells recognize the bacterial cells by microorganisms associated molecular patterns (MAMPs)

3.4.3 Definition of probiotics

The modern definition of probiotic was first introduced in 1965 as a substance secreted by protozoan to stimulate the growth of other microorganisms, which had an opposite effect to antibiotics (Lilly & Stillwell, 1965). From then on, the definition of probiotic has been revised continually as the methodology and technology on probiotic investigation have been developed and improved rapdly. The concept of probiotic was further declared by Afrc (1989) as 'A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'. In 2001, the Food and Agriculture Organization of the United Nation (FAO) and World Health Organization held the first conference focusing on the nutrition and physiological function of the probiotics in food. The conference specified the definition of probiotic as alive microorganisms which had beneficial effect on host when consumed at a certain amount by human. Two important concepts of probiotic now have been accepted worldwide: 1) the probiotic is a viable microorganism; 2) it has a beneficial effect on the host health.

3.4.4 Beneficial effects of probiotics

Probiotics are considered to affect the gut health. Many clinical studies and systematic analyses have shown the positive effects of probiotics on gut health of host, such as relieving constipation (Chmielewska & Szajewska, 2010), promotion of lactose intolerance (Levri, Ketvertis, Deramo, Merenstein, & D'Amico, 2005) and reducing diarrhoea (Van Niel, Feudtner, Garrison, & Christakis, 2002). Probiotic therapy has also been applied for irritable bowel syndrome and inflammatory bowel disease (Hong et al., 2009; Sartor, 2004). Capurso et al (2006) summarised 29 tests involving probiotics on colon cancer or tumor. They found that the preventative effects of

probiotics during the pre-cancer period or early stage of tumor formation were better than the therapeutic effects.

The immune modulation function of gut flora is mainly contributed by probiotics. Two strategies are thought to be applied by probiotics to control the immune response. First, the probiotic bacteria can be directly recognized by the gut-associated lymphatic tissue (GALT), inducing cell differentiation and simulating the secretion of cell factors. Secondly, probiotics can produce organic acids or antibacterial peptides to inhibit the growth of pathogen. Strong immuno-regulatory activity of probiotics has been reported in both adults and infants (Paineau et al., 2008; Taylor, Dunstan, & Prescott, 2007).

Besides the gut health and immune modulation, probiotic has been reported to have promotion effects on a large series of diseases such as controlling blood pressure, reducing serum cholesterol levels and anti-obesity. These numerous effects can hardly be explained by a unifying hypothesis that is based on a single quality or mechanism. The therapeutic effects of probiotics on some of these diseases still need farther study and clinical verification.

3.4.5 Commonly used species: *Bifidobacterium* and *Lactobacillus*

A desirable prebiotic bacterium species should meet several requirements (Afrc, 1989). Firstly, the bacterium should be non-pathogenic and not have any adverse effect. Secondly, the bacterium has the ability to survive and possess certain activity under gastrointestinal condition. *Bifidobacterium* and *Lactobacillus* are the major genera of probiotic bacteria that are most commonly used in the food industry as well as scientific research. Some streptococci and enterococcus species are also used as probiotics (Fuller, 2012).

Lactobacillus is a genus of gram-positive, non-spore-forming, facultative anaerobic or microaerophilic bacteria (Makarova et al., 2006). Presently, 56 species are included in the genus *Lactobacillus* (Gomes & Malcata, 1999).This bacteria often grow in nutrient-rich matrix, such as human or mammal mucous membrane, sewage, milk product and rotten food. Many species of this genus have received much attention as potential probiotics in recent years. They can adjust and improve the biological equilibrium between the beneficial and harmful bacteria, thus benefiting the health of host (Sreekumar & Hosono, 2000). Both *in vivo* and *in vitro* study showed the consumption of lactobacilli leaded physiological benefits, such as promoting lactose absorption (Hyung Soo Kim & Gilliland, 1983) and relieving lactose intolerance (Lin, Yen, & Chen, 1998). Animal models also demonstrated some strains of *L. acidophilus* had the capability of inhibiting tumor development (Mital & Garg, 1992).

Bifidobacterium is a gram-positive and non-sporeforming anaerobic bacteria (Ishibashi, Yaeshima, & Hayasawa, 1997). According to Tissier (1900), bifidobacterium is a pleomorphic rod usually with different shapes, including short, club-shaped rods, curved rods and bifurcated Y-shapes, isolated from the faeces of breast-fed infants. This bacterial genus constitute around 3-7% of intestinal microbiota in adults and up to 91% in newborns (Cheikhyoussef et al., 2009; Salminen & Von Wright, 2004). In human or mammals, there is nearly no bifidobacterium in upper small intestinal but large amount in large intestinal with a level up to 10^8-10^{12} CFU/g. In addition, small amount of this species is found in human oral cavity and vagina. The optimal temperature for this bacteria is 37 ^oC and they cannot grow in condition with pH lower than 5.0 or higher than 8.0. But the choice of optimal condition for bifidobacteria greatly depends on the species. Based on the DNA homology and sugar consumption, bifidobacteria are classified into thirty-nine species (Pokusaeva,

Fitzgerald, & Sinderen, 2011), including previous thirty species and recent additions of nine. Bifidobacterium in human or animal gut is reported to have health-promotion effects on host, such as providing absorbable products by fermentation of non-digestible carbohydrate, defending pathogen and modulating the immune response (Rastall et al., 2005). A significant increasing of research interests in the bifidobacterial genome has impelled scientists to sequence over twenty strains of this particular bacterial group during the last several years. These investigators also devote to reveal the genetic basis of these bacteria for their health benefits on the hosts.

3.4.6 Carbohydrate metabolism by *Bifidobacterium*

Bifidobacteria are saccharolytic in their fermentation strategies. The capability of utilizing complex and nondigestible carbohydrates from diet and/or host gives the ability of bifidobacteria to survive and colonize in GIT. The majority of bifidobacteria is anaerobic except for some species isolated from insect (Bottacini et al., 2012). Therefore they can just obtain energy by sugar fermentation in host.

The transportation and uptake of carbohydrates in the bifidobacterial cells are mainly driven by ATP-bind cassette (ABC) transporter system, major facilitator superfamily (MFS) transporter system or phophoenolpyruvate:carbohydrate phosphotransferase (PEP-PTS) transporter system (Pokusaeva, Fitzgerald, & van Sinderen, 2011)(Figure 3-6). MFS transporters act as symporters to transport the sugars by H^+ or Ca^+ gradient while ABC transporter system contains cell surface binding proteins to recognize the sugars and then transports the sugars by ABC transporters meanwhile ATP is consumed. PEP-PTS system applies facilitated diffusion together with phosphorylation cascades to phosphorylate the imported sugars, which prevent the sugars from diffusing back. Until now ABC-type transporter systems have been

frequently identified in most bifidobacterial genomes to act predominantly for carbohydrate uptake while PEP-PTS systems are less identified (Barrangou et al., 2009). Oligosaccharides uptake greatly depends on the ABC-type transporters (Schneider, 2001) while PEP-PTS system is only applied for the internalization of monosaccharide and some simple disaccharide (Deutscher, Francke, & Postma, 2006; Lengeler, Mayer, & Schmid, 1982), which explains the reason that complex carbohydrates rather than monosaccharides are available in the large intestine as substrates for gut flora.

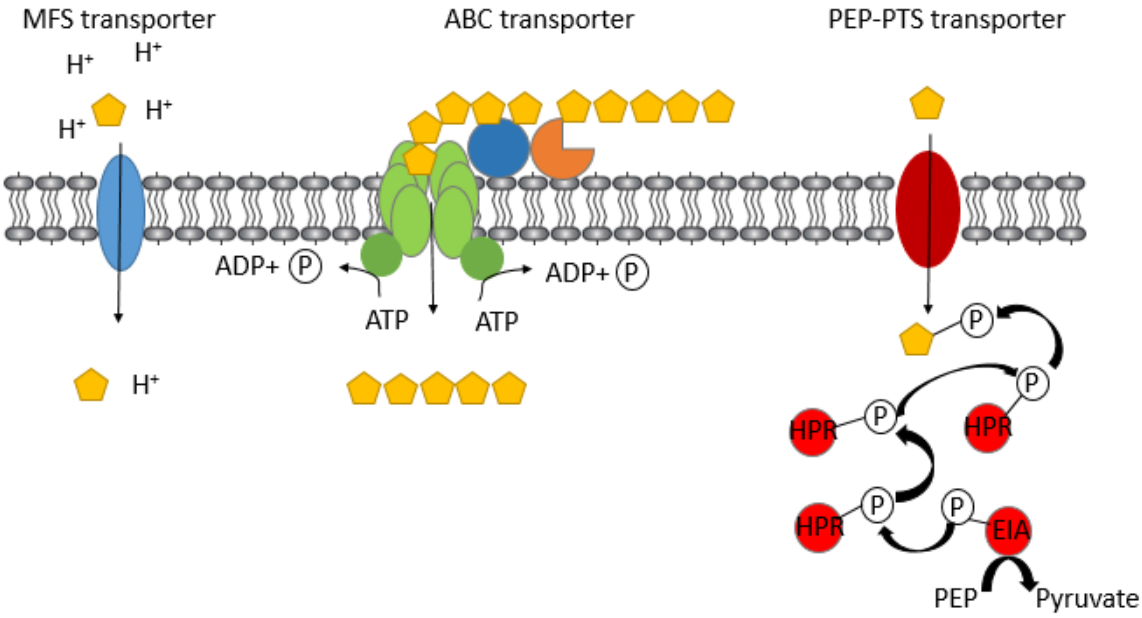

Figure 3-7 Sugar transport systems in bifidobacterium.

Most of the gut microbes go through the pentose phosphate pathway and glycolytic pathway for heterofermentation and homofermentation of carbohydrates, respectively (Kandler, 1983), while bifidobacteria is assumed to employ the fructose-6-phosphate phosphoketolase (F6PPK) pathway which is also called bifid shunt (de Vries & Stouthamer, 1968; Scardovi, 1965). F6PPK also found in all other bacteria belonging to the family *Bifidobacteriaceae* is the key enzyme for the pathway and is considered

as a taxonomic marker of the family (Felis & Dellaglio, 2007). It should be noticed that both pentose and hexose can be assimilated through F6PPK pathway to produce short-chain fatty acids (SCFAs) which are considered to be beneficial for the host (Palframan, Gibson, & Rastall, 2003).

PS from natural sources have many pharmacological functions. Although the exact structures of these macro-molecular polymers are still unclear, the basic information on their chemical composition and linkage has been intensely studied. These PS may perform their functions through two strategies, directly acting on the related cells or modulating the balance of gut flora. Investigation on the structures of PS and their interactions with gut microbiota is of great importance to reveal the function mechanisms.

Chapter 4 General materials and methods

The whole project process and some commonly used materials and experimental methods are described in this chapter. Some conditions of the experimental methods may vary based on the different materials applied. These specific conditions are recorded detailed in the related chapters.

4.1 Outline of the project

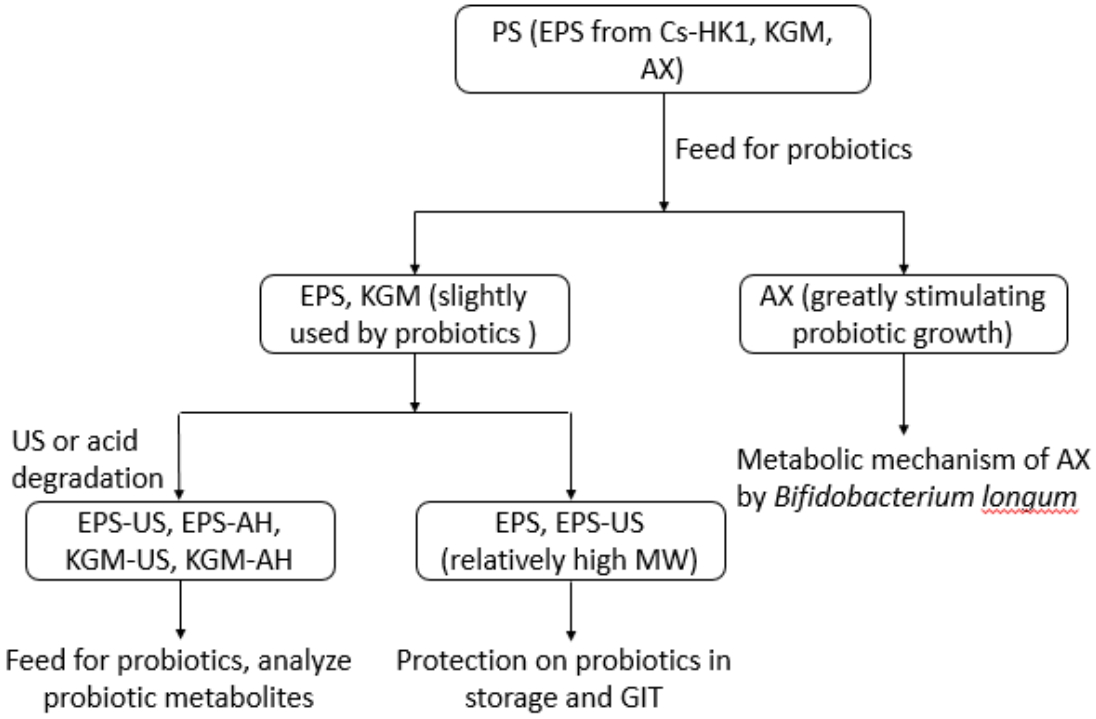

Figure 4-1 Outline of the project

Three types of PS were used in this project, KGM, AX and EPS from *C. sinensis* fungus Cs-HK1 mycelial fermentation. Their prebiotic functions were evaluated on the growth of selected bifidobacterial species. Preliminary results showed that both native EPS and KGM could only slightly support the bacterial growth which was probably

due to the high MW and viscosity of these large polymers. Therefore lower MW fractions were prepared by ultrasound (US) degradation and acid hydrolysis. When the US-degraded EPS (EPS-US) was used as a carbon source, a very high bacterial viability was observed, while the acid-hydrolysed EPS (EPS-AH) with much lower MW did not show this effect. Therefore, the EPS samples with relatively high MW including native EPS and EPS-US were further examined of their protective effect on probiotic bacteria during cold storage as well as in gastrointestinal condition. AX, unlike EPS and KGM, had a strong stimulation effect on the growth of *Bifidobacterium longum* while the other four species of bifidobacteria could not use AX. Therefore the metabolic mechanism of AX by *B. longum* was then investigated by combination of cellular and molecular approaches.

4.2 Bacterial species, culture conditions and inoculum preparation

Six species of *Bifidobacterium* and one *Lactobacillus* were used in the experiments of this project including *B. adolescentis* (CICC 6070), *B. bifidum* (CICC 10395), *B. breve* (CICC 6079), *B. infantis* (CICC 6069), *B. longum* (CICC 6186), *B. infantis* (R33) and *L. casei*. The first five bifidobacteria were obtained from China Centre of Industrial Culture Collection (CICC), the sixth was from Biostime Inc. (Guangzhou, China) and the *L. casei* was gift from Peter CK Cheung's lab at the Chinese University of Hong Kong. The bacterial species were stored in 30% glycerol at -80 ^{o}C. The bacterial culture was initiated by inoculation of each species from the storage in Reinforce Clostridial Medium (RCM) agar for bifidobacterium or de Man, Rogosa and Sharp (MRS) agar for lactobacillus in a petri dish and incubation for about 2 days. A single colony on the agar plate was picked out and inoculated into 5 mL of RCM or MRS liquid broth in a 10 mL centrifuge tube to prepare the starter culture, followed by shaking incubation for 18-22

h at 200 rpm. The culture period for the starter culture was determined based on the preliminary experiments when the culture reached the early stage of stationary phase. All bacterial cultures were incubated at 37 ^{o}C under anaerobic condition in air-tight jar with anaerobic gas generating sachets (AnaeroGen TM, Thermo Scientific Oxoid, USA) (Tanner et al., 2014).

RCM broth contained 10.0 g/L beef extract, 10.0 g/L peptone, 3.0 g/L yeast extract, 1.0 g/L soluble starch, 5.0 g/L glucose, 0.5 g/L cysteine HCl, 5.0 g/L NaCl, 3.0 g/L sodium acetate, and 0.5 g/L agar. MRS broth contained 10.0 g/L enzymatic digest of casein, 10.0 g/L beaf extract, 4.0 g/L yeast extract, 2.0 g/L triammonium citrate, 5.0 g/L sodium acetate, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $MnSO_4 \cdot 4H_2O$, 2.0 g/L K_2HPO_4, and 20.0 g/L glucose, and 1.08 g/L Tween 80. MRS or RCM agar was prepared by adding agar (A 4675, Sigma-Aldrich, USA) in the broth to get final concentration of 1.5% (w/v).

4.3 Preparation of medium with alternative carbon source

For the experiments on various carbohydrates as alternative carbon sources to glucose for the bifidobacterial growth, all carbohydrate samples were added at 5 g/L (~0.5% w/v) to a glucose-free RCM broth (5 mL) in 10 mL centrifuge tubes and stirred over night at room temperature. Undissolved portion of the carbohydrates, if any, was removed by centrifugation at 4000 rpm (1780×g). Galacto-oligosaccharide (GOS), inulin or glucose at 5 g/L was tested as prebiotic reference and glucose-free RCM broth was used as control. In the experiments on combination of PS with glucose or the prebiotic GOS, the two carbohydrates were each added at 5 g/L to make a total of 10 g/L in the culture medium. All the mediums were sterilized by autoclaving at 121 ^{o}C for 20 min.

4.4 Measurement of bacterial growth and viability

The growth of bacterial cultures on various carbon sources was evaluated based on the final concentration and the colony-forming unit (CFU) (viability) of bacteria in the culture medium over a selected culture period. Bacterial concentration in the liquid culture medium was determined by measuring the optical density (OD) at 600 nm with a spectrophotometer. For CFU measurement, the bacterial suspension was diluted with 0.85% (w/v) saline sequentially from 10^{-1} to 10^{-10}, and then inoculated onto a solid RCM or MRS agar plate. After incubation in anaerobic atmosphere at 37 ^{o}C for 48 h, the number of viable colonies on the plate was counted, and valid counts in the range of 20-300 colonies were recorded.

4.5 Partial degradation of PS

Ultrasound and acid were applied to degrade PS in this project. Ultrasonic degradation mainly occurred by shear force generated through cavitation effect (Basedow & Ebert, 1977; Mason, Cobley, Graves & Morgan, 2011). This depolymerisation process involved two possible mechanisms, mechanical degradation of polymers by bubble collapse and chemical degradation due to the reaction between polymers and OH/H radicals. According to the recent reports focusing on ultrasonic process of PS (Koda, Taguchi & Futamura, 2011; Zhang et al., 2013), the degradation of these water-soluble polymers mainly induced midpoint scission on a large chain. Therefore sonication was considered to have drastic results in decreasing aqueous viscosity and unique effects on the modification of PS. Acid degradation was a much intenser method which resulted in random cleavage of both backbones and branches. It was usually applied to generate monosaccharides and oligosaccharides.

4.5.1 Ultrasonic degradation

Ultrasonic degradation and the recovery of the degraded carbohydrates were carried out as reported previously with minor changes (X. Chen, Siu, Cheung, & Wu, 2014; J. Li, Li, Geng, Song, & Wu, 2017). Power US was generated with a VCX 750 processor with a fixed frequency of 20 kHz and a maximum output power of 750 W (Sonics and Materials Inc., Newton, USA). PS solution was prepared in a glass beaker and the US probe (with a tip diameter 13 mm) was inserted into the sample solution for irradiation. During the US treatment, the sample beaker was surrounded with ice to avoid overheating. The US power was fixed at 80% amplitude and the treatment period was 30 min. After the US treatment, sample solution was concentrated by vacuum evaporation at 40 ^{o}C and then lyophilised to yield US-degraded samples.

4.5.2 Acid hydrolysis

For more extensive degradation of the PS into much lower MW fractions, the samples were hydrolysed with trifluoroacetic acid (TFA). Each 0.15 g of the PS sample mas mixed with 60 mL of 2 M TFA in a 100 mL round bottom flask with vigorous stirring. The time and temperature of hydrolysis process were dependent on different PS. The hydrolysate was evaporated to dryness at 40 ^{o}C under vacuum in a rotary evaporator, washed several times with methanol to completely remove TFA and then re-dissolved in 10 mL water. The solution was freeze dried to give the acid hydrolysed samples.

4.6 Monosaccharide composition and molecular weight analysis

Monosaccharide composition was analysed by the 1-phenyl-3-methyl-5-pyrazolone (PMP)-high-performance liquid chromatograph (HPLC) method as described previously (Siu et al., 2014) with minor modifications. The analysis was

performed with an Agilent Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm) on an Agilent 1100 instrument at 25 ^{o}C. Potassium phosphate buffered saline (0.05 M, pH 6.9) containing 15 % (solution A) and 40% acetonitrile (solution B) was used as mobile phase. The sample (~5 mg) was hydrolysed with 2 mL of 2 M TFA at 110 ^{o}C for 4 h, followed the evaporation under vacuum. The residual solid was re-dissolved in 2 mL DI water and mixed 0.5 M PMP solution in methanol and 0.3 M NaOH solution at an equal volume (450 μL) and maintained at 70 ^{o}C for 30 min. The reaction was stopped by adding 450 μL of 0.3 M HCl, followed by washing thrice with chloroform, and the aqueous layer was collected for the HPLC analysis.

