ANTI-INFLAMMATORY ACTIVITIES OF EXOPOLYSACCHARIDES FROM A MEDICINAL FUNGUS *CORDYCEPS SINENSIS* Cs-HK1 AND ENHANCED BY BIFIDOBACTERIAL FERMENTATION

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CERTIFICATE OF ORIGINALITY

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Abstract

This research project aims to investigate the anti-inflammatory potential of exopolysaccharides (EPS) produced by a medicinal fungus *Cordyceps sinensis* Cs-HK1, to fractionate the bioactive EPS fraction, and study the interaction of EPS fermentation by probiotic *bifidobacterium*, as well as the modified EPS molecular properties and enhanced bioactivity after bifidobacterial metabolization.

In the first part of this project, the whole and crude EPS (CEPS) was recovered from the Cs-HK1 mycelial fermentation liquid by ethanol precipitation in a single step. After deproteinization and dialysis of the crude EPS, the partially purified EPS had a total sugar content of 74.8% with a maximum average molecular weight (MW) over 10^7 Da, consisting mainly of glucose, mannose, and a trace amount of galactose and ribose. In THP-1 and RAW264.7 cell models *in vitro*, the EPS fraction significantly inhibited lipopolysaccharide (LPS)-induced inflammatory responses including the release of NF-κB and several pro-inflammatory factors such as NO, TNF-α and IL-1β. In the murine model of LPS-induced acute intestinal injury, the oral gavage of EPS alleviated the intestinal injury and also suppressed the expression of major inflammatory cytokines TNF-α, IL-1β, IL-10 as well as iNOS in the treated animals. These results suggest that the EPS from Cs-HK1 has notable anti-inflammatory activity and can be a potential candidate for further development of new anti-inflammatory therapeutics.

The second part of the project was then to assess the anti-inflammatory activities of different EPS fractions, to isolate and purify the most active EPS fraction and to characterize its physicochemical properties. The EPS was first isolated from the Cs-HK1 fermentation liquid by two-step ethanol precipitation, using 40% ethanol in the first step to attain the higher MW EPS fraction (EPS-HM) and 80% in the second step to attain the lower MW EPS (EPS-LM). As the EPS-LM fraction showed higher anti-inflammatory activity than both the higher MW fraction EPS-HM from the two-step precipitation and the whole EPS from single step precipitation at 80% v/v ethanol, it was chosen for further characterization and anti-inflammatory assessment. The low-MW EPS-LM was further purified by columns, giving a purified EPS designated EPS-LM-1 with a homogenous average MW of 360 kDa. EPS-LM-1 was composed of three monosaccharide residues including mannose, glucose, and galactose at 3.9:6.9:1 mole ratio. EPS-LM-1 had a main chain of [6)-β-D-Glc*p*-(1→4)-α-D-Man*p*-(1→4)-α-D-Glc*p*-(1]$_2$→3,6)-α-D-Glc*p*-(1→[3)-α-D-Man*p*-(1→4)-α-D-Glc*p*-(1]$_2$, which was branched at the O-3 position of 3,6)-α-D-Glc*p*-(1→ with β-D-Gal*f*-(1→ side chains (~8% degree of branching). In addition, the anti-inflammatory effects of EPS-LM-1 in *in vitro* were evaluated. In human monocytic THP-1 cell model, EPS-LM-1 significantly inhibited LPS-induced release of major pro-inflammatory factors with IC50 values below 5 μg/mL. Excessive protein expression levels of IKBα (nuclear factor-kappa-B inhibitor alpha) and NF-κB complex were both dose-dependently inhibited by EPS-LM-1 as well. In summary, EPS-LM-1 showed a notable anti-inflammatory activity probably through suppressing NF-κB signalling pathway. EPS-LM-1 may play a

crucial role for its be a promising candidate for further development of anti-inflammatory therapeutics or nutraceuticals.

The third part of the project was to assess the anti-inflammatory activity of fermented EPS and metabolic products by selected *Bifidobacterium* species. The results indicated that the digesta (supernatant) from the bifidobacterial fermentation of EPS-LM showed much higher activity than the EPS-LM alone on LPS-stimulated proinflammatory responses in THP-1 cell model. This suggests that some metabolic products derived from the bifidobacterial fermentation of EPS-LM may produce stronger anti-inflammatory activity. The EPS-LM could enhance the growth of two bifidobacteria strains (*B. breve* and *B. longum*) at 1 g/L ($p < 0.05$) and also increased the acetic acid production significantly. Analytical results from the fermentation digesta suggested that EPS-LM was partially degraded to lower molecular weight (MW) products with modified structures during the bifidobacterial fermentation. More interestingly, the higher MW digesta fraction containing the partially degraded EPS-LM showed even stronger inhibiting activity than the original EPS-LM on the LPS-induced inflammatory THP-1 cell model. These results indicated that the fermentation by selected bifidobacterial strains is effective to modify natural polysaccharides with enhanced bioactivities. Another interesting effect from the bifidobacterial fermentation of EPS-LM was the notable antibacterial activity on the growth and biofilm formation disruption of *Escherichia coli* compared to the conventional digesta (RCM) as well as EPS-LM without fermentation, indicating the EPS-LM metabolism by bifidobacteria is responsible for potent bioactive components production.

This thesis presents the interesting and useful effects of Cs-HK1 EPS and its metabolite products from bifidobacterial fermentation and the new findings of highly active EPS molecular characteristics and the possible mechanisms for their anti-inflammatory effects. These results and findings will help pave the way for further development and application of Cs-HK1 EPS as new and effective ingredients for anti-inflammation therapy as well as maintaining human gut health. The findings will also make new and valuable contributions for discovery of novel nutraceutical and therapeutic products from probiotic fermentation of natural polysaccharides.

List of Publications

Journal papers

1. **Li, L. Q.,** Song, A. X., Wong, W. T. & Wu, J. Y. (2021). Modification and enhanced anti-inflammatory activity by bifidobacterial fermentation of an exopolysaccharide from a medicinal fungus Cs-HK1. International Journal of Biological Macromolecules,188, 586-594.

2. **Li, L. Q**., Song, A. X., Wong, W. T. & Wu, J. Y. (2021). Isolation and assessment of a highly-Active anti-Inflammatory exopolysaccharide from mycelial fermentation of a medicinal fungus Cs-HK1. International Journal of Molecular Sciences, 22(5), 2450.

3. **Li, L. Q.**, Song, A. X., Yin, J. Y., Siu, K. C., Wong, W.T. & Wu, J. Y. (2020). Anti-inflammation activity of exopolysaccharides produced by a medicinal fungus *Cordyceps sinensis* Cs-HK1 in cell and animal models. International Journal of Biological Macromolecules, 149, 1042-1050.

4. Zhang, B., **Li, L. Q.**, Liu, F., & Wu, J. Y. Human milk oligosaccharides and infant gut microbiota: Molecular structures, utilization strategies and immune function. Carbohydrate Polymers, (2021) 118738.

5. Song, A. X., **Li, L. Q**., Yin, J. Y., Chiou, J. C. & Wu, J. Y. (2020). Mechanistic insights into the structure-dependant and strain-specific utilization of wheat arabinoxylan by Bifidobacterium longum. Carbohydrate Polymers, 116886.

6. Mao, Y. H., Song, A. X., **Li, L. Q.**, Siu, K. C., Yao, Z. P. & Wu, J. Y. (2020). Effects of exopolysaccharide fractions with different molecular weights and compositions on fecal microflora during in vitro fermentation. International journal of biological macromolecules, 144, 76-84.

7. Mao, Y. H., Song, A. X., **Li, L. Q.**, Yang, Y., Yao, Z. P. & Wu, J. Y. (2020). A high-molecular weight exopolysaccharide from the Cs-HK1 fungus: ultrasonic degradation, characterization and in vitro fecal fermentation. Carbohydrate Polymers, 116636.

Paper in preparation

1. **Li, L. Q.,** Song, A. X., Wong, W. T. & Wu, J. Y. (2021). Potential antibacterial activity of metabolites by bifidobacterial fermentation of an exopolysaccharide from a Medicinal Fungus Cs-HK1.

Conference presentations and posters (International)

1. **Li, L. Q.,** Mao, Y. H., Yin, J. Y., Sui, K. C. & Wu, J. Y. (2019). Chemical composition and Anti-inflammation Activity of a Fungal Exopolysaccharide from *Cordyceps sinensis* Cs-HK1. International Conference on Polysaccharides for Nutraceuticals and Biomaterials (ICPNB-2019), Shenzhen, China.

2. 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.

Patent in application

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List of Abbreviations and Symbols

EPS Exopolysaccharides

FID Flame ionization detector

GC Gas chromatography

GC-MS Gas chromatography-mass spectrometer

HPGPC High performance gel permeation chromatograph

HPLC High performance liquid chromatography

LPS lipopolysaccharides

MS Mass spectrometry

MW Molecular weight

NMR Nuclear magnetic resonance

NF-κB Nuclear factor kappa B

NO Nitric oxide

SCFA Short chain fatty acid

SEM Scanning electron microscopy

Chapter 1 Introduction

Inflammation, which is tightly regulated by the immune system, is a complex physiologic defensive response to protecting the body against noxious stimuli and microbial invaders (Majno & Joris, 2004; Kumar, Abbas & Aster, 2017). Inflammation involves a sequence of molecular, cellular, and physiological alterations. The typical inflammatory reaction can cause tissue dysfunction, so that the inflammatory cascade reaction cannot reach the decomposition state, and eventually lead to organ disorder and failure. In recent years, inflammation, which includes various cell processes (including phagocytosis, chemotaxis, mitosis and cell differentiation), is one of the main target areas in biomedical research. There are many studies on how the immune system leads to inflammatory responses via the regulation of cellular immunity and antibody. Inflammatory disorders may lead to the overexpression of inflammatory cytokines, which are small molecular weight proteins acting as soluble mediators and as 'messengers' secreted by cells, such as interleukin (IL)-1β, IL-8 and tumour necrosis factor (TNF)-α, which are all implicated in the pathogenesis of inflammatory diseases (Mantovani, Allavena, Sica & Balkwill, 2008), and cause some prevalent human diseases such as arthritis, inflammatory bowel disease, and even cancer. The effective control of the inflammatory reactions is crucial for prevention and treatment of these diseases. Therefore, the modulation of inflammatory responses is a crucial strategy for the treatment of chronic inflammatory diseases.

Conventionally, antibiotics and steroids are applied for the control and treatment of inflammation infections (Sweeney, Wong & Khatri, 2016). Increasing concerns with their serious side effects and adverse reactions have motivated the considerable effort in recent decades toward the discovery of safer and more effective anti-inflammation agents (Barochia et al., 2011). A favourable and effective strategy in the development of new therapeutics is based on molecular targeting on the mediators that can disrupt specific cellular signalling pathways involved in the diseases (Castellarin, Zorzet, Bergamo & Sava, 2016).

Polysaccharides from edible and medicinal fungi are recognized as biological response modifiers with immunomodulatory and anti-inflammatory activities (Sindhu et al., 2021), which are promising candidates for new anti-inflammation agents. Another attractive aspect of the fungal polysaccharides is the selective inhibition of pro-inflammatory molecules secreted by cells without significant cytotoxicity (Schepetkin & Quinn, 2011; Dumlupinar, 2021). Polysaccharides as complex biomacromolecules have various physicochemical characteristics such as monosaccharide composition, glycosidic linkage, degree of branching, molecular weight, bonding of proteins or peptides, which can affect their properties and bioactivities (Song et al., 2013).

Cordyceps sinensis (= *Ophiocordyceps sinensis*), generally known as the Chinese caterpillar fungus (or Dong-chong-xia-cao in Chinese), is a unique and esteemed medicinal fungus in traditional Chinese medicine and has also been used as a health food or tonic to promote health and alleviate a variety of diseases. Since the natural Chinese caterpillar fungus is very rare and expensive, mycelial fermentation has been

widely applied for the production of the fungal materials. Cs-HK1 used in this study is a fungus species isolated from the fruiting body of a natural *C. sinensis* strain, and the mycelial culture has been found to produce significant amount exopolysaccharide (EPS) in liquid fermentation. This EPS had a complex composition and a wide molecular weight (MW) distribution, some of the EPS components showed significant antioxidant and moderate immunomodulatory activities in previous study (Yan, Wang & Wu, 2014). However, the anti-inflammation potential of EPS from the Cs-HK1 mycelial fermentation remains unknown. To date, there are only a few studies reported the potential anti-inflammatory effect of exopolysaccharides from some other *Cordyceps* strains (Smiderle et al., 2014), but there is still little information on their molecular properties and relationship to the bioactivity, and the underlying molecular mechanism.

In addition, human gastrointestinal tract (GIT) is the largest immune organ and maintains systemic immune homeostasis in the presence of its unique and complex microbial ecosystem. Host immunity regulates the microbiome by altering bacteria-associated signalling to influence tumour surveillance. It has been well known for more than two decades that *Bifidobacterium* is a major health promoting genus of bacteria contributing to host health and reducing the risk of diseases (Gibson & Roberfroid, 1995; Wong, Odamaki & Xiao, 2020). On the other hand, natural polysaccharides with non-toxicity and high resistance to human gastrointestinal condition have also been demonstrated to provide a rich source of potential prebiotics (Singdevsachan et al., 2016). Prebiotics are the non-digestible fibers that can be selectively fermented by certain bacteria in the gut microbiota and produce beneficial effects on human health

(Roberfroid et al., 2010; Saad et al., 2013; Gibson et al., 2017; Peredo-Lovillo, Romero-Luna & Jiménez-Fernández, 2020). Another new term "postbiotics" refers to the soluble factors (products or metabolic by-products) secreted by live bacteria or released after bacterial lysis, that are beneficial to the host health by providing additional bioactivity (Aguilar-Toalá et al., 2018; Cuevas-González et al., 2020; Moradi et al., 2020). Such soluble factors which have been collected from many bacteria species may include short chain fatty acids (SCFAs), enzymes, peptides, exopolysaccharides, vitamins (Tsilingiri & Rescigno, 2013; Russo et al., 2019). Despite the fact that the mechanisms implicated in the health beneficial effects of postbiotics are not fully elucidated, scientific data have provided evidence that postbiotics possess different functional properties including, but not limited to, antimicrobial, antioxidant, and anti-inflammatory potential (Sharma & Shukla, 2016; Cuevas-González et al., 2020).

Based on the above-mentioned background, the molecular properties and health benefits of bioactive polysaccharides from edible and medicinal fungi, and their association with the gut microbiota affecting on human health are worthy further exploration. This thesis mainly focuses on investigating the potential anti-inflammatory activity of Cs-HK1 EPS on both *in vitro* cell model and *in vivo* animal models, revealing the potential activity mechanism and its relationship to the composition and molecular properties of EPS fraction. Additionally, the *in vitro* bioactive properties of EPS metabolites fermented with bifidobacteria as a novel source of prebiotic or postbiotic will also be addressed as current trends in food and future pharmaceutical applications.

Chapter 2 Objectives and Significance

2.1 Objectives

This research project aims to investigate the potential anti-inflammatory activity and mechanisms of exopolysaccharides (EPS) from Cs-HK1 mycelial fermentation, and the bioactive metabolites derived from bifidobacterial fermentation of the Cs-HK1 EPS. These will be achieved through the following studies:

1) Evaluation of the potential anti-inflammatory functions of EPS fractions in lipopolysaccharide (LPS)-induced inflammatory cell and animal models.
2) Fractionation and purification of the crude and complex EPS, analysis of the molecular properties of EPS fractions including chemical composition, molecular weight (MW) distribution, and molecular structure and assessment of the relationship between the molecular properties and anti-inflammatory activity.
3) Investigation of the effects and metabolic products derived from probiotic fermentation of the EPS by selected bifidobacteria strains, including the bifidobacterial growth, short chain fatty acid production and changes in the EPS molecules and other major culture nutrients, and metabolites, and exploration of the novel metabolites and activities including anti-inflammatory and anti-bacterial activities.
4) Discovery of the possible mechanisms for the anti-inflammatory activity of EPS, particularly the molecular events in the LPS-induced pro-inflammatory

signal pathways and the connection with the prebiotic action in the gut microbiota.

2.2 Significance

Inflammation is closely associated with various diseases, and the potential mechanisms have been widely studied (Hussain, Hofseth & Harris, 2003; Medzhitov, 2008). Conventional treatments using antibiotics and steroids still have the limitation and side effects, drawing the wide interest for safer natural anti-inflammatory products. Natural polysaccharides (PS), especially those from medicinal fungi are recognized as an important class of natural biological response modifiers (BRMs) having immunomodulatory functions. In this connection, many natural PS have potential anti-inflammatory activity as a potent source for promotion of human health. Moreover, because of their non-digestible nature, bioactive fungal PS also have great potential as prebiotic/postbiotic function, which could become more effective through fermentation by the probiotic bacteria such as bifidobacteria.

Cs-HK1 is a fungus species isolated from the fruiting body of a natural *Cordyceps sinensis* and the Cs-HK1 mycelial culture has been found to produce significant amount of exopolysaccharide (EPS) in liquid fermentation. Previously studies also have shown that EPS has notable antioxidant activity and immunoregulation potency. This thesis will provide evidence that EPS from *C. sinensis* fungi Cs-HK1 liquid fermentation has significant anti-inflammatory activity, and the physicochemical characteristics of its

crucial fraction EPS-LM-1 was further investigated which could have potential efficacy for the treatment of inflammatory-related diseases. Meanwhile, the relationship between active EPS-LM fraction and their interaction with *Bifidobacterium* was also investigated. In addition, the investigation on the metabolisms of EPS-LM by two bifidobacteria strains (*B. breve* and *B. longum*) in this project indicated that the EPS-LM could regulate bifidobacteria metabolizing products and enhance the bioactivity of bifidobacterial digesta as postbiotics.

This thesis will give a foundation for understanding the possible anti-inflammatory mechanism of an exopolysaccharide from Cs-HK1 fungus as a potential pharmacological agent based on both *in vitro* and *in vivo* studies, as well as prebiotics function for further study on the metabolic effects to enhance the bioactivities of intestinal microbiota metabolites, which could be useful for both foundational studies and commercial applications on functional food production, and give a promising new function food and therapeutic products.

Chapter 3 Literature Review

3.1 Sources and bioactivities of natural polysaccharides

Polysaccharide is a kind of complex chain polymer which contains different composition of monosaccharides, glycosidic bonds linkage, branched chains, molecular weight, and structural conformation. It is not only a direct source of energy supplement, but an important part for organism as an important biological macromolecule besides nucleic acid and protein (Schepetkin & Quinn, 2006; Yu et al., 2018). Polysaccharides are abundant in most living organisms such as plants, fungi, bacteria, algae, and animals, and perform a wide range of physiological functions in these organisms (Hasnain & Nayak, 2019). Polysaccharides are divided into homoglycans of single monosaccharides, heteroglycans of two or more monosaccharides and glycoconjugates with peptides, proteins or lipids. In recent years, reports on the biological functions of bioactive polysaccharides mainly include anti-tumor, hypolipidemic, antiviral and immune enhancement (Chakraborty, et al., 2019). At present, polysaccharide, envisaged as a natural bioactive ingredient, is favored by world widely because of its multiple biological activities, non-toxic, harmless, non-residue, non-drug resistance, as well as a hot issue in the research fields of medicine, agriculture and food industry. Among them, fungal polysaccharides have attracted widely attention due to their unique biological activities (Li et al., 2020).

Polysaccharides from higher fungi especially the edible and medicinal fungi or mushrooms are a large group of bioactive polysaccharides with multiple and high

biological activities and benefits to human health. The fungal polysaccharides can be attained from fungal fruiting body (mushroom), fungal mycelium and the fermentation liquid. In recent years, it has been found that fungal polysaccharides have a wide range of biological activities, such as immunoregulation, anti-tumor, hypolipidemic, hypoglycemic, anti-hepatitis, anti-aging, antiviral and so on (Schepetkin & Quinn, 2011; Sindhu et al., 2021; Dumlupinar, 2021). Some fungal polysaccharide components, especially d-glucan derivatives, in *Cordyceps sinensis*, *Ganoderma lucidum*, *Flammulina cocos*, *Agaricus blazei* and other medicinal fungi have the notable ability to increasing the function of macrophages, monocytes and neutrophils, stimulating the production of antibodies, so as to improve the human immunity and enhance the anti-tumor ability of the human body (Sindhu et al., 2021). However, the relationship between the bio-function and potential mechanisms of polysaccharides is still insufficient, and the study of the structure-activity relationship of bioactive polysaccharides needs further exploration.

3.2 Structural features and influence on the bioactivities of polysaccharides

3.2.1 Composition and glycosidic bond type

The composition of the main chain units of polysaccharide determines the type of polysaccharides, physicochemical characteristics, as well as varies their pharmacological activities with nontoxic properties. The active polysaccharide

obtained from the natural source is generally composed of glucans, mannans, pectic polysaccharides, arabinogalactans, fucoidans and so on, which may have different relevance depending on the type of polysaccharides and their combination to be the basic inducement of the host immune defense mechanism. For instance, *Lentinan* and *Schizophyllum* polysaccharides have the main chain structure of dextran (Klaus, Kozarski & Niksic et al., 2011; Lyu, Xu & Zhang, 2020), both of which have antitumor effects, and can improve the cellular and humoral immune functions. The main chain of *Tremella* polysaccharide is α-(1→3) glucoside linked mannan, which has the functions of immune regulation, antitumor, anticoagulant and antithrombotic (Du et al., 2009). In addition to homopolysaccharide, heteropolysaccharide also has some biological activities. The monosaccharide composition of *Cordyceps sinensis* polysaccharide is mannose, galactose and glucose, which can improve the immune function of the body, inhibit tumor cells, and improve the adverse reactions caused by chemotherapy (Yan et al, 2014). The *Grifola frondosa* polysaccharide with immunomodulatory activity is also a heteropolysaccharide containing glucose, galactose, and mannose (Li et al., 2018). The type of glycosidic bond refers to the connection mode of adjacent glycosyl groups on the main chain of polysaccharide, and the type of glycosidic bond on the main chain of polysaccharide is also an important factor determining the activity of polysaccharide. Most of the bioactive glucan are linked by (1→3) glycosidic bonds (Huang & Huang, 2021). β-D-glucan linked by β-(1→3) bond also has obvious immunoregulation, antitumor and anti-inflammation activity, as well as the glucan linked by (1→6) glycosidic bond has certain activity. The

immunomodulatory activity of *Grifola frondosa* polysaccharide mainly comes from β-(1→3) or (1→6) glucan (Wu, Siu & Geng, 2021). The mannan with activity is (1→6) linked, while the active galacto-polysaccharide is (1→3) linked.

3.2.2 Chain configuration

The activity of polysaccharide can be influenced by the linkage between the sugar units, glycoside bond, presence of branches, as well as the main chain configuration. In general, D-glucopyranosyl is mainly present in polysaccharides with different types of glycosidic bonds originating linear or branched α/β-D-glucans (Synytsya & Novák, 2013; Singh et al., 2021). Most of the polysaccharides with bioactivity have the main chain structure of β-(1→3)-D-glucan/β-(1→6)-D-glucan (Nakashima et al., 2018). The active polysaccharides in edible and medicinal fungi, such as lentinan and *Polyporus umbellatus* polysaccharide, are β-(1→3)-D-glucan with branches (Mulloy, Mourao & Gray, 2000), while the polysaccharide from *Aconitum carmichaeli* is α-(1→4)-glucans proved with immunostimulation (Yang et al., 2020). Meanwhile, a galactoglucomannan found in *Cordyceps militaris* is composed by β-(1→6)-D-Man*p* with random coil conformation also having high immunostimulatory functions (Lee et al., 2010), while another triple-helix mannan composed by β-(1→2)-D-Man*p* showed less activity, which indicated the different chain types of polysaccharides, such as glycan compositions, helical and random coil conformations, may also influence their immunostimulation (Mueller et al., 2000).

3.2.3 Length of branch chain and position

The degree of branching (DB) also affects the activity of polysaccharides. Only when the polysaccharide reaches a certain degree of branching can it obtain biological activity (Chen et al., 2019). Each functional polysaccharide may have its best DB, which makes its bioactivity reach the ideal state. Studies on the structure of polysaccharides revealed that water-soluble D-glucans had multiple biofunctions, such as immunomodulation, anti-tumor, and anti-inflammation, especially those with a linear chain of backbone and no overlong branched chains (Vannucci et al., 2013). The *Poria cocos* polysaccharides showed no bioactivities due probably to its long branch chain with insoluble property (Jia et al., 2016), and some other natural polysaccharides attained bioactivities after controlled oxidative hydrolysis to reduce the length of the branch chains. For instance, when the branching degree of β-(1→3)-D-glucan was 0.2 ~ 0.33, its biological activity was strong, and the best DB of antiviral activity was about 1.5 when the dextran sulfate was replaced by the sulfate group (Mulloy, Mourao & Gray, 2000).

The position of DB also affects the activity of polysaccharide. The anticoagulant activity of chondroitin sulfate B depends on the position of the sulfuric acid substituent. The anticoagulant activity of chondroitin sulfate B will be completely lost if the sulfuric acid group at the 0-4 position of galactosamine is changed to the sulfuric acid group at the 0-6 position even with a high degree of sulfation (Mukherjee et al., 2019). The branches hydrolysis of α-D-heteroglucans or β-D-heteroglucans also proved to the loss of immunostimulation due to the missing side chains (Huang & Nie, 2015; Zhao et al.,

2005).

3.2.4 Influence of functional groups

The type and quantity of functional groups in polysaccharides have a great influence on their charges, solubility, conformation, and biological activities. The substitution position and content of sulfate group, acetyl group and alkyl group on the sugar chain and the type of complex elements determine the bioactivity of polysaccharides. These functional groups can be added or eliminated by certain chemical methods, so the modification of functional groups in polysaccharides has become powerful means to study the structure-activity relationship of polysaccharides. However, for different polysaccharides, various functional groups have diverse effects on their biological activities (Simsek et al., 2021).

3.2.4.1 Sulfate group

Sulfation of polysaccharides has become an important direction of polysaccharide structural modification recently. There is clear evidence that the profound functional properties of the sulfated polysaccharides are probably due to the position of sulfation and glycosidic branching (Huang et al., 2008). There are kinds of methods to modify the polysaccharides, including sulfur trioxide–dimethylacetamide, chloro-sulfonic acid–pyridine (CSA–Pyr), sulfur trioxide–pyridine as well as sulfuric acid methods. Sulfate groups are usually attached to the polysaccharide at C-position 1,2,3,4 and 6 where hydroxyl groups present (Xie et al., 2020). Sulfated polysaccharides have shown

enrich antioxidant activities as compared to their native polysaccharides (Liu et al., 2018). Another sulfated polysaccharide from *Zizyphus jujuba cv.* also presents remarkable immunomodulatory and anticoagulant activities (Cai et al., 2018).

3.2.4.2 Acetyl group

Acetylation is one of the most commonly used chemical methods for polysaccharides modification. The number and position of acetyl group had significant effects on the activity of polysaccharide. Acetyl groups can change the orientation and transverse order of polysaccharide molecules, thus changing the physical properties of polysaccharide (Du et al., 2014). The position and degree of O-acetylation are genetically determined and varies of different polysaccharides. O-acetyl substituents can increase polysaccharide hydrophobicity and lead to conformational changes that influence interactions with other polymers, which is also crucial for food and other industrial applications (Pauly & Ramírez, 2018). The introduction of the acetyl group changed the molecular stretching, eventually leading to the exposure of polysaccharide hydroxyl group, increasing its water solubility, so it has an impact on its bioactivity. For instance, the antitumor activity of *P. obliquus* polysaccharide after acetylation is higher than that before modification because of the modifying group as well as the change of water solubility (Carlucci et al., 1997).

3.2.4.3 Alkyl group

Alkylation modification is the introduction of the alkyl group to the terminal end

of the main chain, and the effect of alkylation on the activity of polysaccharides has also been reported. It has been reported that the modified oligosaccharides had the ability to inhibit HIV-1 by molecular modification and the introduction of alkyl and sulfate (Sashiwa & Aiba, 2004). Sulfated alkyl oligosaccharide may inhibit HIV-1 from two aspects: on the one hand, its oligosaccharide part has high affinity with the membrane glycoprotein of HIV, which is consistent with other sulfated polysaccharides; on the other hand, its alkyl part is similar to the surfactant, which can double-layer function with the membrane lipid of virus, breaking its membrane (Han et al., 2010). Acylating agents mainly include halogenated alkane, higher fatty aldehyde, and long-chain fatty acid. Also, some polysaccharides need to be modified by a methyl or ethyl group before the gas chromatograph is analyzed (Zúñiga, Matsuhiro & Mejías, 2006).

3.2.4.4 Chain conformation

Up to now, there are few studies focused on the conformations of polysaccharides associated with their function properties, while establishing the relationship among the advanced structure with its biological function has arisen much attention. The specific spatial conformation of polysaccharide is essential for its pharmacological activity. Because polysaccharides have multiple biological activities in solution, they have a variety of conformational forms, including helix (Saito, Ohk & Sasaki, 1977; Guo et al., 2021) aggregates (Pieczywek et al., 2021), random coil (Douglas-Gallardo et al., 2019), rod-like structures (Jana, Gearheart & Murphy, 2001), and sphere like structures (Gu & Catchmark, 2012), among others. For instance, *Lentinan* with antitumor activity

has a triple helix structure, while their activity will be lost when the structure was disrupted by urea or DMSO. The specific rotation of the two polysaccharides in aqueous solution is different from that in urea or DMSO, which indicates that the stereo-structure of the polysaccharide changed, resulting in the loss of activity (Hamuro et al., 1971). The triple helix conformation of a β-D-glucan from *Ganoderma lucidum* was also closely associated with its immunostimulatory activity (Liu et al., 2018). Therefore, accurately identifying the stereoscopic configuration of polysaccharides is critical to gain insight into their functional and biological activities.

3.3 The relationship between physicochemical properties and biological activities of polysaccharides

3.3.1 Solubility

The solubility of polysaccharide also has a great influence on biological activity. Water soluble is the primary condition for its biological activity, where the increase of the water solubility could promote the transformation of polysaccharides into an ideal injection or other dosage forms, and then their bioactivities were enhanced. For example, the water solubility of the polysaccharides in *Pleurotus eryngii* was significantly improved after modification by sulfation, which increasing up to over 80%, with a rising degree of substitution, where its biological properties are also improved (Jung et al., 2011).

3.3.2 Molecular weight

Molecular weight is also crucial for polysaccharides to have biological activity, which may be related to the high-level configuration of polysaccharides. Polysaccharides have a large range of molecular weights (Greenwood, 1956), which of those with lower molecular weight could easily pass-through multiple cell membrane barriers and show better bioactivity. Moreover, the range of the best relative molecular mass for the biological activity of different polysaccharides is diverse. It has been reported that the antiviral effect of sulfated polysaccharide is also closely related to its molecular weight. The anti-HIV-1 activity of sulfated dextran increases with the increase of its molecular weight, which is the highest when Mw = 10,000 and the highest activity between 10,000 and 500,000. The ability of sulfated dextran against other enveloped viruses also has a similar trend (Witvrouw & De Clercq, 1997).