Molecular weight (MW) of the PS samples was determined by high-pressure gel permeation chromatography (HPGPC) as reported previously (Q.-L. Huang, Siu, Wang, Cheung, & Wu, 2013). The analysis was performed with a Waters 1515 isocratic pump, a 2414 refractive index detector, and three columns in series, Ultrahydrogel 120, 250 and 2000 (7.8 mm × 300 mm) (Waters Co., Milford, MA, USA) at 50 ^{o}C. Milli-Q water was used as the mobile phase at a flow rate of 0.6 mL/min. All samples were dissolved in Milli-Q water (0.2 mg/ml for EPS-US and KGM-US, 3 mg/ml for EPS-AH and KGM-AH), centrifuged at 6000 rpm (4020×g) for 15 min and filtered through 0.45 μM membrane before injection. Calibration curve was derived with dextran MW standards 1, 5, 12, 25, 50, 80, 270, 410 and 670 kDa and computated with the Breeze V3.3 software.

4.7 Statistical analysis

Student *t-test* was the only statistical method applied in this project to analyse the difference between control and sampling groups.

Chapter 5 Bifidogenic effects of KGM and Cs-HK1 EPS

5.1 Introduction

The gut microbiota or microflora plays an important role in human health and disease. Prebiotics are non-digestible food ingredients such as carbohydrate fibres that can selectively stimulate the growth and metabolic activity of certain bacteria in the colon in a way beneficial to the host health (Gibson & Roberfroid, 1995). The undigested carbohydrate fibres enter the large intestine or colon and may be utilized as the carbon sources by the gut bacteria via anaerobic metabolism (Glenn R. Gibson & Fuller, 2000). The chief end metabolite products of bacterial fermentation in the gut are short chain fatty acids (SCFAs) including acetate, propionate and butyrate, which have important physiological functions and health benefits to the host. The ingestion of prebiotic foods and supplements has been increasing taken as a viable measure for restoring and maintaining the healthy balance of gut microbiota so as to improve human health (Roberfroid, 2007; Rouzard & Gibson, 2004). However, only a small number of carbohydrate polymers or oligomers are generally accepted and applied as probiotic ingredients including fructooligosaccharides (FOS), GOS and inulin extracted from chicory root (Gibson & Roberfroid, 1995; Roberfroid, 2007).

Natural PS from edible plants and fungi are regarded as potential sources of prebiotics (Aida, Shuhaimi, Yazid, & Maaruf, 2009). Because of the important role of the gut microbiota in human health and disease and the increasing demand for prebiotic products, it is of significance to evaluate the prebiotic function of new and alternative probiotic materials. A common and feasible approach for evaluating the prebiotic function of samples is assessment of their effects on the growth and SCFA production of probiotic bacterial species belonging to the *Bifidobacteria* and *Lactobacilli* genera

in vitro cultures. Many previous studies have evaluated the effects of natural PS from food on the cell growth and production of SCFAs during *in vitro* fermentation of probiotic bacteria. Among the various molecular properties MW is an important factor affecting the fermentability and prebiotic activity of natural PS and their hydrolysed low-MW derivatives (Hughes & Kolida, 2007). In general, carbohydrate fibres with lower MW were more fermentable in bacterial cultures as most of the prebiotic fibers belong to low to medium MW carbohydrate polymers. Therefore, high MW natural PS have been hydrolyzed by enzymes or acids for improving the prebiotic activities in many previous studies (Rouzard & Gibson, 2004).

Konjac glucomannan (KGM) is a water-soluble PS from the root of *Amorphophallus konjac* plant which has been traditionally cultivated and used as a food ingredient in China and some other Asian countries. KGM or konjac flour has been widely used as a thickening and gelling additive to food products because of its desirable physical properties. KGM is a linear copolymer of (1→4) linked β-D-mannose and β-D-glucose at molar ratio of 1.6:1 (Dave, Sheth, McCarthy, Ratto, & Kaplan, 1998). More recently, KGM has been recognized as a dietary fibre and functional food supplement for relieving constipation, lowering the risk of type 2 diabetes and obesity and cholesterol (Arvill & Bodin, 1995; Chen et al., 2008) and other health benefits (Behera & Ray, 2016). As a major source of dietary fibre, the health effects of KGM are most probably associated with its potential prebiotic function (Connolly, Lovegrove, & Tuohy, 2010). Enzymatic hydrolysates of KGM have been shown to promote the growth of *Lactobacilli* and *Bifidobacteria* in culture (Al-Ghazzewi et al., 2007).

Edible and medicinal fungi or mushrooms provide a rich source of health promoting PS with notable antitumor, immunomodulatory and other bioactivities. Most

of the bioactive PS from mushrooms are non-starch with complex structures and various glycosidic linkages. They are usually non-digestible in the human gastrointestinal tract and can be potential prebiotic fibres for the gut microbiota (Synytsya et al., 2009). Therefore, mushroom has been recognized as a potential source of prebiotics (Aida et al., 2009). However, the potential of mushroom as a valuable source of prebiotics remain to be widely explored. To date only a small number of PS from mushrooms have been evaluated for prebiotic function compared to the many of bioactive PS documented in the literature. *Cordyceps sinensis* generally called Chinese caterpillar fungus or Dong-Chong-Xia-Cao is a special and highly-valued medicinal fungus which has been used in traditional Chinese medicine mainly as a tonic with numerous health benefits (Zhu et al., 1998b). PS are the major bioactive constituents of *C. sinensis* fungus with antitumor, immunomodulatory and some other medicinal properties (Yan et al., 2014). As natural *C. sinensis* caterpillar fungus is very rare and expensive, mycelial fermentation is mainly applied for commercial production of *C. sinensis* biomass and PS. We have established the mycelial culture of a *C. sinensis* fungus Cs-HK1 and applied it to liquid fermentation for the production of exopolysaccharide (EPS) (Leung, Zhang, &Wu, 2006; Yan et al., 2014). However, little or no information is available on the prebiotic activity of PS originated from the *C. sinensis* species.

This chapter was to evaluate the bifidogenic effects in relationship to the MW range of KGM and the EPS of Cs-HK1 based on their utilization as an alternative carbon sources to glucose in liquid cultures of several common bifidobacterial species. The native KGM and EPS were first degraded with high-intensity power ultrasound for higher water solubility, and then further hydrolysed with trifluoroacetic acid to much lower MW range. Their effects on the growth, viability and SCFA production of the bifidobacteria were measured in comparison with some known prebiotic OS.

5.2 Materials and methods

5.2.1 Production of crude EPS from Cs-HK1 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 (Leung et al., 2006). As described previously, the liquid medium for Cs-HK1 mycelial fermentation consisted of 40 g/L glucose, 5 g/L peptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 10 g/L yeast extract. The liquid fermentation was carried out in 1 L Erlenmeyer flasks each filled with 200 mL of the liquid medium at 20 ^oC 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. Ethanol (95%) was added into the solid-free liquid medium at 5:1 volume ratio and maintained at 4 ^oC overnight for precipitation. The precipitate was separated by centrifugation at 12,000 rpm (21,612×g) for 15 min and then freeze-dried, yielding crude EPS. The whole experimental procedure for isolation and purification of EPS and the preparation of partially degraded EPS fractions is shown with a flowchart in Figure 5-1.

5.2.2 Degradation of EPS and KGM

The native EPS produced by the Cs-HK1 fermentation and the KGM attained from the commercial supplier had very high MW and low water solubility, they were exposed to power ultrasound (US) for partial degradation to lower MW and higher solubility. The raw KGM material with a purity over 90% was attained from Hubei Konson Konjac Gum Co., Ltd. (Hubei, China) and used without purification. For preparation of US-degraded of EPS, the supernatant from centrifuged Cs-HK1 mycelial fermentation liquid (1 L) was transferred into a glass beaker and the US probe was inserted into the

sample liquid for irradiation. After the US treatment, ethanol precipitation was performed and the precipitate was recovered and dried all as described above, yielding the US-degraded EPS, EPS-US. The crude EPS-US was further purified through a series of steps as shown in Supplemental data, Fig. 1. The crude EPS-US (5 g/L) was first deproteinized by Sevage treatment (1-butanol/chloroform at 1:4 v/v) at 25 ^{o}C with constant stirring for 30 min and then centrifuged to remove denatured protein. This step was repeated several times until no protein layer was visible. The deproteinized EPS-US solution was dialysed using a 3.5 kDa membrane against water for 48 h. The purified EPS-US solution was concentrated by vacuum evaporation and then lyophilised. The KGM solution (10 g/L in deionized water) was treated by ultrasound and recovered to yield degraded KGM-US

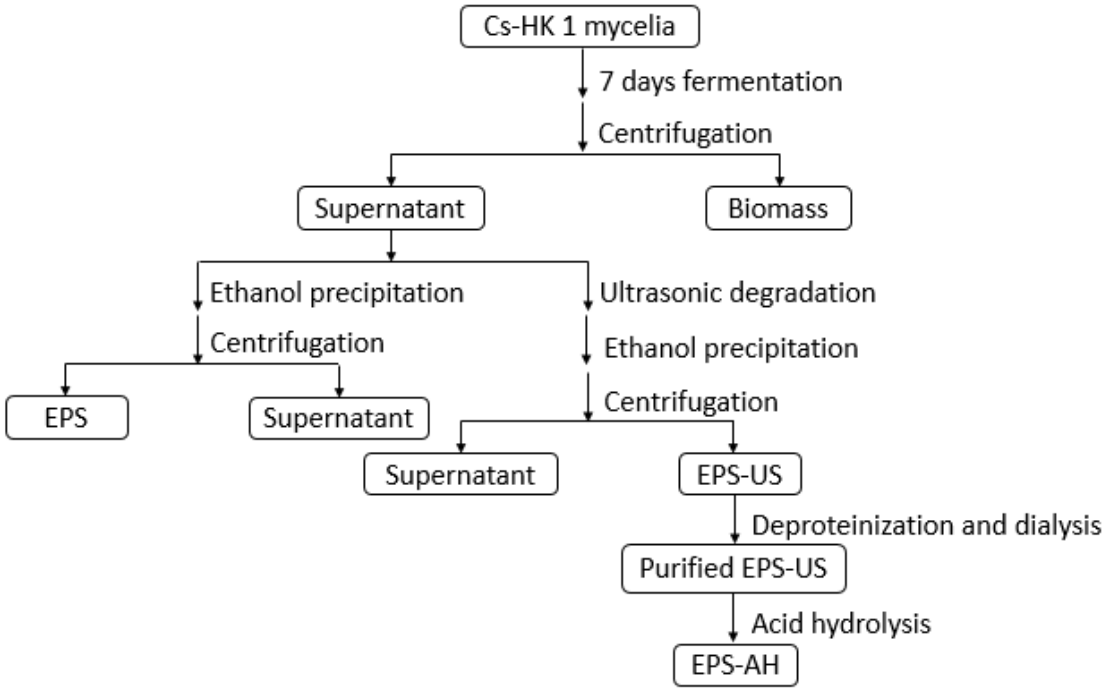

Figure 5-1 Experimental procedure for isolation of EPS from Cs-HK1 mycelial fermentation medium and for preparation and purification of ultrasound-degraded and acid-hydrolysed EPS fractions.

For more extensive degradation of the PS into much lower MW fractions, the purified EPS-US and KGM-US were hydrolysed with trifluoroacetic acid (TFA) at 70 ^{o}C for 4 h as described in Chapter 4.5.2.

5.2.3 Chemical composition and molecular weight analysis

The total carbohydrate content of all PS samples (native and degraded EPS and KGM) was determined by Anthrone test, involving the acid hydrolysis of PS samples in the presence of anthrone agent to yield a yellow green colour at 100 ^{o}C (Chaplin & Kennedy, 1994). The absorbance of sample solution was measured at 620 nm with a spectrophotometer and glucose was used as a standard. The protein content was determined by Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), through the reaction with copper ions at 100 ^{o}C under alkaline condition to generate a blue colour. The absorbance of sample solution was measured at 750 nm and bovine serum albumin (BSA) was used as a standard.

Monosaccharide composition of EPS and KGM was analysised by PMP-HPLC method and MW was determined HPGPC (Chapter 4.6).

5.2.4 Bacterial growth on EPS and KGM

Five bifidobacteria species were used in this study, *B. adolescentis* (CICC 6070), *B. bifidum* (CICC 10395), *B. breve* (CICC 6079), *B. infantis* (CICC 6069) and *B. longum* (CICC 6186). Bacteria from starter culture was inoculated at 1% (v/v) into RCM medium containing EPS or KGM sample as single carbon source. The cultures were incubated at 37 ^{o}C, 200 rpm for 2 days, followed by OD and CFU determination.

5.2.5 Measurement of medium pH and SCFAs

The culture medium pH was measured before and after bacterial fermentation with a pH meter. Short chain fatty acids (SCFAs) in the bacterial culture medium were analysed by gas chromatograph (GC) according a documented method with minor modifications (Zhao, Nyman, & Åke Jönsson, 2006). In brief, the bacterial culture liquid was diluted with 4 volumes of milli-Q water and the pH was adjusted to 2-3 with 1 M HCl, followed by centrifugation at 5000 rpm (2790×g) for 20 min. The supernatant was added with 2-ethylbutyric acid as an internal standard at 1 mM final concentration before injection into the GC system. The GC analysis was performed on 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) coated with a free fatty acid phase of 0.25 film thickness (DB-FFAP 123-3232, Agilent Technologies Inc.). Nitrogen gas was used as the carrier gas flowing at 0.6 mL/min. The initial oven temperature was 80 ^{o}C, maintained for 2 min, then raised gradually at 6 ^{o}C/min to 180 ^{o}C, and held for 4 min. The detector was controlled at 220 ^{o}C and the sample liquid (1 μL) was injected at 200 ^{o}C. Several SCFA standards (Aladdin®, Shanghai, China) were used for identification and quantification including acetic acid (A116165), propionic acid (P110443), *n*-butyric acid (B110439), *i*-butyric acid (I103521), *n*-valeric acid (V108269), and *i*-valeric acid (I108280).

5.3 Results and discussion

5.3.1 Chemical composition and physical properties of EPS and KGM

Table 5-1 shows the major chemical contents of EPS, KGM and their partially hydrolysed products. The EPS samples had a similar carbohydrate content (~70%) but different protein contents, which were lowest in the native EPS (6.97%) and highest in EPS-US (18.4%). A possible explanation for the relatively low protein content of the

native EPS was that EPS with a very high MW had a high viscosity and formed large aggregates which trapped the protein molecules. The US treatment of EPS disrupted the aggregates and lowered the viscosity, freeing the protein molecules (Chen, Siu, et al., 2014). In contrast, all native and degraded KGM samples had the similar carbohydrate content of 65-70% and protein content of ~2.5%. The HPLC analysis (Appendix, Figure A1) shows that EPS had three monosaccharide constituents, mannose, glucose and galactose at a molar ratio of 1.73:1.81:1, and KGM had two, mannose and glucose at molar ratio of 1.93:1, which were consistent with those reported previously (Katsuraya et al., 2003; Yan et al., 2014).

Table 5-1 Major chemical contents of EPS, KGM and their partially degraded products

Sample	Carbohydrate (wt %)	Protein (wt %)
EPS	71.34 ± 3.82	6.97 ± 0.15
EPS-US	70.72 ± 3.10	18.43 ± 1.44
EPS-AH	70.07 ± 6.41	13.04 ± 2.82
KGM	64.51 ± 3.36	2.38 ± 0.78
KGM-US	67.44 ± 1.04	2.43 ± 1.56
KGM-AH	70.32 ± 1.13	2.56 ± 2.05

Figure A2 shows the GPC profiles of native and partially degraded EPS and KGM samples. The native EPS exhibited several peaks, a large broad peak group, peak1 around 27.167 min with very high MW (~10^8) and several small peaks, e.g. peak 2 and peak 3 at 42.333 min and 50.867 min, respectively. The native KGM exhibited two major peaks at 26.283 min (MW~10^8) and 30.883 min, respectively. The GPC results indicate that both native EPS and KGM were composed of different MW fractions. Compared to the native EPS, the US-degraded EPS-US also had three major peaks but fewer small peaks, implying a more uniform MW distribution. In the GPC of EPS-AH,

the high MW group peak 1 had a delayed elution time and reduced peak area; the two lower MW peaks peak 2 and 3 had similar elution time, and peak 3 become sharper and higher. The changes in the GPC profile and MW distribution of KGM after US and acid treatment were similar to those of EPS. All these changes were mainly attributed to polymer degradation and reduction of MW moderately by US and dramatically by acid hydrolysis.

Table 5-2 presents the MW distribution data of all EPS and KGM factions derived from the GPC analysis. Both native EPS and EPS-US consisted mainly of high MW fractions (over 10^8) and a small fraction of reducing sugar (MW 210-220) based on the relative peak areas. The acid hydrolysis resulted in significant reduction of the maximum MW (to ~7×10^5) and increase in the percentage of low MW fractions. The MW distribution and composition of KGM were affected more significantly by US degradation and acid hydrolysis with notable MW reduction and increase in the percentage of low MW fractions. In particular, nearly 100% of KGM-AH was composed of low MW oligomer about 1400 (Vodeničarová, Dřímalová, Hromádková, Malovíková, & Ebringerová, 2006). Overall, the MW results show that US is mainly suitable for moderate reduction of MW and acid hydrolysis is a much more vigorous method for effective degradation of large PS to small molecules such as oligomers.

5.3.2 Bacterial growth on glucose

Figure 5-2 shows the growth curves of the five bifidobacterial species in the normal RCM liquid medium containing glucose as the carbon source, which were all in a sigmoid pattern. Most of the bacterial species started with a lag growth phase for about 6 h with little or no increase in the cell concentration and then underwent a rapid growth period for 10-12 h with a notable increase in the cell concentration. Four of the bacterial

species reached the stationary phase in about 18 h post inoculation, while *B. bifidum* reached the stationary after 24 h. The maximum cell concentration (OD) as well as the average growth rate differed among the different species, being the highest (~1.0) with *B. adolescentis* and the lowest with *B. bifidum* (<0.5), and ~0.7 with the other three species. The maximum OD of all species remained at the similar level after 48 h of culture (data not shown).

Table 5-2 Major molecular weight (MW) peaks on the GPC profiles of EPS and KGM samples.

Sample	Retention time (min)	MW (Da)	% Area
EPS	27.167	2.768×10^{8}	85.75
	42.333	1.858×10^{5}	4.61
	50.867	213	9.65
EPS-US	28.633	2.252×10^{8}	82.75
	42.833	1.226×10^{4}	5.10
	50.069	217	12.15
EPS-AH	35.719	7.098×10^{5}	25.85
	40.353	5.641×10^{4}	27.94
	45.700	3,981	19.02
	50.868	214	20.82
KGM	26.283	8.773×10^{8}	69.83
	30.883	2.781×10^{7}	27.88
KGM-US	26.600	8.885×10^{7}	4.29
	29.100	2.009×10^{7}	1.39
	34.100	1.774×10^{6}	78.04
KGM-AH	47.367	1,369	98.89

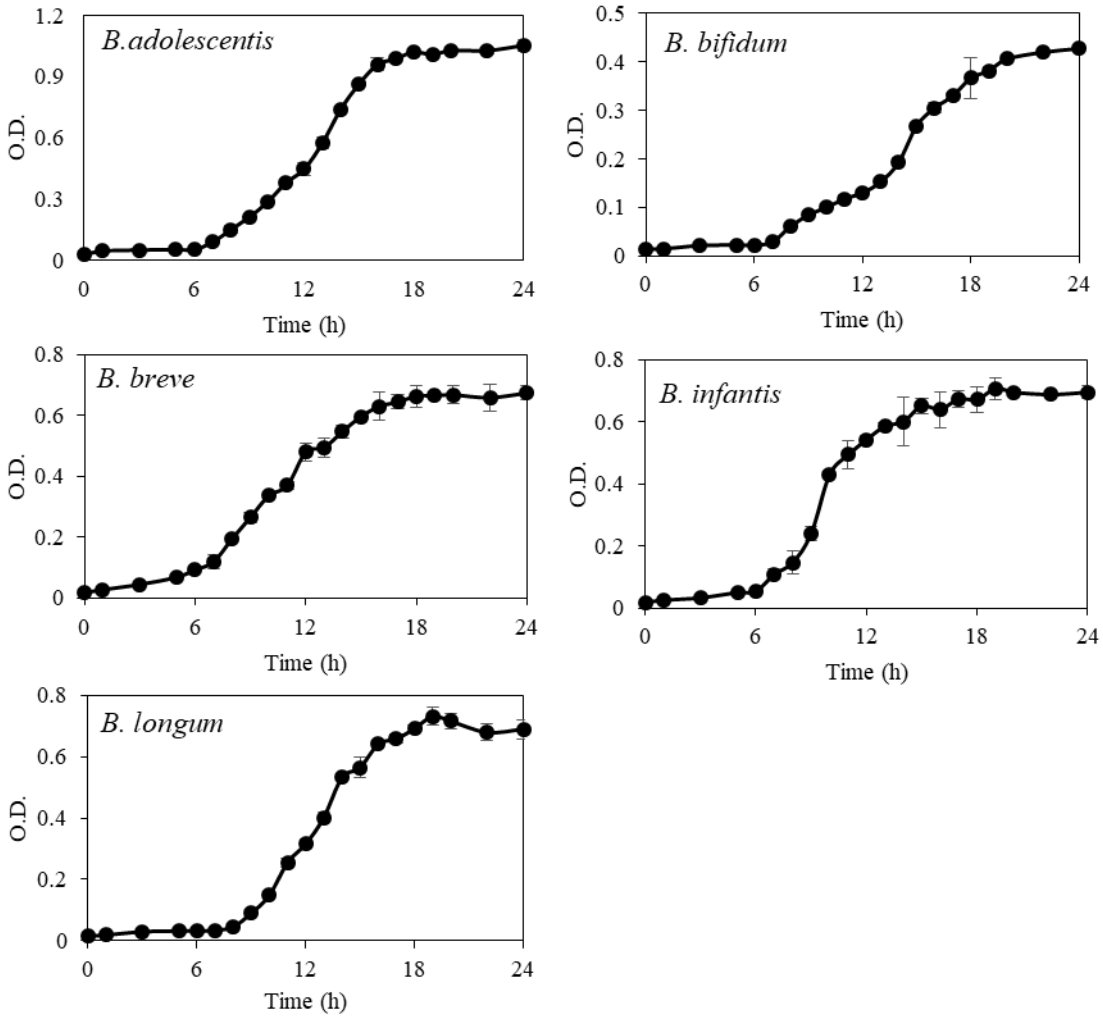

Figure 5-2 Growth curves of five *Bifidobacteria species* cultured in RCM medium.