3.3.3 Viscosity

The viscosity of polysaccharides is mainly due to the hydrogen bond interaction between the molecules of polysaccharides, as well as affected by the molecular weight. It is not only positively related to its solubility to a certain extent, but crucial in clinical efficacy (Li & Feke, 2015). It is not conducive to the diffusion and absorption of polysaccharide medicines if the viscosity is too high. Anti-inflammatory activity of polysaccharide from *Schizophyllum* can be enhanced after degrading by ultrasonication to reduce its viscosity (Du, Yang, Bian & Xu, 2017).

3.4 Polysaccharides from medicinal fungus *Cordyceps sinensis*

3.4.1 Bioactive components of *C. sinensis*

A broad spectrum of natural polysaccharides (PS) from plants and fungi has broadly been used to promote health or for prevention and treatment of pharmacotherapy using for a long time. Many natural polysaccharides, of different structural types, isolated from different species have been shown to have various immunomodulating effects, such as immunoregulation, anti-cancer, anti-tumor and anti-oxidant activities.

The Chinese caterpillar fungus *Ophiocordyceps* or *Cordyceps sinensis* (Cs) is a unique and precious medicinal fungus in traditional Chinese medicine (TCM) with a number of health-promoting and therapeutic effects. The worldwide research on *Cordyceps* started from 1947, which professor Mains analyzed and verified the related components and pharmacological effects of *Cordyceps* for the first time. The nutrients provided by *Cordyceps* attracted their attention especially on improving physical performance and endurance (Zhu, Halpern, Jones & Medicine, 1998), and many research have focused on studying the growth condition of *Cordyceps* in both natural and artificial ways. Since then, lots of studies have also explored the bioactive components of *Cordyceps sinensis*, such as cordycepin, adenosine, polysaccharides, ergosterol, mannitol and amino acids (Chen, Wang, Nie & Marcone, 2013), as well as anti-tumor, anti-cancer and immunomodulatory effects (Chen et al., 2006; Wu et al.,

2014). Up to now, there are seven main chemical components of *Cordyceps sinensis*, including macromolecular proteins (peptides, amino acids and polyamines) and polysaccharides (carbohydrate derivatives), as well as small molecules of sterols, nucleoside compounds, organic acids, vitamins and inorganic substances (Zhu et al., 1998b). However, the biological mechanism of these compounds is still not clear.

Figure 3-1 Natural *Cordyceps sinensis*

Nucleosides such as cordycepin, cytosine and uracil are regarded as the major bioactive constituents of *C. sinensis* (Li, Li, Dong & Tsim, 2001). Several nucleosides and their derivatives have been isolated from *C. sinensis*, one of which is the endogenous purine nucleoside cordycepin (Zhu et al., 1998b; Ashraf et al., 2020). The content of nucleosides and their derivatives in *C. sinensis* produced by natural and artificial fermentation is different.

Cordyceps also contains proteins, peptides, and all essential amino acids. The literature shows that the protein content of fungal fruiting bodies can be as high as 30%, while the content of mycelium from fermentation is about 15% (Shashidhar et al., 2013). Amino acid profile analysis also showed that aspartic acid, arginine and glutamic acid were the highest in the larval body. *C. sinensis* contains 18 kinds of amino acids,

including the essential amino acids for human body, and some cyclic dipeptides with antitumor and antibacterial activities, such as cyclo-(Val-Pro) and cyclo-(Ala-Leu). **Table 3-1** shows the major bioactive components of *C. sinensis*.

Among the above bioactive components, polysaccharides from *C. sinensis* are one of the most crucial pharmacological active ingredients for its potential therapeutic effect. Cordycepin was originally considered as one of the main bioactive components of *C. sinensis*. Its chemical structure is D-Mannitol, which is also a sugar derivative (Chen & Chu, 1996). There are mainly two kinds of polysaccharide produced by *C. sinensis* fermentation, which namely intracellular polysaccharide (IPS) and extracellular polysaccharide (EPS). The commonly used extraction methods for IPS from mycelium include hot water, acid, and alkaline extraction. EPS is secreted from the fungal mycelium to the culture medium and can be obtained by ethanol precipitation. Iron-exchange chromatography and gel-filtration column chromatography are commonly employed for further purification. In general, although many different IPSs and EPSs from *C. sinensis* have been obtained, the monosaccharide composition is both mostly composed of mannose, glucose, and galactose with various ratios, and is often mixed with proteins (Yan et al., 2014). The molar ratio of PS monosaccharides of different strains was also diverse. Some of the PS purified from mycelium or culture medium of *C. sinensis* has been summarized and listed in **Table 3-2**. The current researches all showed that the molecular weight and connection mode of PS from the same strain might be different due to the different extraction processes.

Table 3-1 Major bioactive components of *Cordyceps sinensis.*

Bioactive components		Contents	Bioactivities	References
	Biomass	10-54 g/L	cardiovascular diseases, anti-tumours, anti-aging, anti-hyposexuality and hyperlipidaemia.	Ding et al. (2011); Marchbank, Ojobo, Playford & Playford, (2011). Yan et al. (2014).
	Polysaccharides	0.4-48.9 g/L	Immunomodulation, anti-tumour, anti-oxidation, hypoglycaemic effect.	Cheung et al. (2009). Sheng, Chen, Li & Zhang, (2011).
Nucleosides (μg/g)	Cordycepin	36.3–57.1	Inhibition of various tumour cells growth.	Fan, et al. (2006). Müller et al. (1977).
	Cytosine	9.2-12.1	Regulation and modulation of various physiological processes in the central nervous system.	Gong, et al. (2004).
	Uracil	16.7-66.2		Guo, et al. (2006).
	Cytidine	20.8-123.6		Li, et al. (2004).
	Hypoxanthine	103.9-204.6	Maintenance of the immune response.	Fan, et al. (2006).
	Guanine	55.2-192.4		Carver. (1994).

Table 3-2 Polysaccharides from *C. sinensis* fungi.

PS type	Extract methods	Monomer	MW	Major linkage	Reference
IPS	Hot water	Glu:Man:Gal= 2.8:2.9:1	13.6 kDa	(1→2) and (1→4)-linkage of mannose, (1→3)-linkage of galactose, (1→) and (1→3, 6)-linkage of glucose (1→4)- -d-glucosyl residue	Wang et al. (2009)
IPS	5% Alkaline extract	Man:Glc:Gal = 56.7:8.8:34.5	23 kDa	glucogalactomannan, with a backbone of (1→2)-linked-α-d-Manp,	Smiderle, Sassaki, Van Griensven & Iacomini. (2013)
IPS	Hot water extraction and gel-filtration column	Man:Glc:Gal = 1:28.63:1.41	1402 kDa	a backbone of (1 → 4)-linked α-d-glucopyranosyl residues	Liu et al. (2016)
IPS	Hot water extraction and gel-filtration column	Man:Glc:Gal = 1:12.41:0.74	1273 kDa	a backbone of (1 → 4)-linked α-d-glucopyranosyl residues	Liu et al. (2016)
IPS	Hot water	-Glc	1180 kDa	(1→4)-linked -D-Glcp backbone with (1→6)-D-Glcp side chains	Yan, Wang, Li & Wu (2011)
IPS	1.25 M NaOH and 0.04% NaBH4 aqueous solution	-Glc	1150 kDa	(1→4)-linked -D-Glcp backbone without branches	Yan et al. (2011)
EPS	95% ethanol, collected by filtration through 0.45 μm Whatman filter paper	Man:Glc:Gal = 65.12:6.12:28.72	36 kDa	1,6-branched-β-heteromannan	Lee et al. (2010)
EPS	Ethanol precipitation and fractionation by ion-exchange chromatography	Glc:Man:Gal = 1:0.6:0.75	210 kDa	--	Li et al. (2006)

3.4.2 Bioactivity of polysaccharides from *C. sinensis*

Polysaccharides are an important class of bioactive components of *Cordyceps sinensis*. A large number of *in vitro* and clinical studies have proved that health care and pharmacological activities of *C. sinensis* are attributed to its polysaccharides. The immunostimulatory and immunosuppressive properties of polysaccharides from *C. sinensis* (both IPS and EPS) have been evaluated in several immune system responses on cells, such as natural killer cells, T cells, and macrophages (Paterson, 2008; Zhou et al., 2009). Since the immune regulation has no side effect on the hemopoietic system and lymphocyte toxicity, natural *C. sinensis* and its fermentation product PS have been used in the treatment of immunocompromised and immunologic diseases. Recent reports showed that polysaccharides isolated from various natural or cultured *C. sinensis* have the same immunoregulatory activity, which can stimulate the release of several major cytokines in mouse macrophage line RAW264.7 and mouse spleen cells by activating IκB-NF-κB pathway (Akaki et al., 2009; Chen, Zhang, Shen & Wang, 2010; Wang & Peng et al., 2011).

Polysaccharides from *C. sinensis* also have been proved to have anti-tumour activity. Previous study in mice showed that IPS extracted from *C. sinensis* mycelium can inhibit colon cancer cell line HCT116 growth via mTOR signalling pathway (Qi et al., 2020). Some studies have shown that EPS obtained from *C. sinensis* culture medium also can significantly inhibit the growth of melanoma in mice lung and liver (Yang et al., 2005), as well as protective effects on rats with liver damage (Nguyen et al., 2021), which may become a potential adjuvant for cancer treatment. This information may be

helpful in the discovery of new immunostimulatory-related polysaccharides from *C. sinensis* with potential for therapeutical use or functional foods.

3.5 Inflammation

3.5.1 Introduction

Inflammation is the defensive response to harmful stimulus, manifested as redness, swelling, heat, pain and dysfunction of human body. In general, inflammation caused by the body automatic defense response is beneficial, but sometimes excessive inflammation is also harmful, such as failure to regulate the immune system, attacks on the body's own tissues, and so on. Inflammation can be divided into acute and chronic inflammation depending on its duration. Acute inflammation is mainly characterized by redness, swelling, and pain. It contains vasodilation, blood slow, blood plasma and blood ingredients such as neutrophils seeping into tissues. Some substances produced by host system can enhance the role of vascular permeability: a). Histamine, 5-hydroxytryptamine and other amines which can lead to immediate responses to inflammatory stimuli; b). polypeptides represented by bradykinin, kallidin, methionyl-lysyl-bradykinin. Its common characteristic is to make hemal permeability hyperlink, smooth muscle contracts, hemal dilate, promote leukocyte to swim. The structure of release lysyl has been identified; c). protease (plasmin), kallikrein, globulin-p and other proteases cannot become the acting material of vascular permeability by themselves. But it can make kininogen turn into kinin. However, the role of these substances on

blood vessels and the mechanism of action are mostly unknown. Histologically, the vascular exudation and repair process that occurs in acute inflammation can be seen mixed together. Macrophages, lymphocytes, plasma cells infiltration and fibroblast proliferation were also seen.

The main tissue changes of inflammation can be classified as follows: (1) metamorphic inflammation. (2) exudative inflammation (serous inflammation, cellulose inflammation, pyogenic inflammation, hemorrhagic inflammation, necrotizing inflammation, catarrh). (3) proliferative inflammation. (4) specific inflammation (tuberculosis, syphilis, leprosy, lymphoid granuloma, etc.)

3.5.2 Inflammasome

The natural immune response is the first response gate which the body needs to defend against the invasion of pathogenic organisms by ingesting phagocytes (such as macrophages, neutrophils). It kills invading pathogens and then activates the acquired immune system by secreting relative cytokines and chemokines. The rapid recognition of host natural immune cells to various pathogenic microorganisms and their products of intracellular infection depends on the presence of a class of polyprotein complexes within the cytoplasm, which is well known as inflammatory complexes (Mariathasan & Monack, 2007). Besides identifying various pathogenic bacteria, inflammatory complexes can also sense the metabolic stress stimulation of the host itself. By activating cysteinyl aspartate-specific protease, such as caspase-1, caspase-5, and other inflammatory complexes, the process and apoptosis of interleukin (IL)-1 temperament,

IL-18, IL-33 and other pro-inflammatory cytokines can be further induced to regulate immune response and inflammatory response. Recent studies suggest that the activation of inflammatory complexes has important regulatory effects on the acquired immune response of the body, which is related to the secretion of IL-6 and IL-12, as well as affects the activity of T helper cell-1 (Th-1), Th17 and regulatory T cells (Graham, Longhi & Heneghan, 2021). Its functional status is of great significance to the occurrence, development, and prognosis of various clinical diseases. More and more studies in recent years have shown that inflammatory complexes play a vital role in the natural immune response.

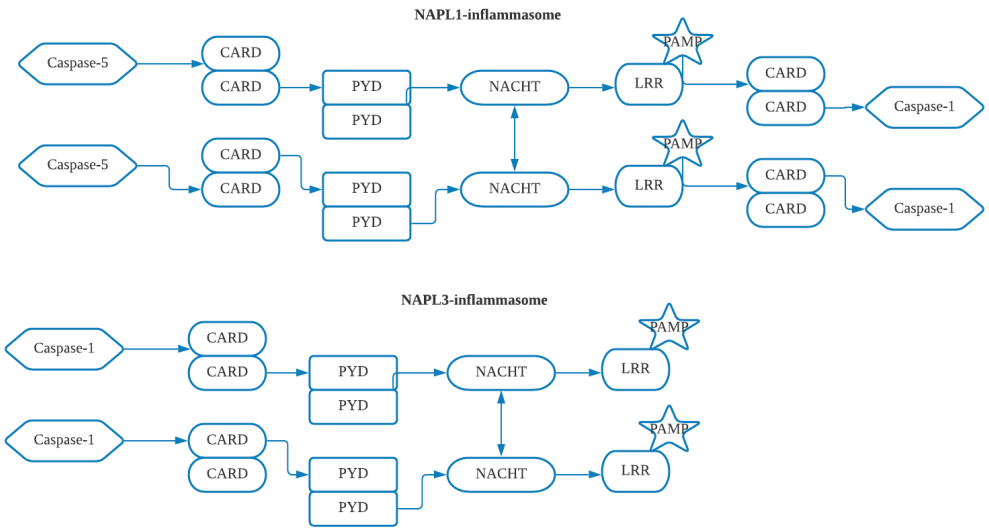

Figure 3-2 Stucture of the NALP1- and the NALP3-inflammasome

The inflammatory complex is a necessary response platform for cell caspase activation, and a number of different inflammatory complexes have been identified to assemble inflammation combined with a variety of complex of protein composition, including nucleotides oligomerization domain structure (NOD) like receptor (NOD like

receptors, NLRs) members of the family, including NALPs (NACHT, LRR and PYD containing proteins), interleukin invertase (interleukin - converting enzyme, ICE) activation factor (ICE protease-activating factor, IPAF), and neuron apoptosis inhibiting protein (neuronal apoptosis inhibitor protein, NAIP), which can feel the stimulation of the signal to start inflammation complex assembly, forms the core of inflammation of the complex composition (Tschopp, Martinon & Burns, 2003). The study of inflammatory complexes began with a genetic analysis of patients with "familial cold autoinflammatory syndrome". Study has found that the cold sensitivity trait had an autosomal genetic pattern, where the patient had a mutation in the same gene, named "cold induced self". The cold-induced auto-inflammatorys-1 (cias-1) gene, which encodes a protein called cryopyrin (an important member of the NALPs family, NALP3), subsequently discovered chronic infantile dermal joint syndrome. People also have the mutation of this gene (Feldmann et al., 2002). Subsequent studies have shown that the activation of this protein binds to the intracellular procaspase-1 protein through the interaction between proteins, which is very important for the processing and activation of pro-caspase-1 (Kanneganti et al., 2006; Martinon et al., 2006; Wang et al., 2021). Martinon et al. (2002) identified a multiprotein complex composed of apalp1 and apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1/5, which is very important for caspase activation and named "inflammasome".

3.5.3 Inflammatory mediators

The major cell types like neutrophils, monocytes/macrophages and mast cells can

response to acute inflammation through producing a series of chemical factors. The molecular factors mediating the inflammatory reaction are called chemical mediators or inflammatory mediators, which are involved in the physiological events associated with the inflammatory responses (Sugimoto et al., 2019). The mechanism of vasodilatation, permeability and leukocyte exudation in acute inflammatory response is an important topic. The released mediators in inflammatory conditions can initiate and amplify the inflammatory response by regulating the relevant signaling pathways. Some inflammatory factors can damage endothelial cell directly, causing vascular permeability increase, while many other inflammatory factors do not directly impact on local organizations, and mainly through the role of endogenous chemical factor and cause inflammation, which is also called chemical medium or inflammatory mediator (Medzhitov, 2010). Below are some major inflammatory mediators used in the inflammation study:

Table 3-3 Functions of various inflammatory mediators.

Functions	Inflammatory mediators
Hemangiectasis	Serotonin, histamine, bradykinin, PGE2, PGD2, PGF2, PGI2, NO
Increased permeability of the vessel	histamine, bradykinin, C3a, C5a, LTC4, LTD4, LTE4 PAF, active oxygen metabolites, substance P, platelet activators
Chemotaxis	C5a, LTB4, bacterial metabolites, neutrophil cationic proteins, cytokines
Fever	Cytokines (IL-1、IL-6、and TNF-α)、PG
Pain	PGE2, bradykinin, substance P
Tissue Damage	Oxygen free radicals, lysosomal enzymes, NO

3.5.4 Vasoactive amine

Vasoactive amine mainly includes histamine and serotonin (5-HT). Histamine is found mainly in the granules of mast cells and basophils, as well as in platelets. The stimuli that induce the release of histamine in mast cells include physical factors such as trauma or heat; the immunoreactions according to the interaction of the antigen with the IgE on the surface of the mast cells, allows the mast cells to release particles; footage of complement, such as anaphylatoxin; the neutrophil lysosome cationic protein; and some neuropeptides. In humans, histamine causes the arterioles to dilate and the venous endothelial cells to constrict, leading to increased vascular permeability. Histamine can be inactivated by histaminase. Histamine also acts as a chemotaxis to eosinophils. Serotonin can induce multiple effects in different tissues due to the presence of different receptors, which will activate distinct signaling pathways metabolism. Therefore,

serotonin was proved to contribute to the regulation of inflammatory responses.

3.5.5 Arachidonic acid metabolites

Arachidonic acid metabolites include prostaglandin (PG) and leukotriene (LT), which are metabolites of arachidonic acid (AA). AA is an unsaturated fatty acid, produced by activating phospholipase under the action of inflammatory stimulation and inflammatory mediators (Das, 2018). In inflammatory activity, the lysosomes of neutrophil are an important source of phospholipase. AA is metabolized through cyclic oxygenase and lipid oxygenase pathways to produce various products.

In brief, inflammation stimulates arachidonic acid metabolism and releases its metabolites, leading to inflammatory responses such as fever, pain, vasodilatation, increased permeability, and leukocyte exudation. On the other hand, anti-inflammatory agents such as aspirin, anti-inflammatory pain and steroid hormones can inhibit arachidonic acid metabolism and reduce inflammation.

3.5.6 Leukocytic metabolites

When activated by the inflammatory cytokines, neutrophils and monocytes can release oxygen free radicals and lysosomal enzymes, promoting the inflammatory response and destroying tissues, and becoming the inflammatory mediators.

For reactive oxygen metabolites, three aspects are involved. Superficial injury to vascular endothelial cells can lead to increased vascular permeability; inactivated

antiprotease (e.g., inactivated α-1 antitrypsin) can disrupt structural components such as elastic fibers by increasing the protease activity, and lesion of red blood cells or other parenchyma cells. For neutrophil lysosomal components, due to the death of neutrophil, exhalation and exocytosis during the formation of phagocytes, lysosomal components can be released to mediate acute inflammation. Among them, neutrophil protease, such as elastase, collagenase and cathepsin can mediate tissue damage.

3.5.7 Inflammatory cytokines

Inflammatory cytokines are endogenous polypeptides produced mainly by immune system cells with many strong biological effects, which can mediate a variety of immune responses. Inflammatory cells in the body can cause a variety of highly expression phenotype, which closely depend on binding to specific receptors to mediate communication between leukocytes. Although there are a variety of cytokines with different functions and effects, they are synthesized in response to external stimuli (Curfs, Meis & Hoogkamp-Korstanje, 1997). Many studies have focused on the serum inflammatory cytokines of patients with heart failure, mainly including tumor necrosis factor (TNF), interleukin-6 (IL-6) and IL-1 family cytokines. IL is short for interleukin, which function as an expression and regulation of immune responses, with the involvement of many factors such as lymphocytes or macrophages. Lymphocyte cytokines are derived from lymphocytes, and macrophages are collectively called monokine. The biological activity of each factor is different (such as macrophage activation, promoting T cell proliferation). Molecular biological studies have shown

endothelial dysfunction, decoupling of receptor and adenosine cyclase, embryonic gene expression activation, and cardiomyocyte apoptosis.

Among many inflammatory cytokines, TNF-α and interleukin play a major role in inflammatory activity. TNF-α is the earliest and most important inflammatory mediator in the process of inflammatory reaction, which can activate neutrophils and lymphocytes, increase vascular endothelial cell permeability, regulate metabolic activity of other tissues and promote the synthesis and release of other cytokines (Ruddle, 1992). Interleukin is a kind of cytokine produced by many cells and used in many cells. Originally it referred to the cytokines produced by white blood cells and regulated among white blood cells, now refers to a class of cytokines whose molecular structure and biological functions have been basically defined and uniformly named with important regulatory effects. The two coordinate and interact with each other to complete the function of hematopoiesis and immune regulation. Interleukins play an important role in transmitting information, activating, and regulating immune cells, mediating the activation, proliferation and differentiation of T and B cells, and in inflammatory responses (Smith et al, 1980). For instance, IL-6 can induce the differentiation and production of B cells and antibodies, and induce T cells to activate, proliferate and differentiate, and participate in the immune response of the body. It is the initiator of inflammatory response, and IL-8 can stimulate the chemotaxis of neutrophils, T lymphocytes and eosinophils, promote neutrophils degranulation, release elastase, and damage endothelial cells, causing microcirculation blood stasis, tissue necrosis, and organ function damage.

3.6 Gut microbiota, probiotics and postbiotics

3.6.1 Gut microbiota

Gut microbiota refers to the essential, complex community of microorganisms that live in the human gastrointestinal tracts (GIT). The human gastrointestinal tracts live more than 10 trillion bacteria that influence digestion, the risk of infections, autoimmune diseases, and control the body's response to medicines. The gut microbiome is made up of about 500-1000 known species of bacteria. Normally, the gut microbiome is dominated by anaerobic bacteria, which is 100 to 1,000 times more than aerobic and facultative anaerobic bacteria. The most abundant phyla in the human gut are *Firmicutes* and *Bacteriodetes*, then follows by *Proteobacteria*, *Verrumicrobia*, *Actinobacteria*, *Fusobacteria*, and *Cyanobacteria* (Gevers, et al., 2012; Qin et al., 2010). This vast microflora provides a wide variety of biochemical and metabolic activities to complement host physiology. The gut microbiome metabolism is comparable to other organs such as livers (Gill et al., 2006). These bacteria are essential for host health.

3.6.2 Probiotics

The term probiotic is defined by FAO/WHO (2002) as “live, non-pathogenic microorganisms in the gastrointestinal tract which attribute to the host health as well as improving microbial balance”. Probiotics have been used to treat a variety of clinical

conditions such as pouchitis, diarrheal diseases and irritable bowel syndrome (Williams, 2010; Hojsak et al., 2018). Some of their beneficial effects are well documented, while others still have limited evidence. Not only is probiotic species-specific, but due to the complexity of microbial population control, the use of one probiotic may be more beneficial than the use of another. In addition, microbial combinations can make it challenging to quantify specific clinical benefits (Górska, et al., 2019; Wang et al., 2020). Probiotics also contribute to the roles in boosting immune responses, reducing the risk of colon and bladder cancer, allergic and atopic diseases, and treating upper respiratory tract infections (Goldin & Gorbach, 2008; Reid et al., 2003). *In vivo* studies have found that some probiotic strains can enhance immunoregulation by stimulating the phagocytic activity of lymphocytes and macrophages. Probiotics also increase immune globulin A (IgA), which stimulates the production of cytokines by monocytes (Williams, 2010). Secondly, the production of various metabolic substances by probiotic bacteria can also suppress the growth of pathogenic, thereby reestablishing the balance of the gut microbiota (Sánchez et al., 2017). Although many of these treatments are well proven, probiotics used to treat diseases are still regarding lacking efficacy data.

3.6.2.1 *Bifidobacterium*

Bifidobacterium are gram-positive, anaerobic, catalase negative, non-pathogenic bacteria with high G+C content (Ventura et al., 2004), which are considered probiotic and are associated with various health benefits to the host, such as pathogen protection,

including production of acetate to protect against enteropathogenic infection (Fukuda et al., 2011), competing for epithelial binding sites with pathogens (Vazquez-Gutierrez et al., 2016), immune modulation, and reducing the risk of contracting rotaviral diarrhea (Moreno Muñoz et al., 2011).

Bifidobacterium was first isolated from the feces of breast-fed infants in 1899 (Tissier, 1899). Subsequently, the Bergey's Manual (2004) reclassified them as a separate taxon and designated the genus *Bifidobacterium*, consisting of 11 species. To date, there are 31 proposed species that have been isolated (Lee & O'Sullivan, 2010). The constitution of *Bifidobacterium* in adults is around 3-7%, and up to 91% in newborn intestinal microbiota, respectively (Salminen & Von Wright, 2004), which mainly colonized in large intestinal with a level up to 10^8-10^{12} CFU/g.

3.6.3 Postbiotics

Postbiotics are soluble substances released by or produced through the bacterial metabolism of the microorganisms which directly offer physiological effects to the host, these are bioactive molecules or fractions of different components derived from the microbiome (Konstantinov, Kuipers & Peppelenbosch, 2013; Cuevas-González, F., Liceaga & Aguilar-Toalá, 2020). Postbiotics are mainly produced during the fermentation or fragments of intestinal bacteria, where short-chain fatty acids (SCFA), enzymes, peptides, teichoic acid, endo- and exopolysaccharides, vitamins, plasma, and organic acids are the main products of interest and have been studied *in vivo* to have a great influence on host physiology including immunomodulation, anti-inflammatory,

anti-obesogenic, anti-hypertensive, anti-proliferative, and antioxidant effects (Nakamura et al., 2017)

At present, cell-free products obtained from the metabolites of probiotics have been applied to potential pharmaceutical applications (Klein et al., 2013), activities in *in vitro* and *in vivo* models exposed to specific probiotic digested exopolysaccharides (EPS) are also reported. Studies found that fractions and extracts from *Bifidobacterium* and *Lactobacillus spp*. contained high levels of microbial carbohydrates, which showed profound anti-tumor activities *in vitro* (Raman, Ambalam & Doble, 2016). Zhang et al. (2018) found that *Lactobacillus plantarum* NCU116 fermentation on *Asparagus officinalis* polysaccharide can enhance its immunoregulatory activities. Lee et al. (2002) found that cell free supernatants from *B.bifidium* BGN4 have immunomodulation activity, and Amaretti et al. (2013) also found intracellular contents from seven *Bifidobacterium* strains have antioxidant activity as postbiotic potential. *Bifidobacterium breve* fermented milk also proved efficacy in maintaining the remission of ulcerative colitis (Matsuoka et al., 2018). Additionally, several postbiotics such as cell wall components, intracellular bacterial enzymes (ex: glutathione peroxidase GPx, superoxide dismutase SOD, nicotinamide adenine dinucleotide (NADH)-oxidase and NADH-peroxidase) are also associated with *in vitro* immunomodulatory properties as well as other beneficial effects (Konstantinov et al., 2013).

Postbiotics have demonstrated inducing anti-inflammatory, immunomodulatory, hypocholesterolemic, anti-proliferative, and antioxidant activities, which may

contribute to the improvement of host health by providing specific physiological effects. However, the exact mechanisms still need full elucidation.

3.6.4 Carbohydrate utilization of *Bifidobacterium*

3.6.4.1 Single sugar metabolism

Bifidobacteria can utilize a wide variety of hexose substrates, such as glucose, fructose, galactose, N-acetylglucosamine, N-acetylgalactosamine, arabinose, xylose, ribose, sucrose, lactose, cellobiose, mannose and so on. Parche et al. (2007) demonstrated the variety of carbohydrate substrates utilization system by B*ifidobacterium longum subsp. Longum* NCC2705. Furthermore, a recent study by Pokusaeva et al. (2010) revealed the ribose utilization by *B. breve* UCC2003 because of the presence of a functional complete ribose transfer and utilization gene cluster.

There are four major types of carbohydrate transportation systems in the bifidobacterial cell, including the ATP-bind cassette (ABC) transport system, major facilitator superfamily system (MFS), glucose-specific phosphotransferase transport systemV (PTS) and the glycoside-pentoside hexuronide (GPH) cation symporter family (Lee & O'Sullivan, 2010) (**Fig. 3-4**). Among them, ABC transporters exist in all living organisms as the most efficient one for up-taking sugars, while the PEP-PTS only detected in bacteria for now. A wide spectrum of sugar transport systems provides bifidobacteria with flexibility in utilizing different types of sugars, with a selective advantage over other bacteria in the intestinal ecosystem.

Bifidobacterium is one of the few bacterial genera that contain the fructose-6-

phosphoketase (F6PPK), which is the key enzyme of hexose metabolic pathway in bifidobacterium, also referred to as the "bifid shunt". Hexoses such as glucose, galactose or fructose are metabolized in this way to acetate and lactate for energy production (Bezkorovainy, 1989; De Vries & Stouthamer, 1967). Since F6PPK enzyme is specific for bifidobacteria, it is commonly used for identification. The completely sequenced bifidobacterial genomes revealed all the required genes for the F6PPK-mediated bifid shunt, as well as some genes for the trichloroacetic acid (TCA) cycle.