5.3.3 Bacterial growth on degraded EPS and KGM as alternative carbon sources

Figure 5-3 shows the results from the growth experiments of bifidobacterial species on different carbon sources. The acid hydrolysed PS fractions EPS-AH and KGM-AH supported a better growth of all five bacteria species (reaching a higher OD or bacterial concentration) compared with the control without a carbon source ($p < 0.05$ or $p < 0.01$). The ultrasound degraded fractions, EPS-US and KGM-US, only supported the growth of some species, *i.e.* EPS-US by *B. adolescentis* and *B. bifidum* ($p < 0.05$), and KGM-US by *B. bifidum* ($p < 0.05$). The results suggest that lower MW fractions from acid hydrolysis were more easily utilized or fermentable by the bifidobacteria.

However, these partially hydrolysed PS were much less favourable for bacterial growth than the glucose and the prebiotic oligosaccharide GOS. In addition, GOS was more favourable than glucose for most of the bifidobacterial species especially *B. bifidum* and *B. breve* to grow to higher concentrations. When the acid hydrolysed EPS and KGM fractions were applied to the medium containing glucose or GOS (i.e. Glc or GOS+EPS-AH or KGM-AH), the growth of some bacterial species were enhanced, suggesting the ability of the bacterial species to metabolize different carbohydrates. However, addition of the US degraded EPS-US and KGM-US fractions significantly suppressed the growth of most bacterial species.

The better growth of bifidobacteria with lower MW KGM fractions derived from acid hydrolysis is in agreement with that reported previously on acid hydrolysed glucomannans as carbon sources for growth of probiotic bacteria (Connolly et al., 2010; Huang, Liu, Yang, & Huang, 2007; Pan, Chen, Wu, Tang, & Zhao, 2009). As reported by Chen et al. (2005), acidic hydrolysates of glucomannans increased the cecal bifidobacterial counts in mice more significantly than the native glucomannans. Although KGM-AH could increase the growth of bifidobacteria in the present study, the effect was not as significant as in many previous reports. This may be attributed to the different bacterial species used. Moreover, most previous studies were carried out in faecal cultures or animal models while pure cultures of single bacterial species were used in this study. The collaboration and interaction among different bacterial speices may contribute to a better use of PS in mixed faecal cultures than in pure bacterial cultures with limited number of PS degrading enzymes.

The fermentability of a PS by the gut bacteria is species specific and may require the collaborative action of different species to provide all the enzymes for cleaving the different glycosidic linkages. Therefore, the complex carbohydrate polymers are less

likely to be metabolized by a single bacterial species in pure culture. As no enzymes for hydrolyzing the carbohydrate polymers have been found in the extracellular media, PS and OS are mainly metabolized inside the bacterial cells, probably after being hydrolyzed by cell-associated glycoside hydrolases into monosaccharides. The uptake of carbohydrates by the bifidobacterial cell is mediated by certain carbohydrate transport mechanisms such as ATP-binding cassette (ABC) transporters, permeases and proton symporters (Pokusaeva, Fitzgerald, & van Sinderen, 2011; Ventura et al., 2009). The high MW and complex structure can make the carbohydrate more difficult to be transported through the cell and metabolized by the cell. The suppressed bacterial growth by EPS-US or KGM-US combined with glucose or GOS was due probably to the interference of these high MW polymers with the transport of nutrients to and through the bacterial cell.

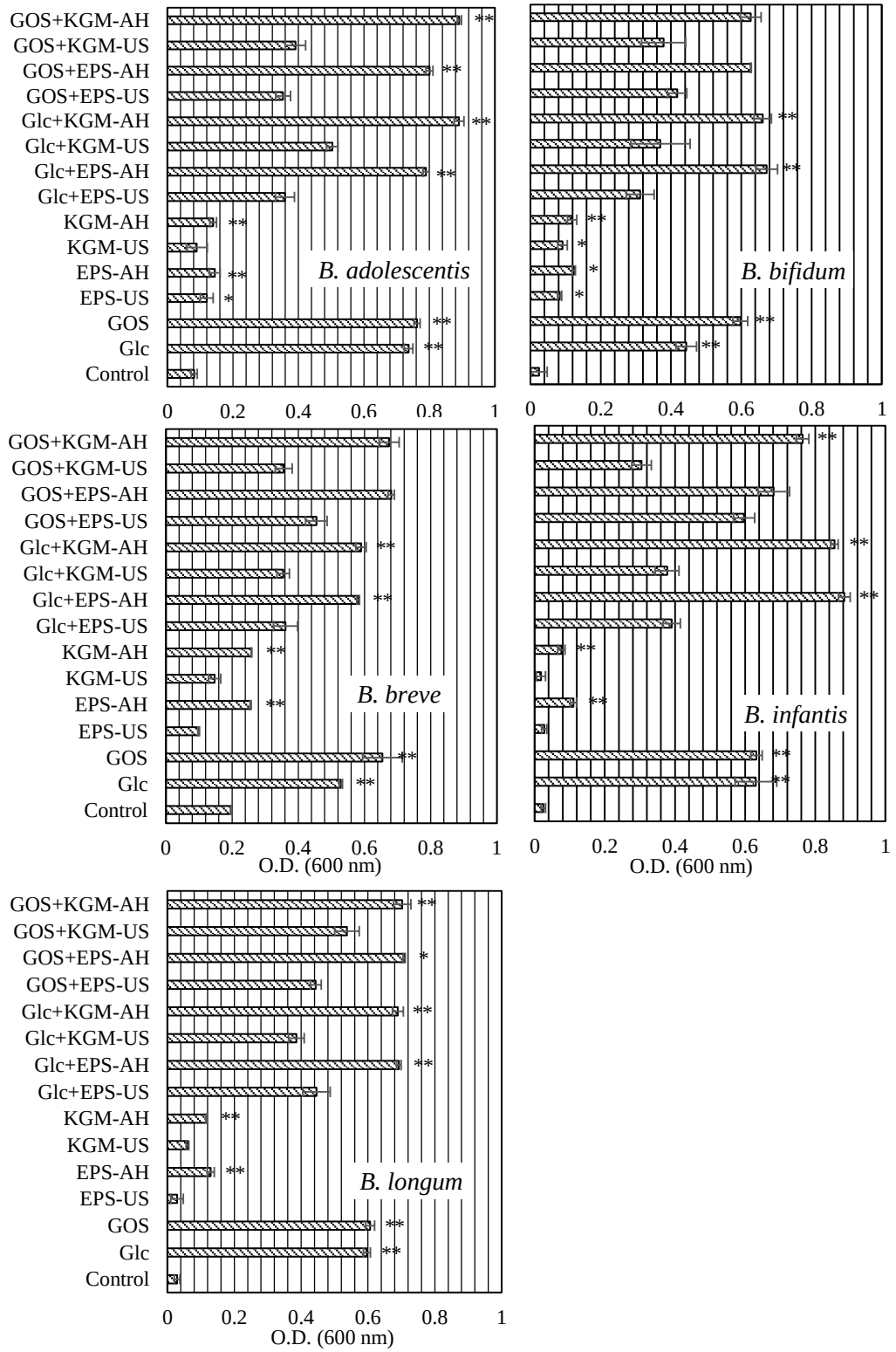

Figure 5-3 Optical density (600 nm) of five *Bifidobacteria* species cultured in RCM medium with different carbon sources (no C source in control group; concentration of single C source 5 g/L; total concentration of mixed C source 10 g/L and ratio of two C source 1:1; 48 h cultured period; *****: $p < 0.05$; ******: $p < 0.01$ by student *t*-test).

Table 5-3 Colony forming units (CFU) 10^8/mL of *Bifidobacteria* cultured with different carbon sources (culture period 48 h; C source concentration 5 g/L)

C source	*B. adolescentis*	*B. bifidum*	*B. breve*	*B. infantis*	*B. longum*
Control	0.77 ± 0.315	0.22 ± 0.09	(2.50 ± 0.46) × 10^3	0.70 ± 0.34	0.17 ± 0.14
Glc	0.50 ± 0.39	0.24 ± 0.04	(2.69 ± 0.43) × 10^3	1.8 ± 0.45	0.55 ± 0.07
GOS	0.78 ± 0.13	0.25 ± 0.10	(2.12 ± 0.54) × 10^3	1.53 ± 0.35	0.71 ± 0.33
EPS-US	**> 3 × 10^5**	**10.9 ± 5.72**	**> 3 × 10^5**	**> 3 × 10^5**	**(1.72 ± 0.38) × 10^4**
EPS-AH	1.07 ± 0.44	**1.24 ± 0.83**	**> 3 × 10^5**	1.20 ± 0.35	**19.43 ± 9.34**
KGM-US	**19.5 ± 7.09**	0.20 ± 0.04	**> 3 × 10^5**	**(1.48 ± 0.06) × 10^3**	**(5.30 ± 3.90) × 10^3**
KGM-AH	0.81 ± 0.20	**2.65 ± 0.62**	(1.56 ± 0.19) × 10^3	1.3 ± 0.69	0.19 ± 0.10

Bod-faced numbers highlight the significantly higher CFUs over control, $P < 0.05$, *t*-test.

5.3.4 Effects of EPS and KGM fractions on bacterial viability

Table 5-3 shows the results of CFU measurement from bacterial cultures with different carbon sources. OD is proportional to the total bacterial cell concentration including both viable and dead cells. Colony forming unit (CFU) is a reliable measurement of the viable cells in the culture or the cell viability. Although the OD of all the species cultured with glucose or GOS as the carbon source was much higher than that in control, the CFU values of most bacterial species were similar or only slightly higher. This means that the bacterial cultured in glucose and GOS medium contained a higher proportion of non-viable cells at the time of CFU measurement. This may be attributed to the depletion of limiting nutrients and accumulation of inhibitory metabolites in these cultures. Another possible cause for the higher cell death rate in these cultures is the more significant drop in the medium pH because the survival of probiotic bacteria is very sensitive to pH change during fermentation (Shah, 2000). As shown in Table 5-4, the initial pH of RCM medium was about 6.6-6.8 and was notably lower in most bacterial cultures after fermentation for 48 hours, and the lowest in the cultures with glucose and GOS for most bacteria species. The pH drop was mainly attributed to acid production from anaerobic fermentation, which is more rapid at a higher growth rate.

More interestingly, most bacterial species cultured in medium with the partially degraded EPS and KGM fractions as the carbon sources attained a much higher CFU (Table 5-3). Among these EPS and KGM fractions, EPS-US was the most effective for maintaining bacterial viability, giving rise to the highest CFU for all five species. For most bacterial species, KGM-US was more favourable than KGM-AH to support a higher viability. The high MW PS (EPS-US and KGM-US) appeared very beneficial for the survival of bacterial cells, though they were not well utilized as a carbon source

for the bacterial growth based on the above OD measurement. The results suggest that the EPS and KGM fractions have a protective effect on the survival and viability of probiotic bacteria and the protective effect is stronger at a higher MW.

Table 5-4 pH of the culture medium for *Bifidobacteria* with different carbon sources after 48 h fermentation (initial medium pH 6.6-6.8; C source concentration 5 g/L)

C source	*B. adolescentis*	*B. bifidum*	*B. breve*	*B. infantis*	*B. longum*
Control	5.71 ± 0.21	5.88 ± 0.08	4.93 ± 0.05	5.52 ± 0.06	5.65 ± 0.02
Glc	4.51 ± 0.09	4.92 ± 0.07	4.35 ± 0.03	4.39 ± 0.03	4.35 ± 0.08
GOS	4.40 ± 0.03	4.73 ± 0.26	4.14 ± 0.09	4.24 ± 0.07	4.41 ± 0.10
EPS-US	5.07 ± 0.06	5.72 ± 0.06	4.96 ± 0.12	5.02 ± 0.04	5.13 ± 0.01
EPS-AH	5.09 ± 0.07	5.66 ± 0.08	4.88 ± 0.12	4.96 ± 0.08	5.07 ± 0.12
KGM-US	5.91 ±0.07	6.21 ± 0.03	5.23 ± 0.17	5.86 ± 0.24	6.09 ± 0.03
KGM-AH	5.15 ± 0.07	5.41 ± 0.06	4.87 ± 0.01	5.03 ± 0.04	5.11 ± 0.05

Similar to the protective effect of EPS and KGM on the bifidobacteria in the present study, a previous study has shown that β-glucans produced by *Pediococcus parvulus* significantly increased the survival of *Lactobacillius paracasei* in the acidic environment during gastrointestinal passage (Stack, Kearney, Stanton, Fitzgerald, & Ross, 2010). A few previous studies have also investigated the protective effect of natural PS from different sources on probiotic bacteria in the gastrointestinal condition (Chou, Sheih, & Fang, 2013; Guergoletto, Magnani, Martin, Andrade, & Garcia, 2010; Michida et al., 2006).The protective effect of high MW EPS and KGM fractions on the bifidobacteria may share the similar mechanisms as for the EPS produced by *Lactobacilli* or lactic acid bacteria (LAB) (Caggianiello, Kleerebezem, & Spano, 2016). The bacterial EPS may be beneficial to the probiotic bacteria in adapting to extreme environments and protect the bacterial cell against biotic and abiotic stresses (Donot, Fontana, Baccou, & Schorr-Galindo, 2012). A possibility is that the EPS bind to the

bacterial cell surface to form a physical barrier, protecting the cell against the environment stresses (Chapot-Chartier, Monnet, & De Vuyst, 2011).

5.3.5 Short chain fatty acids (SCFAs)

The chief SCFA produced during the bacterial fermentation was acetic acid (Table 5-5). For most bacterial species, the acetic acid content was highest in the cultures using glucose as the carbon source and lowest in the control culture without a carbon source. The higher acid concentrations in the cultures supplied with the partially degraded EPS and KGM than the control suggest their utilization and metabolism by the bacteria as a carbon source. Overall, there was a weak correlation between the acetic acid contents and the OD values (Figure 5-3) for most of the bacterial species. Specifically, the ultrasound degraded EPS and KGM increased the acetic acid production of most species except *B. adolescentis* though they did not increase the cell concentration (OD) compared with the control. Together with the above CFU results, it can be suggested that the high MW EPS-US and KGM-US were utilized by the bacteria to maintain the metabolic activity.

5.4 Conclusions

In the present study, partially degraded EPS and KGM fractions in different MW ranges were tested as alternative carbon sources to glucose for in vitro culture of five bifidobacteria species. The growth, viability and short chain fatty acid production of bacterial cultures were dependent both on the bacterial species and the MW of EPS and KGM fractions. In general, the lower MW fractions derived from acid-hydrolysis were more favourable for the bacterial growth (with a notable increase in cell concentration). All EPS/KGM fractions, irrespective of their MW, were able to increase the acetic acid

production of some bifidobacterial species. The most remarkable effect of the EPS and KGM fractions was a dramatic enhancement of the cell viability (with a much higher CFU), especially by the high MW PS fractions. It is concluded from these results that the water-soluble EPS and KGM fractions have prebiotic or bifidogenic activity. It is of significance to assess the prebiotic function of EPS and KGM in mixed culture of faecal flora and to analyse quantitatively their fermentability, utilization and metabolism, and to investigate the protective effect of EPS and KGM on the probiotic bacteria.

Table 5-5 Acetic acid accumulation (mM) in liquid cultures of *Bifidobacteria* with different carbon sources (C source at 5 g/L; no C source in control group; 48 h culture in RCM medium).

C source	*B. adolescentis*	*B. bifidum*	*B. breve*	*B. infantis*	*B. longum*
Control	29.75 ± 2.22	26.85 ± 1.82	32.00 ± 2.94	25.70 ± 1.78	26.41 ± 1.67
Glc	**72.19 ± 7.06**	**70.68 ± 1.24**	**68.28 ± 4.09**	**77.90 ± 0.89**	**73.04 ± 3.88**
GOS	**55.75 ± 3.42**	**68.86 ± 3.32**	**55.18 ± 4.36**	**59.20 ± 5.37**	**58.91 ± 1.33**
EPS-US	42.68 ± 10.82	**39.97 ± 2.68**	**39.81 ± 3.37**	**42.24 ± 2.69**	**34.39 ± 3.23**
EPS-AH	34.23 ± 3.17	**34.85 ± 1.20**	**41.93 ± 2.64**	**35.56 ± 4.10**	**31.10 ± 5.11**
KGM-US	34.02 ± 3.01	**35.31 ± 2.17**	**38.67 ± 2.43**	**36.33 ± 3.67**	**34.09 ± 3.77**
KGM-AH	28.97 ± 1.49	**40.76 ± 3.47**	**44.18 ± 2.77**	**30.17 ± 2.23**	29.87 ± 2.27

Bold number highlights the significantly higher concentration over control, $P < 0.05$.

Chapter 6 Protective effect of Cs-HK1 EPS on probiotics in various conditions

6.1 Introduction

Recent years has witnessed an enormous interest worldwide in the development of probiotic, prebiotic and synbiotic products targeting a healthy human gut microbiota (Krumbeck, Maldonado-Gomez, Ramer-Tait, & Hutkins, 2016; Quigley, 2018). Human uptake of probiotic microorganisms has been regarded as an effective strategy for balancing the gut microbiota so as to improve gut health and lower the risk of various diseases such as metabolic syndrome, inflammation the brain and skin (Lee, Slonczewski, & Foster, 1994; Yahfoufi, Mallet, Graham, & Matar, 2018). Other potential health benefits of probiotic ingestion include increase of mineral absorption, immunity enhancement, inhibition of tumor formation and hypercholesterolemia prevention (Fuller, 1989; Schrezenmeir & de Vrese, 2001). *Bifidobacteria* and *Lactobacilli* are the most common probiotic species used in functional foods and dietary supplements (Tripathi & Giri, 2014). As an effective probiotic product, the constituent bacteria should be resistant to the harsh conditions in the gastrointestinal tract (GIT), especially gastric acid and bile salt, reaching the large intestine alive to exert an influence on the gut microflora. The number of viable bacteria is an important quality index of probiotic products and is also essential for achieving the desired effects on the host (Lourens-Hattingh & Viljoen, 2001). Various measures have been explored to enhance the survival rate of probiotics, such as screening for high tolerance strains, encapsulation, and supplementation of PS and oligosaccharides (Heidebach, Först, & Kulozik, 2009; Michida et al., 2006; Tuomola, Crittenden, Playne, Isolauri, & Salminen,

2001). Supplementation of carbohydrate fibers is a relatively simple approach and is also possible to add extra benefits to the products, such as the prebiotic fibers in synbiotic products.

Edible and medicinal fungi or mushrooms provide a rich and diverse source of natural PS which are recognized as new prebiotic candidates (Aida et al., 2009). In addition to the few well-known β-glucans which have been applied to anticancer and immunotherapy (Wasser, 2002), many homo- and hetero-glucans have been isolated from mushrooms or fungal mycelia (Ramberg, Nelson, & Sinnott, 2010; Ruthes, Smiderle, & Iacomini, 2016). Most of the bioactive mushroom PS are resistant to the digestive enzymes in the human GIT and their health benefits and bioactivities such as immunomodulation and anti-inflammation may involve the modulation of gut microbiota (Jayachandran, Xiao, & Xu, 2017; Ramberg et al., 2010).

Cordyceps sinensis, the Chinese caterpillar fungus, is a precious medicinal fungus in traditional Chinese medicine for treatment of diseases related to the lung, kidney, and respiratory systems and for promotion of health and physical performance (Zhu et al., 1998b). Because the wild caterpillar fungus called Dong Chong Xia Cao in Chinese is very rare and expensive, mycelial fermentation has become the major resort for *Cordycpes sinensis* products. The mycelial culture of a *C. sinensis* fungus Cs-HK1 has been established in our lab for fermentation production of the mycelial biomass and PS (Leung, Zhang, & Wu, 2006). The exopolysaccharides (EPS) produced by Cs-HK1 fungus in liquid fermentation have shown notable antitumor, immunomodulatory and antioxidant activities in our previous studies (Yan et al., 2014). In our recent studies, the high MW fractions of Cs-HK1 EPS had protective effect on bacterial viability in normal culture conditions (Song, Mao, Siu, & Wu, 2018) and during exposure to antibiotics (Mao, Song, Wang, Yao, & Wu, 2018a).

The aim of this chapter was to further evaluate the potential of the Cs-HK1 EPS as a protective additive during the storage of probiotic bacteria and as a dietary, prebiotic fiber in the human GI environment. Experiments were performed in liquid cultures of some common probiotic strains of *Lactobacillus* and *Bifidobacterium* bacteria. The original EPS from the Cs-HK1 mycelial fermentation and a partially degraded EPS by power ultrasound were tested together with two well-known prebiotic fibers inulin and galactooligosaccharide (GOS) on the bacterial survival rate during cold storage and in simulated gastrointestinal conditions.

6.2 Materials and methods

6.2.1 Cs-HK1 mycelial fermentation and preparation of EPS fractions

Mycelial fermentation of Cs-HK1, isolation of EPS and preparation of EPS fractions were performed using the same methods as described in Chapter 5.2.1 & 5.2.2.

6.2.2 Physical property analysis of EPS

The molecular weight (MW) of EPS and EPS-US were determined by HPGPC. Intrinsic viscosity [η] of EPS samples were measured with an Ubbelohde viscometer at 25 ^{o}C. The sample were dissolved in water at series of dilute concentration. The intrinsic viscosity [η] of a sample was taken from the intercept of In(η_r/C) versus C line, where η_r is the relative viscosity and C the concentration of sample solution in g/100 mL (Committee, 2010). Apparent viscosity of EPS and EPS-US solutions at 5 g/L was measured by a rheometer (AMETEK Brookfield, USA).

Solubility of EPS and EPS-US in water was determined by the method previously used for starch (Mukerjea, Slocum, & Robyt, 2007). Samples (0.2 g) were suspended in 10 mL of DI water and stirred vigorously by a magnetic stir bar at room temperature

overnight. Sample solutions were centrifuged at 12,000 rpm for 20 min. The undissolved residue was removed from the supernatant and freeze dried.

Particle size distribution of EPS and EPS-US solution was measured at room temperature by a nanoparticle (405 nm) tracking analyzer (Nanosight NS300HSBF). The sample solution (5 mg/mL) was centrifuged at 12,000 rpm for 20 min. For each measurement, a total of 3 runs were performed.

6.2.3 Analysis of dietary fiber content in EPS

The total dietary fiber content was determined by the assay kit (TDF-100A, Sigma-Aldrich, USA). Briefly, EPS samples were first treated by heat stable α-amylase, protease and amyloglucosidase to remove protein and starch. Ethanol (95%) was then added for precipitation. The residues were filtrated and washed by ethanol and acetone. After drying, the residues were analyzed for the protein by Kjeldahl method and ash at 525 ^{o}C. The total dietary fiber was the weight of the residue less the weight of the protein and ash. Calculation equation was provided by the assay kit.

6.2.4 Degradation of EPS in simulated gastric acid

EPS and EPS-US were tested for acid resistance according to Tingirikari et al. (2014) with slightly modification. Simulated gastric acid was prepared using hydrochloric acid buffer containing 8 g/L NaCl, 0.2 g/L KCl, 8.25 g/L $NaHPO_4 \cdot 2H_2O$, 14.35 g/L NaH_2PO_4, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.18 g/L $MgCl_2 \cdot 6H_2O$. The pH of the buffer was adjusted to 2 by 5 M HCl. EPS samples was dissolve in water at 5 g/L. 2 mL simulated gastric acid was added to 1 mL sample solution and the mixture was incubated in 37 ^{o}C for 6 h. Sample was taken at 1, 2, 3, 4, 5, 6 h for reducing sugar and total sugar content determination. Reducing sugar content was determined by DNS

method (Robertson et al., 2001) and total sugar content was determined by anthrone test (Song et al., 2018).

6.2.5 Probiotic bacterial species and culture conditions

Four probiotic species were used in this chapter including *B. adolescentis* (CICC 6070), *B. infantis* (CICC 6069), *B. infantis* (R33) and *L. casei*. The bacteria from starter culture were subcultured at 2% (v/v) into RCM or MRS broth. All bacterial cultures were incubated at 37 ^{o}C, 200 rpm under anaerobic condition (Tanner et al., 2014). The CFU of the bacterial growth was determined at day 1, 2, 3, 7, 14, 21, and 28.

6.2.6 Cold storage of probiotic bacteria and measurement of H_2O_2

The bacteria from starter culture were sub-cultured at 2% (v/v) into RCM or MRS broth. After 24 hours of incubation, the bacterial cells were centrifuged at 5000 rpm for 10 min. The bacterial pellets were washed twice with sterile saline (0.9% w/v NaCl solution) (Michida et al., 2006) and then were re-suspended in 2 mL centrifuge tube containing 1 mL EPS sample solution (5 g/L) or sterile saline (control). These mixture solutions were stored at 4 ^{o}C for 28 d. The viability of the probiotics was determined using RCM or MRS agar at 0, 7, 14, 21 and 28 d. Galacto-oligosaccharide (GOS) and inulin (5 g/L) were tested as prebiotic references. According to the results, EPS had the best effect on bifidobacterial viability at 4 ^{o}C while *L. casei* in all groups had very high CFU. Therefore, EPS solution at different concentration (1, 3, 5 g/L) was only tested on bifidobacteria at 4 ^{o}C. Before usage all the mediums, sample solutions and centrifuge tubes were sterilized by autoclaving at 120 ^{o}C for 20 min. Reduction of death rate was calculated by the following equation,

$$\text{Death rate Rd} = \frac{\text{CFUo} - \text{CFUt}}{\text{t}}$$

$$\text{Reduction of death rate} = \frac{\text{Rd of control} - \text{Rd of sample}}{\text{Rd of control}}$$

It has been suggested that the oxygen-sensitive bifidobacterial species could produce H_2O_2 in the presence of oxygen and it was toxic to the bacteria (Kawasaki, Watanabe, Fukiya, & Yokota, 2018). The concentration of H_2O_2 in the bifidobacterial culture medium after storage at 4 ^oC was determined by H_2O_2 assay kit (BC3595, Beijing Solarbio Science& Technology Co., Ltd, China).

6.2.7 Probiotic bacteria in simulated gastrointestinal conditions

The sample solution suspended with bacteria was prepared in the same way as that described in 6.2.5. The survival rates of the probiotic strains in sample solutions were examined according to Michida et al. (Michida et al., 2006) and Chou et al. (2013) with slightly modified. 0.5 mL of the bacterial suspended solution was mixed with 1 mL simulated gastric acid (HCl solution buffer, pH = 2) or bile juice (0.3% bile salt in saline, pH = 8). pH was adjusted by HCl and NaOH. The bacteria were maintained at 37 ^oC for 3 hours, and the viability of the bacteria was determined using RCM or MRS agar at 0, 0.5, 1, 1.5, 2, 3 h for gastric acid tolerance or at 0 and 3 h for bile juice tolerance. Before usage all the medium and sample solution were sterilized by autoclaving at 120 ^oC for 20 min, and the simulated gastric acid and bile juice were passed through a sterile filter (Minisart, 16532).

6.2.8 Scanning electron microscopy

The structure of the bacteria and EPS samples and their interaction was monitored by scanning electron microscope (SEM). Due to the similar trend of the effect contributed by EPS samples on different bacterial species, only *B. adolescentis* was

applied for SEM detection. *B. adolescentis* was first cultured in RCM broth for 24h as described in 2.4 and then centrifuged at 5000 rpm for 10 min and the pellets were washed twice with sterile saline and resuspended in EPS sample solution (5 g/L). Inulin was used as comparison. The mixtures were immediately freezed by liquid nitrogen, followed by freeze dried. The dried samples were coated with gold. SEM was performed with a JSM 6710 SEM (JEOL Ltd., Tokyo, Japan).

6.3 Results and discussion

6.3.1 Properties of EPS samples

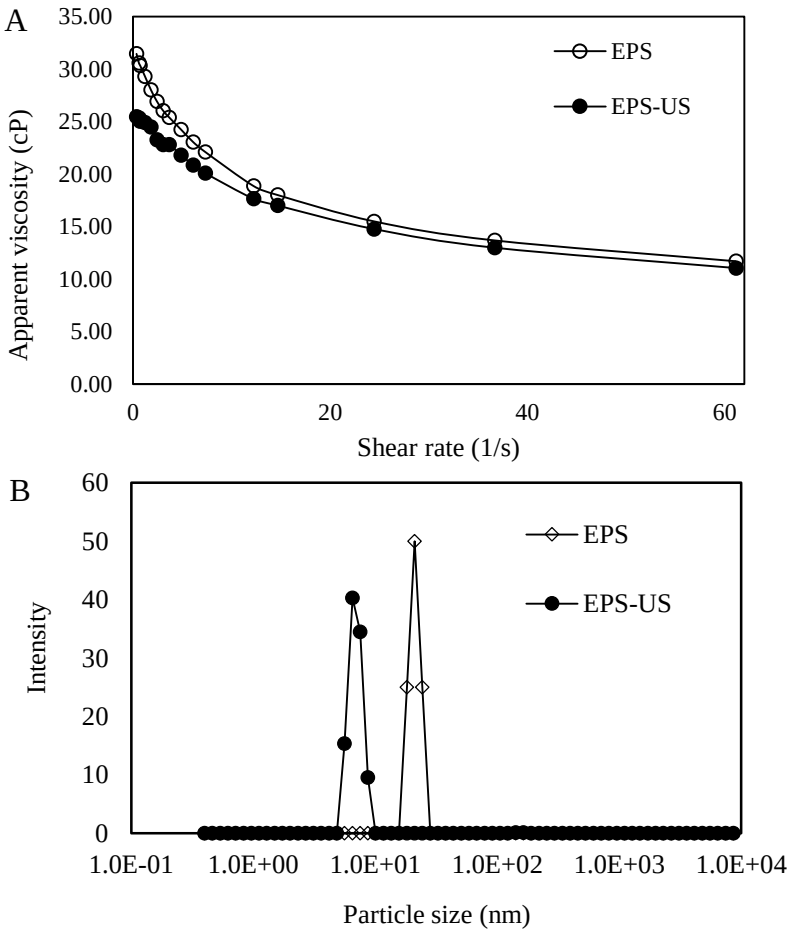

Figure 6-1 Apparent viscosity (A) and particle size distribution (B) of EPS and EPS-US.

Table 6-1 Physical properties of EPS and EPS-US

	Intrinsic viscosity (dL/g)	Solubility (g/L)	Average particle size (nm)
EPS	6.03 ± 0.98	8.83 ± 0.76	17.28 ± 4.93
EPS-US	4.65 ± 0.72	20.08 ± 0.63	9.85 ± 2.53

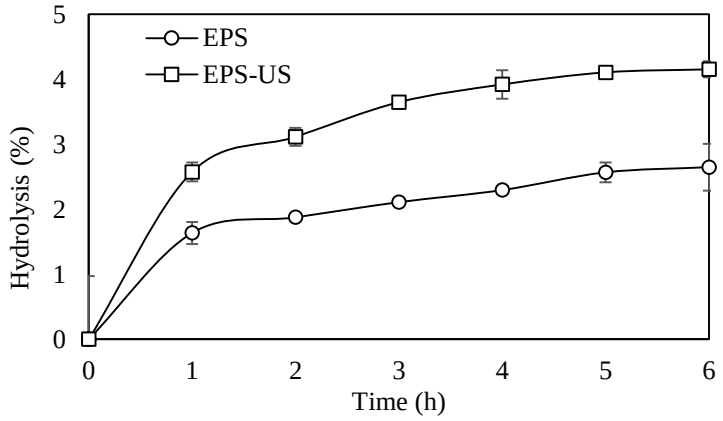

Figure 6-2 Hydrolysis of EPS and EPS-US (to reducing sugar) in simulated gastric acid (37 ^{o}C).

The chemical content, molecular weight distribution and monosaccharides composition were reported in Chapter 5. Both the intrinsic and apparent viscosity of EPS were decreased after the treatment of ultrasound (Figure 6-1A & Table 6-1) and the solubility of EPS was significantly increased from 8.83 to 20.08 g/L. The average particle size (Figure 6-1C & Table 6-2) of EPS (17.28 nm) and EPS-US (9.85 nm) also implied that the samples at 5 g/L were colloidal solution. The total dietary fiber content in EPS and EPS-US was 68.9% and 60.1%, respectively. As the total carbohydrate content of EPS was 71%, most of the EPS carbohydrates belong to non-digestible fiber that are resistant to the digestive enzymes in human digestive system. As shown in Figure 6-2 EPS and EPS-US were very resistant to simulated gastric acid, with no more than 5% (w/w) hydrolyzed to reducing sugar over 6 h. The higher degradation rate of EPS-US (4.15%, w/w) than EPS (2.64%, w/w) was probably due to the fact that the aggregates of EPS-US were much smaller and looser, and more accessible for the acid

in solution. The high intensity ultrasound (US) can cause disruption of the EPS aggregates, reducing the solution viscosity and increasing the water solubility significantly (Wang, Cheung, Leung, & Wu, 2010). Inulin, a commercial prebiotic, was hydrolyzed more than 25% (w/w) in the gastric acidic solution (Tingirikari et al., 2014; Wichienchot, Jatupornpipat, & Rastall, 2010). This suggests that the acidic tolerance of EPS samples were much higher than inulin. The high resistance of EPS and EPS-US to the digestive enzymes and simulated gastric acid warrants their function as dietary fibers or prebiotics useful for the gut bacteria in the large intestine.

6.3.2 Bacterial survival and H_2O_2 accumulation during cold storage

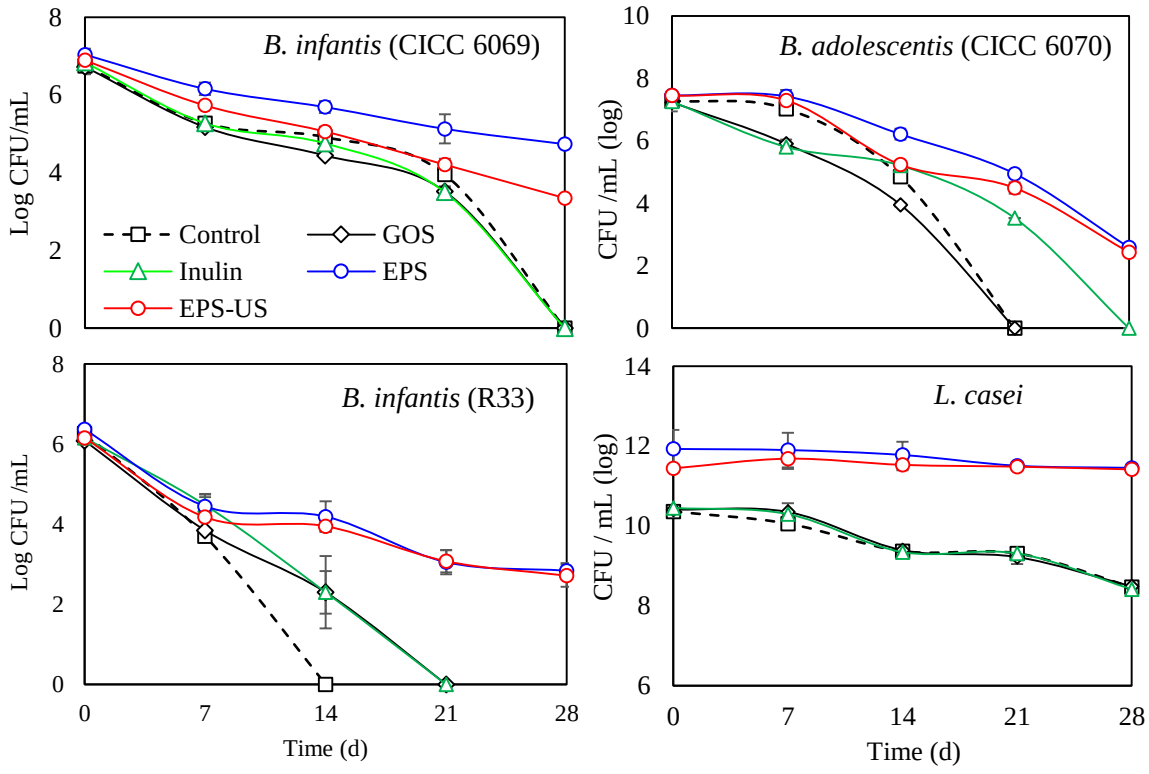

Figure 6-3 Viability of bifidobacteria and lactobacillus with EPS and other carbohydrate fibers (5 g/L) during cold storage at 4°C for 28 d.

Figure 6-3 shows the viability time courses of all probiotic bacteria during cold storage at 4 °C. All three species of bifidobacterium in the control, inulin and GOS

groups died out within 28 days or a shorter period while *L. casei* in all groups maintained a relatively high viability over the cold storage period. As reviewed previously (Ouwehand, Salminen, & Isolauri, 2002), lactobacilli are generally more tolerant than bifidobacteria to stress conditions such as low temperature, oxygen stress and extreme pH. EPS and EPS-US had a significant protective effect on the survival rate of all species. *B. infantis* (R33) was most susceptible among the bifidobacterial species at low temperature and died rapidly within two weeks in the control, but retained a survival rate above 10^2 CFU/mL in the presence of EPS or EPS-US. The protective effect of EPS was slightly stronger than that of EPS-US for *B. adolescentis*.

Table 6-2 Reduction of Bifido-/Lactobacillus bacterial death rate during cold storage and in simulated gastric acid with EPS and other carbohydrate fibres (all at 5 g/L in the culture medium).

Bacterial species	GOS	Inulin	EPS	EPS-US
Cold storage (4°C, 28 days)				
B. adolescentis (CICC 6070)	0.7	25.3	49.9	48.3
B. infantis (CICC 6069)	0.4	-1.3	65.8	47.6
B. infantis (R33)	35.4	34.6	71.8	72.6
L. casei	-3.2	-9.09	74.3	82.9
Simulated gastric acid				
B. adolescentis (CICC 6070)	33.6	26.5	68.0	50.3
B. infantis (CICC 6069)	36.9	-5.5	49.3	40.3
B. infantis (R33)	30.8	6.7	70.8	70.0
L. casei	24.5	25.2	67.9	45.5

For a more quantitative comparison of the protective effects of various carbohydrates, Table 6-2 presents the percentage values of death rate reduction that are derived from experimental data in Figure 6-3 & 6-6. Overall, the protective effects of EPS and EPS-US on the probiotic bacteria were much stronger than inulin and GOS.

EPS and EPS-US showed significant effect on all bacteria species in cold storage. Although inulin has been previously shown to maintain the viability of probiotics (Donkor, Nilmini, Stolic, Vasiljevic, & Shah, 2007), it only increased the survival rate of *B. adolescentis* (CICC 6079) and *B. infantis* (R33) during the cold storage.

A possible cause for the cell death during cold storage is attributed to the oxidative stress produced by the reactive oxygen species such as H_2O_2. Some of the O_2-sensitive bifidobacteria could produce H_2O_2 in the presence of O_2 which was toxic to the bifidobacteria (Kawasaki, Mimura, Satoh, Takeda, & Niimura, 2006; Shimamura et al., 1992). To confirm this postulation, the H_2O_2 concentration in the liquid medium of three bifodonacteria at the end of cold storage was measured. As shown in Table 6-3, *B. adolescentis* and *B. infantis* (R33) produced a much higher level of H_2O_2 than *B. infantis* (CICC6069), suggesting that the first two species were more sensitive to oxygen. Consistently with the results shown in Figure 6-3, the *B. infantis* (CICC6069) species also survived longer than the first two bifidobacterial species. Moreover, the addition of EPS and EPS-US significantly decreased the level of H_2O_2 produced by *B. adolescentis* and *B. infantis* (R33) compared to control. The reduction of H_2O_2 production may be attributable to the high viscosity of EPS, increasing the resistance of oxygen access to the bacteria. Overall, this set of results provided supporting evidence for the postulation that H_2O_2 is a major factor contributing to the cell death during cold storage in previous studies. In this connection, the protective effect of EPS on the bifidobacteria may also be attributable to its antioxidant activity as reported previously (Leung et al., 2009).