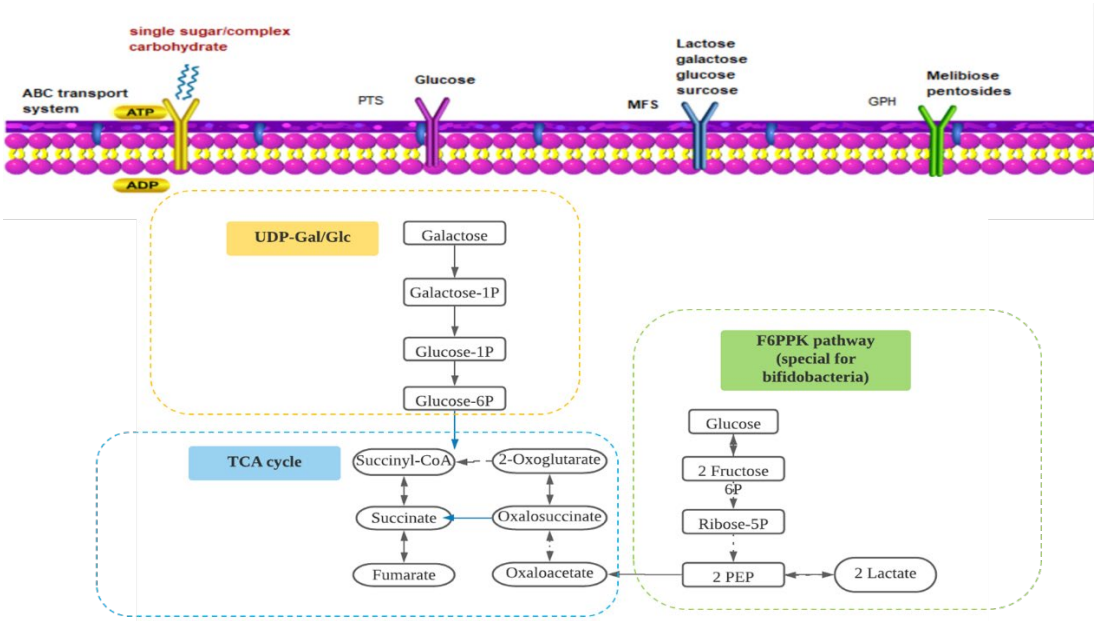

Figure 3-3 Carbohydrate metabolism system of *Bifidobacterium*

3.6.4.2 Complex carbohydrate metabolism

Most of the easily digestible single sugars are metabolized in the upper gut, while the indigestible complex carbohydrates such as plant-derived dietary fibers, host-derived glycans, oligosaccharides, resistant starch, cellulose, pectin, and gums, and are utilized by intestinal bacteria in the lower gut (Hooper, Midtvedt, Gordon, 2002). Among the gut microbiota, numerous gene clusters of *Bifidobacterium* predicted to be

involved in the complex carbohydrate metabolism to efficiently utilize the complex carbohydrate substrates, as it sequenced genomes contain genes predicted to encode many complex carbohydrate utilization enzymes, which known as carbohydrate-active enzymes (CAZy). Although different bifidobacterial strains may possess different carbohydrate utilizing abilities, there are still some commonly encrypted enzymes within this species. **Table 3-4** listed some unique glycoside hydrolase (GH) families of two standard *Bifidobacterium* strains for the complex carbohydrate utilization, which is also commonly represented in *bifidobacterium* species. For increasing interest in the complex carbohydrate utilization on *bifidobacterium*, for the discovery of new prebiotics functions and for understanding why certain oligo- and polysaccharides can be metabolized and modified, a better understanding of the carbohydrate-hydrolyzing enzymes encoded by *Bifidobacterium* species is needed.

Table 3-4 Glycoside Hydrolase Families of *Bifidobacterium longum* JCM 1217 and *Bifidobacterium breve* ATCC15700 may be responsible for hydrolysis of complex carbohydrate.

	Bifidobacterium longum JCM 1217	Reaction
Glycoside Hydrolase Family 13/ Carbohydrate-Binding Module Family	◆ α-glucosidase (EC 3.2.1.20)	Hydrolysis of terminal, non-reducing (1→4)-linked α-D-glucose residues with release of D-glucose
	◆ glucan 1,6-α-glucosidase/exo-1,6-β-glucosidase (EC 3.2.1.70)	Hydrolysis of (1→6)-D-glucosidic linkages in (1→6)-D-glucans and derived oligosaccharides
	◆ 1,4-α-glucan branching enzyme (EC 2.4.1.18)	Transfers a segment of a (1→4)-α-D-glucan chain to a primary hydroxy group in a similar glucan chain
	◆ oligosaccharide 4-α-D-glucosyltransferase (EC2.4.1.161)	Transfers the non-reducing terminal α-D-glucose residue from a (1→4)-α-D-glucan to the 4-position of a free glucose
Glycoside Hydrolase Family 43	◆ galactan 1,3-β-galactosidase (EC3.2.1.145)	Hydrolysis of terminal, non-reducing β-D-galactose
	◆ β-galactofuranosidase (EC3.2.1.146)	Hydrolysis of terminal non-reducing β-D-galactofuranosides, releasing galactose
	Bifidobacterium breve ATCC15700	
Glycoside Hydrolase Family 38	◆ α-mannosidase (EC 3.2.1.24)	Hydrolysis of terminal, non-reducing α-D-mannose residues in α-D-mannosides
	◆ exo-1,3-1,6-α-mannosidase (EC 3.2.1.114)	Removes two mannosyl residues, one linked by α-1,3 linkage, and the other linked by α1,6 linkage
	◆ α-amylase (EC 3.2.1.1)	Endo-hydrolysis of (1→4)-α-D-glucosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units
Glycoside Hydrolase Family 43	◆ β-galactosidase (EC3.2.1.23)	Hydrolysis of terminal, non-reducing β-D-galactose
	◆ α-galactosidase (EC3.2.1.22)	Hydrolysis of terminal, non-reducing α-D-galactose residues in α-D-galactosides

● From CAZy (Carbohydrate Active Enzymes) Database

Chapter 4 General Materials and Methods

This chapter introduces the whole process of the project as well as some commonly used materials and experimental methods. Some conditions of the experimental method may vary depending on the materials used. See relevant chapters for details.

4.1 Outline of the project

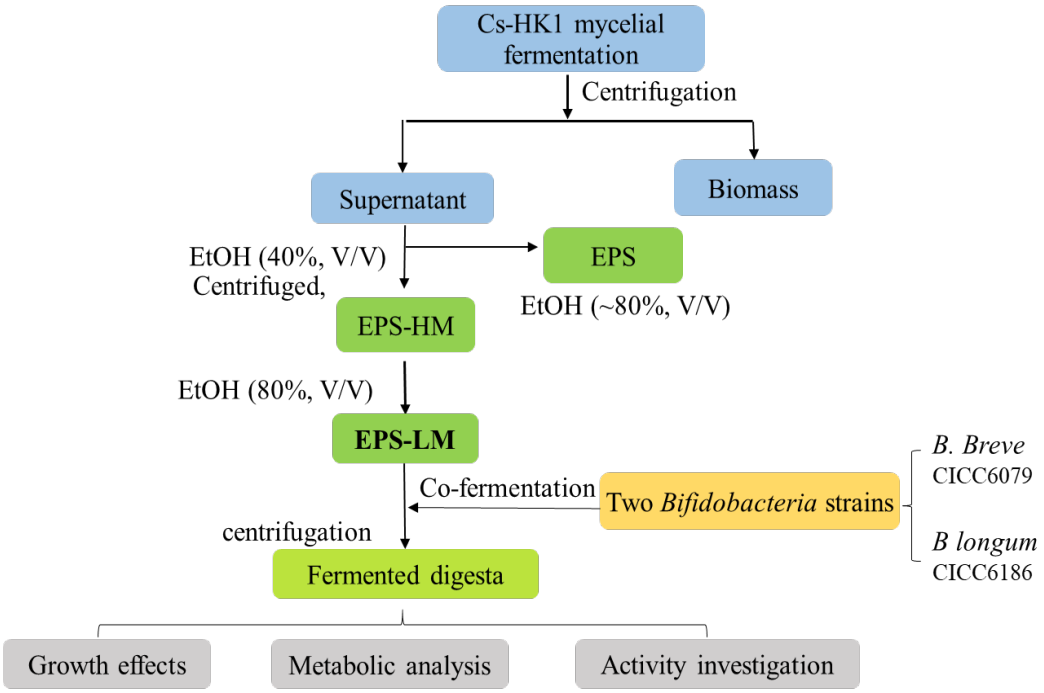

Figure 4-1 Outline of the project

EPS fractions with different MWs and compositions were used in this project, which were all from *C. sinensis* fungus Cs-HK1 mycelial fermentation. The anti-inflammatory activities of Cs-HK1 EPS were evaluated in cell (*in vitro* activity) and animal models (*in vivo* activity). CEPS was a crude EPS isolated from the Cs-HK1 mycelial fermentation medium by a single step of ethanol precipitation at 5:1 volume ratio of ethanol to fermentation medium (~80% v/v). EPS was attained by

deproteinization of the CEPS. Both CEPS and EPS treatment in the cell model significantly inhibited the release of pro-inflammatory cytokines, and EPS had even higher activity than CEPS. For the isolation of EPS with different MWs, two-step ethanol precipitation was applied to the liquid medium of Cs-HK1 mycelial fermentation, yielding a higher-MW EPS fraction EPS-HM in the first step with 40% ethanol and a lower-MW EPS EPS-LM with 80% ethanol in the second step. Since the low molecular weight fraction EPS-LM was the most active fragment among all the EPS fractions, the metabolism potential of EPS-LM fraction by *bifidobacterium* was further studied for its prebiotic and postbiotic functions by evaluating the metabolites and potential anti-inflammatory effects of bifidobacterial digesta through *in vitro* experiments. Moreover, EPS-LM was further purified through different columns to a homogenous EPS-LM-1 and structure-characterized.

4.2 Preparation of different EPS fractions

Cs-HK1 is a fungus species isolated from the fruiting of Chinese caterpillar fungus *Cordyceps sinensis* fruit body by Wu's group (Leung, Zhao, Ho & Wu, 2009). The Cs-HK1 mycelial culture was maintained in a liquid medium consisting 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 Cs-HK1 mycelial fermentation and EPS preparation were carried out as reported previously (Leung, et al., 2009; Mao, et al., 2018). In the present study, the Cs-HK1 mycelial fermentation was carried out in 1 L conical flasks each filled with 200 mL of

liquid medium and placed on a shaking incubator at 200 rpm and 20°C for 7 days. The fermentation liquid was then centrifuged (12,000 rpm and 20 min) to attain a solid-free medium. Ethanol at a volume ratio of 5:1 (v/v) was added slowly to the fermentation medium with moderate agitation and then maintained at 4°C overnight and the precipitate was recovered by centrifugation (12,000 rpm and 30 min) and lyophilized, yielding the crude EPS fraction, CEPS. The CEPS was deproteinized with Sevag reagent (chloroform/1-butanol, v/v = 4:1) for several times till free of precipitates, and the aqueous solution was then collected and dialyzed against distilled water through a 3500 MW cut off membrane for 48 h and finally lyophilized to give the purified EPS.

4.3 Cell lines and cell culture conditions

The cell line used in this experiment was THP-1-Dual cell line, obtained from Invivogen (InvivoGen, San Diego, CA, USA). It was derived from the human THP-1 monocyte cell line by stable integration of two inducible reporter constructs (NF-kB-SEAP and IRF-Lucia reporter monocytes). THP-1 cell line is a human monocytic cell line which could be induced by LPS as a prototypical stimulator for cytokine production (Auwerx, 1991). Briefly, the THP-1 cell culture was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), and penicillin (100 U/mL) and streptomycin (100 μg/mL) with extra 25 mM HEPES (Sigma-Aldrich, Shanghai, China) and 100 μg/mL Nornocin (InvivoGen). The culture was incubated at 37°C in a humidified incubator with 5% CO_2 and kept in logarithmic growth at 5-15 × 10^5

cells/mL through routine sub-culturing, according to standard ECACC protocol. During this study, the culture was sub-cultured every 3 days by inoculating 7 x 10^5 cells/mL and the passage number was kept below 20. The newly recovered stock culture was sub-cultured for at least one passage, and then further sub-cultured in the growth medium to which 100 μg/mL of Zeocin™ and 10 μg/mL of blasticidin (InvivoGen, USA) were added every other passage to maintain the selection pressure.

The most commonly used cell line for immuno- and inflammation assays, macrophage RAW264.7 was also used in some of the experiments to confirm the anti-inflammatory activity of EPS. The RAW264.7 cells were maintained in DMEM (glutamine, high glucose) supplemented with 10% FBS, and penicillin (100 U/mL) and streptomycin (100 μg/mL) and were incubated at 37°C under a humidified atmosphere with 5% CO_2. The maximum passage number was 40 in the experiments.

4.4 Analysis of EPS composition and molecular weight

The total carbohydrate content of EPS samples was determined by Anthrone test, involving the acid hydrolysis of samples at 100°C in the presence of anthrone agents (2 g anthrone dissolved in 80% H_2SO_4). The absorbance of samples solution was measured at 620 nm with a spectrophotometer using glucose as a reference. The protein content was determined by Lowry method and the absorbance of was measured at 750 nm using bovine serum albumin (BSA) as a reference.

The monosaccharide composition of EPS was analyzed by the PMP-HPLC

method as reported previously with minor modifications. In brief, the EPS sample was hydrolyzed with 2 M TFA at 110°C for 4 h, followed by evaporation under vacuum to dryness. The residual solid was re-dissolved in DI water and mixed with 0.5 M PMP solution in methanol and 0.3 M NaOH solution at an equal volume and maintained at 70°C for 30 min. The reaction was stopped by adding 0.3 M HCl, followed by washing thrice with chloroform, and the aqueous layer was collected for the HPLC analysis. The analysis was performed with an Agilent Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm) on an Agilent 1100 instrument at 25°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 MW of EPS samples was determined by HPGPC as reported previously . The HPGPC system consisted of 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°C. Water was used as the mobile phase. The EPS samples were dissolved in Milli-Q water, centrifuged at 6000 rpm for 15 min and filtered through 0.45 μM membrane before injection. Calibration curve was derived with dextran MW standards of 1.0-670 kDa and computed with the Breeze V3.3 software.

Fourier transform infrared (FT-IR) spectroscopy of CEPS and EPS was performed with a Nexus 670 FT-IR spectrometer (Thermo NicoletCo., Cambridge, UK) at the wavenumber range of 500-4000 cm^{-1} with KBr pellets and referenced against air in the Spectrum 6.1 software.

4.5 Bacterial species, culture conditions and inoculum preparation

Two species of *Bifidobacterium* were involved in this project including *B. breve* (CICC 6079) and *B. longum* (CICC 6186). The two bifidobacteria were obtained from China Centre of Industrial Culture Collection (CICC). The bacterial species were stored in 30% glycerol at -80℃. The bacterial culture was initiated by inoculation of each species from the storage in Reinforce Clostridial Medium (RCM) agar for bifidobacterium in a petri dish and incubation for 2 days. A single colony on the agar plate was picked out and inoculated into 5 mL of RCM liquid broth in a 10 mL centrifuge tube to prepare the starter culture, followed by shaking incubation for 18 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. Bifidobacteria were both incubated at 37℃ 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. RCM agar was prepared by adding agar (A 4675, Sigma-Aldrich, USA) in the broth to get final concentration of 1.5% (w/v).

4.6 Preparation of medium with EPS-LM mixed carbon source

For the experiments on ELM-mixed carbon sources for the bifidobacterial growth, ELM sample added at 1 g/L (~0.1% w/v) to a starch-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). Starch-free RCM broth was used as control. All the mediums were sterilized by autoclaving at 121℃ for 20 min.

Chapter 5 Anti-inflammation Activity of EPS Produced by *C. sinensis* Cs-HK1[1]

5.1 Introduction

Inflammation is a defensive reaction of the human body against the harmful stimuli, which is tightly regulated by the inflammatory response system (Majno, 2004, Kumar, 2017). However, excessive inflammatory responses by the regulation system can lead to a wide range of chronic inflammatory diseases, such as rheumatoid arthritis, chronic hepatitis, and inflammatory bowel disease (Medzhitov, 2008). Moreover, inflammatory disorders may be linked with other life-threatening diseases such as cancer, and the effective control of the inflammatory reactions is crucial for prevention and treatment of many diseases including cancer. The overexpression of inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α, is implicated in the pathogenesis of these inflammatory diseases (Mantovani, 2008). Therefore, the modulation of cytokine activities is an important strategy for the treatment of chronic inflammatory diseases.

Conventionally, antibiotics are applied for treatment of infections and steroids for control of inflammation (Sweeney, 2016). Increasing concerns with their serious side effects and adverse reactions have motivated the considerable effort in recent decades toward the discovery of safer and more effective anti-inflammation drugs (Barochia,

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Solomon, Cui & Natanson, 2011). A favourable and effective strategy in the development of new therapeutics is based on molecular targeting on the mediators that can disrupt specific cellular signalling pathways involved in the diseases (Castellarin, Zorzet, Bergamo & Sava, 2016). Polysaccharides from edible and medicinal fungi are recognized as the biological response modifiers to produce immunomodulatory and anti-inflammatory activities (Tsim, 2004), which are promising candidates for new anti-inflammation agents. Another attractive aspect of the fungal polysaccharides is the selective inhibition of pro-inflammatory molecules without significant cytotoxicity (Schepetkin & Quinn, 2011). Polysaccharides as complex biomacromolecules have various characteristics such as monosaccharide composition, glycosidic linkage, degree of branching, molecular weight, bonding of proteins or peptides, which can affect the properties and bioactivities (Song et al., 2013).

Cordyceps sinensis, generally known as the Chinese caterpillar fungus (or DongchongXiacao in Chinese), is a unique and esteemed medicinal fungus in traditional Chinese medicine and has also been used as a health food or tonic to promote health and alleviate a variety of diseases (Zhang et al., 2011; Nie, Cui, Xie & Phillips, 2013). Since the natural Chinese caterpillar fungus is very rare and expensive, mycelial fermentation has widely applied for the production of the fungal materials. Cs-HK1 is a fungus species isolated from the fruiting body of a natural *C. sinensis* and the Cs-HK1 mycelial culture has been found to produce significant amount of exopolysaccharide (EPS) in liquid fermentation (Chen, Wu & Gui, 2016). The EPS had a complex composition and a wide molecular weight (MW) distribution, some of the EPS

components showed significant antioxidant and moderate immunomodulatory activities (Yan, Wang & Wu, 2014). However, the anti-inflammation potential of EPS from the Cs-HK1 mycelial fermentation remains unknown.

This study was to evaluate the potential anti-inflammatory function of Cs-HK1 EPS in both cell culture and animal models and to examine the activity relationship to the EPS composition. EPS was isolated from the Cs-HK1 liquid fermentation broth by ethanol precipitation and purified by deproteinization and dialysis. The EPS was first tested on human THP-1 monocyte cell line as well as RAW264.7 cell as the in vitro model and then on lipopolysaccharide (LPS)-induced acute intestinal injury in mice as an in vivo model. The major inflammation cytokines and NO were detected to reveal the anti-inflammatory effects and the potential signal pathway.

5.2 Materials and methods

5.2.1 Chemical and biochemical agents

Cs-HK1 mycelial fermentation, isolation, and preparation of EPS fractions using the methods as described in 4.2.2.

Analysis of EPS composition and molecular weight were performed using the same methods as described in 4.2.4.

Cell lines used in this chapter and culture conditions were performed using the same methods as described in 4.2.3.

5.2.2 Cell culture experiments

5.2.2.1 Cell viability assay

For viability assessment, cells were first incubated in 96-well flat-bottom tissue culture plates (10^5 cells/well) for 24 h to give a stable growth, and then stimulated with different CEPS/EPS concentrations for 48 h. After 48 h of incubation, cell viability was evaluated by the MTT (methyl thiazolyl tetrazolium, Sigma) assay according to previously study (Mosman,1983; Inui, et al., 2002). The succinate dehydrogenase in mitochondria of living cells reduced exogenous MTT to dirty blue and purple crystals insoluble in water and deposited in cells, but dead cells did not have this function. After 4 h incubated with MTT, Dimethyl sulfoxide (DMSO) was added to dissolve MTT, and the supernatant was shaken well in a plate shaker. Then the optical density (OD) value was determined at 490 nm by a microplate reader. All the experiments and measurements were done in triplicate and arithmetic averages were taken throughout the data analysis and calculations.

5.2.2.2 NF-κB assay

NF-κB-induced SEAP activity of THP-1 cells was assessed using Quanti-Blue agent (InvivoGen). The cells were first fully recovered and remaining stable growth, then were incubated in 96-well flat-bottom tissue culture plates (2×10^5 cells/well) for 48 h and then subjected to different treatments, LPS (Sigma-Aldrich, Shanghai, China) at 1 μg/mL, 1 μg/mL LPS + CEPS or EPS at 50 ~ 500 μg/mL. A control group of cells

was included which was not subjected to any treatment. 48 h later, 20 μL culture supernatant was collected from each well and the alkaline phosphatase activity was assayed by adding Quanti-Blue reagent at 1:4 (v/v). The activation of NF-kB was determined by measurement of absorbance at 625 nm relative to that of LPS-stimulated samples.

5.2.2.3 Nitric oxide (NO) assay

The cells were cultured and subjected to various treatments in the same ways as in section 2.3.3. After 48 h incubation, 100 μL of the culture supernatant was collected from each well and the concentration of nitrite was determined by the Griess assay (Kleinbongard, 2002). All the samples were tested in triplicate by a nitrite detection kit according to the manufacturer's instructions (Beyotime, Shanghai, China).

5.2.2.4 Analysis of cytokines by ELISA

The cells were inoculated in 24-well plates (5×10^5 cells/well) and pre-incubated for 24 h. At the end of the pre-incubation period, the wells were rinsed with phosphate buffer saline (PBS), and the medium was exchanged to RPMI1640 without FBS. The cells were then stimulated subjected to LPS and CEPS/EPS treatments as in section 2.3.2 for 48 h to induce inflammatory cytokines (IL-1β and TNF-α). The conditioned or treated culture medium was collected and centrifuged, and the concentration of

cytokines in the supernatant was measured by DuoSet enzyme-linked immune sorbent assay (ELISA) kits (R&D Systems) (Sigma-Aldrich, Shanghai, China) according to the manufacturer's instructions.

5.2.3 Animal test with the LPS-induced acute intestinal injury mouse model

The LPS-stimulated mouse model is a well-established animal model for the study of inflammation and *in vivo* assessment of anti-inflammation agents (Hozumi, 2006). The LPS-induced pathological process is marked by a range of inflammatory responses such as acute intestinal injury and secretion of inflammatory cytokines by macrophages (Benoit, Desnues & Mege, 2008; Bozza et al., 2007). In this study, male C57BL/6 mice weighing 20-25 g were used. The animals were housed in temperature-controlled rooms and received food and watered libitum. All experiments were performed in accordance with the China National Accreditation Service for Conformity Assessment (CNAS L3623), which were approved according to the Ethics Committee of Guangdong Medical Experimental Animal Centre. The mice were randomly assigned to five treatment groups (n = 10 per group) as shown in **Table 5-1**.

Table 5-1 Animal test groups and treatments (DW: distilled water; Dexameth: Dexamethasone)

Negative control	LPS	Positive control	EPS-L: EPS low dose	EPS-H: EPS high dose
DW oral for 7 days	LPS 25 mg/kg injection on day 7	DW oral for 7 days; Dexameth 100 mg/kg injection on day 7 following LPS injection	EPS 150 mg/kg oral for 7 days. LPS 25 mg/kg injection on day 7	EPS 300 mg/kg oral for 7 days. LPS 25 mg/kg injection on day 7

The EPS samples were dissolved in distilled water with low or high dose (EPS-L or EPS-H) and then was administered to the mice via oral gavage for 7 consecutive days before the LPS stimulation. On the last day LPS (an *Escherichia coli* endotoxin O55:B5, Sigma Aldrich Inc., St. Louis, MO, USA) was injected (at 25 mg/kg) to the mice one hour after the oral administration of EPS. Dexamethasone (Dexameth) was used as a positive control drug and 100 mg of the drug was pre-dissolved in 1 mL DMSO, and the solution was diluted to 0.1% (v/v) with normal saline and the diluted solution was intraperitoneally injected to each mouse. The drug was administered to the mice by oral gavage at 0.1 mg/mL immediately after the LPS injection. At 4 h after LPS injection, the jejunum tissue and peripheral blood of each mouse was collected for histography and cytokine analysis. The major inflammatory factors were measured including the pro-inflammatory cytokines IL-1β, TNF-α and the anti-inflammatory cytokine IL-10 in jejunum tissues and the level of iNOS in serum using commercially available ELISA or NO synthase assay kits (Beyotime, Shanghai, China).

For the intestinal histopathology, animals were taken from each group, and their ileum tissues were rinsed with PBS buffer and fixed in 4% paraformaldehyde solution for 24 h, the tissues were embedded in paraffin and cut into slides, then the sections

were dehydrated and stained with hematoxylin and eosin (H&E) for histopathological examination. The histological structures of ileum of experimental animals in each group were observed under the photographed light microscope (Olympus IX73, Japan) to determine the pathological changes such as edema, shedding of intestinal villi and inflammatory cell infiltration.

5.2.4 Statistical analysis of experiments

All data are presented as the mean ± standard deviation (SD) from at least three independent experiments. The statistical analysis was performed using the SPSS 16.0 statistical software package. Independent-samples T test was performed for comparison the differences among the different treatment groups, * at $p < 0.05$ and ** at $p < 0.01$. The IC50 values were calculated according to Beck et al (2017) using the GraphPad Prism 6.02 software package (GraphPad Software Inc., USA).

5.3 Results and discussion

5.3.1 Composition and properties of EPS

Table 5-2 shows the major chemical contents of EPS and its partially hydrolysed products. The crude EPS (CEPS) had a total sugar content of 45.6% and total protein content of 17.3%. After deproteinization and dialysis of CEPS, the resultant EPS fraction had a much higher total sugar content of 74.8% and much lower protein content of 0.9%. EPS was composed mainly of glucose and mannose, and low contents of ribose

and galactose. On GPC spectrum, CEPS exhibited three major peaks (**Fig. 5-1a**), two high MW peaks in the order of 10^8 and 10^5, and a very low MW peak of 213, while the purified EPS exhibited two major peaks on the GPC (**Fig. 5-1b**), a high MW in the order of 10^7 and a very low MW peak of 213 Da. The low MW may be attributed monosaccharide residues. Wang et al. (2017) previously reported that the polysaccharide fractions extracted from several natural and cultured *C. sinensis* species had a maximum MW of 950 kDa and about 80% of glucose in the monosaccharide composition. In comparison, the EPS from the Cs-HK1 mycelial fermentation has a much higher MW and lower content of glucose but higher contents of mannose and galactose.

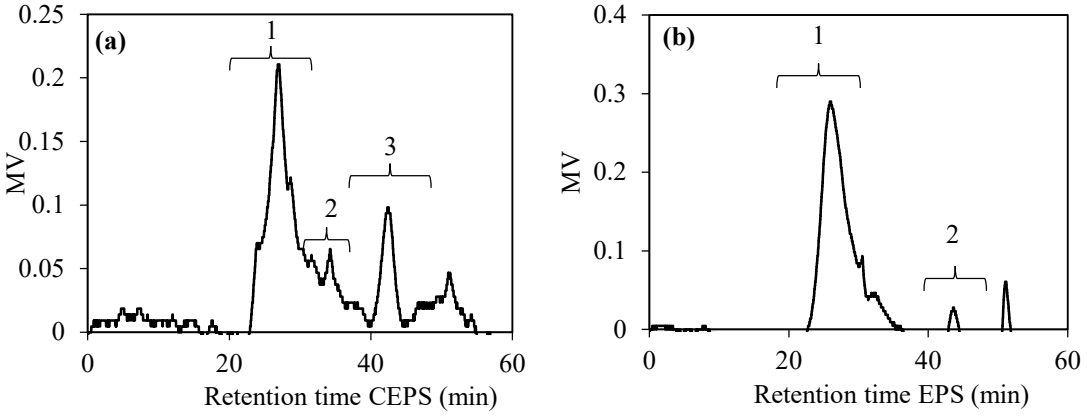


Figure 5-1 GPC profiles of (a) CEPS and (b) EPS.

Table 5-2 Molecular properties and chemical composition of Cs-HK1 EPS.

	MW (Da)	Area (%)	Total content (wt.%)		Monosaccharide molar ratio			
			Sugar	Protein	Man	Rib	Glc	Gal
CEPS	2.768×108	68.67	45.6 ± 3.5	17.3 ± 0.27	1.98	0.44	6.90	1.00
EPS	1.424×107	84.57	74.8 ± 0.44	0.9 ± 0.06	2.65	0.76	5.35	1.00

Fig. 5-2 shows the FT-IR spectrum of EPS. The broad and intense peak around 3400 cm^{-1} is attributed to the axial stretch of -OH group of glycol-chain, and the peak around 1380 cm^{-1} to the OH bending vibration. The peak around 2936 cm^{-1} is ascribed to the weak C-H stretching vibration and that at 1644 cm^{-1}, 1557 cm^{-1} and 1427 cm^{-1} are characteristic of the asymmetric and symmetric vibration of the ring stretching of carboxylate group. The absorption peaks in the region of 1000–1200 cm^{-1} are associated with the stretching vibrations of C-O-H side groups and the C-O-C glycosidic band vibrations (Kacurakova et al., 2000). The peak around 1077 cm^{-1} is attributed to the C-O stretching of pyranoside, especially in the glucose residues (Shingel, 2002). The absence of absorption peak at around 1710 - 1740 cm^{-1} confirms the absence of carboxylic acid.

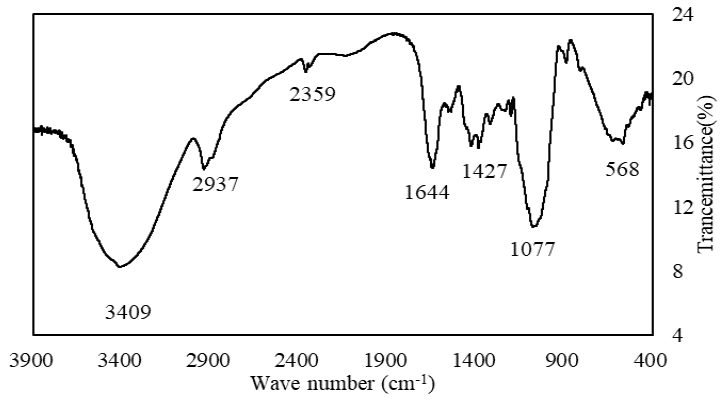


Figure 5-2 FT-IR spectrum of EPS

5.3.2 Effect of EPS on THP-1 cell viability

As shown in **Fig. 5-3**, the cell viability was reduced by 20-30% in the presence of 50-500 mg/mL of CEPS and EPS. The reduction of cell viability by the EPS fractions was most probably attributed to the increase in the viscosity of the liquid culture medium instead of direct cytotoxicity, increasing the resistance to the transfer of nutrients and oxygen to the cells (Yan et al., 2009). This may also explain why CEPS with a higher MW reduced the cell viability more significantly than the EPS. Similarly, in a previous study, EPS caused slight reduction of bifidobacterial growth in liquid culture, which was attributed to the viscosity increase (Song, 2018). Nevertheless, the cell viability remained over 70% with EPS even at a concentration up to 500 μg/mL. Therefore, the EPS fractions did not have significant cytotoxicity.

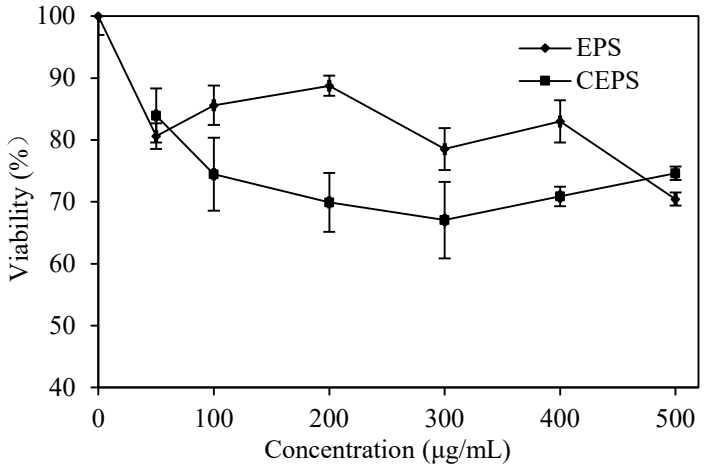


Figure 5-3 Viability of THP-1 cell line treated with EPS after 24 h incubation.