Additional experiments were also performed on the survival of *Bifidobacteria* and *L. casei* stored at the normal culture temperature of 37^{o}C. All four bacteria died out much faster than during cold storage at 4^{o}C. In the EPS solution (without any growth

nutrients), three of the bifidobacteria species died out within a week (data not shown) and only *B. infantis* (CICC 6069) retained a CFU of 413 ± 97 /mL on day 7 but died out in two weeks. Inulin, EPS and EPS-US had a positive effect on survival of *L. casei* (Figure 6-4). EPS maintained *L. casei* alive over three weeks while inulin maintained the bacterial survival over four weeks. Inulin has been shown previously to maintain the viability of probiotics (Donkor et al., 2007).In the normal culture medium (RCM or MRS broth) without EPS, all bifidobacteria died within two weeks and *L. casei* died within three weeks (Figure 6-5). The less significant protective effect of EPS and EPS-US at 37^{o}C than at 4^{o}C was probably attributed to the lower viscosity at the higher temperature.

Table 6-3 Accumulation of H_2O_2 (μmol/mL) by bifidobacteria after cold storage at 4 ^{o}C.

Bacterial strains	Control	GOS	inulin	EPS	EPS-US
B. adolescentis	0.47 ± 0.14	0.55 ± 0.12	0.27 ± 0.12	0.14 ± 0.03*	0.23 ± 0.10
B. infantis (R33)	0.74 ± 0.08	0.59 ± 0.16	0.44 ± 0.11	0.36 ± 0.07*	0.52 ± 0.06*
B. infantis (CICC6069)	0.19 ± 0.16	0.16 ± 0.14	0.14 ± 0.04*	0.16 ± 0.08	0.15 ± 0.09

*: significant at $p < 0.05$ by t-test

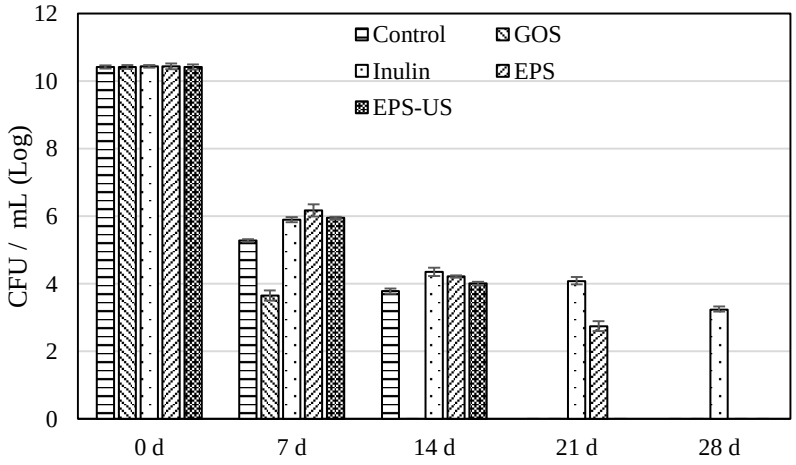

Figure 6-4 Viability of *L. casei* in different carbohydrate fiber solutions (5 g/L) during storage at 37 ^{o}C.

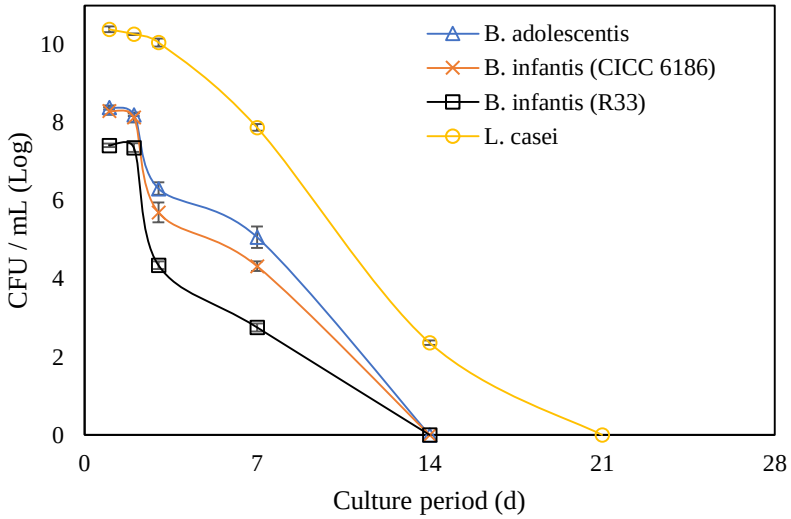

Figure 6-5 Viability change of bacteria maintained in liquid culture medium (RCM or MRS broth) at 37°C and 200 rpm over long period.

6.3.3 Bacterial survival in gastric acid

Figure 6-6 shows the viability trend of probiotic bacteria during incubation in simulated gastric acid for three hours. Although *B. infantis* (R33) was very susceptible to oxygen stress and nutrient deficiency, it was relatively tolerant to the acid stress with slower reduction of viability in the control group. EPS, EPS-US and GOS significantly increased the gastric acid survival of all four probiotic bacteria compared to the control group, while inulin showed some positive effect only on *B. adolescentis* (CICC 6070) and *L.casei*.

For a more quantitative comparison of the protective effects of various carbohydrates, Table 6-2 presents the percentage values of death rate reduction that are derived from experimental data in Figure 6-3 & 6-6. EPS and EPS-US showed significant effect on all bacteria species in both cold storage and gastric acid. Table 6-4 presents the percentage values of death rate reduction of EPS at three different concentrations, 1, 3, 5 g/L. In most cases (bacterial species and conditions), the

protection effect (death rate reduction) with EPS was significantly lower at a lower concentration.

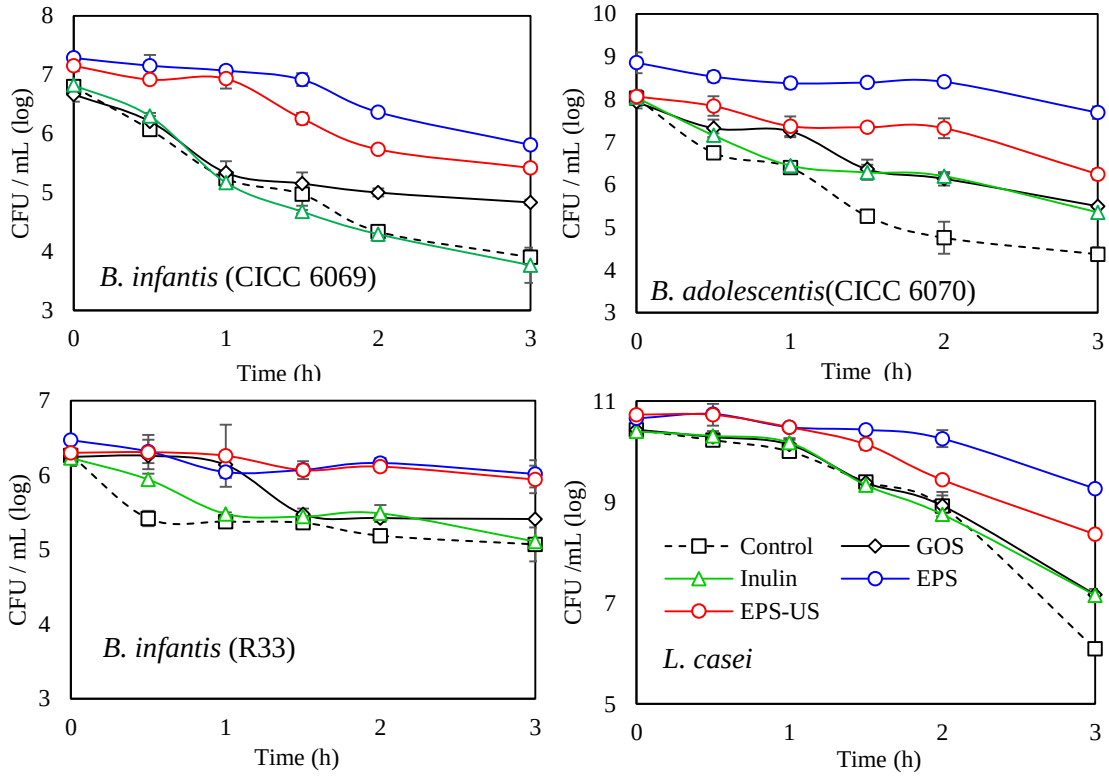

Figure 6-6 CFU of *Bifidobacteria* and *Lactobacilli* in simulated gastric acid with different polysaccharide solution (5 g/L) during 3 h incubation.

Bacterial cells have developed the general stress response (GSR) system to cope with nutrient starvation and various environmental stresses (Brown & Barker, 1999). It has been reported that GSR can be triggered at a high cell density (Hengge-Aronis, 1996; Liu, Ng, & Ferenci, 2000). In this study, the GSR mechanism may be a possible contributor to protective effect of EPS on the probiotic bacteria in various stress conditions as the bacteria cells were agglomerated in the EPS gels to a high density. In addition, the bacterial cell density may also affect their acid tolerance due to the quorum sensing. Besides the GSR, bacterial cells have the acid tolerance response (ATR) system for the better survival in acidic conditions (Cotter & Hill, 2003; Lee et al., 1994).

As reported by Li et al. (2001), the high cell density of *Streptococcus mutans* could modulate ATR to gain a significantly higher resistance to lethal pH. This suggests that cell-cell communication plays an important role in bacterial resistance to acid stress.

Table 6-4 Reduction of Bifido-/Lactobacillus bacterial death rate during cold storage and in simulated gastric acid with different concentrations of EPS solution.

Bacterial species	EPS-1	EPS-3	EPS-5
Cold storage (4°C, 28 days)			
B. adolescentis (CICC 6070)	24.8	23.4	49.0
B. infantis (CICC 6069)	54.2	55.1	67.3
B. infantis (R33)	50.5	72.2	72.4
Simulated gastric acid			
B. adolescentis (CICC 6070)	4.4	76.2	80.9
B. infantis (CICC 6069)	-1.6	32.5	39.2
B. infantis (R33)	16.5	30.6	55.3
L. casei	53.3	54.0	76.4

Note: EPS-1, EPS-3 and EPS-5 for EPS solution at 1, 3, and 5 g/L, respectively.

6.3.4 Bacterial survival in bile juice

Table 6-5 shows the survival rate of probiotic bacteria in bile juice with EPS, EPS-US, inulin and GOS. All of the four bacterial species had a very low survival rate in bile juice, which was mainly attributed to the alkaline environment of bile juice (pH 8) plus the antimicrobial activity of bile salt. Bile salts could cause disruption of the cell membrane and DNA damage (Ruiz, Margolles, & Sánchez, 2013). After incubation in the bile juice for 3 hours, nearly all bifidobacterial cells died out in the control, GOS and inulin groups, and viable cells were only present in the EPS groups. EPS had shown significant protective effect on the four bacterial strains with a notable survival rate, while EPS-US, inulin and GOS only increased the survival rate of *L. casei* in bile juice.

The protective effect of EPS was weak or negligible as the concentration was lowered to 3 g/L and 1 g/L.

Table 6-5 Survival rate (%) of bacterial strains after 3 h incubation in bile juice with EPS and other carbohydrate fibers (all at 5 g/L or specified concentrations in culture).

	B. adolescentis (CICC 6070)	*B. infantis* (CICC6069)	*B. infantis* (R33)	*L. casei*
Control	< 0.001	< 0.010	< 0.010	0.0043 ± 0.002
GOS	< 0.001	< 0.010	< 0.010	0.014 ± 0.007
Inulin	< 0.001	< 0.010	< 0.010	0.010 ± 0.003
EPS	0.063 ± 0.030	0.67 ± 0.20	0.97 ± 0.18	7.9 ± 2.7
EPS-US	< 0.001	< 0.010	< 0.010	0.046 ± 0.012
EPS at different concentrations (EPS-1,-3,-5: EPS at 1, 3, 5 g/L).				
Control	< 0.001	< 0.010	< 0.010	0.0038 ± 0.001
EPS-1	< 0.001	< 0.010	< 0.010	0.0085± 0.002
EPS-3	< 0.001	< 0.010	0.47 ± 0.11	0.67 ± 0.25
EPS-5	0.055 ± 0.020	0.84 ± 0.17	1.01 ± 0.28	8.1 ± 1.6

Both inulin and GOS are well-known prebiotic carbohydrates that can support the growth of probiotic bacteria (Glenn R Gibson, 1999; Huebner, Wehling, & Hutkins, 2007). The increasing viability of some strains by inulin or GOS can be partly attributed to their nutritional effect. As reported previously by our group (Song et al., 2018), GOS was a favorable carbon source as glucose, but EPS, EPS-US or inulin was not well utilized for the growth of bifidobacteria in culture. Therefore, the ability of EPS and EPS-US to sustain the bacterial survival in various conditions can be mainly attributed to protective effect. In a recent study (Mao, Song, Wang, Yao, & Wu, 2018b), a viscous layer formed on the *B. adolescentis* was regarded as a major contributing factor for protecting the bacteria from antibiotic damage and atomic force microscope (AFM) was applied to visualize the layer. Physical interactions of the bacteria cells with the EPS

aggregates and gels in the liquid medium might also contribute to the protective effect in this study. Moreover, the formation of biofilm by bacteria may also increase the resistance to detrimental factors and harsh conditions, which is facilitated by an EPS gel matrix surrounding the bacteria cells (Mah & O'toole, 2001). In the following experiments, scanning microscopy (SEM) was applied to detect these phenomena possibly occurring to the bifiodobacterial cells with the EPS.

6.3.5 Interactions between bacterial cells and polysaccharides

Figure 6-7 shows the SEM images of *B. adolescentis* cells in three PS solutions, EPS, EPS-US and inulin. EPS and EPS-US formed planner sheets of aggregates on which rod-shaped bacteria cells were attached. Inulin appeared as clusters of aggregates with relatively few bacteria cells protruding from the outer periphery. Many bacteria cells were attached on the EPS aggregates but very few on EPS-US and inulin. The immobilization or encapsulation of probiotics by PS gels is recognized as an important factor contributing to the tolerance of probiotic bacteria to environmental stress, thus increasing their survival rate (Guerin, Vuillemard, & Subirade, 2003; Sun & Griffiths, 2000). The bacterial cells attached to or entrapped by EPS and EPS-US aggregates are less exposed to the stress factors and conditions in their surroundings. Compared to EPS-US, EPS was more viscous in liquid solution and more capable to immobilize the bacterial cells, conferring a stronger protective effects. As the concentration of EPS was decreased, the viscosity of solution decreased and the protective effect was also weaker.

6.4 Conclusions

The EPS from Cs-HK1 mycelial fermentation has shown notable protective effect on probiotic bacteria in three practical conditions, cold storage, acidic pH and bile salt.

The protective effect was mainly associated with the physical properties of EPS, namely the high MW and high liquid viscosity, and was weaker after partial degradation by power ultrasound. The commercial prebiotics such as inulin and GOS with much lower MW showed little or no protective effect. Firstly, the viscous EPS surrounding the bacterial cells may slow or block the access of the stress factors to the bacterial cells. Secondly, the immobilization or attachment of bacterial cells to the EPS gel matrix is also favorable for the bacterial survival under stress. With the protective effect on the probiotic bacteria in various conditions plus its high dietary fiber content and high resistance to gastric acid hydrolysis, EPS is a potential health supplement to be used separately or in combination with probiotic bacteria for improving gut microbiota.

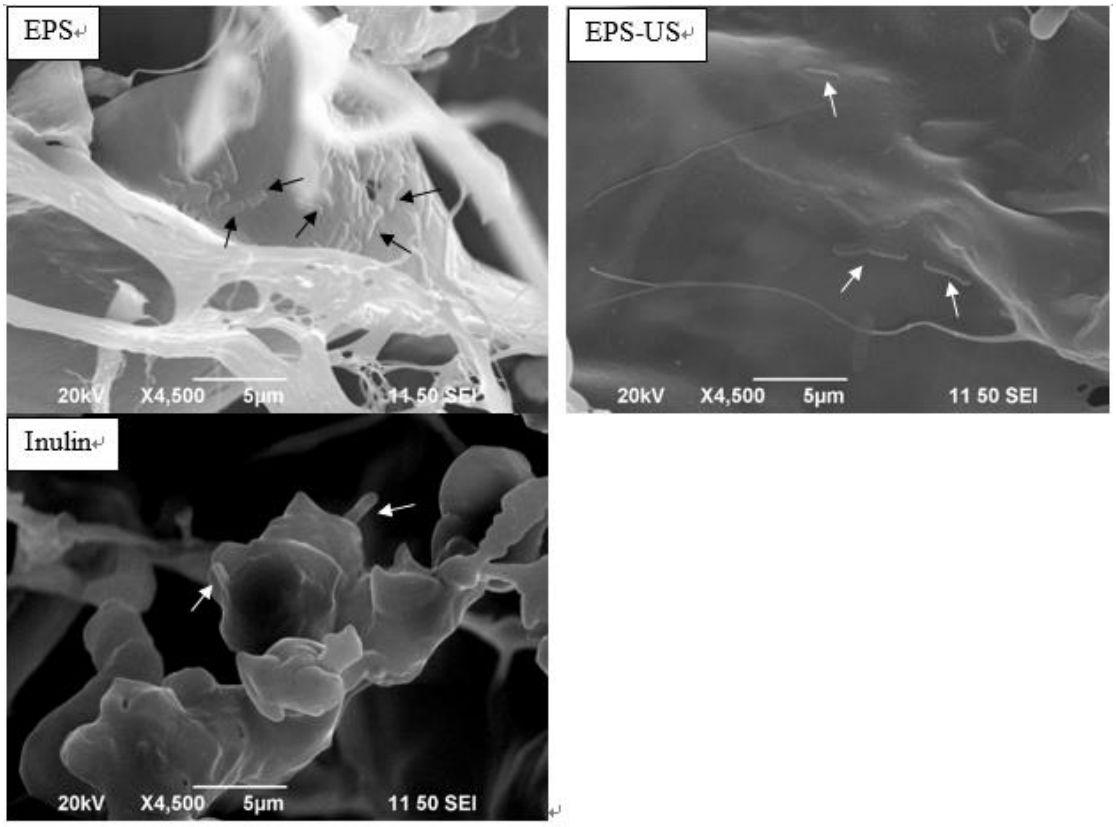

Figure 6-7 SEM image of *B. adolescentis* trapped by different polysaccharides.

Chapter 7 Fermentative metabolism of arabinoxylan by *Bifidobacterium longum*

7.1 Introduction

The human gastrointestinal tract (GIT) is inhabited by a highly diverse microbial community referred to as gut microbes which are essential to its proper functions on the human health (Eckburg et al., 2005). Until now this densely populated ecosystem still remains incompletely characterized for less than half of the microbiota has been cultured or described (Marteau et al., 2001). Five bacterial phyla have been revealed to dominate the human GIT microbiota, with *Firmicute* being the most abundant, followed by *Bacteroides*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (Tap et al., 2009). The *Bifidobacterium* genus belonging to gut *Actinobacteria* is one of the prevalent groups of culturable anaerobic bacteria within mammalian GIT (Pokusaeva, Fitzgerald, & van Sinderen, 2011) and among the first colonizers in human GIT (Favier, Vaughan, De Vos, & Akkermans, 2002). Although bifidobacteria compose only 3-6% of the adult fecal flora, their presence is considered to be associated with health benefits, such as prevention of pathogen colonization, improvement of lactose intolerance and modulation of the immune response (Rastall et al., 2005; Schell et al., 2002). Due to health-promotion effects, certain bifidobacterial strains are commercially exploited as components in functional food (Pokusaeva, Fitzgerald, & van Sinderen, 2011). Many of the health-promotion activities of bifidobacteria as well as their adaptation to the human GIT are associated with their ability to utilize various carbohydrates that are indigestible by the hosts. Therefore, understanding carbohydrate utilization by bifidobacteria may help reveal the mechanisms of beneficial interactions in the human GIT.

Arabinoxylan (AX) is the main non-starch PS found in cereal grains, such as wheat, rye, rice, barley and oats. It consists of a linear β-1,4-linked xylose backbone which can be substituted at the C(O)2 and/or C(O)3 positions with arabinose monomers (Izydorczyk & Biliaderis, 1995). AX and arabinoxylan oligosaccharide (AXOS) cannot be digested by human enzymes and directly enter the colon as energy sources for certain saccharolytic organisms (Rivière et al., 2014). Currently, *Bacteroids* and *Roseburia* are the only known gut xylanolytic taxa that have the ability to hydrolyze the backbone of AX by producing extracellular endo-xylanases (Ejby et al., 2013). However, many bifidobacteria strains are found to be able to utilize AX, AXOS or xylo-oligosaccharides (XOS) although the degradation mechanisms are still unknown. The bifidogenic effect of AX also indirectly affects some other genera in GIT, probably through cross-feeding (Falony, Vlachou, Verbrugghe, & Vuyst, 2006; Van den Abbeele et al., 2011).

Although several studies have demonstrated the utilization of AX by bifidobacteria, the degradation mechanism is still unclear. Many of these studies only investigate the bifidogenic effects of AX or AXOS by determining the bacterial growth, pH change and short-chain fatty acid production (Crittenden et al., 2002; Van Laere et al., 2000). In addition, some of these investigations considered the gut bifidobacteria as a whole (Neyrinck et al., 2011) and little of them involve the influence of AX fine structure on its fermentation by bifidobacteria. A recent study based on 36 bifidobacterial strains pointed out that the AX degradation process is strain-dependent (Rivière et al., 2014). Besides, it was the fine structure of AX, rather than the generalized structure, that mainly governed its fermentation (Mendis et al., 2016). Therefore, understanding the AX metabolism process by individual species is of great importance to reveal the mechanisms adopted by the whole gut bifidobacterial

population and the dynamic effects that different non-digestible carbohydrates have on the intestinal microbiota population. This study investigated the wheat AX degradation process by *Bifidobacterium longum* CICC6186. The genome sequence of this strain was determined and analysed to further understand its metabolic mechanism on AX.

7.2 Materials and methods

7.2.1 Degradation and de-branching of AX

Wheat AX (P-WAXYH, Megazyme) was hydrolysed to lower molecular weight (MW) by trifluoroacetic acid (TFA). 0.25 g of the AX was mixed with 20 mL of 0.1 M TFA in a 100 mL round bottom flask at 90 ^{o}C with vigorous stirring for 10, 30 or 60 min (AX-10, AX-30, AX-60). TFA was removed by evaporation to dryness at 40 ^{o}C under vacuum in a rotary evaporator. Degraded AX samples were washed several times with methanol and then re-dissolved in 10 mL water. The sample solutions were freeze dried to get the acid-hydrolysed arabinoxylan.

α-L-Arabinofuranosidase (E-ABFUM, Megazyme) from *Ustilago maydis* specifically cleaved non-reducing α-L-arabinofuranose from singly substituted xylose residues in AX while α-L-arabinofuranosidase (E-ABFBO17, Megazyme) from *Bacteroides ovatus* could only hydrolyze α-1, 3-arabinofuranose from double substitution. These two enzymes were applied for the de-branching of AX separately. AX (5 mg/mL) in sodium phosphate buffer (100 mM, pH 6.5) or sodium acetate buffer (100 mM, pH 5.0) was mixed with α-L-arabinofuranose from *Bacteroides ovatus* or *Ustilago maydis*, respectively and then incubated at 40 ^{o}C for 30 min with stirring. Enzyme was denatured by boiling for 10 min. De-branched AX was then freeze-dried.

7.2.2 Molecular weight and ^{1}HNMR of AX samples

Molecular weight (MW) of acid-hydrolysed samples was determined by HPGPC. The de-branching of AX was confirmed by ^{1}HNMR. Before NMR analysis, de-branched samples were dissolved in deuterium oxide (10 mg/mL) and then lyophilized (Rose, Patterson, & Hamaker, 2009). The dissolving and lyophilizing steps were repeated twice and samples were re-dissolved in deuterium oxide. ^{1}HNMR analysis was performed on a Bruker AV400 spectrometer.

7.2.3 Bacterial strains and culture conditions

Five *Bifidobacterial* strains from China Centre of Industrial Culture Collection (CICC) were used in this Chapter, including *Bifidobacterium adolescentis* CICC6070, *B. bifidum* CICC10395, *B. breve* CICC6079, *B. infantis* CICC6069 (= ATCC15697) and *B. longum* CICC6186 (= JCM 1217).

For bacterial growth, AX was added at 5 g/L (~0.5% w/v) to a glucose-free RCM broth (5 mL) in 10 mL centrifuge tubes. Glucose and galacto-oligosaccharide (GOS) at 5 g/L were tested as reference and glucose-free RCM broth was used for the control group. All the medium were sterilized by autoclaving at 121 ^{o}C for 20 min Before usage. Bacteria from starter culture were inoculated into the sample medium at 2% (v/v). After 24 hours of incubation, the optical density was measured at 600 nm. Results showed only *B. longum* could use AX. Therefore, degraded and de-branched AX samples, XOS, arabinose, xylose and the mixture of arabinose and xylose at a molar ratio of 1:2 were applied as single carbon source for the growth of *B. longum*.

7.2.4 Preparation of crude enzyme from *B. longum*

AX was used at 5 g/L as single carbon source and glucose at 5 g/L was tested as reference. *B. longum* was cultivated for 16 h to get enough and highly active enzymes.

Enzyme activities in *B. infantis*, which could not use AX for growth, was also determined as comparison. The bacterial cells (~0.3 g wet weight) were separated from culture broth by centrifugation at 4000 rpm for 8 min, washed twice by phosphate buffer (100mM NaH_2PO_4, 0.02% w/v NaN_3, pH=6.0) and resuspended in 30 mL phosphate buffer. Cell extracts were prepared at 4 ^oC using a French press at 1.38×10^5 KPa for three cycles (Derensy-Dron, Krzewinski, Brassart, & Bouquelet, 1999). Cellular debris was pelleted by centrifugation at 10,000 rpm for 30 min and the supernatant was collected. Both the cell extract and cultured medium contained crude enzymes and were stored at -80^oC for further measurement.

7.2.5 Enzyme activity

7.2.5.1 Enzyme assay

β-xylosidase and α-L-arabinosidase activities were determined according to Zeng et al. (2007) with slight modification. *p*-Nitrophenyl β-D-xyloside (*p*NPX), and *p*-nitrophenyl α-L-arabinofuranoside (*p*NPA) were used as substrates for β-xylosidase and α-L-arabinosidase activity, respectively. One unit of activity was defined as the amount of enzyme that required to produce 1 μmol of *p*-nitrophenol per min at 40 ^oC. The reaction mixture containing 100 μL of 10 mM of substrates and 100 μL of crude enzymes was incubated at 40 ^oC for 10 min. 0.6 mL of 1 M Na_2CO_3 was added to stop the reaction, and the optical density was measured at 405 nm. *p*-nitrophenol was used as standard.

Xylanase assay kit (K-XyIX6-1V, Megazyme) was used for endo-xylanase activity determination. Briefly, XyIX6 substrate reagent was mixed with properly diluted crude enzymes and incubated at 40 ^oC for 10 min. Tris buffer (2% w/v, pH 10.0) was added to stop the reaction and the absorbance was measured at 400 nm. One unit

of activity is defined as the amount of enzymes required to release one micromole of *p*-nitrophenol from XyXI6 substrate in one minute.

7.2.5.2 Enzymatic hydrolysis of AX by cell extract

Activities of β-xylosidase and α-L-arabinosidase could not be detected in cultured medium of these two bacteria. Therefore, only cell extracts were applied for AX hydrolysis. 0.4 mL of cell extract was mixed with 1.6 mL of AX solution (5 g/L). The mixture was incubated at 37 ^{o}C and 200 rpm for 24 h and the produced monosaccharides were determined by PMP-HPLC method.

7.2.6 Analysis of carbohydrate in culture medium

In order to determine the degradation of AX by *B. longum*, 5 g/L of AX was applied as single carbon source and soluble starch was removed from the culture medium. *B. longum* was cultured for 24 h. Bacterial cells were separated by centrifugation at 4000 rpm for 8 min and the supernatant was collected.

MW of culture medium was determined by HPGPC and monosaccharide in culture medium and cell extract was determined by the PMP-HPLC method. The concentration and monosaccharide composition of AX in culture medium and bacterial cell extract were determined by analysis of neutral sugar following acid hydrolysis. 1.7 mL of the cultured medium or cell extract was mixed with 0.3 mL of TFA to get a total concentration of 2 M TFA. The mixture was stirred at 110 ^{o}C for 4 h. The TFA was then removed by vacuum evaporation and washed several times by methanol. The hydrolysate was re-dissolved in 1.7 mL water. Monosaccharide composition was determined by PMP-HPLC method.

7.2.7 Genome sequencing and analysis

The genome of *B. longum* was sequenced using an Illumina HiSeq 4000 system (Illumina, San Diego, CA, USA) at Beijing Genomics Institute (Shenzhen, China). The genome analysis was done by Shanghai Majorbio Bio-pharm Technology Co., Ltd. Raw reads of low quality from paired-end sequencing (those with consecutive bases covered by fewer than five reads) were discarded. The sequenced reads were assembled using SOAPdenovo (v2.04) (http://soap.genomics.org.cn/). Glimmer (v 3.02) and GeneMarks (v 4.3) were run to model the coding sequence (CDS) of the gene. Predicted amino acid sequences of CDS were searched against COG, KEGG and CAZy database by BLAST+ and Diamond. When possible, predicted proteins were assigned based on these database. rRNA and tRNA were identified by using Barrnap and tRNA-scan-SE, respectively.

7.3 Results and discussion

7.3.1 Bacterial growth

Table 7-1 showed the growth of five bifidobacterial strains on different carbon sources. All of the bifidobacteria could utilize GOS very well. This result was in agreement with Kondepudi et al (2012) that GOS was efficiently metabolized by bifidobacteria. A mild growth of *B. breve* and *B. bifidum* was observed on glucose. *B. breve* could grow weakly even there was no carbon source in the medium, indicating the ability of this strain to obtain nutrients from other ingredients in the medium for growth. Only *B. longum* was able to use AX and its growth on AX was much stronger than on glucose and GOS. The capability of *B. longum* using AX and AX oligosaccharide (AXOS) had been reported (Crittenden et al., 2002). As shown in Figure 7-1, *B. longum* had a limited usage of arabinose, xylose and their mixture and

the ability of this strain to utilize XOS was the weakest, and its growth on AX-10 was the strongest, followed by AX-30, AX, glucose and AX-60. According to the molecular weight (MW) of these carbohydrates shown in Table 7-2, AX was degraded to lower MW molecules and the content of monosaccharides (~175 Da) increased as the hydrolysis time increased. *B. longum* preferred AX with relatively higher MW. An *in vivo* study on fish showed similar results that AXOS with higher degree of polymerization (DP) had higher effects in stimulating the growth of lactic acid bacteria compared to AXOS with lower DP (Geraylou et al., 2012). In addition, the arabinose side chain content in AXOS was observed to show selectivity of these oligosaccharides by bifidobacterium and the higher side chain content on AXOS backbone was favoured (Jaskari et al., 1998). This might be due to the specific structures of the side chain that could be recognized by bifidobacterium. In this study when AX was degraded by acid into oligosaccharides or monosaccharides, some of these structures were destroyed, resulting in weak growth.

Table 7-1 Growth of five bifidobacterial strains on different carbon sources.

Bacteria	Control	Glc	GOS	AX
B. adolescentis	-	++	++	-
B. breve	+	+	++	+
B. bifidum	-	+	++	-
B. infantis	-	++	++	-
B. longum	-	++	++	+++

Scale: OD < 0.1 (- = no growth), 0.1-0.39 (+ = mild growth), 0.4-0.69 (++ = moderate growth), > 0.7 (+++ =strong growth).

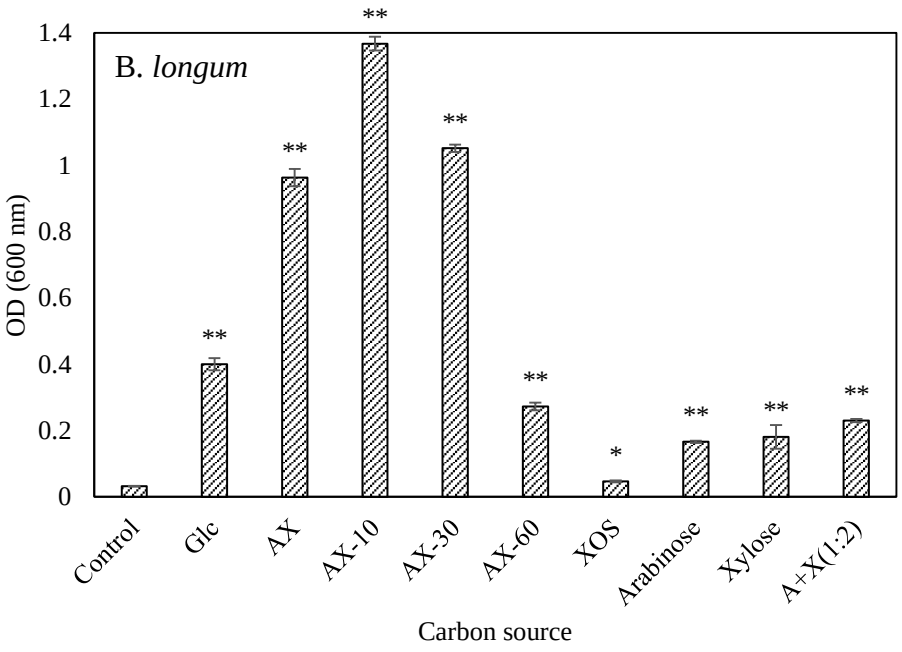

Figure 7-1 Growth of *B. longum* on different carbon sources (A+X (1:2): arabinose and xylose at a molar ratio of 1:2; *: $P < 0.05$, **: $P < 0.01$ by t-test).

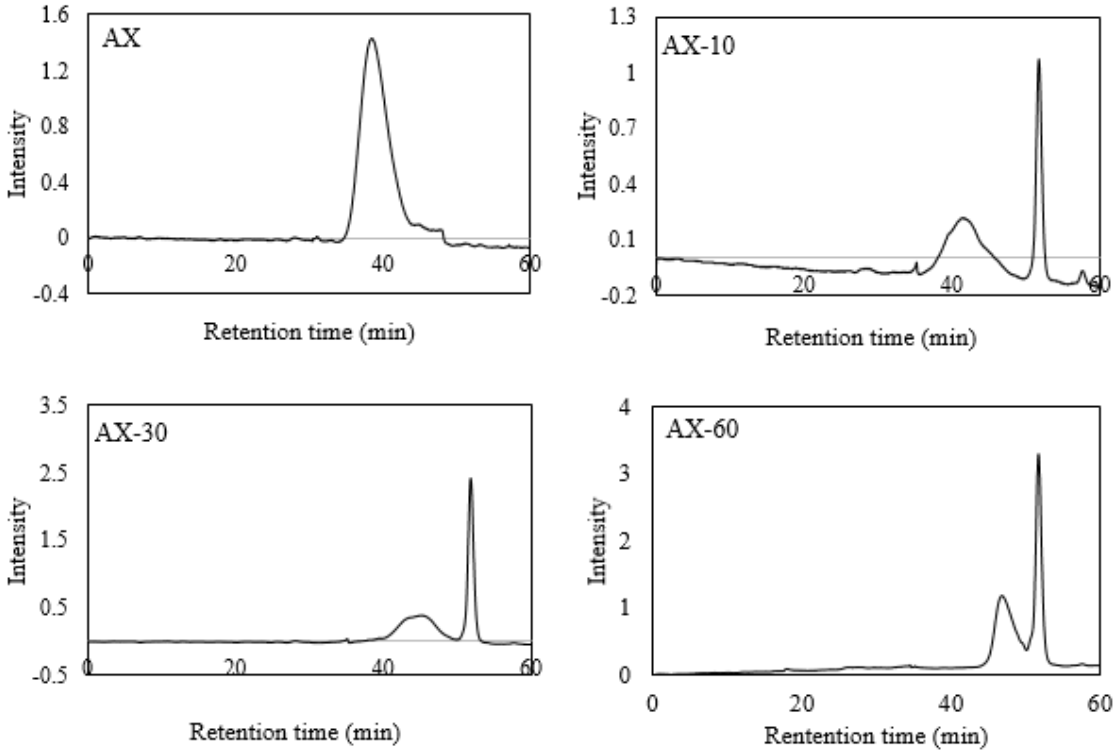

Figure 7-2 GPC profile of AX samples (AX-10, 30, 60: acid hydrolysed for 10, 30, 60min).

Table 7-2 Molecular weight distribution of AX samples.

	Retention time / min	Mw / Da	%
AX	38.517	159829	100
AX-10	41.750	39499	61.06
	51.743	176	38.94
AX-30	45.350	8650	46.66
	51.742	176	53.34
AX-60	46.850	1661	50.18
	51.759	172	49.82

7.3.2 Enzyme activity

Table 7-3 showed the enzyme activities of *B. longum* and *B. infantis*. All of these three enzymes, endo-xylanase, α-L-arabinosidase and β-xylosidase, could be detected inside *B. longum* and only endo-xylanase was detected outside the bacterial cells. Even though many reports had suggested extracellular α-L-arabinofuranosidase was produced by bifidobacteria, until now no such enzymes in *B. longum* had been characterized (Schell et al., 2002; van den Broek, Hinz, Beldman, Vincken, & Voragen, 2008). Activities of endo-xylanase and α-L-arabinosidase produced by *B. longum* could be induced by AX while all the enzyme activities in *B. infantis* remained at the same level no matter glucose or AX was applied. Although *B. infantis* could not utilize AX, it had higher enzyme activities of α-L-arabinosidase and β-xylosidase than *B. longum*. This might be due to the enzyme specificities. Indeed, several arabinosidases were characterized for their specificities. Two arabinofuranohydrolases (AXH-d3 and AXH-m23) in *B. adolescentis* DSM 20083 were reported to be only active on AX (Van Laere et al., 1999) while a GH family 43 arabinofuranosidase (Abf3) in *Lactobacillus brevis* DSM 20054 displayed high specific activity for arabinobiose but could not release arabinose from AXOS (Michlmayr et al., 2013). In this study, the detected enzymes

produced by *B. infantis* might be specific to some other sugars. Therefore they could not be induced by AX, even though they had relatively high activities, they did not work on AX.

No matter glucose or AX was applied as carbon source, only a critical amount of arabinose was detected through the enzymatic hydrolysis of AX by cell extract of *B. infantis* (Table 7-4). This also suggested the α-L-arabinosidase and β-xylosidase in *B. infantis* cells could not be induced by AX, while the enzymes in *B. longum* cells were activated by AX to cleave arabinose and xylose from AX, which showed an intracellular hydrolysis of AX by this strain. Only when AX was applied could the induced enzymes in *B. longum* cleave backbone of AX into monosaccharides although the concentration of the produced xylose was critical. Similar results were reported by Lagaert et al. (2011). They found that two β-xylosidases in *B. adolescentis* could only hydrolyze xylose from XOS but had low activity on AX. This was because the β-xylosidases could only hydrolyse the non-reducing end of the xylan backbone until they were stopped by a substituted residue. Therefore, little activity of β-xylosidases was expected on AX to produce xylose. The induced activities of α-L-arabinofuranosidase and β-xylosidases inside *B. longum*, as well as the production of xylose and arabinose from AX hydrolysis by *B. longum* cell extract, indicated the intracellular degradation of AX or AXOS by *B. longum*.

7.3.3 Carbohydrate in culture medium

MW of AX in the culture medium before and after bacterial fermentation was detected by HPGPC (Figure 7-3). Table 7-2 shows MW of AX was ~1.6×10^5 Da. The peak at around 38 min should indicate AX before bacterial fermentation but it was overlapped with a broad and large peak 2 at around 34.08 min (Figure 7-3A). There

were multiple peaks (peak group 1) before 30 min. These broad and multiple peaks resulted from salts and ingredients in the medium. After fermentation of *B. longum* (Figure 7-3B), the area of peak 1 and 2 decreased and they connected to show multiple peaks (peak group 3). The peak at around 38 min related to AX disappeared. This was due to the consumption of AX and some of the ingredients in the medium by *B. longum*. A new peak 4 at 47.42 min (~2700 Da) appeared and there was no peak at the same retention time when glucose was applied (Figure 7-3C & D), which showed peak 4 resulted from the hydrolysis of AX by *B. longum*. This coincided with the results (Table 7-3) that enzyme activity of endo-xylanase was detected extracellularly.

Table 7-3 Enzyme activity in culture medium and cell extract.

	Carbon source		Endo-xylanase (XylX6 Units/mL)	α-L-Arabinosidase (U/mL)	β-xylosidase (U/mL)
B. longum	Culture medium	Glucose	0.013 ± 0.004	-	-
		AX	**0.044 ± 0.013**	-	-
	Cell extract	Glucose	0.005 ± 0.002	0.017 ± 0.003	0.0133 ± 0.003
		AX	**0.017 ± 0.002**	**0.032 ± 0.004**	0.0130 ± 0.001
B. infantis	Culture medium	Glucose	0.028 ± 0.004	-	-
		AX	0.027 ± 0.002	-	-
	Cell extract	Glucose	-	0.065 ± 0.004	0.287 ± 0.017
		AX	-	0.059 ± 0.002	0.273 ± 0.014

Monosaccharides in the culture medium and cell extract after *B. longum* fermentation on AX were detected by HPLC. There was no xylose or arabinose outside the cell (Figure 7-4A), which meant no enzymes were released in the medium to hydrolyze AX into monosaccharides. This was in agreement with the enzyme activity determination (Table 7-3) that α-L-arabinosidase or β-xylosidase could not be detected in the culture medium. No xylose or arabinose was inside the cell (Figure 7-4B). The

bacteria might use xylose and arabinose immediately after they were released in the cell, thus there were nearly no free monosaccharides existed in cell extract.

Table 7-4 Monosaccharides produced through the enzymatic hydrolysis of AX by bacterial cell extract.