5.3.3 Effect of EPS on LPS-induced NO production of THP-1 cells

As shown in **Fig. 5-4a**, LPS (1 μg/mL) effectively stimulated the NO production,

the activation of NF-κB pathway, and the release of two pro-inflammation cytokines TNF-α and IL-1β in the THP-1 cell culture, in comparison with the negative control group. In human or animal systems, LPS may cause alternation of the barrier function of tissue-resident macrophages and mast cells, resulting in the increased permeability or the disrupted tight junction (Sappington et al., 2003; Medzhitov, 2008). Microbial LPS leaked into the blood stream through the disrupted tight junction causes inflammation by stimulating granulocytes (neutrophils and eosinophils), monocytes, macrophages, and natural killer (NK) cells in non-specific immune system. The LPS-induced inflammatory responses NO production was suppressed both with CEPS and EPS (**Fig. 5-4a** and **Fig. 5-4b**). The suppressing effects of CEPS and EPS on the NO production was concentration-dependent and were increasingly more significant as the concentration was increased. Nitric oxide (NO) is widely distributed in various tissues and organs, acting as a key messenger in the pathogenesis of multiple inflammatory diseases as well as the host defence against tumour cells and microorganisms.

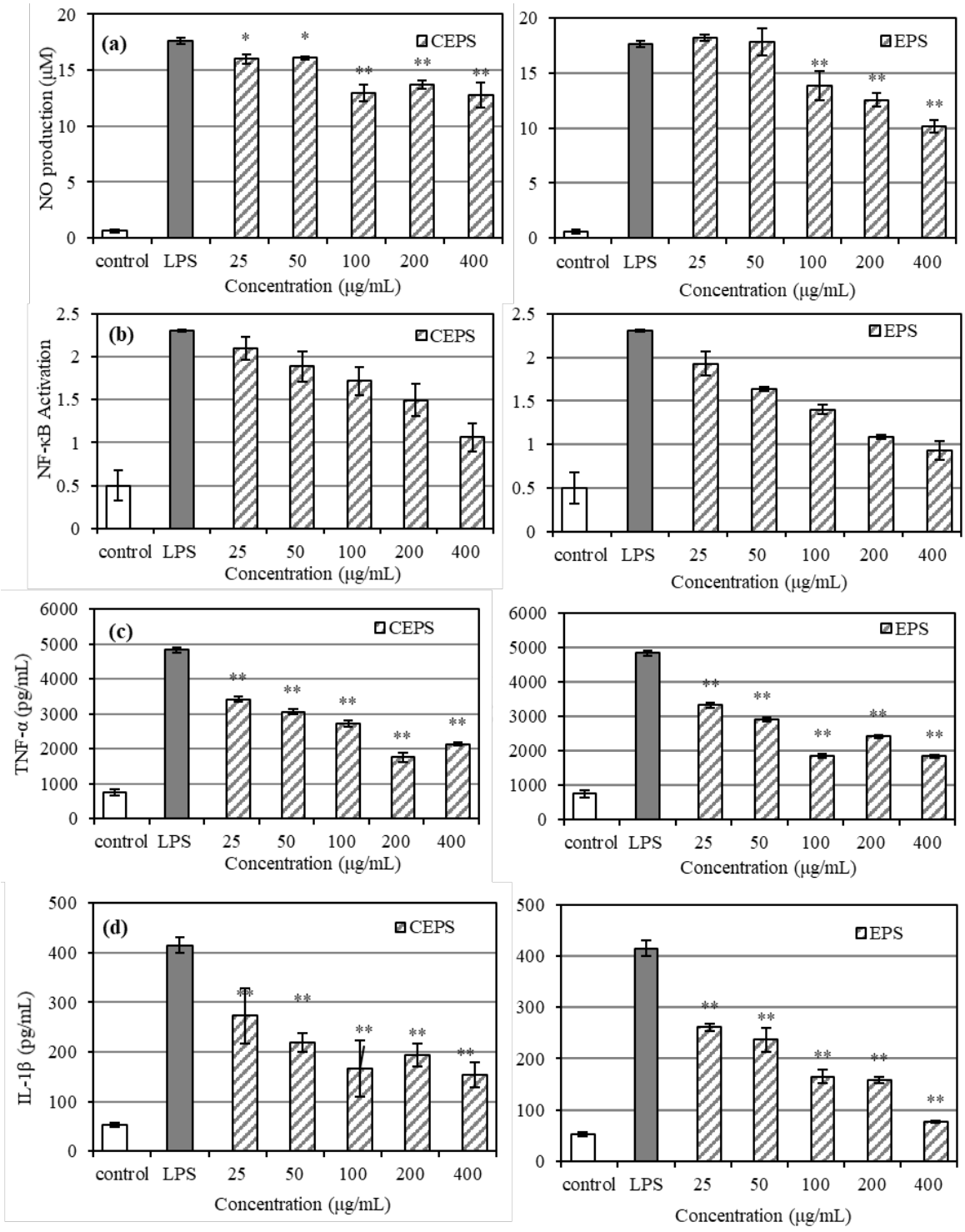


Figure 5-4 Effects of CEPS and EPS on four inflammation cytokine responses on THP-1 cell *in vitro*, (a) NO production; (b)NF-κB; (c)TNF-α, and (d) IL-1β. Data are presented as mean ± S.D. + indicate the group treated with CEPS/EPS + LPS together. * and **: statistically different from LPS group at $p < 0.05$ and $p < 0.01$, respectively.

5.3.4 Inhibition of inflammatory responses in cell cultures by EPS

As shown in **Fig. 5-4b, 5-4c and 5-4d**, CEPS and EPS suppressed the LPS-stimulated activation of NF-κB, the release of TNF-α and IL-1β in a concentration-dependent manner, compared with the LPS group. However, the CEPS or EPS fraction did not completely eliminate the LPS-stimulated inflammatory responses compared with the levels of negative control, which is similar to previous studies such as that by Liu et al. (2017).

Table 5-3 presents the IC50 values of the CEPS/EPS activities derived from the effect versus concentration data. Except for NO, the purified EPS had much lower IC50 values and more potent activities than the crude CEPS. As the major difference of EPS from CEPS is its higher sugar content and negligible protein, the result suggests that the polysaccharide constituent of EPS instead of protein was mainly responsible for the anti-inflammatory activity.

Table 5-3 IC50 values (μg/mL) of CEPS and EPS on different inflammatory indicators (IC50: the half maximal inhibitory concentration, the drug concentration required for 50% inhibition Yung-Chi, 1973).

Pro-inflammation factors	CEPS	EPS
NF-κB	413.6	67.6
NO	41.6	123.3
TNF-α	49.9	51.2
IL-1β	327.0	183.6

Moreover, EPS also showed a significant inhibitory effect on the LPS induced inflammatory responses in RAW264.7 cell culture (**Fig. 5-5a-c**) and the inhibitory

effect was dose-dependent. At the highest dose, EPS reduced the levels of NO by 11.3%, TNF-α by 46.7% and IL-1β by 68.7%. Similarly, Wang et al. (2011) reported that an acidic polysaccharide from *C. sinensis* significantly stimulated the release of several major cytokines, while the EPS had an even more significant effect. These results indicated that the EPS had significant anti-inflammatory activities.

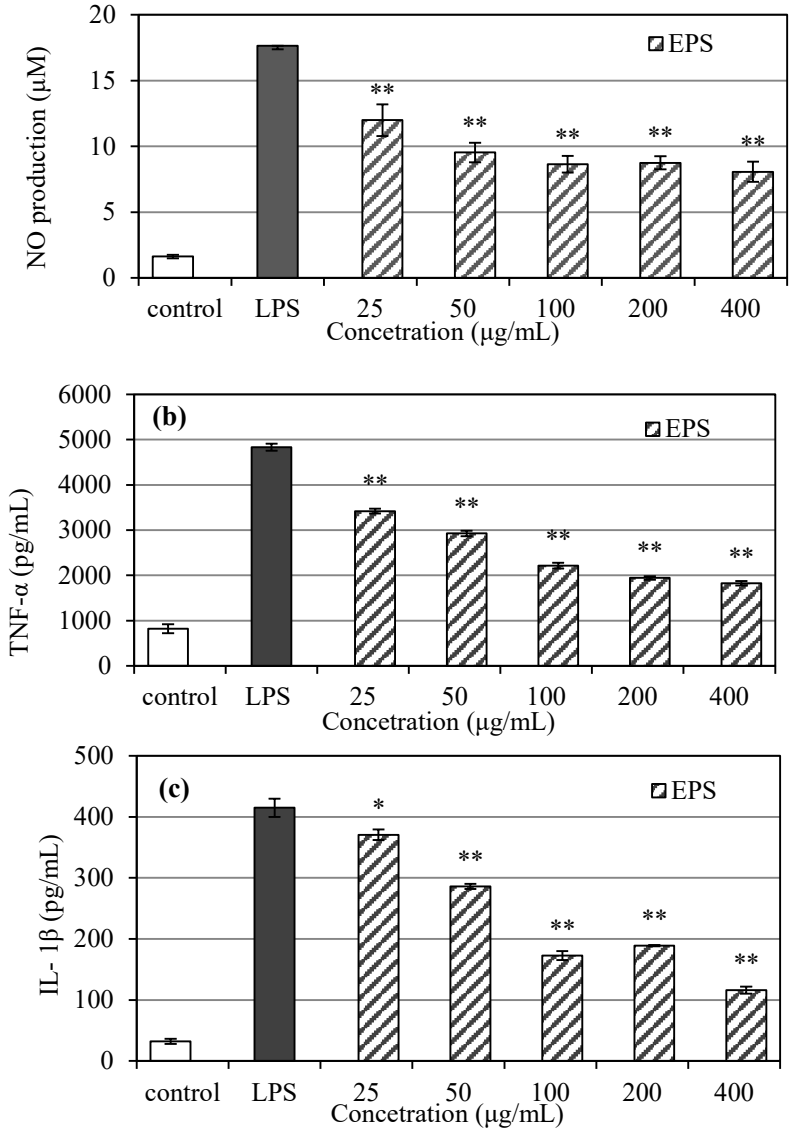


Figure 5-5 Effects of EPS on four inflammation responses on RAW264.7 cell *in vitro*, (a) NO production; (b) TNF-α, and (c) IL-1β. Data are presented as mean ± S.D. + indicate the group treated with CEPS/EPS + LPS together. * and **: statistically different from LPS group at $p < 0.05$ and $p < 0.01$, respectively.

Cytokines are intercellular signalling peptides or proteins with relatively low molecular weight that are released by the cells to alter their own functions (autocrine) or those of adjacent cells (paracrine) (Haddad, Saadé & Safieh-Garabedian, 2002). They are most important in modulating the host immune and inflammatory responses, especially during infection and trauma. The NF-κB family of transcription factors is involved in a classic inflammatory pathway. That is crucial for the activation of numerous cytokines, chemokines and adhesion molecules, which regulate the inflammatory response, immune response and cell survival (Chen & Greene, 2004; Spange, Wagner, Heinzel & Krämer, 2009). The NF-κB signalling pathways can be activated by LPS infection of the granulocytes (neutrophils and eosinophils), monocytes, macrophages, and natural killer (NK) cells, via up-regulation of the DNA-binding activity of NF-κB (Mantovani, 2008). For example, several genes related to the inflammatory cytokines and chemokines were up regulated, as the secretion of the cytokines was promoted (Choudhury et al., 2014). IL-1β and TNF-α are two important pro-inflammatory cytokines that are secreted by inflammatory cells. TNF-α can stimulate the production of genotoxic molecules, such as NO and reactive oxygen species that cause DNA damage and mutations (Hussain, Hofseth & Harris, 2003). According to the signal pathway of inflammation responses in the animal cells, the results suggest that the Cs-HK1 EPS inhibited the inflammatory NF-κB pathway by suppressing the secretion of inflammatory cytokines.

5.3.5 Anti-inflammatory effects of EPS from *in vivo* experiments

As shown in **Fig. 5-6**, LPS effectively stimulated the inflammatory responses in mice, including IL-1β, TNF-α, IL-10 and NO to much higher levels than the control. All these LPS-induced inflammatory responses were significantly suppressed by pre-treatment of the animals with EPS at the higher dose EPS-H, though the levels were still higher than the negative control group. For some of the responses such as IL-1β, the treatment effect of EPS-H was even more remarkable than the positive control drug dexamethasone. The treatment effect of EPS-H on TNF-α was also very significant at $p < 0.01$. IL-10 is a multifunctional negative regulatory cytokine, which is closely related to regulating immune responses to certain infections (Jankovic, Kugler & Sher, 2010; Saraiva & O'garra, 2010). It has been suggested in a previous study that the extracts of *Cordyceps sinensis* could promote the immune regulation activity and enhance bacteriostatic activity of PA-824 via IL-10 in Mycobacterium tuberculosis disease (Li & Ren, 2017). The results from the present study further proved that the EPS from Cs-HK1 has the anti-inflammatory potency by stimulating IL-10 expression. The LPS-stimulated expression of iNOS in the EPS treatment groups was significantly suppressed ($p < 0.05$).

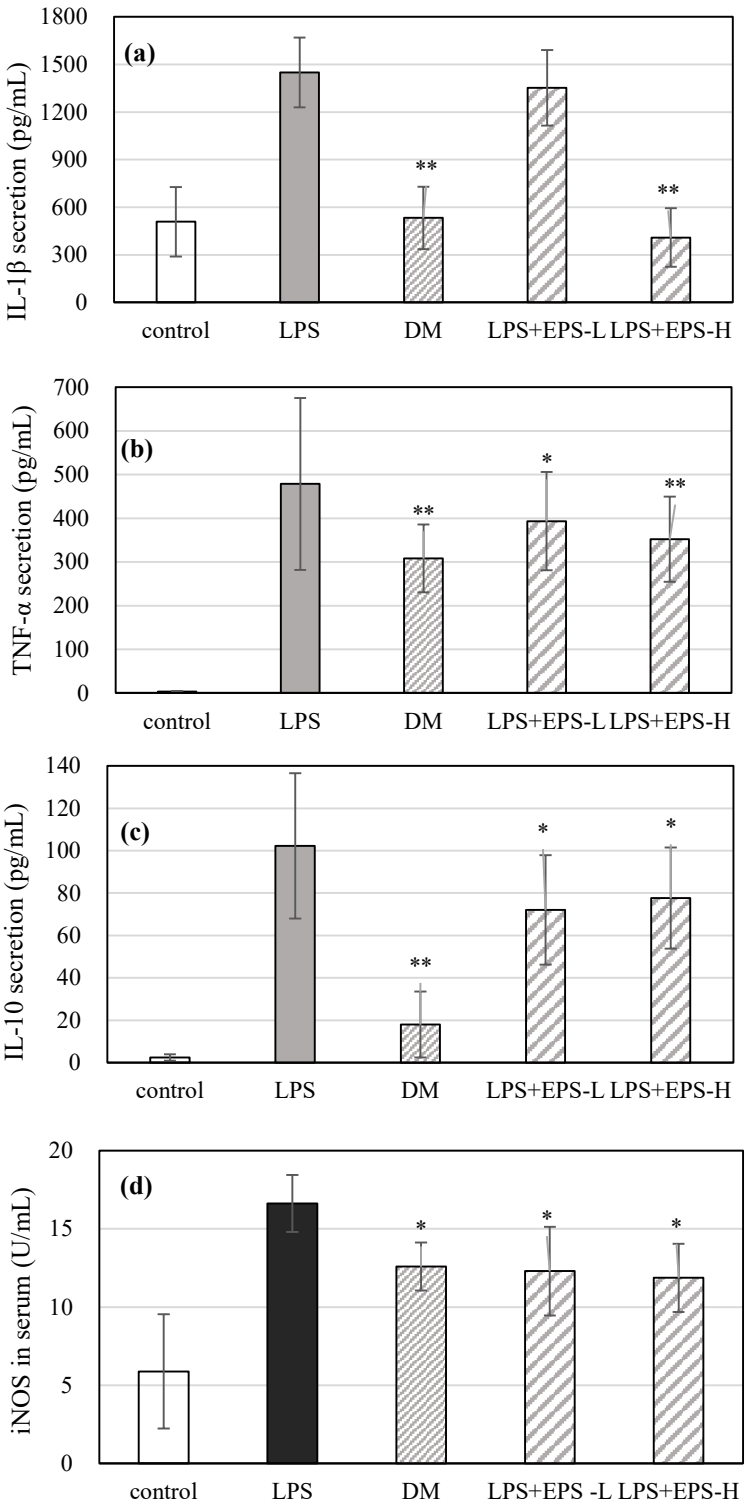


Figure 5-6 Effects of EPS on three inflammation cytokines in jejunum tissues of C57BL/6mice model; (a) IL-1β, (b) TNF-α, (c) IL-10 and (d) iNOS in serum (EPS-L: 150 mg/kg, EPS-H: 300 mg/kg, positive control group: LPS + DM (Dexamethasone)). Data are presented as mean ± S.D. Bars with * indicate statistically different from LPS group ($p < 0.05$); ** indicate statistically different from LPS group ($p < 0.01$).

Fig. 5-7 showed the histological images of colonic mucosa of the test animals in the control and treatment groups. The control group had the normal architecture of the intestinal epithelium and wall (**Fig. 5-7a**). Compared with the normal, intact with smooth and ordered tissue structures in the control animal, the LPS treated group (**Fig. 5-7b**) exhibited the typical features of acute tissue injury, including mild dilation of the chylous duct, short and swollen intestinal villi, edema and sloughing of the villus tips, as well as infiltration of inflammatory cells into the mucosa. The degree of LPS-induced tissue injury and alternations was notably reduced in both the EPS-L and EPS-H treatment groups (**Fig. 5-7d** and **5-7e**) as well as in the positive control group treated with dexamethasone medicine (**Fig. 5-7c**). Therefore, the animal test results provide further evidence for the anti-inflammatory effect of EPS.

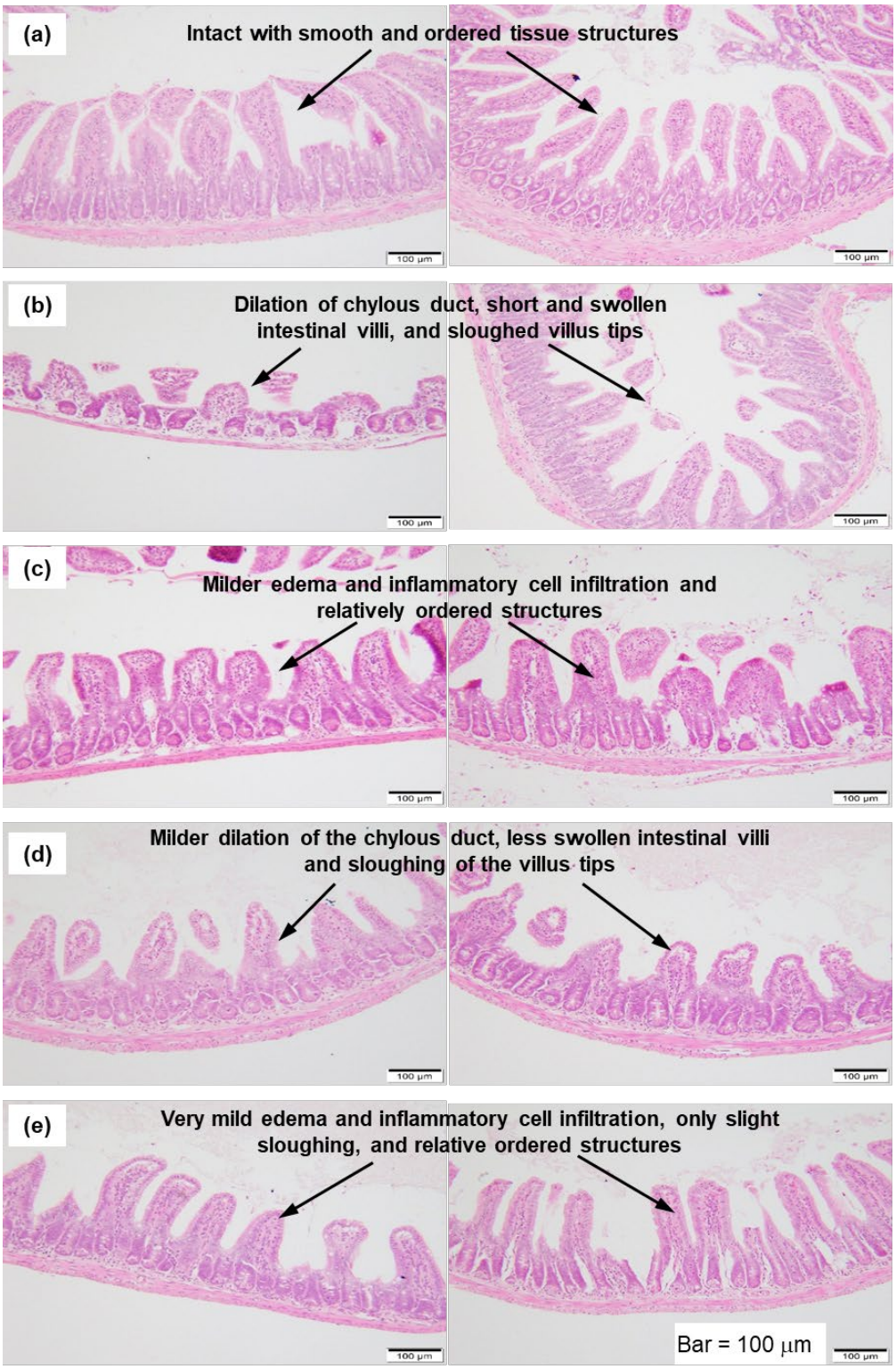


Figure 5-7 EPS ameliorates the severity of LPS-induced intestinal injury in mice. (a) negative control group; (b) LPS-induced intestinal injury group; (c) positive control group (LPS + Dexameth); (d) LPS + EPS-L (150 mg/kg) group; (e) LPS + EPS-H (300 mg/kg) group.

There is a growing concern with gut inflammation due to its prevalence and potential pathogenicity and connection to multiple organ failures. The intestinal injury is an early of the LPS-induced response of gut inflammation which lead to the gut injury and subsequent systemic organ inflammation, and multiple organ failures (Tamion, Richard, Renet & Thuillez, 2007; Yang et al., 2018). Evidence is accumulating for the secretion of inflammatory cytokines during inflammatory bowel diseases (IBDs) (Seo & Chae, 2014; Wu et al., 2017). In the active state of the IBD, macrophages in the intestine are activated to release pro-inflammatory cytokines such as inducible iNOS, IL-1 cytokine family, and TNF-α. The increased production of pro-inflammatory molecules attracts more inflammatory cells to the intestine, amplifying and extending the inflammatory response. On the other hand, the abnormal secretion of these molecules is associated with the survival of intracellular bacteria, inflammatory responses and the onset of IBD (Han et al., 2016). Based on the above *in vitro* and *in vivo* experimental results, we propose that the Cs-HK1 EPS suppressed the expression of inflammatory cytokines and alleviated the inflammation by blocking the activation of NF-κB pathway as illustrated in **Fig.5-8**.

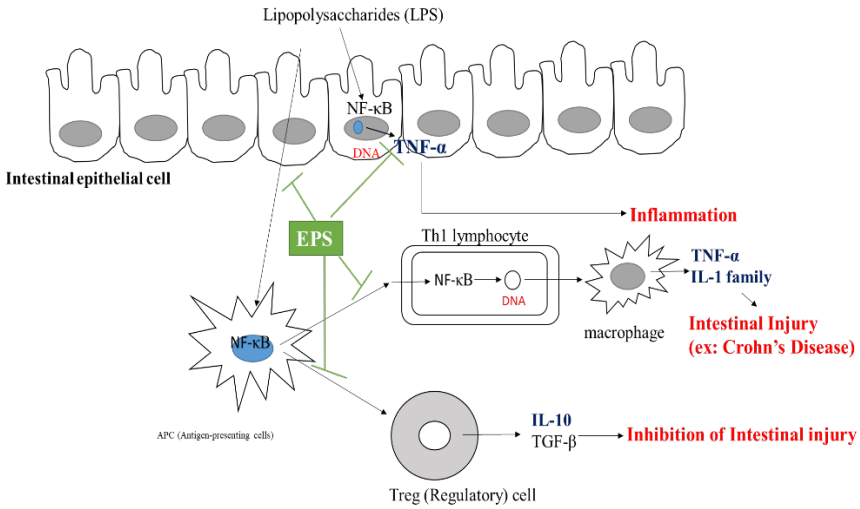


Figure 5-8 A schematic diagram proposing the potential inhibitory role of EPS in inflammatory signalling pathways.

5.4 Conclusions

The extracellular polysaccharide (EPS) produced by the Cs-HK1 fungus had a maximum MW over 10^7 Da and was mainly composed of glucose, mannose and galactose. The Cs-HK1 EPS exhibited significant anti-inflammatory activities in both cell culture and animal models, effectively suppressing the LPS-induced inflammatory cytokine responses and alleviating the intestinal epithelial injury. Therefore, EPS can be a potential candidate for the development of new anti-inflammatory therapy. However, as EPS is a complex of polysaccharides and proteins with a wide MW distribution, further research effort is needed to identify the most active fractions through activity-guided fractionation. Furthermore, purification of the active fractions and characterization of the molecular structures can be performed for establishing the structure and anti-inflammation activity relationship.

Chapter 6 Isolation and Assessment of a Highly-active Anti-inflammatory EPS Fraction[2]

6.1 Introduction

Polysaccharides from various living organisms represent the most abundant and versatile biomacterials for a wide range of applications owing to their structural diversity, biocompatibility and functional versatility (Dumitriu, 2004). Because of the increasing public concerns with the adverse effects of small chemical molecule drugs, natural polysaccharides have been explored as promising candidates for the development of novel nutraceutical products and therapeutic agents (Schepetkin & Quinn, 2006). Many previous studies have shown the notable *in vitro*/*in vivo* anti-inflammatory activities of polysaccharides from various sources such as herbal plants (Wang, Li, Yang, Yao, 2013), macroalga (Phull & Kim, 2017), and edible or medicinal fungi (Ren, Perera, Hemar, 2013; Huang et al., 2018).

Edible and medicinal fungi or mushrooms have multiple health benefits such as anticancer, immunomodulatory, and anti-inflammatory activities which can be attributed at least partially to the polysaccharide constituents (Muszyńska et al., 2018). *Ophiocordyceps sinensis* (previously named *Cordyceps sinensis*), generally known as the Chinese caterpillar fungus, is a treasured medicinal fungus in traditional Chinese medicines for health promotion and therapeutic treatment (Chen, Wang, Nie, Marcone, 2013). However, the wild or natural *O. sinensis* caterpillar fungus is very rare and not

[2] This chapter has been partially published on International Journal of Molecular Sciences, 2021, 22(5), 2450.

an affordable raw material for extraction of polysaccharides. Instead, mycelial fermentation of *O. sinensis* fungi has become a more feasible means for production of the fungal biomass and polysaccharides.

In previous studies, most of the *O. sinensis* fungal polysaccharides have been extracted from the mycelial biomass but very few exopolysacchardies (EPS) from mycelial fermentation (Sheng, Chen, Li & Zhang, 2011; Nie et al., 2013; Yan, Wan & Wu, 2014). To the best of our knwoledge, there is rarely any literature on the molecualr characteristics and anti-inflammatory activities of EPS produced by an *O. sinensis* fungus. Cs-HK1 is a fungal species orignated from the fruiting body of a wild *O. sinensis* caterpiller fungus by Wu's group (Leung, Zhao, Ho & Wu, 2009). The mycelial culture of Cs-HK1 fungus in a liquid medium was able to produce a significant amount of EPS which has a complex compositon and wide molecular weight (MW) range (Huang et al., 2013). In a recent study from Wu's group, a partially purified EPS from the Cs-HK1 mycelial fermenation has shown significant anti-inflammatory activities in both cell culture and animal models (Li et al., 2020). However, the previously reported EPS was a complex mixture containing polysaccharides of different molecular weights and unknown active fractions. It is of significance to fractionate and purify the EPS and to find out the most active polysaccharide fractions, in order for better understanding of the molecular property and anti-inflammation activity relationship as well as the the mechanism of action.

The present study was to acquire an active polysaccharide fraction responsible for the anti-inflammation activity of EPS from Cs-HK1 mycelial fermentation, and to

elucidate its molecular structure. The EPS in the Cs-HK1 mycelial fermentation liquid was roughly fractionated into two MW ranges via two-step ethanol precipitation. A homogenous polysaccharide named EPS-LM-1 was isolated from the lower MW fraction of the precipitated EPS through necessary purification steps. The structure of EPS-LM-1 was elucidated based on analytical experiments, and its *in vitro* anti-inflammation activity was assessed in human monocytic THP-1 cell culture on lipopolysaccharide-induced inflammatory responses.

6.2 Materials and methods

6.2.1 Fungus materials

Cs-HK1 mycelial fermentation, isolation, and preparation of EPS fractions using the same methods as described in 4.2.2.

Analysis of EPS composition and molecular weight were performed using the same methods as described in 4.2.4.

In the present study, a two-step ethanol precipitation was performed using ethanol concentration of 40% (v/v) in the first step and 80% in the second step. In the first step, ethanol was added slowly to the supernatant medium to a final concentration of 40% (v/v) (= 2:3 volume ratio) with stirring and then kept in stationary condition at 4°C for 12 h. The mixture was centrifuged at 10,000 rpm for 20 min to separate the solid from liquid. In the second step, the ethanol was added to the liquid supernatant at 10:3 volume ratio to make up a total volume ratio of 4:1 (2:3 in 1st step + 10:3 in 2nd step)

and the final ethanol concentration of 80% (v/v). All precipitates collected after the centrifugation were re-dissolved in water and lyophilized, yielding the crude EPS fraction in the higher MW range (EPS-HM) from the first step and the lower MW range (EPS-LM) from the second step, respectively.

6.2.2 Purification of EPS-LM

The lower MW fraction EPS-LM attained from the above two-step ethanol precipitation treated repeatedly with the Sevag reagent to remove the protein content (Staub, 1965) as reported previously (Li et al., 2020). The solution was then dialyzed against 3500 Da MW cut-off membrane for 48 h, concentrated by evaporation and lyophilized. About 500 mg of the partially purified EPS-LM was re-dissolved in 2 mL distilled water (250 mg/mL) and fractionated by anion exchange chromatography (AEC) on a DEAE-Sephadex A-25 column (2.6 × 100 cm) (Pharmacia), and eluted stepwise with distilled water and 0.1 M NaCl at a flow rate 0.5 mL/min. Polysaccharide in the elute was detected by the anthrone agent (Sigma). A major fraction was collected during water elution from three fractions, and further purified on a Superdex 200 pg gel-filtration column (2.6×60 cm, Cl^-; Whatman) eluted with 0.3 M NH_4HCO_3 at a flow rate of 0.3 mL/min. The eluate (~3 mL) was collected for detection of polysaccharide by the anthrone method. The main polysaccharide fractions were pooled, concentrated, dialyzed and lyophilized, yielding the final purified fraction EPS-LM-1. **Fig. 6-1** shows the overall procedure for EPS isolation by two-step ethanol precipitation and further fractionation, and purification of EPS-LM through AEC and GPC.