	Carbon source	Monosaccharide	C (g/L)
B. longum	AX	xylose	$(1.41 \pm 0.51) \times 10^{-3}$
		arabinose	0.21 ± 0.03
	Glc	xylose	-
		arabinose	$(0.19 \pm 0.07) \times 10^{-2}$
B. infantis	AX	xylose	-
		arabinose	$(1.13 \pm 0.16) \times 10^{-3}$
	Glc	xylose	-
		arabinose	$(1.64 \pm 0.40) \times 10^{-3}$

AX was composed of xylose and arabinose at a molar ratio of 2.01:1 (Table7- 5) and the concentration of AX was the sum of xylose and arabinose (5.06 g/L). After bacterial fermentation the concentration of xylose and arabinose in the culture medium decreased sharply, suggesting both the backbone and branches of AX were used by *B. longum*. The molar ratio of xylose and arabinose left in the medium after fermentation increased to 3.53:1, showing the preference of the side chains by the bacteria. Figure 5-4B showed there was nearly no free xylose or arabinose in the cell extract, but after acid hydrolysis a small amount of xylose and arabinose was detected. This confirmed that the AX or AXOS could be transported directly into the cell, that the hydrolysis of backbone and side chain into monosaccharides occurred intracellularly and that the monosaccharides were utilized by the bacteria immediately after they were released.

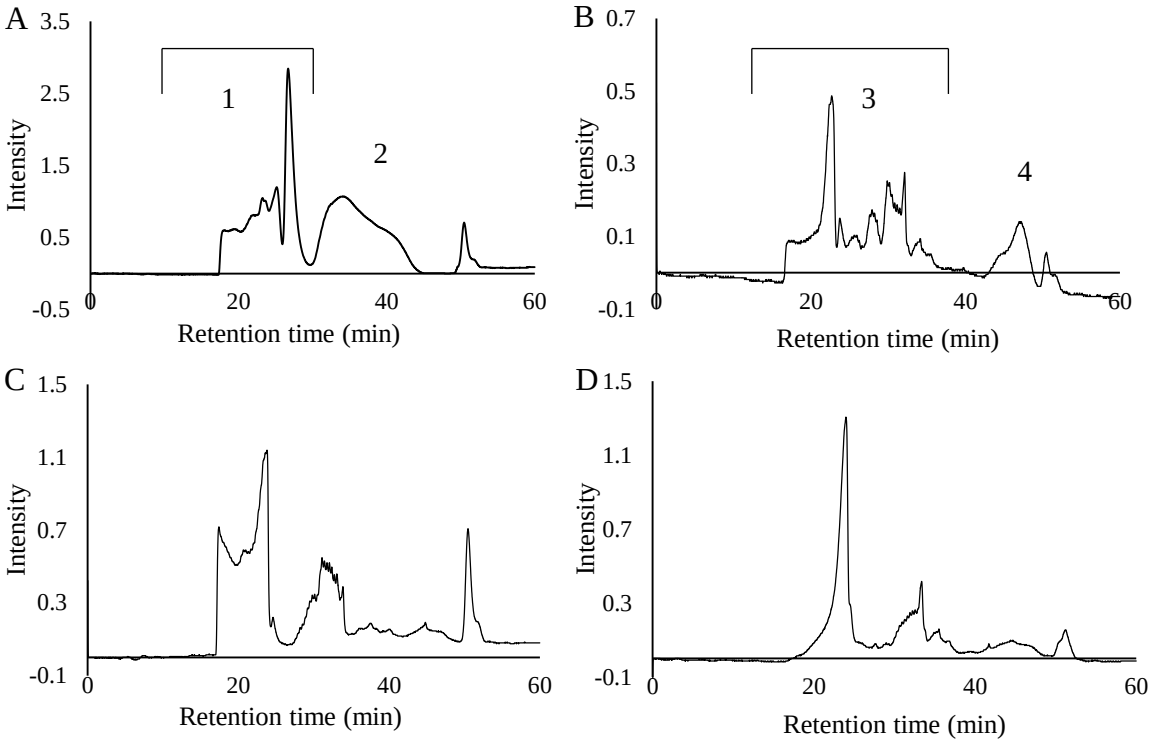

Figure 7-3 GPC profile of the culture medium containing AX (A, B) or glucose (C, D) before (A, C) and after (B, D) fermentation by *B. longum*.

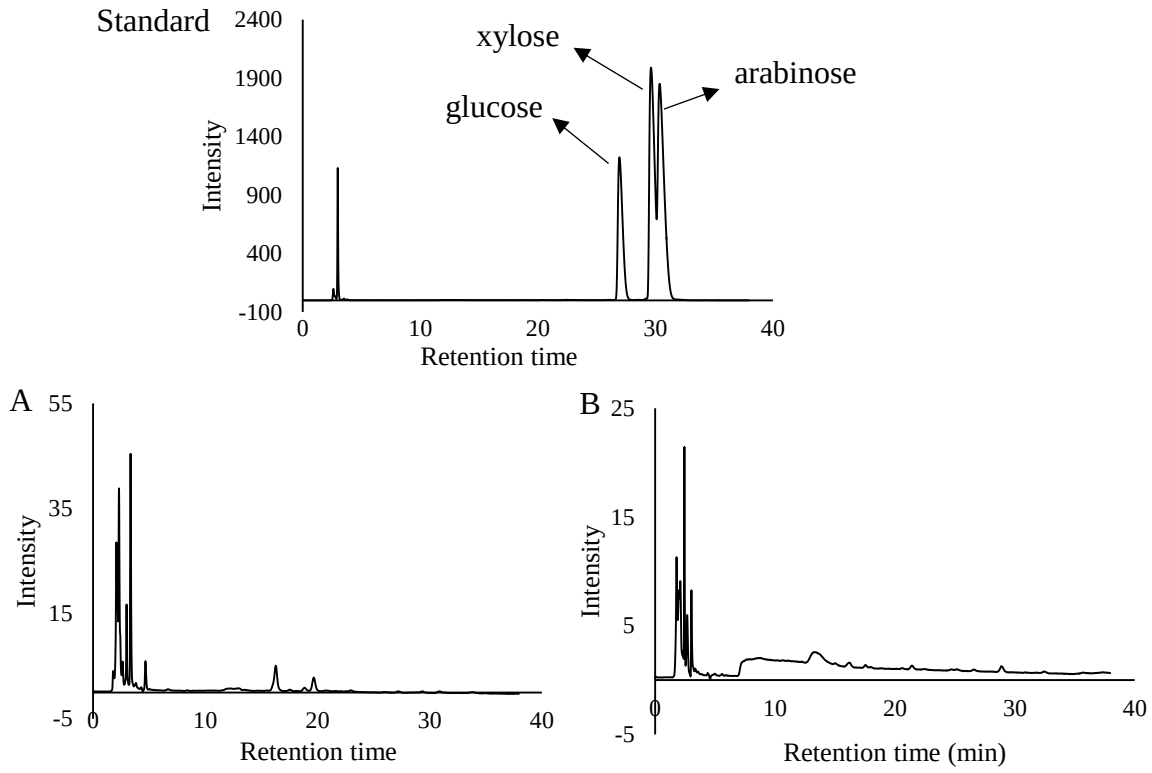

Figure 7-4 Monosaccharides in (A) culture medium and (B) cell extract after *B. longum* fermentation on AX.

Table 7-5 Monosaccharide composition and concentration of AX in cell extract and culture medium before and after *B. longum* fermentation.

		Monosaccharide	C (g/L)
Culture medium	Before fermentation	xylose	3.38 ± 0.22
		arabinose	1.68 ± 0.13
	After fermentation	xylose	0.60 ± 0.02
		arabinose	0.17 ± 0.01
Cell extract	Before fermentation	xylose	-
		arabinose	-
	After fermentation	xylose	0.04 ± 0.004
		arabinose	0.02 ± 0.001

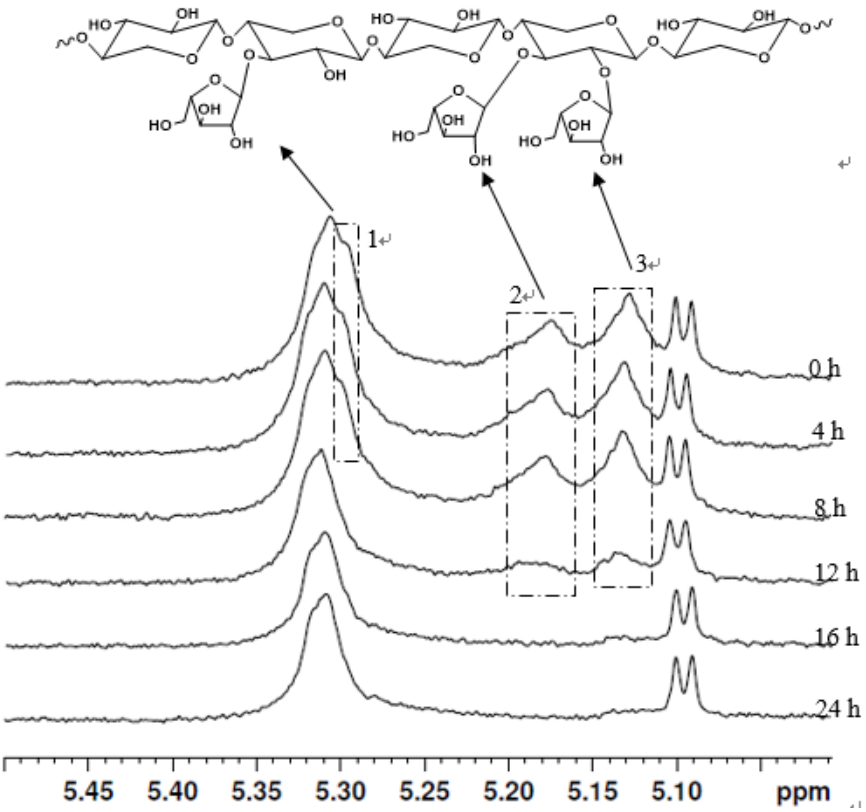

Figure 7-5 [1]HNMR of culture medium containing AX during a 24-hour culture of *B. longum*. The chemical shifts of arabinose mono-substitution at C3, mono-substitution at C2 or di-substitution at C3, and di-substitution at C2 were 5.29, 5.17 and 5.12 ppm, respectively.

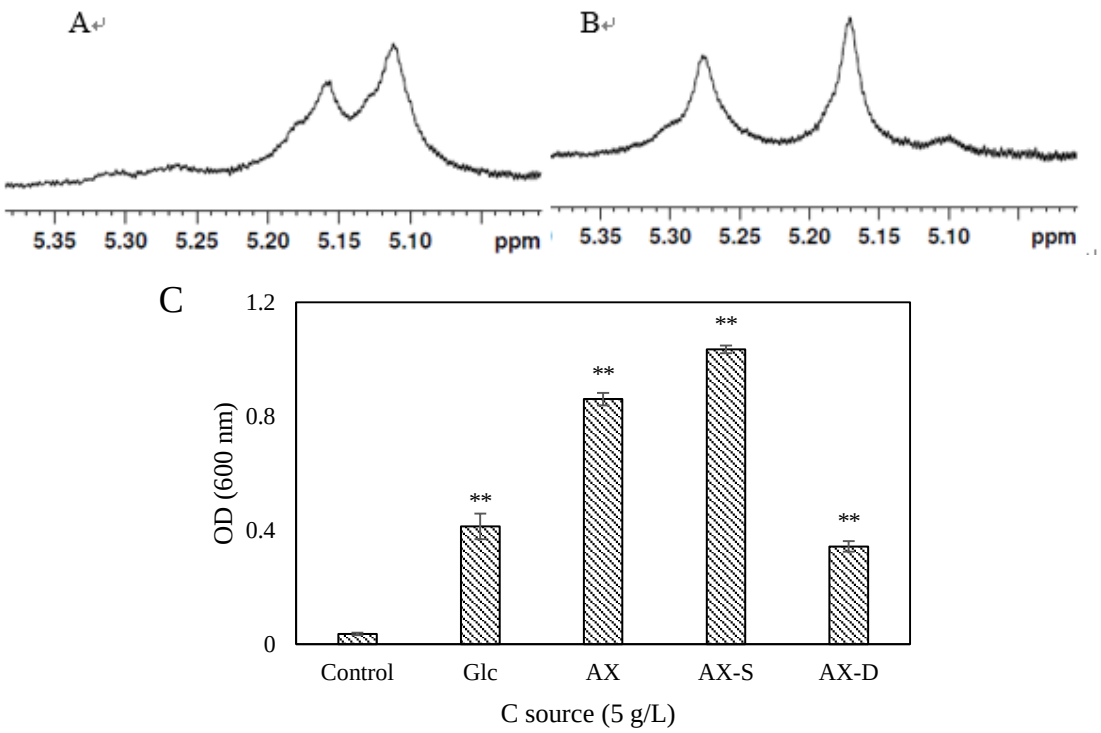

Figure 7-6 [1]HNMR of (A) double substituted AX (AX-D) and (B) single substituted AX (AX-S). (C) Growth of *B. longum* on different C sources (**: P < 0.01 by t-test).

7.3.4 Influence of side chain on bacterial growth

Changes in branches in AX during *B. longum* culture were detected by [1]HNMR (Figure 7-5) and the signals were assigned according to Hoffmann et al (1992). At 0 hour, the first broad peak at around 5.3 ppm was the overlap of two peaks, one of which was caused by the ingredients in medium, the other (peak 1) represented the arabinose mono-substitution on C3 position. The bacteria started to utilize AX after 8 or more hours of incubation. At 12th hour, peak 1 disappeared completely while peak 2 and 3, both represented the di-substitution, still had some signals and 4 more hours later peak 2 and 3 also disappeared, indicating AX was nearly used out. The higher utilization rate of peak 1 indicated that *B. longum* preferred the single branched regions. This could also be confirmed by the results in Figure 7-6C that *B. longum* had the highest OD value in the culture medium containing single substituted AX (AX-S), followed by AX, glucose and double substituted AX (AX-D). Based on the results of bacterial growth on

different carbon sources, a hypothesis could be provided. A specific structure of AX involving side chains might be recognized by the bacteria which initiated the transportation and metabolism processes of AX. Double substitution in AX blocked these specific structures, resulting in less recognition of AX. Acid randomly hydrolysed the blocks at the beginning but the recognised structures were also destroyed as the hydrolysis time going on. Therefore the utilization efficiency of AX-10 and AX-S was higher than that of AX while that of AX-30, AX-60 and AX-D were lower. According to the sidechain linkage of AX-S and AX-D (Figure 7-6A&B), *B. longum* could utilize the side chain with both 1,2- and 1,3-L-arabinofuranosyl linkage. A recent research showed similar results that an intracellular α-L-arabinofuranosidase extracted from *B. longum* had the ability to cleave 1,2- and 1,3-L-arabinofuranosyl linkage on the side chains of both AX and arabinan (Komeno, Hayamizu, Fujita, & Ashida, 2019). This indicated that the spatial structure of the side chain on AX, rather than its linkage, dominated the utilization of AX by *B. longum* and also provided another evidence that *B. longum* hydrolysed the side chain of AX intracellularly.

7.3.5 Genome analysis

The genome size of *B. longum* is 2,382,692 bp containing 60.28% of guanine and cytosine (G+C) content, which was within the typical range of *Actinobacteria*. 2058 probable coding regions comprising 85% of the genome were identified. There were 78 non-coding RNA gene, among which were 76 tRNA and 2 rRNA. 1569 (76%) of the coding regions were annotated to COG family (Figure 7-7), among which 383 (24%) were assigned to function unknown (Figure 7-9). 129 of these unknown genes were identified according to homologs from other bacterial species and the remaining 254 genes were either specific to *B. longum* or unidentified in other species. More than 10.7%

of the total predicted proteins were assigned to carbohydrate transport and metabolism (G group) in COG family. This is 45% more than *Lactobacillus lactis*, *B. halodurans* and *B. subtilis*, 27% more than *B. bifidum* and 22% more than *B. longum* NCC 2705 (Schell et al., 2002; Turroni et al., 2010), suggesting the strong ability of *B. longum* to utilize various non-digestible carbohydrates. This played an important role in its great adaptation to the lower gastrointestinal tract in human. Some other species had similar content of predicted proteins assigned to G group in COG annotation, like *B. infantis* (Sela et al., 2008), which was also tested for comparison on its enzyme activities in this study. The high content of G group proteins in *B. infantis* was due to the extensive capability of human milk oligosaccharides ultilization by *B. infantis*. ATP-binding cassette (ABC)-type transporters related to arabinose, xylose and xylooligosaccahride were found in *B. longum* (Figure 7-10), which confirmed the results in Chapter 7.3.1 that this strain could grow on these carbon sources even the utilization was not efficient. There were 81 predicted proteins annotated to carbohydrate active enzymes in CAZy database (Figure 7-8) and 62% of them, including 3 α-L-arabinofuranosidase, 3 xylanase and 1 arabinosidase, belonged to glycoside hydrolase (Table 7-6). However, there was no β-xylosidase annotated in this strain. β-xylosidase remained to be identified in this strain or the bacteria might have specific hydrolysis process intracellularly for xylooligosaccharide. The former prediction seemed to be more reliable based on the results in Chapter 7.3.2 in which the activity of β-xylosidase inside the bacterial cell was detectable.

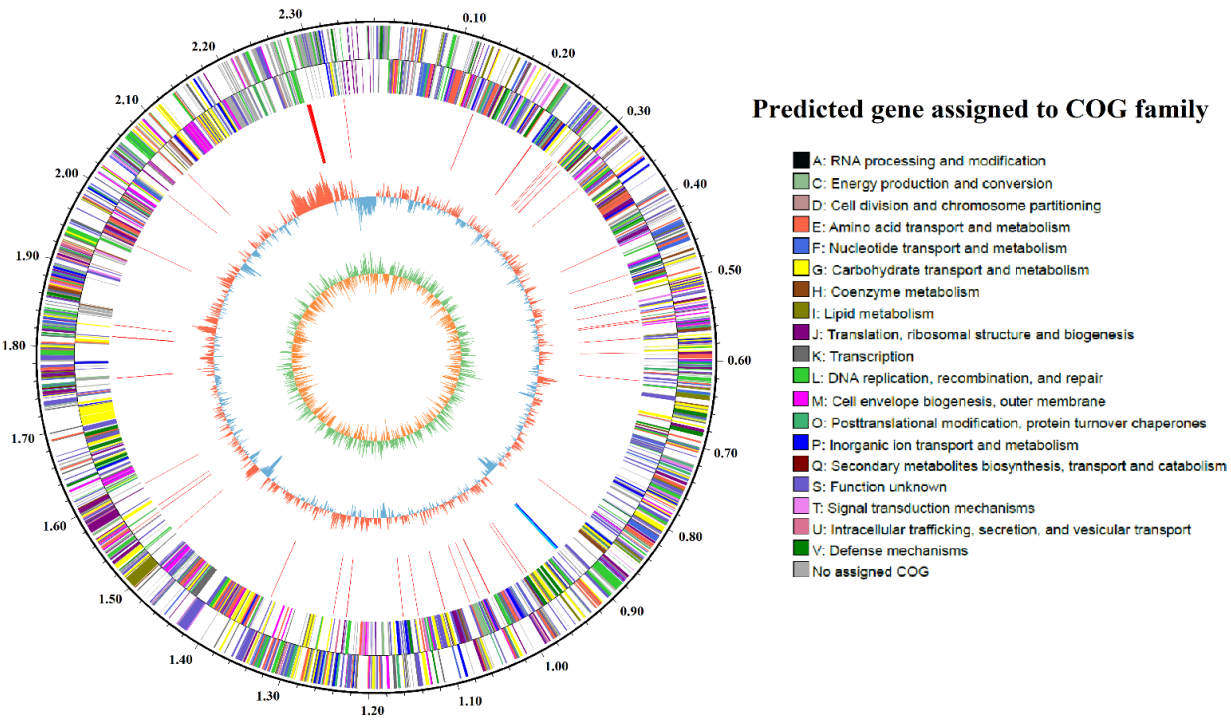

Figure 7-7 Circular genome map of *B. longum* CICC6186. Proceeding from the outer circle inward, circle (a) gene coordinate in Mp. Circle (b) coding regions in color corresponding to the COG functional annotation. Circle (c) RNA genes with tRNA in red and rRNA in blue. Circle (d) (G+C) content with red representing (G+C)% > average (G+C)% and blue < average (G+C)%. Circle (e) GC skew (G-C)/(G+C), values > 0 in green and < 0 in orange.

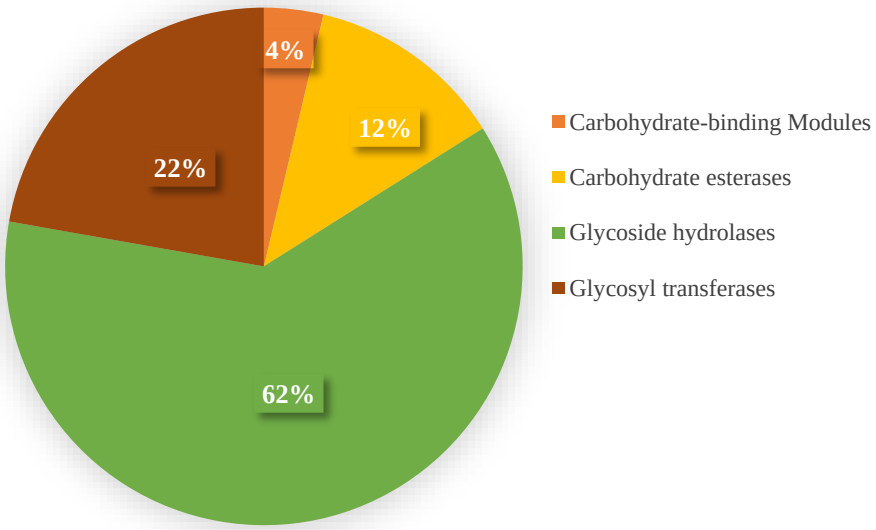

Figure 7-8 CAZy functional categories of predicted genes in *B. longum* CICC6186.