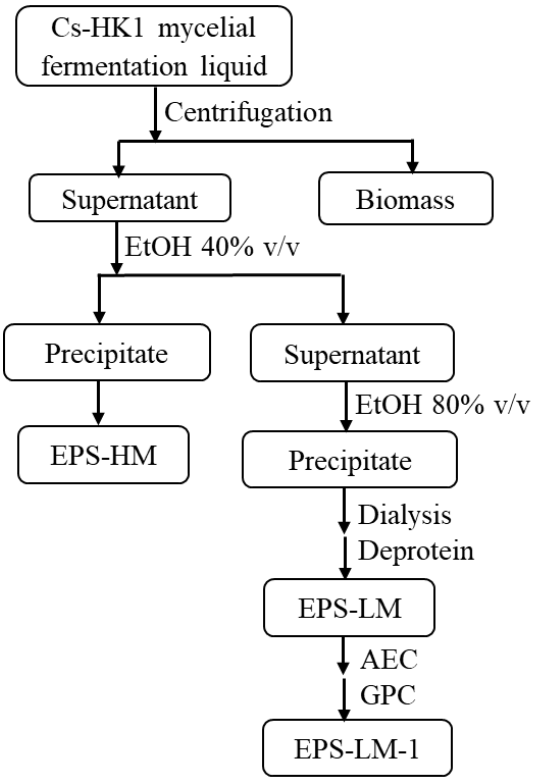


Figure 6-1 Experimental procedure for the isolation of EPS from Cs-HK1 mycelial fermentation by two-step ethanol (EtOH) precipitation and further fraction, and purification to attain EPS-LM-1 (AEC: anion exchange chromatograph; GPC: gel permeation chromatograph).

6.2.3 Analysis of molecular weight and chemical composition

6.2.3.1 Analysis of chemical composition and molecular weight

The MW distribution of EPS-LM-1 was analyzed by high performance gel permeation Analysis of EPS composition and molecular weight were performed using the same methods as described in 4.2.4.

6.2.4 IR and NMR spectrum analysis

The IR spectroscopy was performed as described in 4.2.4.

NMR analysis was performed on a Bruker AVANCE III 600 spectrometer at 25℃ for the ^{1}H and ^{13}C NMR, hetero-nuclear single quantum coherence (HSQC), correlation spectroscopy (COSY) and hetero-nuclear multiple bond correlation (HMBC), respectively. Before the analysis, the EPS-LM-1 sample (10 mg) was co-evaporated with D_2O twice by lyophilization and then dissolved in 600 μL D_2O.

6.2.5 Methylation analysis

EPS-LM-1 was methylated three times as described by Needs and Selvendran (1993). In brief, 5 mg of dry EPS-LM-1 was pre-dissolved in 2.5 mL anhydrous DMSO with constant stirring for 3 h. To the solution and in nitrogen gas, 30 mg of anhydrous sodium hydroxide was added with constant stirring for 30 min, and then 0.8 mL of methyl iodide was added slowly in an ice bath and stirred in the dark at room temperature for 1 h. The reaction was stopped by adding 2.5 mL distilled water and the excess amount of methyl iodide was evaporated off under vacuum at 40℃. The partially methylated sample was extracted with dichloromethane, and the extract solution was then washed with deionized water three times to remove impurities. The partially methylated EPS-LM-1 was then evaporated to dryness under vacuum in a rotatory evaporator at room temperature. The methylation process was repeated three times for completion.

The partially methylated EPS-LM-1 was further hydrolyzed with 2 M TFA (1 mL) in a sealed tube at 110°C for 6 h, and then the excess TFA was removed with a stream of nitrogen in a boiling water bath. The dried hydrolysate was re-dissolved in 1 mL

ammonia-saturated water and reduced with excess $NaBH_4$ at room temperature for 12 h. Excess NaBH4 was reacted with acetic acid (till no bubbles appearing) and the borate acid formed was removed by co-distillation with methanol. The dried residue was acetylated with 1 mL acetic anhydride in a sealed tube at 110℃ for 2 h to form partially methylated alditol acetate (PMAA). The PMAA was evaporated to dryness under vacuum, and re-dissolved in chloroform, and washed three times with water.

The methylation products were analyzed by GC-MS using an Agilent 6890N GC and 5975 VL MSD through a fused silica capillary column (30 mm × 0.25 mm ID, Agilent HP-5MS). The column temperature was fixed at 100℃ for 3 min, then increased to 250℃ at 3℃ /min and fixed for 10 min. The injector and the detector were fixed at 280℃ and 250℃, respectively.

6.2.6 Cell culture conditions

Cell lines used in this chapter and culture conditions were performed using the same methods as described in 4.2.3.

6.2.7 Anti-inflammatory assay of THP-1 cell

Analysis of NF-kB activation, Nitric Oxide (NO) production measurements, and Cytokines IL-1β and IL-10 measurement were performed as previously described in 5.3.3 & 5.3.4.

6.2.8 Western blot analysis

After treatment with LPS and EPS-LM-1, the THP-1 cells were harvested and washed with cold phosphate-buffered saline (PH 7.4). The total cytoplasmic protein (for the detection of NF-κB, I$\kappa B\alpha$) was extracted from the cells using the cell lysis reagent (Sigma-Aldrich) and ice-cold lysis buffer, and β-actin was included as an internal reference. The protein content in the supernatant was determined using the BCA protein assay kit. An equivalent amount of lysate protein (20 μg per lane) was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% (w/v) skimmed milk and the blots were incubated with specific primary antibodies overnight at 4°C followed by incubation with horseradish peroxidase-conjugated secondary antibody. Finally, the blots were probed using enhanced chemiluminescence and autoradiographed.

6.2.9 Statistical analysis

All data were expressed as means ± standard deviation. Statistical analysis was performed using SPSSv.16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Intergroup differences for continuous variables were assessed by multivariate ANOVA.

6.3 Results and discussion

6.3.1 Compositions and anti-inflammatory activities of different EPS fractions

Table 6-1 showed the yields, compositions and average MW distributions of the higher-MW and lower-MW EPS fractions isolated from the Cs-HK1 mycelial fermentation medium by two-step ethanol precipitation. As expected, EPS-HM attained from the first step of ethanol precipitation had a much higher average MW than the EPS-LM from the second step. EPS-LM had much higher protein content but lower sugar content than EPS-HM. Both EPS-HM and EPS-LM were composed mainly of three monosaccharides including mannose, glucose and galactose, and EPS-HM also contained a detectable amount of ribose. The contents of monosaccharides were very different between the two EPS fractions with mannose being the most abundant in EPS-HM, and glucose the most abundant in EPS-LM.

SEM is a powerful analytical tool for detecting the morphological properties of biopolymers, including polysaccharide, protein, and peptide. The SEM micrographs of EPS-LM at 1000, 1500 and 2000 magnification were shown in **Fig. 6-2**. As it was shown the morphological structures, EPS-LM exhibited numerous spherical and rodlike particles piled into a network structure with a corrugated surface, and it was more obvious with the enlargement of magnification. However, EPS-LM demonstrated a quite different morphological structure with respect to EPS previously presented by Mao et al. (2018), which showed a flake-like structure with smooth surface. The possible explanation might be that the different extraction methods leading to the

different morphological structures as well as the molecular weight differences, which may lead to the diverse bioactivity.

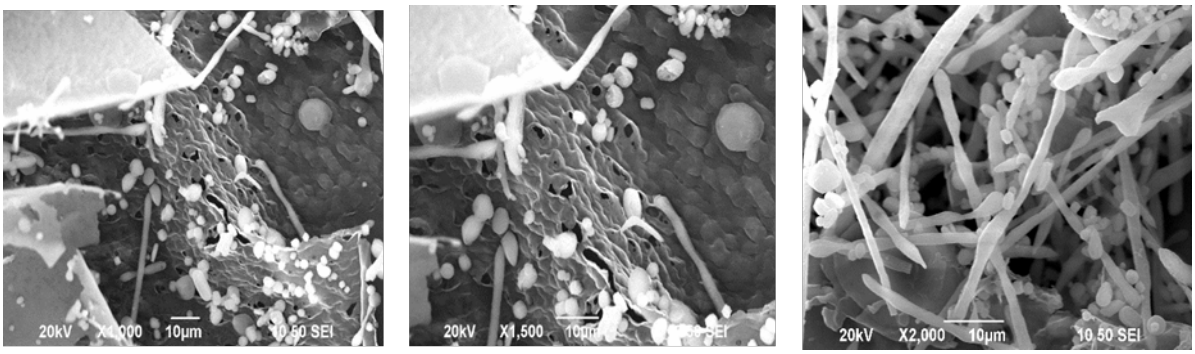


Figure 6-2 SEM micrographs of EPS-LM exopolysaccharides from *Cordyceps sinesis*.

Table 6-1 Yield and composition of EPS fractions isolated from Cs-HK1 fermentation medium by two step ethanol precipitation and further fraction of EPS-LM.

Fraction	Yield (g/L)	Sugar (wt %)	Protein (wt %)	MW in kDa (GPC peak area %)
EPS-HM	2.27 ± 0.19	64.5 ± 5.70	9.0 ± 0.39	6250 (84.76%) 18.5 (15.24%)
EPS-LM	0.93 ± 0.15	25.5 ± 0.60	20.1 ± 0.06	360 (68.7%) 39.2 (12.3%) 1.61 (19.0%)
EPS-LM-1	0.074 ± 0.003	100	--	360 (100%)

Monosaccharide composition (molar ratio)

Fraction	Mannose	Ribose	Glucose	Galactose
EPS-HM	1.71	0.09	0.94	1.00
EPS-LM	7.76	--	13.87	1.00
EPS-LM-1	3.88	--	6.93	1.00

Figure 6-3 showed the results of *in vitro* anti-inflammatory tests on EPS-HM and EPS-L production (**Fig. 6-3a**), NF-κB activation (**Fig. 6−3b**), and release of TNF-α (**Fig.**

6-3c) and IL-1β (**Fig. 6-3d**) in the LPS-induced THP-1 cell culture of the three EPS fractions. The lower-MW EPS, EPS-LM, showed the most notable inhibiting effects on all the LPS-induced inflammatory responses. As EPS-LM was the most active EPS fraction, it was chosen for further purification, characterization and anti-inflammatory assessment.

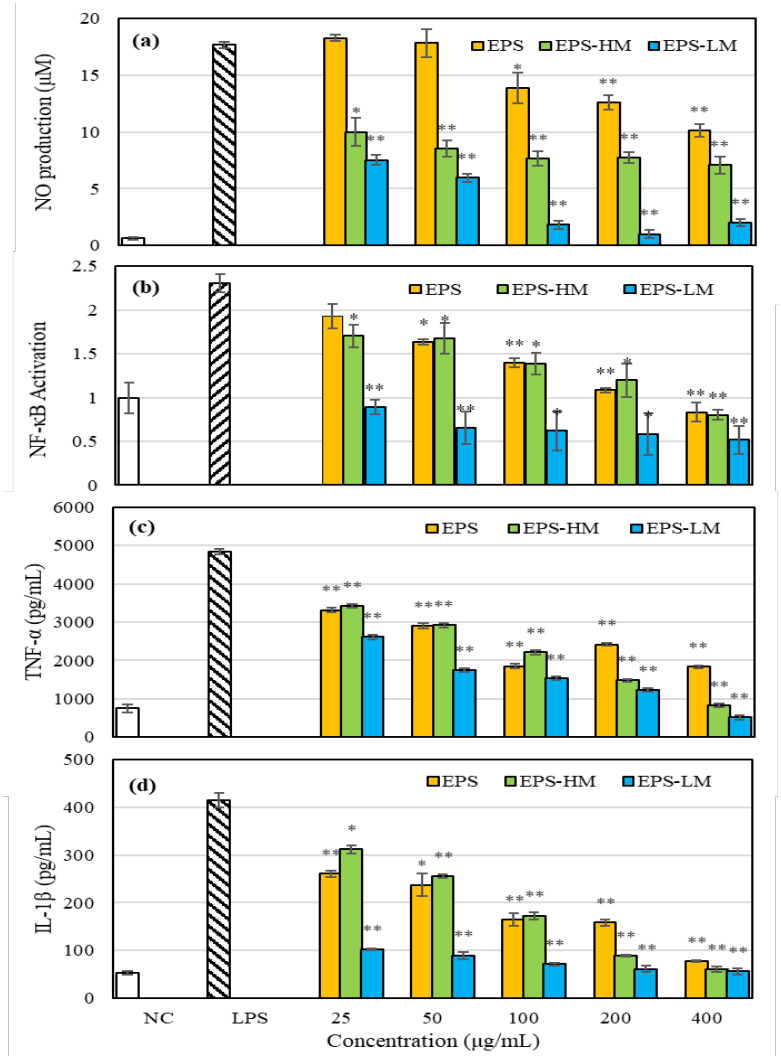


Figure 6-3 Effects of three different EPS fractions on LPS-induced inflammatory responses in THP-1 cell culture: (a) NO production; (b) NF-κB activation; (c) TNF-α cytokine release; (d) IL-1β cytokine release. * and ** indicating statistically significant differences from LPS group at $p < 0.05$ and $p < 0.01$ by student test, respectively.

From the anion exchange chromatograph (AEC), EPS-LM was fractionated into three major fractions, EPS-LM-1, EPS-LM-2 and EPS-LM-3 (**Fig.6-4a**). As EPS-LM-1 showed the most active *in vitro* anti-inflammatory effect (**Fig. 6-4b**), it was further purified by Superdex 200 pg column (yield ~ 2.3% of EPS) (**Fig. 6-4c**). EPS-LM-1 exhibited a single and sharp peak on HPGPC, corresponding to an average MW of 360 kDa by calibration. It had a total carbohydrate content of 95.4% and negligible protein content, and was composed of three monosaccharide residues, Man, Glc and Gal at 3.9:6.9:1 mole ratio (**Table 6-1**). Compared with the partially purified EPS-LM (before AEC and GPC separations), EPS-LM-1 had a much lower content of glucose and mannose.

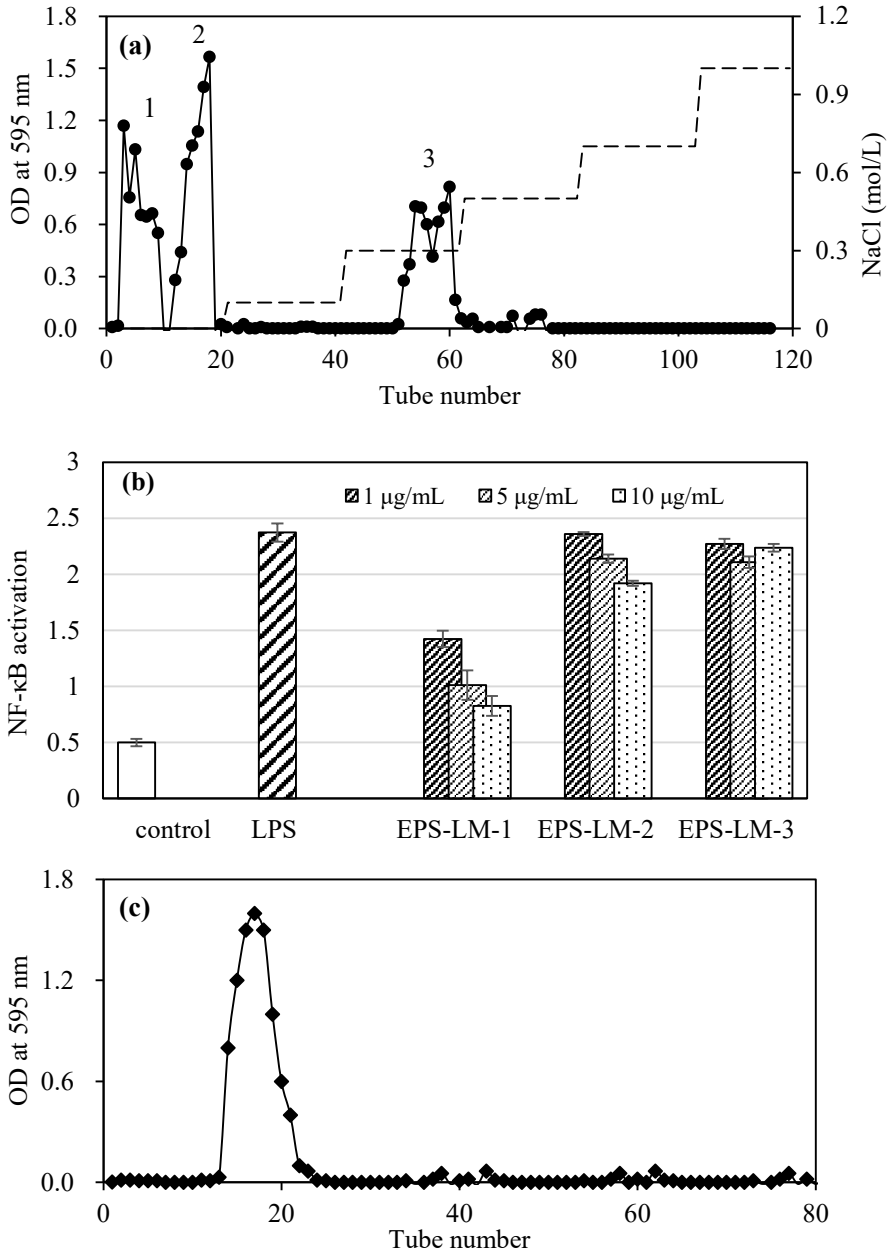


Figure 6-4 Purification and anti-inflammatory activity of EPS-LM fractions: (a) fractionation of EPS-LM by anion exchange on a DEAE-Sepharose column (0.1 M NaCl elution; peaks 1, 2 and 3 representing EPS-LM-1, -2 and -3 fractions, respectively); (b) anti-inflammatory activity test on EPS-LM fractions: effect on LPS-induced NF-κB activation in THP-1 cell culture (* and ** indicating statistically significant differences from LPS group at $p < 0.05$ and $p < 0.01$ by student test, respectively); (c) GPC spectrum of EPS-LM-1 eluted from a preparative Sephadex 200 pg column.

In a previous study by Nie et al. (2011), the polysaccharide extracted from *C. sinensis* mycelium was mainly composed of glucose (95.19%) with trace amount of mannose (0.91%) and galactose (0.61%). As reported by Sheng et al. (2011), an exopolysaccharide from a cultivated *C. sinensis* was mainly composed of mannose, glucose, and galactose in a ratio of 23:1:2.6 and had an average MW of 104 kDa. A galactomannan has also been isolated from natural *Cordyceps sinensis* by Wang et al. (2018) which was composed of galactose (68.65%), glucose (6.65%) and mannose (24.02%). The present and previous studies have all shown that mannose, glucose and galactose are the three major constituent monosaccharides of polysaccharides from the *O. sinensis* fungus, while the differences in the relative contents could be attributed to the different fungal species, extraction conditions and intracellular or extracellular location.

6.3.2 IR spectral characteristics of EPS-LM-1 structure

Table 6-2 summarized the major peaks and the corresponding bonding characteristics derived from the FT-IR spectrum of the EPS-LM-1 fraction. The large absorption peak near 3413 cm^{-1} was attributed to the -OH stretching vibration of sugar chains (Fang et al., 2015). The weak absorption peaks at 2930 cm^{-1} and 1638 cm^{-1} were attributed to C-H stretching vibration and bound water on the sugar chains, respectively. Moreover, several weak absorption peaks around 1000 cm^{-1} indicated the vibrations of the C-O-C bond and the existence of pyran configuration (Albuquerque et al., 2014). There were no absorption peaks at 1740 and 1250 cm^{-1}, confirming the absence of

uronic acid and sulfate groups (Mei et al., 2015). All the spectral data showed that EPS-LM-1 contained the major functional groups in sugars. Overall, the IR spectral characteristics of EPS-LM-1 were very similar to those of the whole EPS isolated from the Cs-HK1 by a single-step ethanol precipitation as reported previously (Li et al., 2020), suggesting the similarity in the functional groups.

Table 6-2 FT-IR peaks and bonding characteristics of EPS-LM-1 (according to Shingel, 2002)

Peak wavenumber (cm-1)	Functional groups
3400	Axial stretch of -OH group
2930	weak C-H stretching vibration
1638 , 1539	Asymmetric and symmetric vibration of the ring stretching of carboxylate group, respectively
1380	-OH bending vibration
1065	Pyranoside (e.g. in the glucose residues)

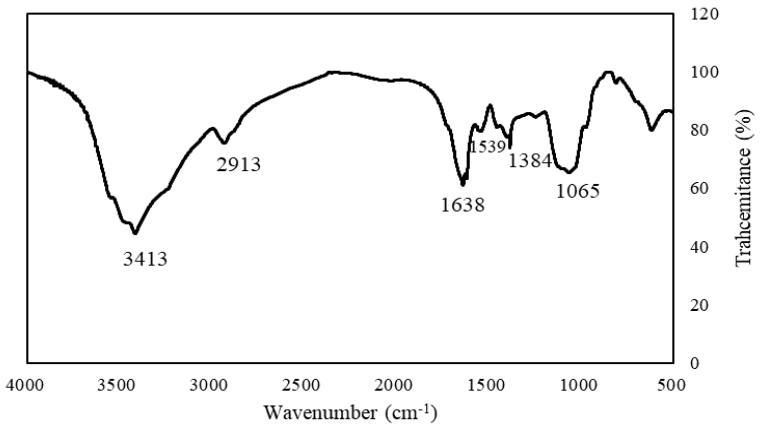


Figure 6-5 FT-IR spectrum of EPS-LM-1.

6.3.3 EPS-LM-1 structural characteristics from GC-MS analysis and NMR spectra

Further structural information of EPS-LM-1 was acquired from GC-MS analysis of the methylated products. All the free OH groups were completely methylated, and the permethylated polysaccharides then underwent hydrolysis, reduction and PMMA, which were analyzed by GC-MS. The signals and mass fragments were analyzed through comparison with the NIST and CCRC database. From the GC-MS comparison analysis of the methylation products, six types of sugar linkages were identified, including 2,3,5,6-Me_4-Gal*f*, 2,4,6-Me_3-Man*p*, 2,3,6-Me_3-Man*p*, 2,4-Me_2-Glc*p*, 2,3,4-Me_3-Glc*p* and 2,3,6-Me_3-Glc*p* at the mole ratio of 1.0:2.2:1.9:1.2:2.0:4.3 (**Table 6-3**). The mole ratio was in close agreement with the monosaccharide composition of EPS-LM-1 as shown in **Table 6-1**, suggesting that EPS-LM-1 was composed of (1→3,6), (1→6), (1→4) linked glucopyranosyl, (1→3), (1→4) linked mannopyranosyl and (1→) linked galactofuranosyl residue.

Table 6-3 GC-MS result of partially methylated alditol acetates of EPS-LM-1.

Methylated sugar	Mass fragments (m/z)	Mole ratio	Linkage
2,3,5,6-Me4-Galf	59,75,89,101,117, 129,145,161,205	1.0	1 →
2,4,6- Me3-Manp	59,71,87,101,118,129,174,190,234	2.2	1 →3
2,3,6- Me3-Manp	59,71,87,102,118, 129,173,189,233	1.9	1→ 4
2,4- Me2-Glcp	59,73,87,118,129,189,234	1.2	1→ 3,6
2,3,4- Me3-Glcp	59,71,87,99,118,129,162,173,189,233	2.0	1→ 6
2,3,6-Me3-Glcp	59,71,87,99,118,129,159,161,173, 233	4.2	1→ 4

The ^{1}H NMR and ^{13}C NMR spectrum were given in **Fig. 6-6a** and **Fig. 6-6b.** The signals were assigned according to the two-dimensional spectra (HSQC, HMBC) (**Fig. 6-6c & d**) and literature data (Bock & Pedersen, 1983; Lu et al., 2019). In the ^{1}H NMR spectrum, the terminal proton peaks were distributed in the 4.90-5.10 ppm region. Combined with the ^{13}C NMR results, seven protons were assigned at 5.08 ppm, 5.02 ppm, 4.98 ppm, 4.93 ppm and 4.91 ppm. The chemical shifts from 3.2-4.1 ppm were assigned to the protons of H2, H3, H4, H5 and H6 of sugar units.

^{13}C NMR spectrum has a wider chemical shift dispersion and provide more structural information than ^{1}H NMR spectrum. As shown in **Fig. 6-6b**, the ^{13}C NMR spectrum of EPS-LM-1 captured signals for anomeric carbons at δ 107.1, 105.9 and 100.7 ppm could be assigned to C1 of terminal β-Gal*f* (A), 1,6-linked β-Glc*p* (E), and 1,3,6-linked α-Glc*p* (D), respectively. Similarly, the resonances at 97.0, 98.2, and 102.3 ppm may be attributed to the C1 of 1,4-linked α-Man*p* (C), 1, 3-linked α-Man*p* (B), and 1,4-linked α-Glc*p* (F). Signals detected at 67.0-71.7 ppm suggesting the existence of (1→6) glycosidic linkages (Yu et al.,2017). All the sugar residues were confirmed by labeling C1 to C6 on the ^{13}C NMR spectrum and summarized in **Table 6-4**. The structural characteristics derived from NMR corroborated those from GC-MS.

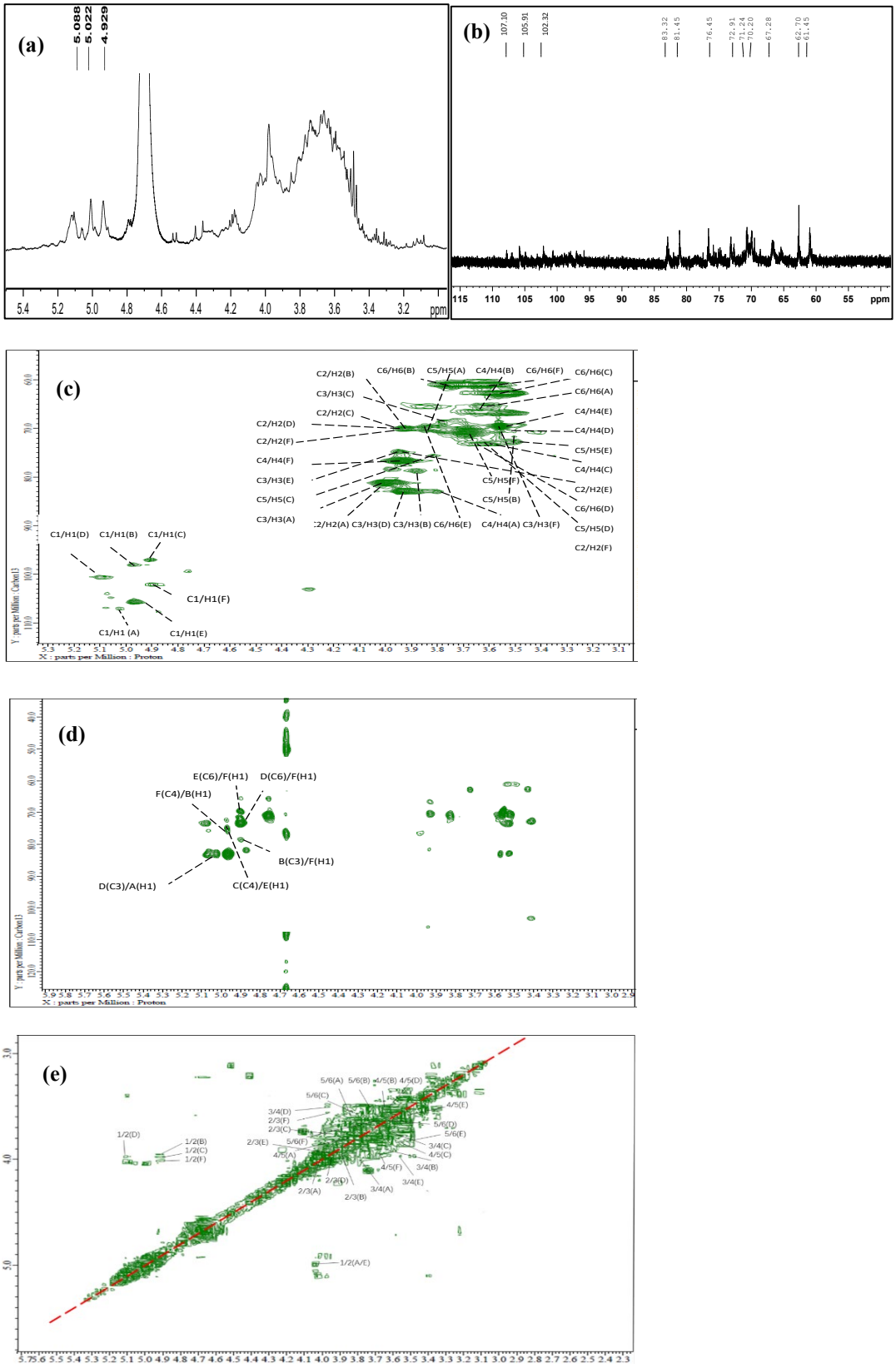


Figure 6-6 NMR spectra of EPS-LM-1: (a) ^{1}H NMR (b) ^{13}C NMR; (c) HSQC NMR (2D); (d) HMBC NMR (2D); (e) COSY spectrum.

Table 6-4 Assignment of ^{1}H NMR and ^{13}C NMR chemical shifts of EPS-LM-1.

Sugar residue	Chemical shifts (ppm)					
	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
(A): β-D-Galf-(1→	5.02/107.1	4.01/82.0	4.02/80.9	3.78/83.1	3.82/71.0	3.62/65.0
(B):→3)-α-D-Manp-(1→	4.93/98.2	3.92/70.0	3.90/79.0	3.64/67.0	3.50/72.1	3.72/61.1
(C):→4)-α-D-Manp-(1→	4.91/97.0	3.95/70.0	3.76/68.0	3.58/73.2	3.80/74.9	3.56/62.3
(D):→3,6)-α-D-Glcp-(1→	5.08/100.7	3.92/70.0	3.94/82.9	3.51/71.0	3.62/72.5	3.64/73.0
(E):→6)- β-D-Glcp-(1→	4.98/105.9	3.94/74.5	3.96/74.5	3.55/69.6	3.50/73.0	3.84/69.5
(F):→4)-α-D-Glcp-(1→	4.91/102.3	3.96/70.1	3.56/70.2	3.92/76.2	3.66/71.0	3.62/61.0

6.3.4 Linkage and structural characteristics of EPS-LM-1 from 2D NMR

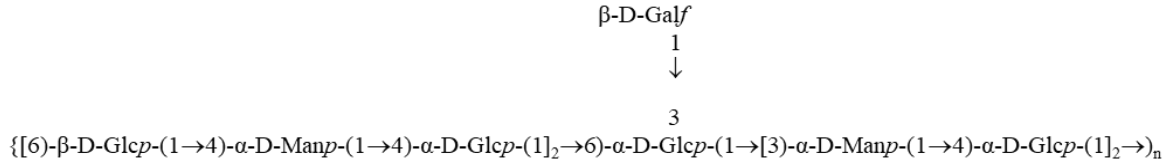
The heteronuclear single quantum coherence (HSQC) spectrum and correlation spectroscopy (COSY) were conducted for responding proton and carbon signals. The HSQC spectrum (**Fig. 6-6c**) exhibited the corresponding carbon chemical shifts at δ71.0 ppm and 65.0 ppm may be attributed to the C-5 and C-6 of 1-β-D-Gal*f* residue according to reference data (Smiderle, Sassaki, Griensven & Iacomini, 2013; Carbonero et al., 2006). In parallel, the COSY spectrum (**Fig. 6-6e**) shows correlative signals from the H1 δ5.02 ppm to the H2 at δ4.01 ppm and the H3 at δ4.02 ppm, from the H3 to the H4 at δ3.78 ppm and the H5 at δ3.82 ppm, and from the H5 to the H6 at δ3.62 ppm. Based on the proton assignments, the corresponding ^{13}C chemical shifts can be readily assigned from the HSQC spectrum between the carbons and protons of C-H pairs. Other residues were subsequently determined by similar approach and assigned in the COSY spectrum. Signals were observed at δ82.9, 76.2, 73.0 and 69.5 ppm, which may be attributed to the downfield shift caused by substitutions of C3, C4 and C6 of

glucopyranosyl residues, and δ79.0 ppm was attributed to the downfield shift caused by substitutions of C3 of 1, 3 linked α-D-Man*p* residue (Bock & Pedersen, 1983; Ieranò et al., 2009). In addition, the downfield shift for C4 (δ73.2 ppm) was attributed to 1,4 linked α-D-Man*p* residue. Compare with the chemical shifts for standard monosaccharides available in literature (Agrawal, 1992), all these down-filed carbon resonances were further confirmed the glycosidic linkage forms of the residues as well as indicated that these carbon positions were replaced. The other corresponding carbon and hydrogen signals of sugar residues were assigned from the HMBC spectrum (**Fig. 6-6d**).

Heteronuclear multiple bond correlation (HMBC) is a powerful spectrum for determining the glycosidic linkages between sugar residues (Hu, 2009). Based on the ^{1}H-^{13}C HMBC spectrum (**Fig. 6-6d**), the linkage sites and sequences among residues were established showed in **Table 6-4**. A cross peak was observed between C6 (δ73.0 ppm) of residue D and H1 (δ4.91 ppm) of residue F [D(C6)/F(H1)], indicating that C6 of residue D was linked to H1 of residue F. Similarly, the cross peaks at δ69.5/4.91 ppm for [E(C6)/F(H1)], δ76.2/4.93 ppm for [F(C4)/B(H1)], δ73.2/4.98 ppm for [C(C4)/E(H1)], δ79.0/4.91 ppm for [B(C3)/F(H1)], and δ82.9/5.02 ppm for [D(C3)/A(H1)] suggested that C6 of residue E was linked to H1 of residue F, C4 of residue F linked to H1 of residue B, C4 of residue C linked to H1 of residue E, C3 of residue B linked to H1 of residue F, and C3 of residue D linked to H1 of residue A, respectively.

The combination of GC-MS and 2D NMR results further revealed that the

polysaccharide backbone of EPS-LM-1 had a branch chain consisting of galactose residues. With a comprehensive consideration of all the analytical results, a hypothetical overall structure of EPS-LM-1 is established as a main chain of [6)-β-D-Glc*p*-(1→4)-α-D-Man*p*-(1→4)-α-D-Glc*p*-(1]$_2$→3,6)-α-D-Glc*p*-(1→[3)-α-D-Man*p*-(1→4)-α-D-Glc*p*-(1]$_2$→, and side chains of β-D-Gal*f*-(1→ linked to the main chain at the O-3 position of 3,6)-α-D-Glc*p* -(1→, as represented by,



6.3.5 Anti-inflammatory activities of EPS-LM-1 in THP-1 cell culture

6.3.5.1 Suppression of LPS-induced inflammatory responses

As shown in **Fig. 6-7**, EPS-LM-1 significantly suppressed the LPS-stimulated activation of NF-κB (**Fig. 6-7a**), NO production (**Fig. 6-7b**), the release of IL-1β (**Fig. 6-7c**) and IL-10 (**Fig. 6-7d**). At the maximum concentration of 25 μg/mL, EPS-LM-1 decreased to the LPS-stimulated NO by 76.7%, IL-1β by 92.4% and IL-10 by 74.3%. The inhibitory effects of EPS-LM-1 on most of the inflammatory markers followed a dose-dependent trend, giving rise to the minimum effective concentrations (MEC) for 50% inhibition in **Table 6-5**. Compared with the whole EPS from a single-step ethanol precipitation reported previously (Li et al., 2020), all the three MEC values of EPS-LM-1 were notably lower, implying that EPS-LM-1 was much more potent on the inflammatory responses.

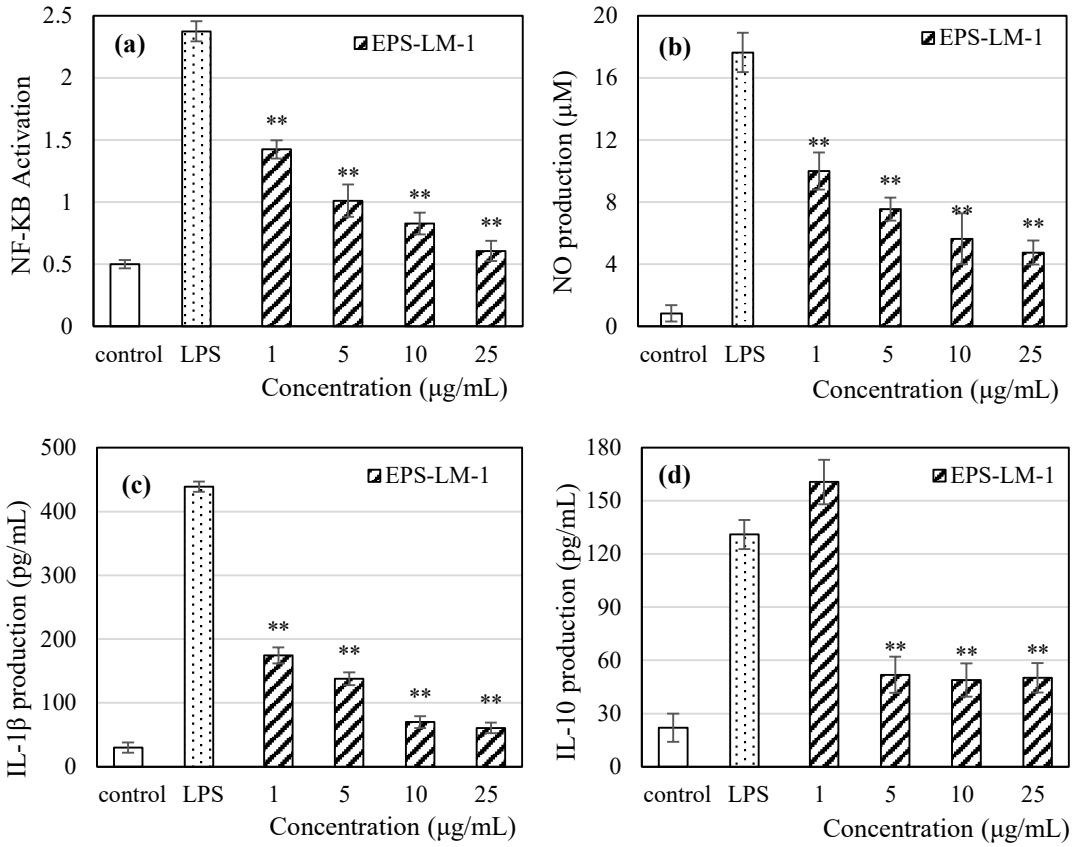


Figure 6-7 Effects of EPS-LM-1 on four inflammation responses on cell in vitro, (a) NO production; (b)NF-κB; (c)IL-1β, and (4) IL-10. Data are presented as mean ± S.D. + indicate the group treated with EPS-LM-1 + LPS together.

Table 6-5 Relative potency indexes of EPS-LM-1 compared with EPS [14] on three major inflammatory markers (MEC: minimum effective concentration for 50% inhibition).

Pro-inflammation factors	MEC (μg/mL)	
	EPS	EPS-LM-1
NF-κB	67.6	4.77
NO	123.3	3.74
IL-1β	183.6	1.90

The NF-κB signaling pathways can be activated by LPS in the granulocytes, monocytes, macrophages and natural killer (NK) cells via up-regulation of the DNA-

binding activity of NF-κB (Choudhury et al., 2014; Mantovani, Allavena, Sica & Balkwill, 2008). NO, IL-1β and IL-10 are important signaling elements for mediation of the host immune and inflammatory responses, especially during infection and trauma (Schepetkin & Quinn, 2006; Moore et al., 2001). Therefore, the experimental results suggest that EPS-LM-1 inhibited the inflammatory NF-κB pathway by regulating the secretion of inflammatory cytokines.

6.3.5.2 Suppression of inflammatory protein expressions by EPS-LM-1

Fig. 6-8 shows the LPS-stimulated expression levels of inflammatory proteins in the NF-κB pathway, including NF-κB, IκBα and phospho-IκBα (NF-kappa-B inhibitor alpha) in THP-1 cells and the effects of EPS-LM-1 treatment. EPS-LM-1 significantly inhibited the phosphorylation and proteolytic degradation of IκBα. Although the expression of IκBα was not notably changed, the expression of p-IκBα was significantly downregulated at sufficient EPS-LM-1 concentrations. Concomitantly, LPS-induced NF-κB activation was strongly inhibited with EPS-LM-1, suggesting that EPS-LM-1 suppressed the LPS-induced inflammatory responses of THP-1 cells by inhibiting the NF-κB pathway.

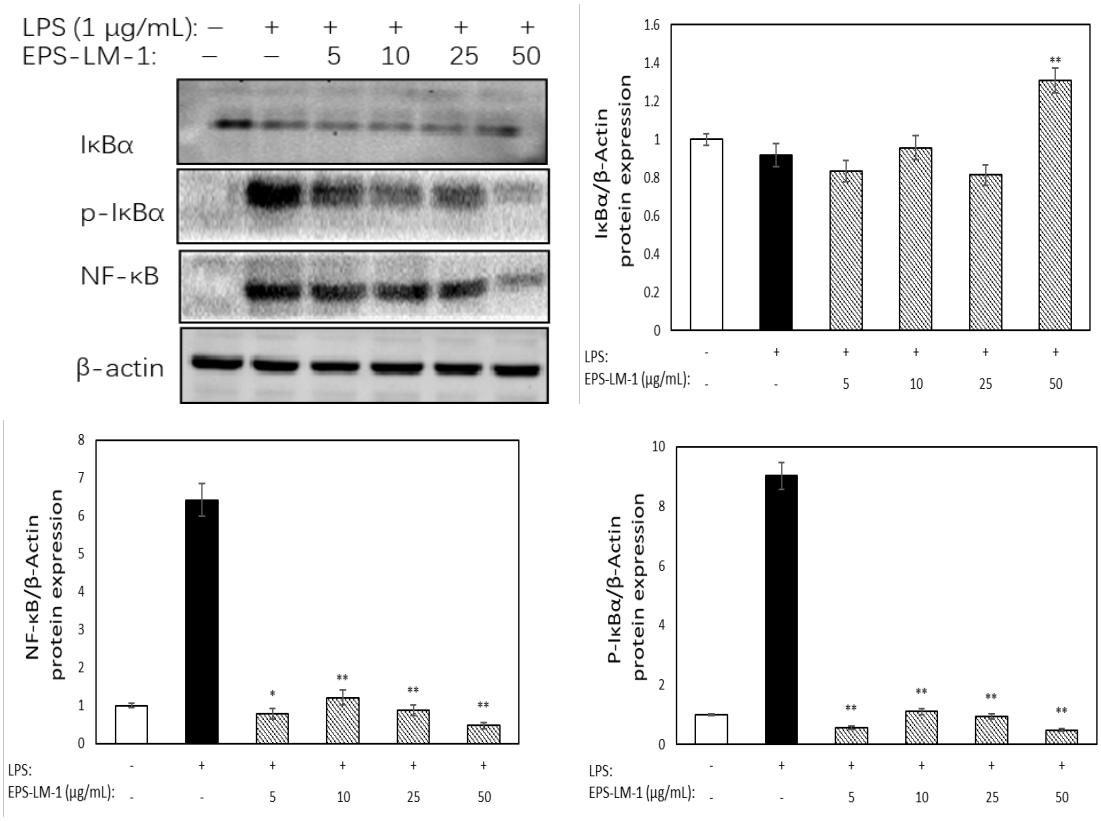


Figure 6-8 Effects of EPS-LM-1 on the activation of the NF -*κ*B signaling pathway in LPS-stimulated THP-1 cells (β-actin: internal reference).

The results from many previous studies in the cell culture and animal models have suggested that homo- and hetero-polysaccharides modulate the cell immunity and inflammatory responses through different membrane receptors, particularly the sugar-rich or glycan binding receptors such as the mannose receptor (MR) to recognize the mannose at the polysaccharide terminals (Ezekowitz, 1990) and a specific galactose lectin on the surface of inflammatory macrophages involved in the interaction of relative inflammatory glycoprotein regulation (Kawakami, et al., 1994; Raes et al., 2005). NF-*κ*B is widely distributed in animal tissues, participating in the transcriptional regulation of many inflammatory genes in response to stimulation, and also plays a key role in a variety of physiological and pathological processes such as host immunity, cell

proliferation and apoptosis (Gilmore et al., 2006; Karin & Greten, 2005). Generally, the inactive form of the NF-κB complex associated with IκBα is silenced in the cytoplasm, which prevents the translocation of NF-κB to the nucleus. However, LPS treatment of the cells triggers the IKK-mediated phosphorylation and degradation of IκBα, leading to the release of the NF-κB complex and its translocation to the nucleus, which ultimately results in the transcription of inflammation-related genes (Ghosh & Hayden, 2008). The results revealed that the upregulated expression of p-IκBα in LPS-stimulated THP-1 cells was significantly inhibited by treatment with EPS-LM-1 in a dose-dependent manner. In addition, the LPS-induced secretion of IL-1β, IL-10, and NO were also markedly reversed by EPS-LM-1. Therefore, it is proposed that EPS-LM-1 inhibited the LPS-stimulated inflammatory responses in the THP-1 cells via suppression of the NF-κB pathway as illustrated in **Fig. 6-9**.

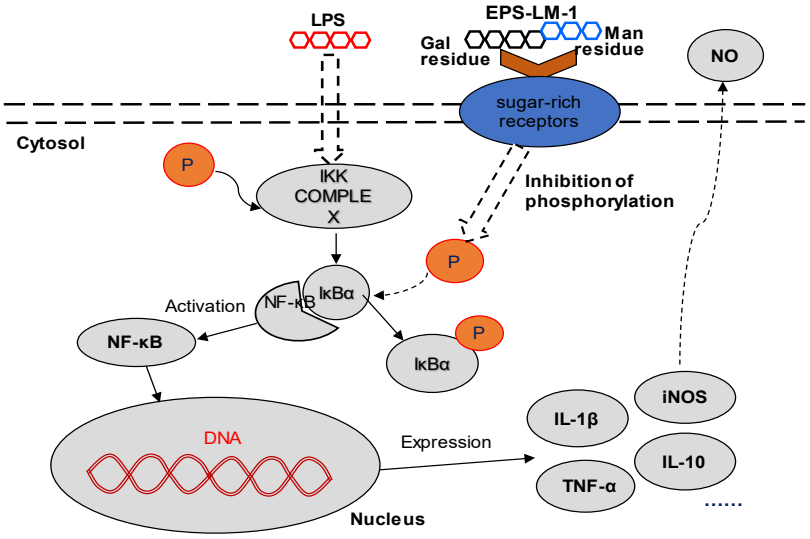


Figure 6-9 Schematic diagram proposing the potential inhibitory role of EPS-LM-1 in inflammatory signaling pathways.

6.3.5.3 Anti-inflammatory activity of EPS-LM-1 versus other polysaccharides

The three EPS fractions isolated from the Cs-HK1 mycelial fermentation liquid showed different levels of inhibition on the LPS-stimulated inflammatory responses (**Fig. 6-3**). The major differences among these three EPS in the chemical composition (total carbohydrate and protein content) and MW range may be responsible for the different activity levels. In comparison with EPS-HM, EPS-LM had a much lower MW and was perhaps more accessible to the cells, to produce a faster and higher activity. Moreover, compared with many polysaccharides from various sources that have been shown active to suppress the LPS-stimulated inflammatory responses in the literature, EPS-LM-1 is among the most potent. For examples, a crude polysaccharide from a cultured *C. sinensis* fungus inhibited the LPS-stimulated IL-1β effectively in human proximal tubular epithelial cells (HK2 cells) at 750 μg/mL (Huang et al., 2018). Lentinan, a well-known fungal β-glucan claimed for notable anticancer and immunomodulation activity, inhibited LPS-induced IL-8 expression in Caco-2 cells significantly at 500 μg/mL (Nishitani et al., 2013). The effective concentration ranges were all much higher than the effective dosage of EPS-LM-1 in the present study (IC50 < 5 μg/mL). This suggests that EPS-LM-1 is significantly more potent and active in anti-inflammation than other crude or purified polysaccharides from edible and medicinal fungi reported previously.

The function and bioactivity of a polysaccharide are dependent upon its molecular structure which is characterized by several factors such as monosaccharide composition, linkage patterns in the main and side chain, and degree of branching. These basic

structural characteristics plus the degree of polymerization, chain length and molecular weight determine the higher-order molecular characteristics and physical properties including conformation and aggregation in solution. Through the present study, the structure of a high-MW fungal polysaccharide EPS-LM-1 has been fully characterized as a branched heteropolysaccharide composed of three monosaccharide residues, glucose mannose and galactose. The results from many studies in the cell culture and animal models have suggested that natural homo- and hetero-polysaccharides modulate the cell immunity and inflammatory responses through different membrane receptors such as toll-like receptors, dectin-1, and complement receptor (Schepetkin & Quinn, 2006). Other specialized receptors have been found in the immune cells, such as the mannose receptor (MR) to recognize the mannose at the polysaccharide terminals (Ezekowitz, 1990) and a specific galactose lectin on the surface of inflammatory macrophages involved in the interaction of relative inflammatory glycoprotein regulation (Kawakami et al., 1994; Raes et al., 2005).

6.4 Conclusion

In the present study, a highly active polysaccharide EPS-LM-1 with a homogenous MW of 360 kDa has been isolated and purified from the crude exopolysaccharide (EPS) produced by Cs-HK1 mycelial fermentation. EPS-LM-1 was a heteropolysaccharide composed of glucose, mannose and galactose residues in the main chain and galactose residue in the side chain. It showed remarkable activity against the LPS-induced activation of NF-κB pathway and the related inflammatory responses in THP-1 cells.

The inhibiting effects were very potent with IC50 values as low as 2-5 μg/mL on some major pro-inflammatory factors including NF-κB, NO and IL-1β. The modulation of the NF-*κ*B signaling pathways by inhibiting the phosphorylation of IκBα has been detected as a possible route for these *in vitro* anti-inflammatory effects of EPS-LM-1. The results from the present study encourage further research and development of the Cs-HK1 EPS as the promising therapeutics for the treatment of inflammation-related diseases. It is imperative to investigate the relationship between the molecular properties such as the MW ranges and the anti-inflammatory activity of EPS and the pharmacological efficacy and effects mechanism of action in animal models.

Chapter 7 Modification and Enhanced Anti-inflammatory Activity by Bifidobacterial Fermentation of Cs-HK1 EPS[3]

7.1 Introduction

Fermented foods are increasingly recognized as healthy or functional foods because of their special health benefits such as anti-hypertensive, anti-inflammatory, immunomodulatory and other activities (Linares et al., 2017). These health-beneficial activities of fermented foods are largely attributed to the presence of probiotic bacteria and bioactive products derived from the fermentative metabolism of food components by the probiotic bacteria. Lactic acid bacteria, especially the *Lactobacilli* and *Bifidobacterium* species are the most important probiotic species in commercial fermented foods.

Some recent studies have attempted to utilize probiotic bacterial fermentation to modify and improve the bioactivities of natural and medicinal products. The water extract of a medicinal fungus *Ganoderma lucidum* showed an enhanced inhibiting activity on LPS-induced NO production and pro-inflammatory cytokines in RAW264.7 cells after sequential fermentation with a *Bifidobacterium* followed by a *Lactobacillus* bacterium (Yang et al., 2015). However, because of the complex crude extract, it was not clear about the molecules responsible for the higher anti-inflammatory activity. The fermentation with a probiotic *Bacillus* bacterium altered the MW and monosaccharide

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composition and enhanced the *in vitro* immunostimulatory activity of a heteropolysaccharide extracted from a Chinese herbal plant of *Dendrobium officinale* (Tian et al., 2019). Similarly, the *Lactobacillus* bacterium fermentation of a polysaccharide from vegetable *Asparagus officinalis* yielded polysaccharides with different molecular properties and enhanced antioxidative, and immunomodulatory activities (Zhang et al., 2018).

Natural polysaccharides (non-starch) from various sources have been shown a variety of health benefits and pharmacological activities (Schepetkin & Quinn, 2011; Lovegrove et al., 2017). As most of these polysaccharides are non-digestible as the dietary fibres, they can reach the large intestine to be metabolized by the gut bacteria. It has been suggested that the beneficial activities of natural polysaccharides are acted through the gut microbiota (Tian et al., 2019; Thongaram et al., 2017). While the specific mechanisms are very complex and unclear, they can be collectively attributed to the prebiotic effects of the polysaccharides which can be selectively metabolized by certain gut bacteria so as to produce a health benefit (Binns, 2013).

Edible and medicinal fungi have been recognized as the attractive sources of prebiotic polysaccharides (Nie, Cui & Xie, 2017). *Cordyceps* (or *Ophiocordyceps*) *sinensis*, an insect-parasitic fungus generally known as the Chinese caterpillar fungus, is a valuable medicinal fungus in traditional Chinese medicine (TCM) (Chen et al., 2013). Since natural *C. sinensis* is very rare and expensive, mycelial fermentation is a more feasible means for production of the fungal polysaccharides and other useful components. Cs-HK1 is a *C. sinensis* fungus and an effective producer of

exopolysaccharides (EPS) in mycelial fermentation. The crude and partially purified EPS fractions isolated from the Cs-HK1 fermentation liquid have shown notable prebiotic (Mao et al., 2018; Song et al., 2018) and anti-inflammatory activities (Li et al., 2020). Very recently, a lower molecular weight (MW) fraction with an average MW of 360 kDa, designated EPS-LM, was separated from the complex EPS, which was mainly composed of three monosaccharide residues including glucose, mannose and galactose (Li et al., 2020). This EPS-LM has been found more active than the whole EPS and the higher MW EPS fraction to inhibit the LPS-induced proinflammatory responses in THP-1 cell culture. More interestingly, the *in vitro* anti-inflammatory activity was notably enhanced after the fermentation of EPS with selected bifidobacterial strains in our preliminary experiments.

In view of the attractive potential for the generation of novel functions and enhanced bioactivities from fermented food and natural products, this study was to investigate the effects of probiotic bacterial fermentation on the molecular properties and *in vitro* anti-inflammatory activity of Cs-HK1 EPS. According to our previous studies, a highly active EPS fraction, EPS-LM, was chosen in this study and fermented by two bifidobacterial strains in a liquid culture medium. The fermented EPS-LM liquid or digesta and its separated fractions were analysed for the chemical composition, and molecular properties and assessed for their inhibiting activity on LPS-inducted pro-inflammatory responses in THP-1 cell culture.

7.2 Materials and methods

7.2.1 Bifidobacterial fermentation of EPS-LM and digesta collection

Bifidobacterial growth medium preparation and fermentation were conducted according to 4.5.1.

Starch-free RCM medium was used in all experiments to avoid the interference of starch with the EPS-LM and its metabolite products. The EPS-LM fraction was dissolved in RCM liquid at 1 g/L final concentration by stirring vigorously for overnight and the solution was sterilized by autoclaving at 121°C for 20 min. To initiate the liquid culture, a starter culture of each bifidobacterial strain was inoculated into RCM broth and incubated for about 18 h. The bacterial cell suspension from the starter culture was inoculated at 2% (v/v) into 10 mL centrifuge tubes, each filled with 5 mL of fresh RCM with or without EPS-LM. The culture tubes were all contained in airtight jars enclosed with anaerobic sachets and incubated at 37°C with constant shaking at 200 rpm for a period of 24 h as reported previously (Mao et al., 2018; Song et al., 2018).

After the 24 h fermentation, the bacterial suspension was collected for analysis and further experiments. The bacterial concentration was determined by measurement of the optical density (OD) at 600 nm with a spectrophotometer. The bacterial suspension was centrifuged at 12,000 rpm for 20 min and the supernatant was collected as the digesta for analysis of soluble metabolite products and for the following experiments on the in vitro anti-inflammatory activity.

7.2.2 Preparation and analysis of Cs-HK1 EPS fractions

Cs-HK1 mycelial fermentation, isolation, and preparation of EPS fractions using the method as described in 4.2.2.

Analysis of EPS composition and molecular weight were performed using the same methods as described in 4.2.4, and the EPS-LM was prepared according to 6.2.1.

7.2.3 Analysis of short chain fatty acids (SCFAs)

As reported previously (Song et al., 2018), SCFAs in the pure bifidobacteria culture were analyzed by gas chromatograph with flame ionization detection (GC-FID) using an Agilent 7980B GC system equipped with a fused silica capillary column (Agilent Technologies Inc., CA, USA). The pure culture supernatant was 5-fold diluted with Milli-Q water and adjusted pH value to 2-3 by 1 M HCl. The 2-ethyl butyric acid was added as internal standard before injection. Nitrogen gas was the mobile phase with flow rate of 0.6 mL/min. The initial oven temperature was maintained at 80°C for 2 min and then raised linearly to 180°C at 6°C/min, and maintained for 4 min. The injection volume was 1 μL and the injection temperature was 200°C. The detector temperature was set at 220°C. The major SCFAs including acetic acid, propionic acid and butyric acid were identified and quantified with the chromatogram of standards.

7.2.4 Fractionation of digesta

For detection of the changes in EPS-LM composition and structure from the

bifidobacterial fermentation and for identification of the major molecular components contributing to the anti-inflammatory activity, the bifidobacterial digesta as well as the initial RCM medium containing EPS-LM was roughly separated into two MW fractions with 10 kDa MWCO ultrafiltration (UF) membrane. The separation was performed using Amicon Ultra centrifugal tubes of 10 kDa MWCO (Millipore Amicon Ultra, Germany) at 4,000 rpm for 30 min at 4℃. The filtrate was collected as the lower MW faction < 10 kDa, while the retentate was diluted with milliQ water, and centrifuged repeatedly in the UF tubes to remove the small molecules more completely. The final retentate was collected as the higher MW fraction > 10 kDa and diluted to the same volume as before separation for the anti-inflammatory test in cell culture.

7.2.5 Analysis of digesta composition and MW profiles

The chemical composition and molecular weight (MW) of digesta with or without the EPS-LM as well as the liquid medium before the bifidobacterial fermentation were determined by the methods as reported previously (Li et al., 2020). The MW distribution and monosaccharides composition was analyzed according to 4.2.4.

Infrared (IR) and NMR spectral analyses were performed of the high MW fractions (> 10 kDa) of the bifidobacterial medium attained from above UF separation. In order to eliminate the interference of glucose and other components, the UF retentate fraction was further purified by chloroform extraction and the aqueous phase was collected, and lyophilized for the spectral analysis. The FT-IR analysis was conducted according to 4.2.4. For the NMR analysis, the purified samples were subject to dissolution deuterium

oxide and lyophilization for twice, and then re-dissolved in deuterium oxide. The NMR analysis was performed on a Bruker Avance-III 400 MHz instrument.

7.2.6 Treatment of on cell culture with different bifidobacterial digesta

7.2.6.1 Cell lines and culture conditions

Cell lines used in this chapter and culture conditions were performed using the same methods as described in 4.2.3.

7.2.6.2 Anti-inflammatory test on THP-1 cell line

Analysis of NF-κB activation, Nitric Oxide (NO) production measurements, and Cytokines IL-1β and IL-10 measurement were performed as previously described in 5.3.3 & 5.3.4.

7.2.7 Statistical analysis

All the microplate assays were performed at least in six replicates and all other experiments were performed in triplicate or more times. Independent samples t-test was performed to determine or compare the statistical significance of the treatment effects. The statistical analysis was performed using the SPSS16.0 statistical software.

7.3 Results and discussion

7.3.1 Effects of EPS-LM on bifidobacterial growth and acid production

As shown in **Table 7-1**, the EPS-LM added to the culture medium had significant

effects on the bacterial growth (with higher OD values) and the acetic acid production of both bifidobacterial strains, *B. breve* and *B. longum*. The results suggested that EPS-LM was utilized by the bifidobacteria as a carbon source for growth and acid production. Acetic acid is one of the major short chain fatty acids (SCFAs) derived from fermentative metabolism of carbohydrates by gut bacteria. In our previous study, the lower-MW EPS fraction as well as the whole EPS could be well utilized, and nearly completely consumed by human gut bacteria during *in vitro* fermentation, resulting in a significant increase in the acetic acid production (Mao et al., 2020). In the human GI tract, non-digestible prebiotic polysaccharides can reach the large intestine and be catabolized by certain gut bacteria, generating SCFAs including acetate, propionate and butyrate which are beneficial to the human health (Fukuda et al., 2011; Peng et al., 2017).

Table 7-1 Bacterial cell growth (OD) and acetic acid production in *B. breve* and *B. longum* cultures after 24 h fermentation with or without EPS-LM in the culture medium.

EPS-LM, g/L	Growth (OD at 600 nm)		Acetic acid (mM)	
	B. breve	*B. longum*	*B. breve*	*B. longum*
0 (control)	0. 52 ± 0.01	0. 53 ± 0.01	48.2 ± 1.0	60.9 ± 1.1
1.0	0. 63 ± 0.03*	0.60 ± 0.01*	66.7 ± 0.8**	68.0 ± 1.6**

Note: * and **: statistically significant different from the control at $p < 0.05$ and $p < 0.01$, respectively. Each data value is represented by mean ± standard deviation (SD) at $n > 3$.

7.3.2 Partial consumption and degradation of EPS-LM during bifidobacterial fermentation

Table 7-2 shows the analytical results of monosaccharides in the bacterial culture medium before and after fermentation (**Fig. 7-1** for the HPLC spectra). For both bifidobacterial strains in the RCM culture medium, there was only consumption of glucose but no consumption of mannose or galactose during the fermentation. While in the RCM+EPS-LM medium, the concentrations of mannose and galactose were significantly decreased after fermentation, as well as the glucose concentration of *B. longum*. This set of experimental results provide more convincing evidence for the consumption and utilization of EPS-LM as a carbon source by the two bifidobacterial strains.