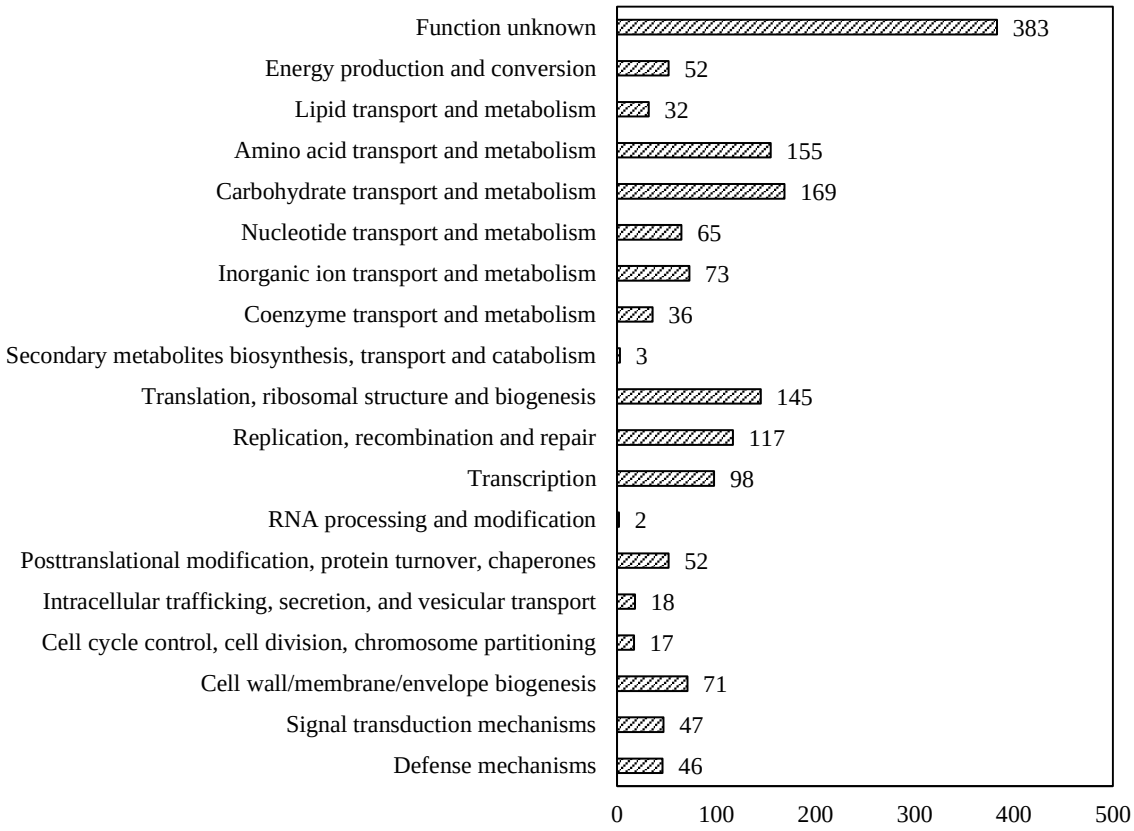

Figure 7-9 COG functional categories of predicted proteins in *B. longum* CICC6186.

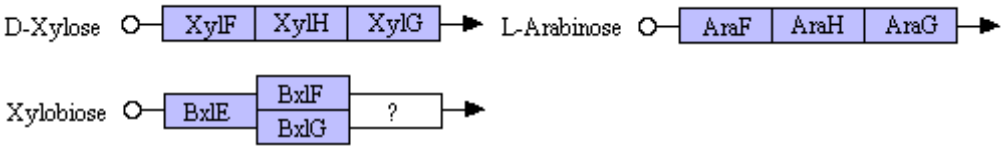

Figure 7-10 Membrane transport pathway in *B. longum* CICC6186 involving ABC transporters (AraF, L-arabinose transport system substrate-binding protein; AraH, L-arabinose transport system permease protein; AraG, L-arabinose transport system ATP-binding protein; BxlE, xylobise transport system substrate-binding protein; BxlF & BxlG, xylobiose transport system permease protein; XylF, xylose transport system substrate-binding protein; XylH, xylose transport system permease protein; XylG, xylose transport system ATP-binding protein).

Table 7-6 Predicted protein related to hydrolysis of AX in *B. longum* CICC6186.

BL[a]	Predicted enzymatic activity	Enzyme homolog[b]	% ID[c]
BL0471	α-L-arabinofuranosidase	SEB34400.1/WP_074709703.1	99.1
BL1158	α-L-arabinofuranosidase	WP_010081386.1	100
BL1366	1,4-β-xylanase	WP_118282429.1	98.4
BL1445	Exo-α-L-arabinofuranosidase	WP_008783615.1	99.8
BL1521	arabinosidase	WP_013583011.1	99.8
BL1367	1,4-β-xylanase	WP_081297354.1	99.8
BL1916	xylanase	WP_074710046.1/EPE39960.1	98.9

a: Protein annotation number of the *B. longum* genome.

b: Accession number of the closest verified enzyme homolog.

c: identical percentage with verified enzyme.

7.3.6 Metabolic strategy of AX by *B. longum*

ABC-type transport system was reported to be employed by bifidobacteria for transportation of multiple carbohydrates, especially for oligosaccharides and PS (Koropatkin et al., 2012).To summarize the findings of this study, a model based on ABC transport system was developed to describe a possible strategy of *B. longum* for AX metabolism (Figure 7-11). All the experiments in this study did not involve the cell wall enzymes. Therefore, cell wall endo-xylanase could not be excluded. The large molecules of AX was first partially hydrolyzed by free endo-xylanase or together with a cell wall endo-xylanase in the culture medium to high-ordered oligosaccharides. A specific structure involving side chains in AXOS was then recognized by the solute binding protein on the cell surface and transported directly into the cell by ABC transporters. AXOS in the cell was further hydrolysed by endo-xylanase into low-ordered oligosaccharides. α-L-arabinosidase and β-xylosidase acted in synergy to release monosaccharides or a specific pathway was performed on the backbone to produce xylose. The strategy shown here was different from the findings in some recent

investigations on the metabolism of AX, AXOS and XOS by bifidobacteria. They suggested that cleavage of arabinose substituents by *B. longum* took place extracellularly and short fraction of XOS was preferential, while longer backbone of xylose could not be used by most of *B. longum* strains (Crittenden et al., 2002; Feng et al., 2018; Rivière et al., 2014). However, in this study arabinose was suggested to be hydrolysed inside the cell and long chain AX was preferred by *B. longum* while XOS and arabinose could not be efficiently utilized. This could be explained by the different characteristics of various *B. longum* strains, which showed that the ability of bifidobacteria to degrade AX and AXOS was strain-dependent.

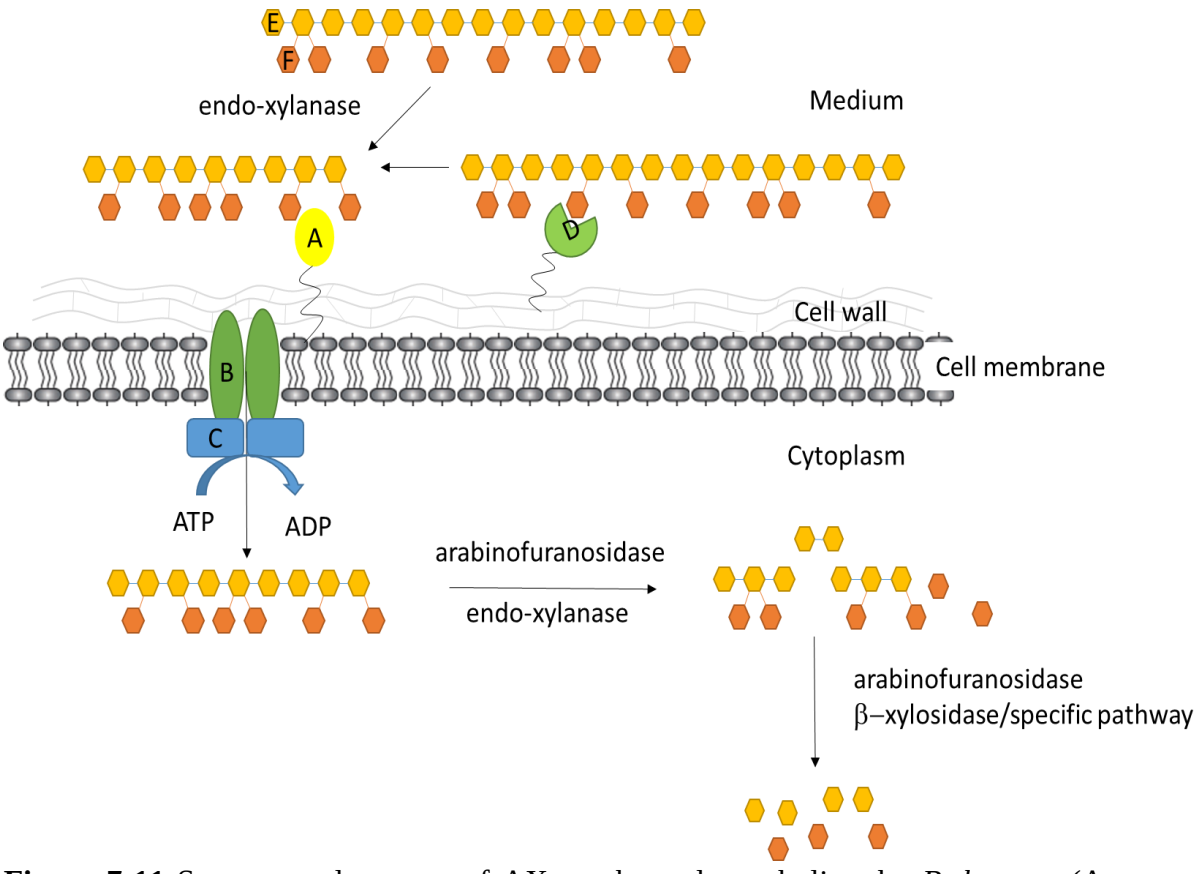

Figure 7-11 Strategy and process of AX uptake and metabolism by *B. longum* (A, solute binding protein; B, membrane permease subunit; C, ATPase; D, cell wall endo-xylanase; E, xylose; F, arabinose)

7.4 Conclusions

The study in this chapter provided a model for the degradation process of AX by *B. longum* CICC6186. Both the backbone and side chain could be utilized by this strain. The degradation of xylose backbones occurred both outside and inside the bacterial cell while the cleavage of arabinose substitutions was intracellular. The fine structure of AX played an important role in governing its fermentation efficiency. AX with higher degree of polymerization and side chain contents had a better effect in stimulating the growth of *B. longum*. The strain could grow on single- and double-substituted AX but it preferred the single-substituted one. The gene results showed that there was no predicted β-xylosidase found in *B. longum*. Therefore, a novel enzyme might remain to be identified or a specific degradation pathway of XOS by this bacteria should be further investigated. In addition, further study should be performed to reveal the membrane recognition and transportation mechanism of AX by the strain.

Chapter 8 General conclusions and future studies

8.1 General conclusions

This research project has accomplished an experimental study on with the potential prebiotic functions of three natural polysaccharides including exopolysaccharide (EPS) from Cs-HK 1 fungal mycelial fermentation, konjac glucomannan (KGM) and arabinoxylan (AX). The chief findings from this study are summarized below.

1) EPS from Cs-HK1 mycelial fermentation was a polysaccharide-protein complex with ~70% carbohydrate content and ~10-20% protein content. EPS was composed of mannose, glucose and galactose at a molar ration of 1.73:1.81:1. The molecular weight (~10^8) and intrinsic viscosity ([η]~6 dL/g) of EPS were very high and ultrasound was an effective method to degrade EPS and reduce its viscosity. EPS had ~68% of dietary fiber content and had strong resistance to gastric acid.

2) All EPS and KGM fractions were able to increase the acetic acid production of some bifidobacterial species. The lower MW fractions of EPS and KGM derived from acid-hydrolysis were more favourable for the probiotic growth while fractions with higher MW had a dramatic enhancement of the bacterial cell viability.

3) EPS from Cs-HK1 mycelial fermentation had notable protective effect on probiotic bacteria in three practical conditions, cold storage, acidic pH and bile salt. The protective effect was mainly associated with the physical properties of EPS, namely the high MW and high liquid viscosity. The viscous EPS surrounding the bacterial

cells might slow or block the access of the stress factors to the bacterial cells. Meanwhile, the immobilization or attachment of bacterial cells to the EPS gel matrix was also favourable for the bacterial survival under stress. The protective effect of EPS was weaker after partial degradation by US.

4) Arabinoxylan (AX) could only be efficiently metabolised by *Bifidobacterium longum* CICC6186 as a carbon source but not by another four species of bifidobacteria. The monosaccharides of AX, arabinose and xylose, and linear xylooligosaccharide (XOS) had very weak stimulation on *B. longum* growth. AX with single substitution on branches was more favourable by *B. longum* than the double substituted one.

5) Based on the enzyme activities, carbohydrate analysis in culture medium and the genome sequence, a model was proposed for the utilization mechanism of AX acquired by *B. longum*.

8.2 Future studies

The results from this research project provide useful reference and foundation for further study on development and application of fungi and plants PS as prebiotics. Specifically the following studies are recommended for future work.

1) The prebiotic activity of EPS, KGM and AX has been examined in this project by pure culture of bifidobacterium. Fecal fermentation and *in vivo* test should be approached to further study the prebiotic function of these three PS.

2) Chemical structure of EPS should be further investigated to reveal the interaction between this bio-polymer and gut microbiota.

3) Chemical modification of EPS or combination with microencapsulation can be applied to improve the protective effect of EPS on probiotics from harsh condition. A comprehensive *in vitro* model such as the SHIME and *in vivo* animal model are also suggested to further confirm the protection of EPS.

4) RNA transcription sequencing can be applied to find out the regulated gene related to AX metabolism by *B. longum*. Due to the fine structure of AX was suggested in this project to play a key on governing its fermentation by bifidobacterium, AX from different sources should be further tested on their bifidogenic effects and degradation mechanism.

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Appendix

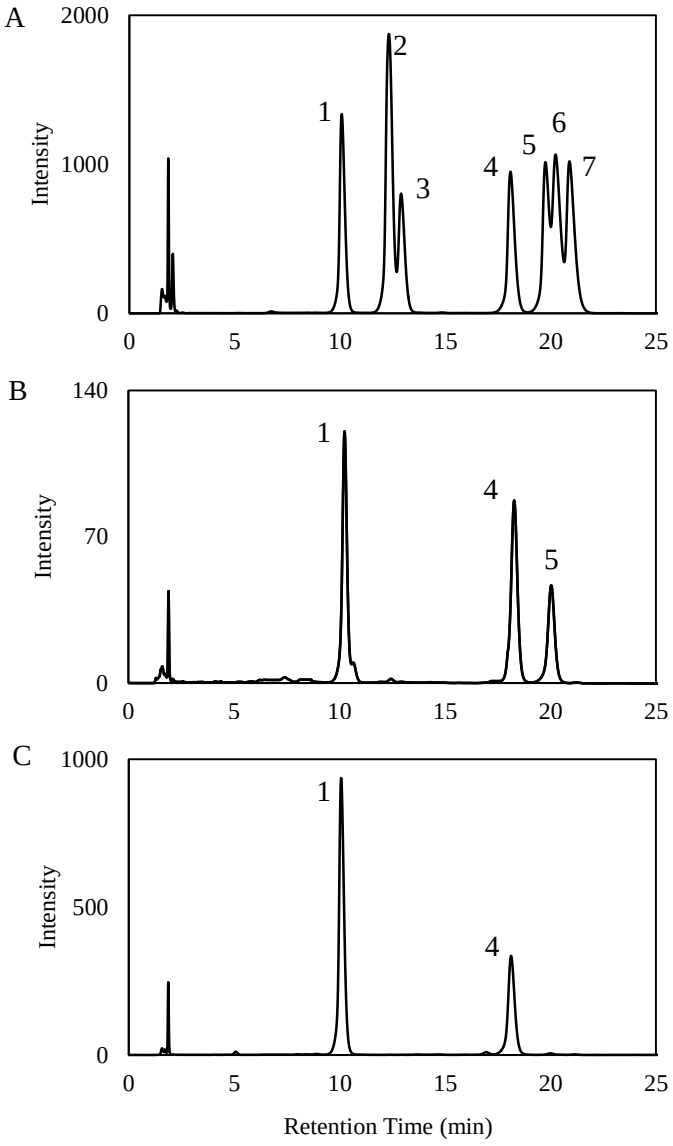

Figure A1 HPLC profiles of (a) monosaccharides standards, (b) EPS and (c) KGM (Peaks: 1, mannose; 2, ribose; 3, rhamnose; 4, glucose; 5, galactose; 6, xylose; 7, arabinose).

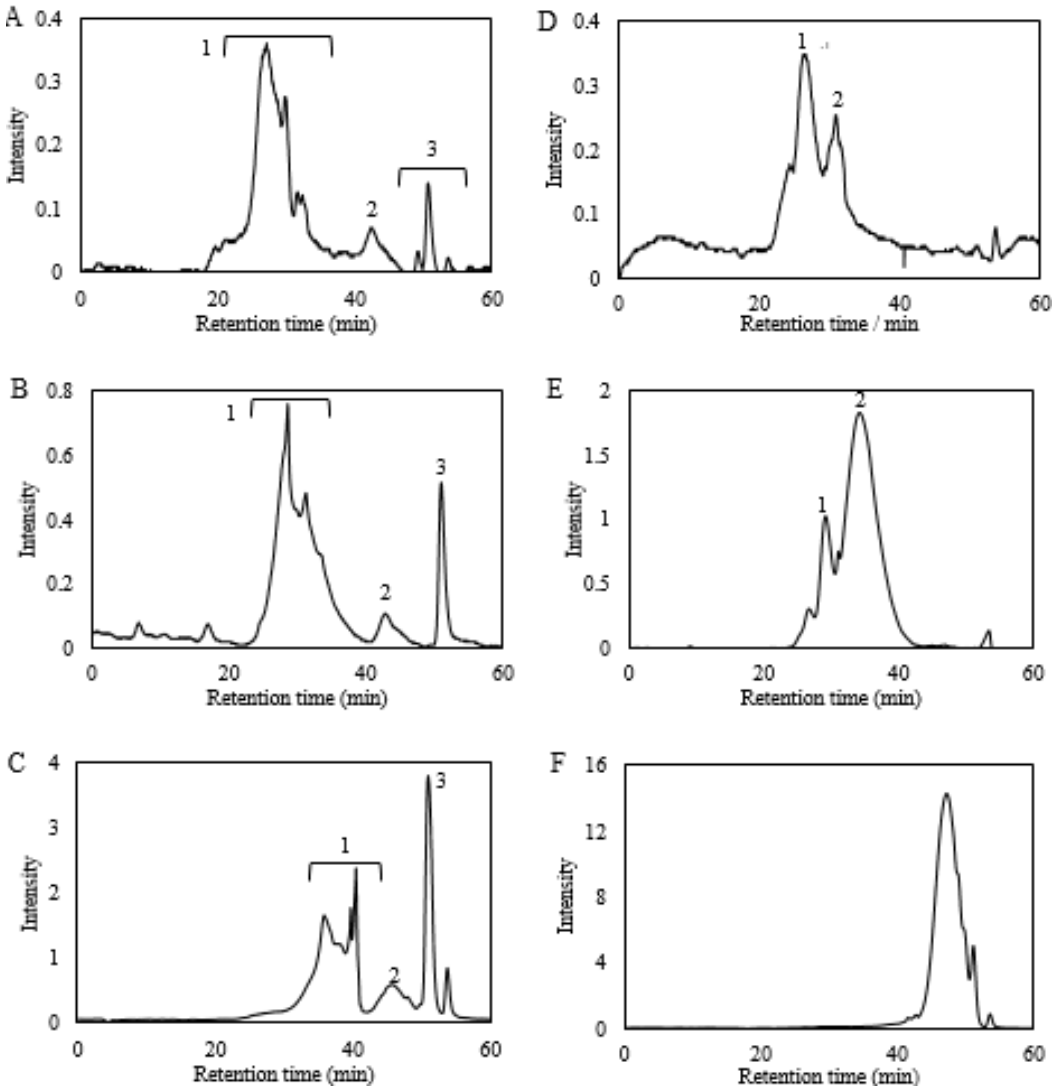

Figure A2 GPC profiles of (A) EPS, (B) ultrasound degraded EPS (EPS-US), (C) acid hydrolyzed EPS (EPS-AH), (D) KGM, (E) ultrasound degraded KGM (KGM-US), and (F) acid hydrolyzed KGM (KGM-AH).