Table 7-3 shows the GPC MW profiles of fresh RCM medium and the bifidobacterial digesta (culture supernatants) after 24 h fermentation with or without EPS-LM (**Fig. 7-2** for the GPC spectra). Because of the highly complex medium composition and limited resolution of GPC analysis, the results are only good for rough and approximate comparison as follows. The higher MW peaks, e.g. 14.5 kD-620 kDa, may be attributed to the peptides and proteins in the RCM components such as beef extract, peptone and yeast extract. In the RCM+EPS-LM medium before fermentation, the peak area (19.3%) corresponding to the maximum MW (~735.7 kDa) was much larger than that of RCM (5.01% at 620 kDa), which may be attributed to the mixture EPS-LM and the high-MW medium components with overlapping peaks on the GPC. After the bifidobacterial fermentation, the top two MW peaks in all culture groups were

all decreased but more notable in the EPS-LM culture groups, e.g. from 620.0 and 193.7 kDa in RCM to 480.4 and 126.9 kDa in *B. longum*-RCM without EPS-LM but from 735.7 and 520.4 kDa to 349.0 and 174.7 kDa in *B. longum*-EPS-LM. The overall shifting of the MW distribution to a lower MW range can be attributed to the partial degradation and consumption of EPS-LM and the higher MW medium components by the bifidobacterial fermentation. The more significant decrease in the higher MW peaks of the EPS-LM culture groups was consistent with the enhanced bacterial cell growth and production of acetic acid by EPS-LM in the culture medium.

Table 7-2 Monosaccharide residues in bifidobacterial medium before and after fermentation (analyzed by HPLC after TFA hydrolysis of the medium samples).

	Glc (g/L)	Man (g/L)	Gal (g/L)
RCM before	4.22 ± 0.10	0.03 ± 0.01	0.18 ± 0.01
RCM after, *B. breve*	1.66 ± 0.01 (consumed 2.56)	0.02 ± 0.01	0.17 ± 0.01
RCM after, *B. longum*	2.20 ± 0.03 (consumed 2.02)	0.04 ± 0.01	0.17 ± 0.01
RCM EPS-LM before	4.70 ± 0.04	0.29 ± 0.02	0.22 ± 0.01
RCM+EPS-LM, *B. breve*	2.07 ± 0.02 (consumed 2.63)	0.15 ± 0.02*	0.18 ± 0.01*
RCM+EPS-LM, *B. longum*	1.63 ± 0.02** (consumed 3.07)	0.07 ± 0.01*	0.10 ± 0.01*

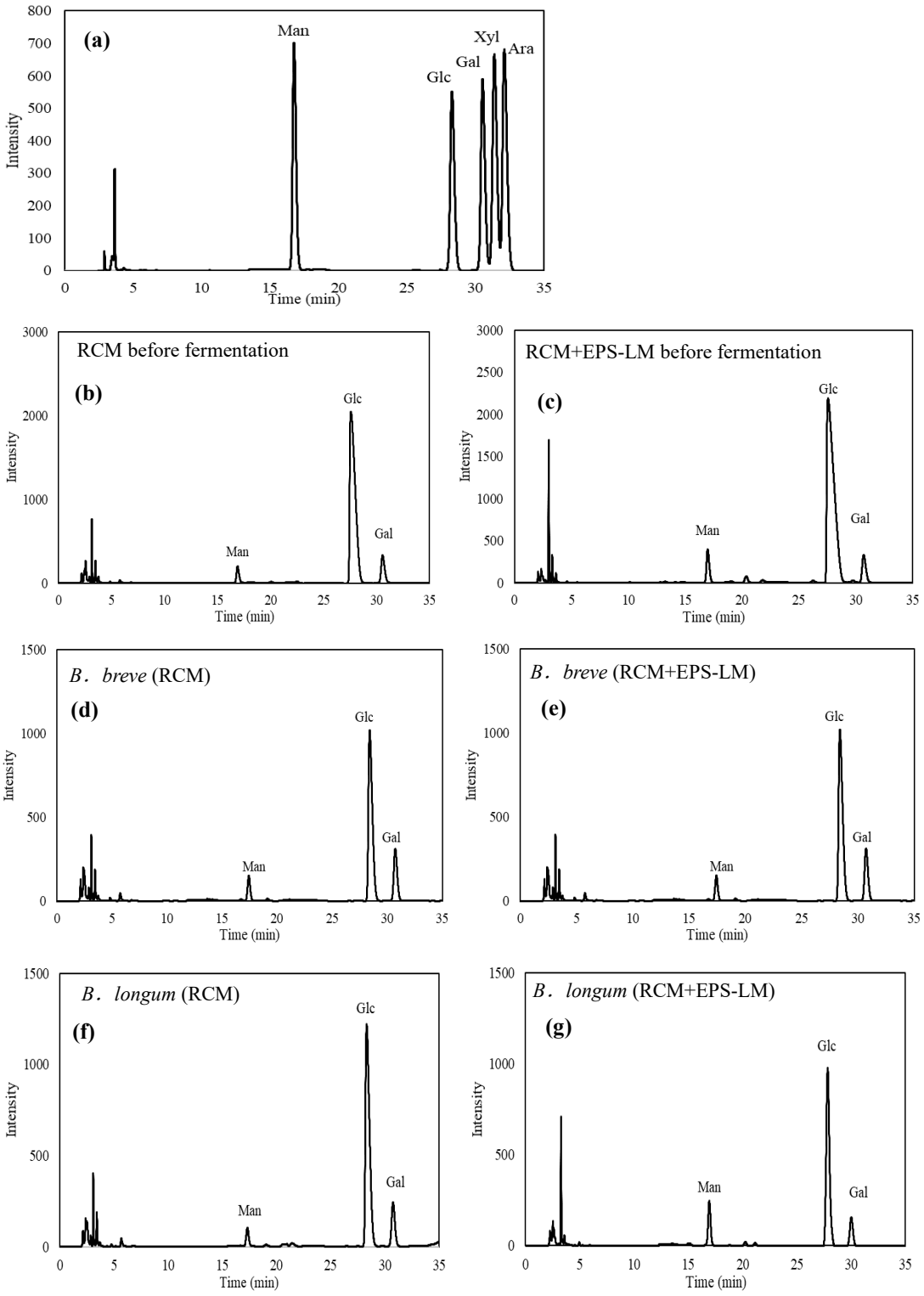

Figure 7-1 HPLC profiles of (a) monosaccharides standards (b) RCM medium before fermentation, (c) RCM+EPS-LM medium before fermentation, (d) *B. breve* fermented RCM supernatants, (e) *B. breve* fermented RCM+EPS-LM supernatants, (f) *B. longum* fermented RCM supernatants, and (g) *B. longum* fermented RCM+EPS-LM supernatants.

Table 7-3 Molecular weight (MW) profiles of bifidobacterial media before and after fermentation (RT: retention time in HPGPC).

	RT (min)	MW (kDa)	Peak area (%)
RCM before fermentation	28.25	**620.0**	5.01
	39.98	193.7	23.6
	44.66	14.5	28.5
	46.02	2.59	9.29
	47.28	1.81	15.2
RCM after fermentation by *B. breve*	28.70	**523.8**	5.83
	41.45	130.5	33.9
	44.25	11.8	27.3
	45.76	3.64	7.49
	46.65	2.37	14.9
	49.00	1.34	12.9
RCM after fermentation by *B. longum*	28.62	**480.4**	5.53
	40.08	126.9	33.9
	42.69	22.6	13.0
	43.65	7.59	18.7
	45.75	3.56	26.7
	53.03	1.11	2.20
RCM+EPS-LM before fermentation	27.67	**735.7**	**19.3**
	36.67	520.4	4.84
	43.65	11.5	57.9
	46.96	2.39	15.1
RCM+EPS-LM after fermentation by *B. breve*	29.05	**419.9**	**9.54**
	40.58	85.4	24.5
	43.57	15.3	20.1
	44.67	4.61	21.7
	47.30	2.73	24.0
RCM+EPS-LM after fermentation by *B. longum*	28.79	**349.0**	**13.6**
	40.23	174.7	18.8
	43.88	12.0	18.1
	45.38	3.56	16.5
	47.96	2.90	28.6

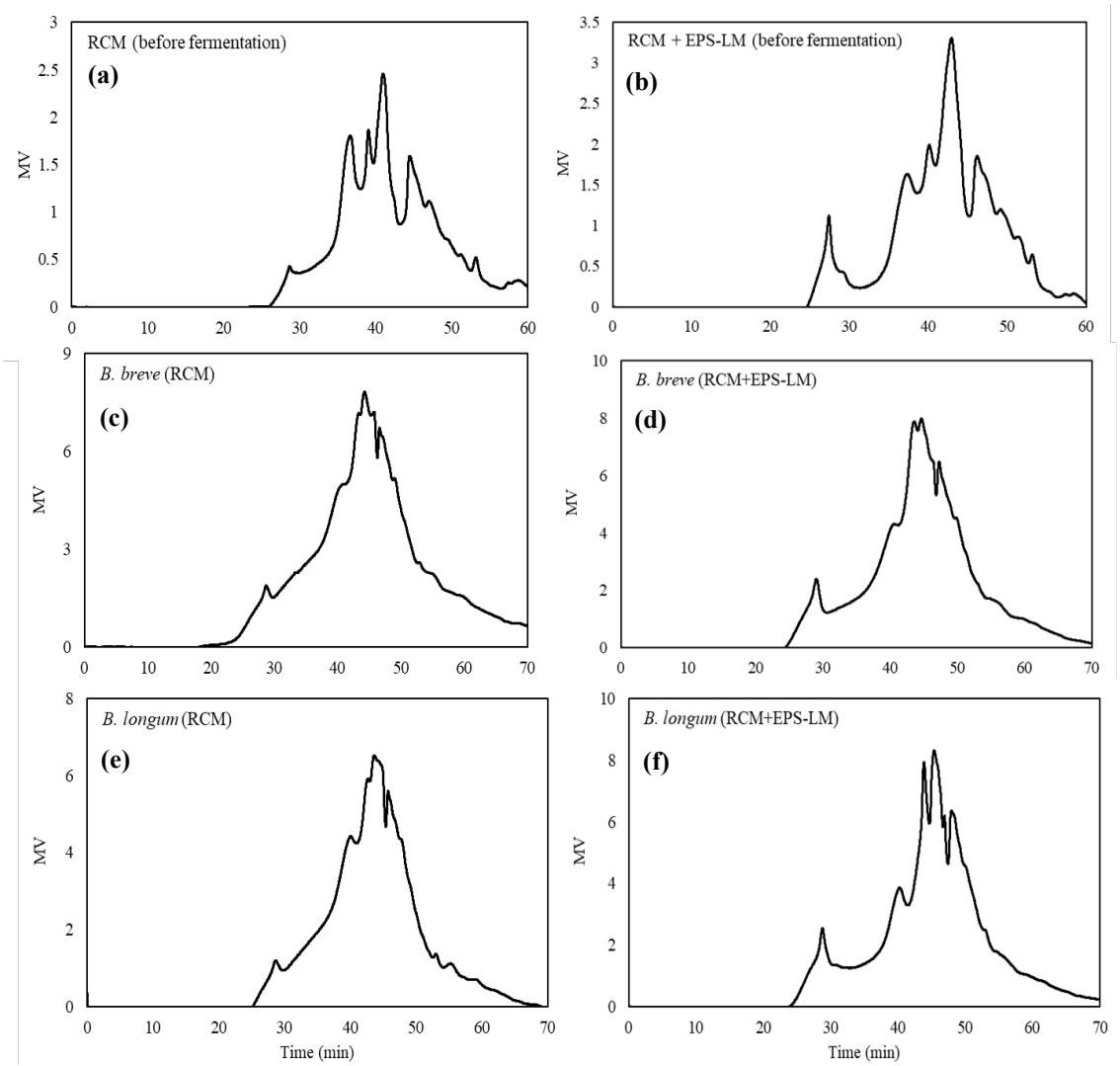

Figure 7-2 GPC profiles of (a) RCM medium before fermentation, (b) RCM+EPS-LM medium before fermentation, (c) *B. breve* fermented RCM supernatants, (D) *B. breve* fermented RCM+EPS-LM supernatants, (e) *B. longum* fermented RCM supernatants, and (f) *B. longum* fermented RCM+EPS-LM supernatants.

7.3.3 Utilization and modification of EPS-LM by bifidobacterial fermentation

Fig. 7-3 shows the IR spectra of the higher-MW fraction (retentate of 10 kDa UF membrane) of bifidobacterial medium and digesta with or without the EPS-LM in the initial medium. As the spectrum of RCM medium was almost flat after purification, all the major peaks in the spectra of RCM+EPS-LM medium or bifidobacterial digesta can be attributed to the presence of EPS-LM or partially degraded EPS-LM. All EPS-LM medium or digesta exhibited the characteristic absorption peaks of carbohydrates around 3400, 2900, 1650, 1412, 1380, 1250, 1042 and 810 cm^{-1}. For example, the absorption peak around 3400 cm^{-1} was attributed to the hydroxyl stretching vibration, the peaks around 2900 and 1650 cm^{-1} were attributed to the C-H expansion ratio and bound water on the sugar chains, respectively. Moreover, the absorption bands at around 1400 cm^{-1} could correspond to the carbonyl C-O stretching vibrations, and the absorption peak around 1250 cm^{-1} demonstrated C=O stretching vibration, respectively (Huang et al., 2018). In addition, the peak at 1042 cm^{-1} and 810 cm^{-1} indicated the pyranose form of sugar and the α-type glycosidic linkages, respectively (Li et al., 2020; Borazjani et al., 2018). Although the IR spectra can hardly tell the structural changes of EPS-LM from fermentation, it is helpful to confirm the presence of polysaccharides originated from EPS-LM in the digesta.

The utilization and modification changes in EPS-LM caused by the bifidobacterial fermentation were further detected by comparing the ^{1}H NMR spectra of the RCM medium with or without EPS-LM before and after the fermentation (**Fig. 7-4**). Before

the fermentation, the first peak on the ^{1}H spectra around 5.3 ppm were attributed to the medium components (**Fig. 7-4a**) and the other two small peaks (peak 1 at 5.11 and peak 2 at 5.13 ppm) were attributed to the C1 position of glucose, both of which overlapped with the C1 position of hexosyl glycosidic residues of EPS-LM fraction with a higher signal intensity (**Fig. 7-4b**). The RCM+EPS-LM medium also contained another C1 position at 5.04 (peak 3) and 4.97 (peak 4) ppm, respectively (**Fig. 7-4c**). After the bifidobacterial fermentation for 24 h, the C1 peaks of these glycosidic pyranosyl residues (peaks 1 and 2) were notably suppressed in all digesta samples, indicating the consumption of glucose constituent as well as the pyranosyl residues of EPS-LM. For the RCM+EPS-LM groups, peak 4 split into 3 new peaks (4.95, 4.97 and 4.98 ppm) in the B. longum digesta (**Fig. 7-4d**), while a new peak 5.16 ppm (peak 5) appeared in the B. breve digesta (**Fig. 7-4e**), which all suggested chain modification of EPS-LM after the fermentation. Moreover, new signals around 4.0-4.1 ppm and 3.6-3.7 ppm conspicuously appeared only in the RCM+EPS-LM digesta (**Figs. 7-4d & f**), which should assign to the protons of hexosyl glycosidic ring from digested EPS-LM residues, respectively (Agrawa, 1992; Cheong et al., 2016). This also indicated the utilization and modification of the constituents of the EPS-LM fraction.

Taken together, the above analytical results confirmed the structure modification and consumption of EPS-LM during the bifidobacterial fermentation. The ability to catabolize non-digestible complex carbohydrates is a major characteristic of *Bifidobacterial* species, though the activity and specificity on specific chain structures and sugar residues are highly strain dependent (Crociani et al., 1994). Since EPS-LM

was only partially fermented and consumed during the bacterial fermentation, the digesta at the end of fermentation contained partially degraded EPS-LM, which probably contributed to the anti-inflammatory activity in the THP-1 cell culture to be assessed in the following experiments.

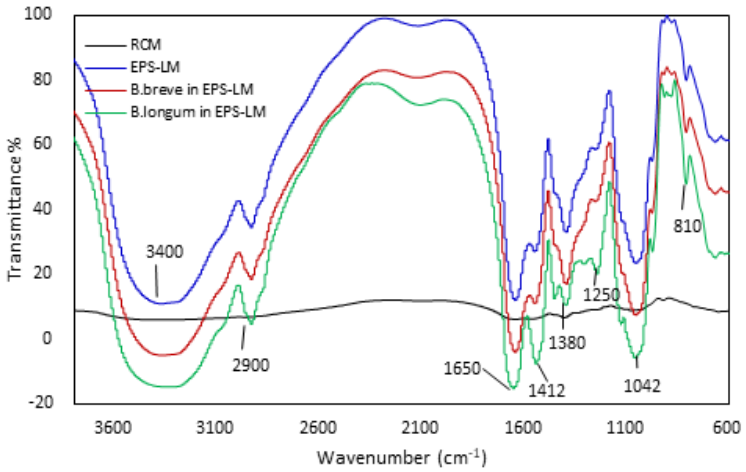

Figure 7-3 FT-IR spectra of RCM and RCM+EPS-LM before, and after fermentation with *B. breve* or *B. longum* bacterium (all medium samples collected from the high MW retentate of 10 kDa UF).

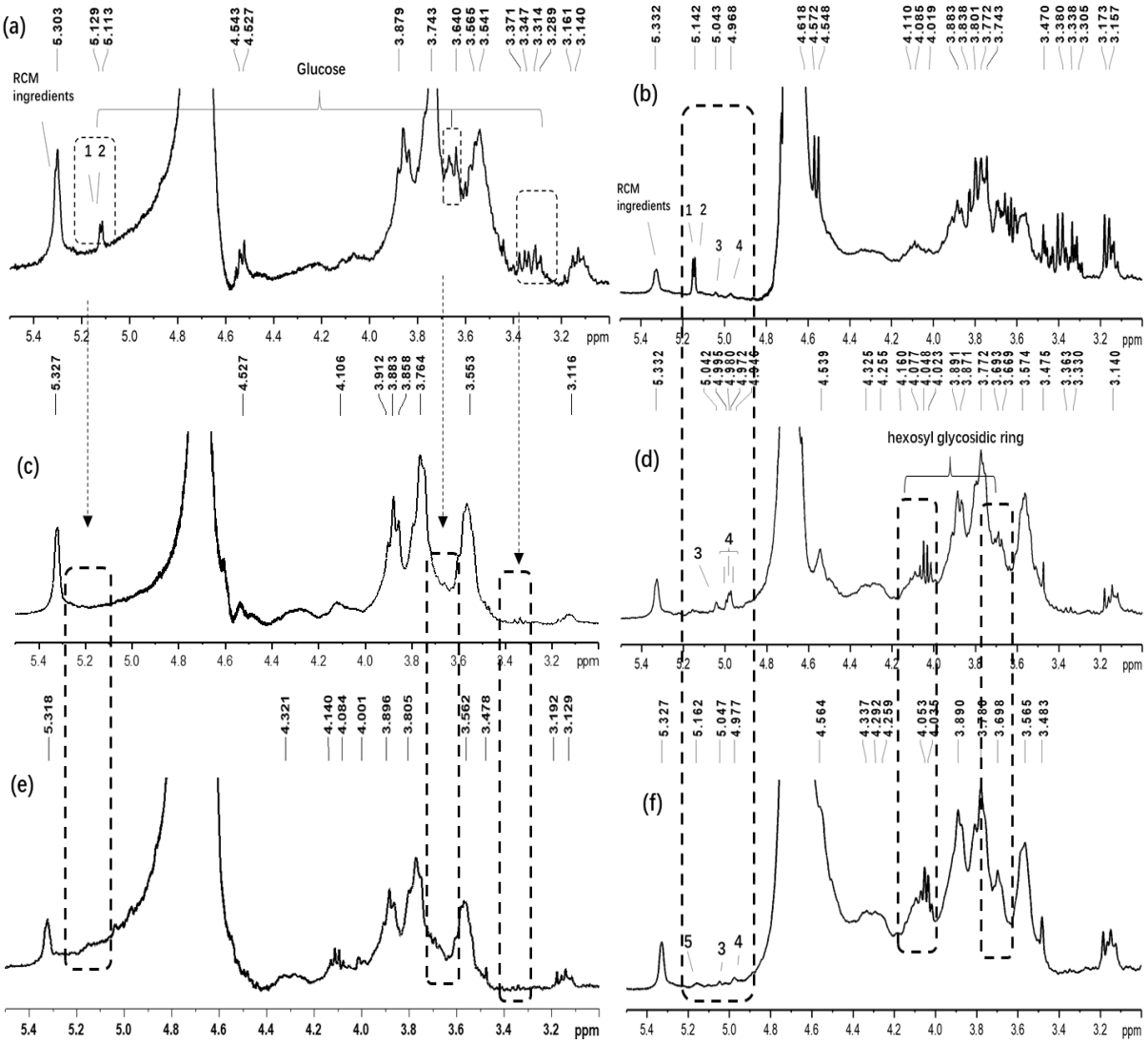

Figure 7-4 ^{1}H NMR spectra of bifidobacterial culture media (> 10 kDa): (a) Fresh RCM; (b) RCM+EPS-LM before fermentation; (c) *B. longum* fermented in RCM; (d) *B. longum* fermented in RCM+EPS-LM; (e) *B. breve* fermented in RCM; (f) *B. breve* fermented in RCM+EPS-LM. The signals were assigned according to Agrawal (1992) and Cheong et al. (2016).

7.3.4 Inhibiting effects of EPS-LM digesta on proinflammatory responses of THP-1 cells

As shown in **Fig. 7-5**, the EPS-LM digesta of both Bifidobacterial strains suppressed the LPS-stimulated proinflammatory responses of THP-1 cells *in vitro* including NF-κB inflammatory activation (**Fig. 7-5a**), nitric oxide (NO) production (**Fig. 7-5b**), release of TNF-α (**Fig. 7-5c**) and IL-8 (**Fig. 7-5d**). In contrast, the RCM digesta (PC groups) at 100 μg/mL had only slight or insignificant effects on these proinflammatory responses. The results confirmed that the fermented EPS-LM and other products derived from the bifidobacterial fermentation of EPS-LM were mainly responsible for the significant *in vitro* anti-inflammatory activity of the bifidobacterial digesta. **Table 7-4** presents the effective doses for 50% inhibition computed from the concentration-dependent effects of EPS-LM digesta in **Fig. 7-5**. For most of the proinflammatory markers except TNF-α, the values of both bacterial digesta were much lower than those of the unfermented EPS-LM, implying that the bifidobacterial fermentation of EPS-LM enhanced the *in vitro* anti-inflammatory activity.

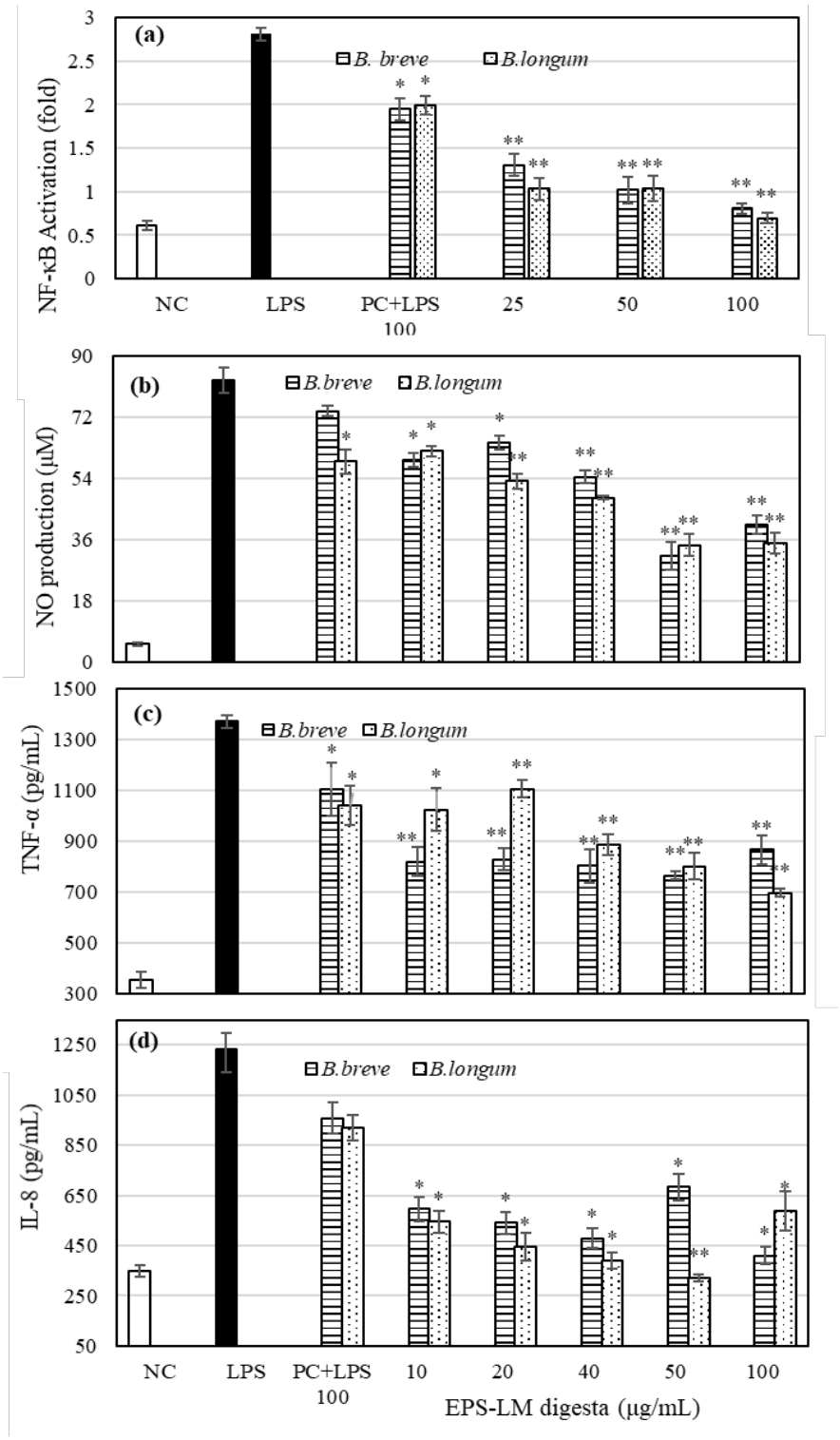

Figure 7-5 Effect of EPS-LM bifidobacterial digesta on LPS-induced responses in THP-1 cell cultures: (a) NF-κB activation; (b) NO production; (c) TNF-α release; (d) IL-8 release. (LPS at 0.2 μg/mL; EPS-LM at 1.0 g/L; PC: positive control with RCM digesta at 100 μg/mL; * and **: statistically significant differences compared with LPS-treated group at $p < 0.05$ and $p < 0.01$, respectively).

Table 7-4 Relative potency indexes of EPS-LM and the fermented EPS-LM digesta on the major pro-inflammatory markers as represented by the effective doses for 50% inhibition in μg/mL of the LPS-induced response (computed by the AAT Bioquest, Inc. Calculator, 2019).

Inflammat. markers	EPS-LM	*B. breve* digesta		*B. longum* digesta	
		Whole	MW > 10 kDa	Whole	MW > 10 kDa
NF-κB	19.84	12.71	3.58	6.29	3.88
NO	34.74	21.18	n.d.	18.76	n.d.
TNF-α	63.75	73.24	30.25	93.79	19.67
IL-8	n.d.	17.33	10.54	17.96	6.01

Note: Digesta, the supernatant medium collected after 24 h culture of each bacterial strain in RCM + 1.0 g/L EPS-LM. The values of EPS-LM were from our previous study (Li et al., 2020).

7.3.5 Identification of the active molecular fractions of EPS-LM digesta

For further identification of the active molecular fractions, the bifidobacterial digesta was separate by a 10 kDa UF membrane into two fractions, MW < 10 kDa and MW < 10 kDa (**Fig. 7-6** for GPC spectra), and **Fig. 7-7 s**how their effects on the LPS-stimulated responses in THP-1 cell cultures. While the MW < 10 kDa digesta fractions showed little or even negative effect (**Figs. 7-7a1-a3**), the MW > 10 kDa digesta fractions of both bifidobacterial strains showed a remarkable activity on the proinflammatory responses (**Figs. 7-7b1-b3**). It is noticeable that the MW > 10 kDa fraction had a strong and notable inhibiting effect on most of the proinflammatory markers at a very low concentration of 5 μg/mL. As shown in **Table 7-4** above, the effective doses for 50% inhibition of the proinflammatory markers of the MW > 10 kDa

fractions were mostly lower than those of the whole EPS-LM digesta.

As predicted from the analytical results of the digesta, MW (**Table 7-3**), monosaccharide composition (**Table 7-2**) and NMR spectra (**Fig. 7-4**), the higher-MW components contained partially degraded EPS-LM and other higher MW metabolite products from the bifidobacterial fermentation. The partially degraded EPS-LM not only had a lower MW but also a modified structure than the original EPS-LM based on the H NMR spectrum analysis. It is envisaged that the partially degraded EPS-LM is a chief contributor to the anti-inflammatory activity of digesta, while contributions from other unknown components are also possible.

A major characteristic of prebiotic fibers including oligosaccharides and polysaccharides is their selective fermentation by certain bacterial species in the gut microbiota which is beneficial to the host health (Holscher, 2017). Furthermore, the beneficial effects of prebiotic polysaccharides the host health can be attributed to the metabolite products derived from the gut bacterial fermentation as postbiotic potential. On the other hand, the selective fermentation of prebiotic carbohydrates is not only beneficial to the host health but also favorable to the growth of probiotic bacteria. *Bifidobacterium* is one of the most important probiotic species and also the most active gut bacteria for fermentative metabolism of complex carbohydrates (Pokusaeva, Fitzgerald & van Sinderen, 2011; Ndeh & Gilbert, 2018). Most of the oligo- and polysaccharides will ultimately be degraded to monosaccharides, and these will be converted to intermediates of the hexose fermentation pathway, also called fructose-6-phosphate shunt or “bifid shunt”. Amaretti et al. (2006) found the substrate preference

of *Bifidobacterium*, where carbohydrate utilization mechanism of *bifidobacterium* on single and mixed carbohydrates substrates were different. The trends of carbohydrate consumption suggested the operation of unusual catabolite regulatory mechanisms, being triggered by exceptionally high concentration of sugars. The preference for utilizing di- and oligosaccharides, rather than monosaccharides, is likely an evolutionary adaptation to an environment that is rather devoid of monosaccharides but rich in complex carbohydrates.

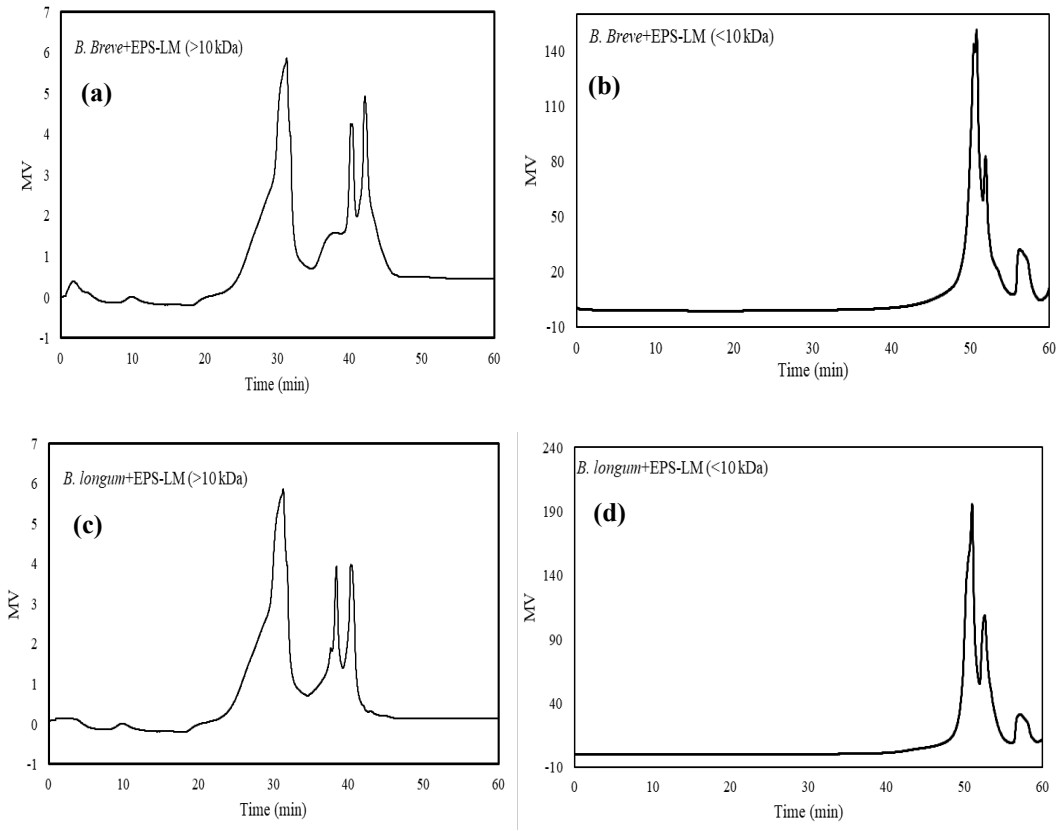

Figure 7-6 GPC profiles of (A) *B. breve* fermented RCM+EPS-LM digesta (> 10 kDa), (B) *B. breve* fermented RCM+EPS-LM digesta (< 10 kDa), (C) *B. longum* fermented RCM+EPS-LM digesta (> 10 kDa) (D) *B. longum* fermented RCM+EPS-LM digesta (< 10 kDa).

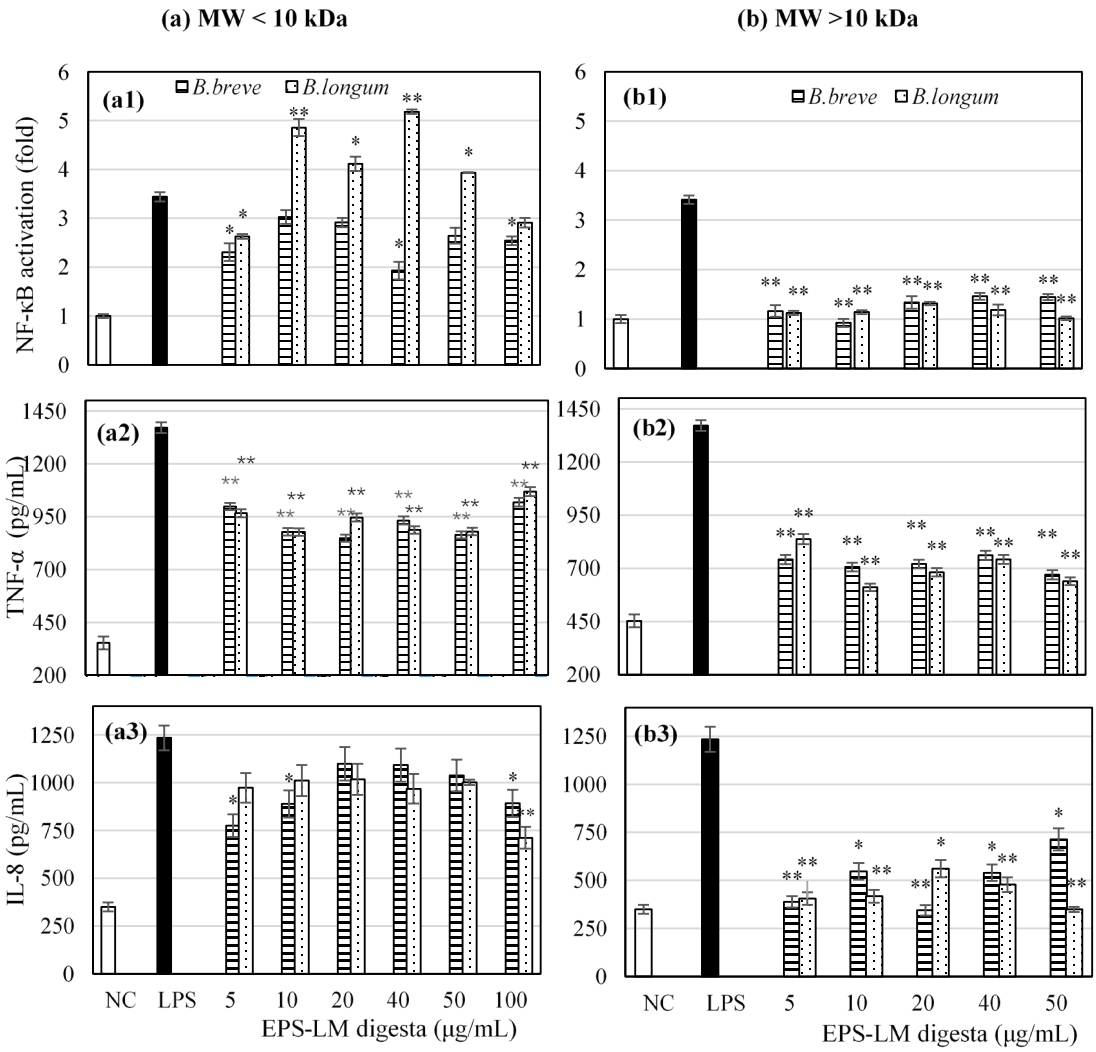

Figure 7-7 Effects of lower-MW (a) and higher-MW (b) digesta fraction separated by a 10 kDa UF membrane on LPS-induced responses in THP-1 cell cultures: NF-κB activation (a1, b1), release of TNF-α (a2, b2) and IL-8 (a3, b3). (1.0 g/L EPS-LM added to RCM medium; * and **: statistically significant difference compared with LPS treated group at $p < 0.05$ and $p < 0.01$, by Student-t test).

7.4 Conclusion

The above experimental results have shown that the EPS from Cs-HK1 mycelial fermentation was partially degraded and consumed as a carbon source by two

bifidobacterial strains, *B. breve* and *B. longum*, for growth and acetic acid production during anaerobic fermentation. The bifidobacterial fermentation modified the structure and reduced the MW of EPS-LM and also enhanced the anti-inflammatory activity significantly. It is of significance to isolate, purify and characterize the fermented EPS-LM for the discovery of novel and active polysaccharide structures and for better understanding of the structure modification mechanism. The present study has demonstrated that bifidobacterial fermentation of bioactive polysaccharides is a promising process strategy for generation of new functional food and therapeutic ingredients.

Chapter 8 Antibacterial Activity of Digesta From Bifidobacterial Fermentation of a Cs-HK1 EPS

8.1 Introduction

The health-beneficial activities of fermented foods are largely attributed to the presence of probiotic bacteria and bioactive products derived from the fermentative metabolism of food components by the probiotic bacteria. Lactic acid bacteria, especially the *Lactobacilli* and *Bifidobacterium* species, are the most important probiotic species in commercial fermented foods and short chain fatty acids (SCFAs) are the most common beneficial metabolites (Guarner & Schaafsma, 1998). Although the microbial composition of human intestinal microbiota changes constantly with age, (Derrien & Vlieg, 2015; Sharma, Mobeen & Prakash, 2018), *Bifidobacterium spp.* are common and important inhabitants of the human intestinal tract throughout life, their metabolites may play a key role on bioactivity and bioavailability modification. Moreover, *Bifidobacterium* can be added to various foods to provide a beneficial effect to the host by improving the intestinal microbial balance (Guarner & Schaafsma, 1998; Isolauri et al., 2002). This beneficial action is the result of several mechanisms such as metabolism of prebiotic and nutrients, production of organic acids, antibacterial synthesis and physical interaction (Loucif & Melzi, 1998; Fukushima, Hoshina & Gomyoda, 1999). Previously work by Kim et al. (2001) revealed that soluble substances present in the metabolites of *Bifidobacterium longum* strains reduced the effect of *E. coli* cytotoxins on mice utilizing intestinal receptor protection. Recently, Ayyash et al.

(2018) showed that bifidobacterial fermentation could enhance the health properties of grains with greater anticancer and antihypertensive activities. These findings are supportive to propose *Bifidobacterium* for technological and therapeutical applications.

Fungal polysaccharides have attracted extensive attention because of their multiple biological activities including antioxidant, antimicrobial, antitumor, anti-inflammatory as well as immunomodulatory activities (Ferreira et al., 2015; Huang, Siu, Wang, Cheung & Wu, 2013; Li et al., 2020). Recent studies have shown that the physicochemical properties and biological activities of polysaccharides are strongly related to the sources, compositions, as well as fermentation products by gut probiotic bacteria (Pallav et al., 2014; Lee et al., 2020). Their relationship with gut microbiota has become a hot topic in the area of macromolecular carbohydrate research. Previous reports have demonstrated exopolysaccharides (EPS) from *Cordyceps sinensis* Cs-HK1 having strong biological activities including anti-tumor, immunomodulatory, anti-inflammatory, and prebiotic activities (Mao et al., 2018; Song et al., 2018), while there is still no reported study on the metabolic impact of Cs-HK1 EPS on *Bifidobacterium.*

Our previous studies have shown that the EPS from *Cordyceps sinensis* Cs-HK1 fermentation had notable protective effects on bifidobacteria against antibiotic inhibition (Mao et al., 2018). However, the information about the influences of EPS on the bifidobacterial metabolites is still limited. In this study, for in-depth research and exploitation of bioactive exopolysaccharides on the bifidobacterial metabolism, the products of EPS co-fermented with bifidobacteria were analyzed, and the potential antibacterial activity of metabolites after fermentation was also assessed. This study

may provide technical support and experimental basis for the further development for the fermented food investigation of the bioactive metabolites of probiotic bacteria in novel carbon sources, as well as to be used as food and pharmaceutical ingredients.

8.2 Materials and methods

8.2.1 Preparation and analysis of EPS fractions from Cs-HK1 mycelial fermentation

Cs-HK1 mycelial fermentation, isolation, and preparation of EPS fractions have been described in section 4.2.2.

Analysis of EPS composition and molecular weight was performed with the same methods as described in section 4.2.4, and the EPS-LM was prepared according to section 6.2.1.

8.2.2 Antibacterial activity measurement

8.2.2.1 Bacteriostatic ring analysis

Escherichia coli (*E. coli*) ATCC 25922, was used to evaluate the antibacterial activity analysis (Meng et al., 2017). Briefly, all the fermented digesta were firstly sterilized by filtration using 0.22 μm Millipore filters. Filter paper disks (diameter: 6 mm) containing 30 μL of samples or penicillin solution (1 mg/mL, positive control) were placed on the surface of agar plates, which were previously seeded by spreading 0.1 mL of *E. coli* bacterial suspension (10^6 CFU/mL) and then incubated at 37°C for 24

h. The antibacterial activity was evaluated by measuring the diameters of the inhibition zones against the test organisms and compared to penicillin and phosphate buffer solution (PBS, negative control).

8.2.2.2 Suspension quantitative bacteriostatic analysis

Suspension quantitative bacteriostatic analysis was conducted according to **WS/T 650-2019** protocol with minor revision. *E.coli* suspension was firstly diluted with PBS to about 5.0×10^5 CFU/mL ~ 4.5×10^6 CFU/mL bacterial suspension for later use. 5.0 mL fermented digesta samples and PBS (control) were first added into sterile test tubes respectively, placed in a water bath at 20 ± 1°C for 5 minutes, and then 0.1 mL bacterial suspension was added and quickly mixed. After 24 h incubation, the mixture of 0.1 mL of all the samples was absorbed and inoculated into agar plates. All the samples were cultured at 37°C for 24 h to observe the final results. The experiment was repeated for at least 3 times and the bacteriostatic rate was calculated according to the standard protocol.

Bacteriostasis rate (%) $=(A_0 - A_1) /A_0*100\%$, A_0: CFU/mL of PBS group, A_1: CFU/mL of sample treatment groups.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) were applied to evaluate the antibacterial potential of bifidobacterial digesta on *E.coli* in microtiter plates as described previously (Cao et al., 2019). In brief, each digesta solution was subject to serial dilution and the diluted solution was transferred at 100 μL aliquots into a 96-well microtiter plate containing

100 μL of PBS in each well. The optical density at 600 nm of *E.coli* cell suspension in the mid-log growth stage was adjusted to 0.1 with fresh RCM medium and the EPS tested, and 100 μL of the standardized bacterial suspension was inoculated into each plate well and incubated for 24 h. PBS was included as a control and bacterium-free PBS as a blank for each plate test. The growth of *E. coli* was assessed in triplicate using a microplate reader at 600 nm. MIC was defined as the lowest concentration of the digesta that completely inhibited the growth of the bacterial inoculum, while MBC was defined as the lowest concentration of the digesta that killed the bacterial inoculum. For the MBC assay, 20 μL of the bacterial suspension from each plate well was inoculated on LB agar and the lowest concentration of a tested antibacterial activity with no visible growth after 24 h incubation was taken as the MBC value. All plate assays were performed in four replicates and the median MIC and MBC values were recorded.

8.2.2.4 Detection of biofilm formation of *E.coli*

The possible mechanism for the infection of pathogens *Escherichia coli* should be biofilm formation (Sharma et al., 2016) to surfaces in culture tubes, microplates as well as cell monolayers. In the tube test, an *E.coli* strain cultured on LB agar plates was inoculated into glass tubes (13 × 100 mm) filled with 4 mL LB broth containing 1 mL bifidobacterial digesta (20%), and 3 mL LB broth containing 2 mL bifidobacterial digesta (40%), respectively. The broth was gently mixed, and 0.6 mL of the broth was used for the microtiter plate test. The tubes containing LB supplemented without bifidobacterial digesta were included in the test as negative control. After incubation

aerobically at 37°C for 24 h, the liquid was removed from the tubes, followed by addition of 2 mL of 0.25% safranin solution into each tube for staining. After removal of the liquid, the tubes were placed upside down at room temperature overnight. The amount of bacterial adhered on the inner tube wall was compared by visible observation and recorded as absent (0), weak (+), moderate (++), or strong (+++).

For microtiter plate test, 200 μL of bacterial suspension from above tube test was filled in each of three wells of a 96-well microtiter plate (for suspension culture) and the wells filled with 200 μL of LB but no bacteria were included as the control. The covered plates were incubated aerobically at 37°C for 24 h, and then the liquid content was removed washed three times with saline. The plates were shaken vigorously to remove all planktonic bacteria. Then 200 μL of 99% methanol was added into each well to fix the attached bacteria. After 15 min, the plates were emptied and dried with a hair drier. Then, 200 μL of 2% crystal violet solution was added to each well for 5 min, rinsed off the excess stain with running tap water. After drying, another 160 μL of 33% (v/v) glacial acetic acid was added to each well to redissolve the dye bound to the adherent bacteria. Finally, OD was recorded with an automated microtiter plate reader at 570 nm. The results of the microtiter plate test were classified into four categories according to Mao et al. (2018).

Caco-2 cell monolayer is commonly used as an *in vitro* model for intestinal epithelium to screen for adhesive strains (Tuomola & Salminen, 1998). Briefly, the Caco-2 cell culture methods and conditions for the assays have been described in detail previously (Mao et al., 2018). For *E.coli* adhesion assays, DMEM without antibiotics

was used in the last two rounds of medium replacement. At 1 h after the last medium replacement, different fermented bifidobacterial digesta were added into the microplate wells with 10 or 20 μL/per well. After 1 h incubation, *E.coli* suspension (10 μL) was added to all the wells, maintaining a multiplicity of infection ratio of 100 bacteria to one Caco-2 cell. Prior to the test, the *E.coli* bacterial suspension was centrifuged and washed for three times with phosphate buffered saline (PBS), and then diluted with DMEM to 5×10^9 colony forming units (CFU)/mL before the following assy. After incubation for 2 h under 5% CO_2 at 37°C, the Caco-2 cells were washed thrice with pre-warmed PBS to remove loosely adherent bacteria and lysed for 10 min with 1% trypsin. Total viable bacteria adherent to or internalized by the cells as well as the initial number of bacteria added were quantified by serial dilution and plating on LB agar. Control cells were treated with the medium alone. The percentage change in the number of viable adherent bacteria was represented by the average CFU in the treated cells/average CFU in the untreated control × 100.

8.3 Results and discussion

8.3.1 *In vitro* inhibition of *Escherichia coli* growth by bifidobacterial digesta

As a potential foodborne pathogen, *Escherichia coli* may cause hemorrhagic colitis and hemolytic uremic syndrome. The low dose of infection and severity of the disease represent a concern to public health (Kaper, Nataro & Mobley, 2004). Therefore,

the growth inhibition of *E.coli* was expressed in terms of the diameter (mm) of the inhibition zones, and penicillin (1 mg/mL) is used as positive control for antibacterial activity comparison. As shown in **Table 8-1**, compared to the PBS treatment group (control), all the digesta showed remarkably antibacterial activity against *E. coli* ($p < 0.001$). For the EPS-LM digesta fermented by *B. breve* (*B. breve* + EPS-LM), the inhibition zones of *E. coli* were 12.67 ± 0.44 mm, which showed significance ($p < 0.01$) compared with *B. breve* digesta group (9.00 ± 0.67 mm). Similarly, the inhibition zones of *E. coli* by *B.longum* + EPS-LM digesta was 13.33 ± 0.39 mm, which was marked significance compared to the *B.longum* group as well ($p < 0.05$). The activity of the digesta in combination against *E.coli* growth in suspension quantitative test was also shown in **Fig. 8-1** and **Table 8-2**. After 24 h incubation, the viability of *E. coli* reduced over 50% of CFU/mL, especially for EPS-LM digesta groups, the fermented digesta showed 89.8% inhibition rate for *B. breve* + EPS-LM digesta and 97.2% for *B. longum* + EPS-LM digesta, which were much more notably higher than the reduction occurred in the RCM digesta groups (47.2% for *B. breve* and 75% for *B. longum*, respectively).

Moreover, **Table 8-2** also presents the MIC and MBC values of *E. coli* strain treatment with different bifidobacterial digesta with PBS and EPS-LM at 1 g/L using as negative control and positive control, respectively. In PBS and EPS-LM alone treatment groups, there is no obvious inhibition effects on growth of *E. coli*, while MIC and MBC values of both RCM digesta and EPS-LM digesta of two bifidobacteria strains can significantly inhibit the growth of *E. coli*. The EPS-LM digesta were much more effective on inhibition of the *E. coli* compared to the RCM digesta groups. The

MIC against *E. coli* was 125 and 250 μg/mL for RCM digesta (*B. breve* and *B. longum*, respectively), while the MIC for the EPS-LM digesta groups against *E. coli* was decreased to 40 and 60 μg/mL, implied that the utilization of EPS-LM by bifidobacteria may generate some potent metabolic factors for the antibacterial activity.

Many microbes produce important metabolites such as acids, alcohols, diacetals and various proteinaceous molecules with antimicrobial potential commonly called bacteriocins secreted by various Gram-positive and Gram-negative bacteria (Desriac et al., 2010; Szkaradkiewicz & Karpinski, 2013). Previously report has demonstrated that bifidobacterial metabolites can protect from enteropathogenic infection (Fukuda et al., 2011). Studies have shown that both probiotics and some prebiotics (polysaccharides) can alter the intestinal microbial balance by blocking adhesion sites, by competition for nutrients and by antimicrobial effects (Meng et al., 2017; Trop, 2014). They directly act by increasing the production of SCFA, which lowers the colonic pH and inhibits the growth of pathogenic bacteria (Jonkers, Penders, Masclee & Pierik, 2012). Moreover, our recent study proved that the potent anti-inflammation potential of EPS-LM digesta is also attributed to the partial utilization and modification of EPS-LM by bifidobacteria strains (Li et al., 2021). Based on the above, some modified EPS-LM fraction may disrupt the permeability of the cell wall and membrane of *E. coli*, which would result in some adverse consequences including a rapid increase in the water-soluble intracellular proteins or bacterial growth inhibition by blocking the import of nutrients (Quan et al., 2015). However, the further antibacterial mechanism of probiotic metabolites combining with prebiotics remains unclear and needs further research.

Table 8-1 Bacteriostasis activity on *E.coli* of EPS-LM digesta (t-test, n > 3).

Groups	Inhibition Diameter (mm)
PBS (control)	0
Penicillin (PC, 1 mg/mL)	35.00 ± 0.67
B. breve	9.00 ± 0.67
B. breve + EPS-LM	12.67 ± 0.44**
B. longum	11.33 ± 0.44
B. longum + EPS-LM	13.33 ± 0.39*

The outside diameter of the cylinder is 78 mm.

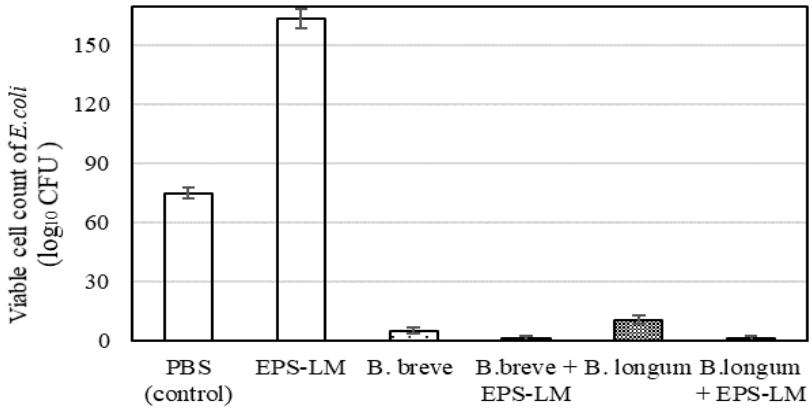

Figure 8-1 Numbers of *E.coli* colony in different bifidobacterial digesta treatment groups after 24 h incubation (CFU: colony-forming unit).

Table 8-2 Bacteriostasis rates and MIC/MBC values (μg/mL) of EPS-LM and its different digesta against *E. coli*.

	Bacteriostasis rate (%)	MIC (μg/mL)	MBC (μg/mL)
PBS (control)	--	--	--
EPS-LM	-123.97 ± 6.8	--	--
B. breve	47.2 ± 8.6	125	500
B. breve + EPS-LM	89.8 ± 2.9**	40	60
B. longum	75.0 ± 1.9	250	500
B. longum + EPS-LM	97.2 ± 1.9**	60	90

8.3.2 Effects of bifidobacterial digesta on adhesion of *E.coli in vitro*

In **Table 8-3**, both tube test and microtiter-plate test showed positive results with moderate or weak adherence ability of *E.coli* strain to the polystyrene surface, while the microtiter-plate test showed more obvious results with rarely adherence of bacteria. The EPS-LM digesta from *B.breve* and *B.longum* could both remarkably inhibit the biofilm formation for *E.coli* by two degrees (from "+++" to "+") at 40% concentration. The decreased biofilm formation of *E.coli* with the addition of 40% concentration of EPS-LM digesta was quite consistent with its antibacterial activity. The different results from tube and microtiter-plate tests indicated that the material property influences the bacteria adhesion. Moreover, the intestinal epithelial cell model established by colonic carcinoma (Caco-2) cells was also used to assess the adherence of bacteria in intestine to mimic the function of large intestine conditions (Parkar et al., 2010).

As shown in **Fig. 8-2**, both RCM and RCM+EPS-LM digesta can sharply decrease the adhesion of *E.coli* to Caco-2 cell monolayer compared to the no treatment group, while the RCM+EPS-LM digesta has more effective inhibition at the same concentration, where only about 8.03% and 9.24% *E.coli* could still attach to the cell monolayer (compared to the control) for EPS-LM fermented *B.breve* and *B.longum* digesta, respectively. EPS-LM treatment probiotic digesta had statistically significant effects on the inhibition of the pathogen adhesion, which is in accordance with the tube test as well as microplate test. Overall, the result suggests that the EPS-LM digesta have the potential ability to inhibit pathogen adhesion to intestinal epithelium.

Table 8-3 Adhesion test of *E. coli*

Concentration of digesta	Tube test		Microtiter-Plate test	
	20%	40%	20%	40%
control	++		+++	
B. breve	++	--	+++	++
B.breve + EPS-LM	+	--	+	+
B. longum	+	--	++	+
B.longum + EPS-LM	+	--	++	+

Note: --: not adherent, +: slightly adherent, ++ moderately adherent, and +++ strongly adherent, compared to the negative control. Cultured for 24 h at 37°C.

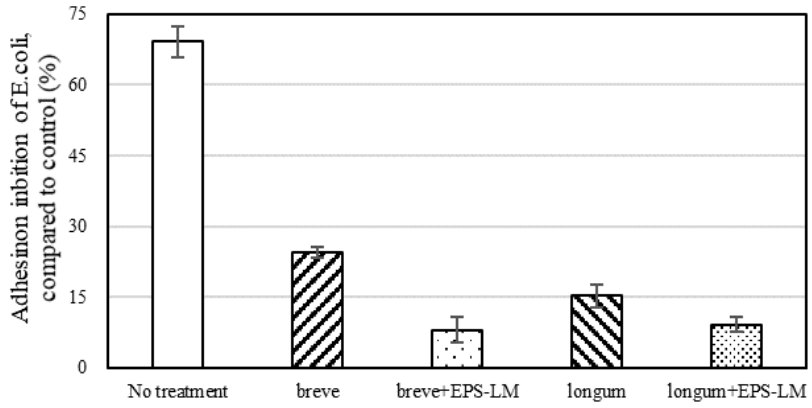

Figure 8-2 Effects of EPS-LM fermented digsta on adhesion and survival of *E.coli* to Caco-2 cells. The cells were pretreated with different digesta for 1h and then incubated with *E.coli* for 2 h. The number of adherent *E.coli* was counted by the plated count method. The values are the mean of triplicates ± SD. The adhesion decreases of bacteria = (average log10 (CFU in treated cells) / average log10 (CFU in untreated control) - 1) × 100.

Studies have indicated that acetate produced by probiotic bifidobacteria improves intestinal defense mediated by epithelial cells and thereby protects the host against lethal infection (Fukuda et al., 2011). Moreover, based on molecular techniques that the polysaccharide can also influence probiotic bacterial surface properties to affect the

adhesion to the intestinal epithelial cell monolayer (Mao et al., 2018; Castro-Bravo et al., 2017), while the promotion of acetic acid and EPS-LM modification were both indicated in our previous results. The potential regulating mechanism of EPS-LM digesta may have distinctive benefits on the modulation of host defense responses and protection against infectious diseases. Another possible mechanism for the inhibition of the growth of *E.coli* by EPS-LM digesta may be decreasing the formation of a viscous layer surrounding the bacterial cell.

8.4 Conclusions

In this study, the potential antibacterial effects of EPS-LM digesta after fermentation by *Bifidobacterium spp.* were investigated. Besides growth enhancement and increasing the acetic acid production previously, EPS-LM may also attribute to the dramatical antibacterial activity against *E.coli* after being modified by two bifidobacteria strains to form the EPS-LM digesta. In addition, the adsorbance ability and biofilm formation effects of EPS-LM digesta against *E.coli* may be partially responsible for the antibacterial activity. The results suggested that natural EPS-LM has regulating potential on probiotic bacteria metabolism, and its metabolites are responsible for promoting the protective effects against the pathogen infection, which provide the industrial potential as functional ingredients for food. Further investigations are required on metabolomics studies to identify the structures of the principal fermentation products for the health-promoting properties.

Chapter 9 General Conclusions and Future Studies

9.1 General conclusions

This research project has accomplished an experimental study on the exopolysaccharide (EPS) from *Cordyceps sinesis* Cs-HK1 fungal mycelial fermentation. The chief findings from this study are summarized below.

1) Crude exopolysaccharides (CEPS) from Cs-HK1 mycelial fermentation were polysaccharide-protein complex, and the EPS contain 78% carbohydrate content and ~ 0.9% protein content after deproteinization. EPS had a very high MW over 10^7 Da, and was mainly composed of glucose, ribose, mannose and galactose at a molar ratio of 2.65:0.76:5.35:1.

2) CEPS and EPS fractions both have remarkable anti-inflammatory activities *in vitro* and *in vivo*. The purified EPS fraction has even higher anti-inflammatory activity and lower inhibition for cell viability. In acute intestinal injury mice model *in vivo*, the gavage administration of EPS to the animals showed a notable effect on suppressing the expression of major inflammatory cytokines and alleviated the intestinal injury.

3) A high anti-inflammatory potential EPS-LM-1 fraction was identified and purified from Cs-HK1 mycelial fermentation. The gradient ethanol precipitation was conducted to get a relative low MW EPS-LM fraction with potent anti-

inflammatory activity, and after purification by different columns, a high purity fraction EPS-LM-1 (360 kDa) was separated with significant anti-inflammatory activity, and the potential mechanism was proved via the modulation of the NF-κB signalling pathways by inhibiting the phosphorylation of IκBα.

4) EPS-LM fraction can be partially utilized by *Bifidobacterium breve* CICC6079 and *Bifidobacterium longum* CICC6186 as a favourable carbon source in mixed carbon sources medium. The EPS-LM had stimulation on two bifidobacteria growth, as well as acetic acid production with statistical significance.

5) The EPS-LM digesta of two bifidobacterial strains showed much more potent anti-inflammatory effects than the polysaccharides alone and RCM digesta, and the highest MW digesta fraction (> 10 kDa) was contributed to its bioactivity. Based on these results, the active EPS-LM polysaccharide should be modified by two bifidobacteria strains to generate the potent anti-inflammatory activity.

6) The EPS-LM digesta possessed notable antibacterial activities than the polysaccharides alone and RCM digesta, indicated the health properties of EPS-LM could be enhanced by probiotic fermentation. The change of metabolites, including SCFA and EPS-LM modification may both attribute to the adsorbance ability and biofilm formation disruption effects of EPS-LM digesta against *E. coli* for its potential antibacterial activity.

9.2 Future studies

The results from this research project provide useful reference and foundation for further study, development and application of fungi EPS with strong anti-inflammation potential as well as prebiotics for modulating the bifidobacterial growth and metabolism. Specifically, the following studies are recommended for future work.

1) Fecal fermentation and *in vivo* tests should be approached to further study for the health promoting function of these active exopolysaccharides (EPS and EPS-LM).

2) Chemical structure of EPS and bifidobacterial modified exopolysaccharides should be further investigated to reveal the interaction between this biopolymer and gut microbiota.

3) Chemical modification of EPS or combination with microencapsulation can be applied to improve the health benefits of EPS on probiotic metabolites. A comprehensive *in vitro* model such as the simulator of the human intestinal microbial ecosystem (SHIME®) and *in vivo* animal model are also suggested to further confirm the modulating function of EPS.

4) Untargeted and targeted time-of-flight mass spectrometry (TOF/MS) can be applied to investigate the potential bioactive metabolite profiles of probiotic bacteria. Since EPS-LM polysaccharide was suggested in this project to play a key on modulating its fermentation and metabolism by *bifidobacterium*, EPS-LM digesta should be further tested on their degradation mechanism as well as metabolites analysis.

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