

Sulfated oligosaccharide of *Gracilaria lemaneiformis* protect against food allergic response in mice by up-regulating immunosuppression

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ARTICLE INFO

Keywords:

Polysaccharide degradation
Sulfated oligosaccharide of *Gracilaria lemaneiformis*
Anti-food allergy
Gut microbiota
Immunosuppression
Regulatory T cells

ABSTRACT

Sulfated oligosaccharide of *Gracilaria lemaneiformis* (GLSO) was prepared from sulfated polysaccharides which possessed antiallergic activity by degradation with high temperature and pressure combined with vitamin C treatment. The present study demonstrated that GLSO could attenuate food anaphylaxis, and inhibit the production of immunoglobulin E, histamine, and related cytokines in both prevention and therapy ovalbumin-induced mice model. Additionally, the gut microbiota analysis revealed that GLSO markedly rescued OVA-induced changes in the *Firmicutes* to *Bacteroidetes* ratio. Following flow cytometry, GLSO was found to suppress the subpopulation of T helper 2 and B cells, and significantly up-regulate regulatory T cells (Tregs) differentiation. Furthermore, GLSO-mediated immunosuppression could be verified by co-culturing Tregs sorted from GLSO-treated mice and CD4⁺ T cells or mast cells. In a word, GLSO attenuated food anaphylaxis through the regulation of gut microbiota and induction of immunosuppression. GLSO had the potential to be used as a nutrient component against food allergy.

1. Introduction

The prevalence of food allergy (FA) has been increasing over the past few decades and is currently a major public health issue worldwide (Du Toit et al., 2018). The prevalence of food allergies in both infants and young children is higher than that of adults, and eggs represent one of the primary foods that can cause food allergies in infants and young children (Dhanapala et al., 2015). A reported survey found that 8.9% of infants could develop an allergic reaction to eggs (Osborne et al., 2011). Four different allergens were found to be the main cause of egg-induced allergies that exist in egg whites, of which ovalbumin (OVA) is the main allergen (Mehmet, 2013).

To date, since there is no effective cure for FA, the current standard of care for patients with FA is complete avoidance of the respective food; however, drug alleviation methods for food allergies often have

shortcomings, especially in infants (Ballmer-Weber & Beyer, 2018). At present, several scientists have turned to research a develop various new, safe, and efficient natural sources of hypoallergenic or anti-allergic drugs or foods (Burks et al., 2018). Marine algae are rich in effective active substances and plays a substantial role in the field of anti-food allergies. It has been reported that marine polysaccharides have anti-allergic properties and their potential application as bioactive food ingredients has been emphasized in addition to nutraceuticals for the prevention of allergic disorders (Vo et al., 2015). Our previous research has also found that sulfated polysaccharides of *Gracilaria lemaneiformis* (GLSP) can prevent the FA response by promoting the secretion of interleukin (IL)-10 and transforming growth factor (TGF)- β in a mouse model of food allergy (Liu et al., 2016).

Compared with polysaccharides, oligosaccharides are more conducive to the absorption and utilization of the body due to their low

Abbreviations: DCs, dendritic cells; FA, food allergy; GLSO, sulfated oligosaccharide of *Gracilaria lemaneiformis*; GLSP, sulfated polysaccharide of *Gracilaria lemaneiformis*; HPT, high temperature and pressure treatment; MACS, magnetic activated cell sorting; MCs, mast cells; mMCP, mouse mast cell proteinase; SEM, scanning electron microscopic; Th, T helper; Tregs, regulatory T cells; VT, vitamin C treatment

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<https://doi.org/10.1016/j.carbpol.2019.115567>

Received 24 August 2019; Received in revised form 27 October 2019; Accepted 1 November 2019

Available online 06 November 2019

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molecular weight and simple structure (Thurl et al., 2017). Previous studies have shown that dietary supplementation with nondigestible oligosaccharides can reduce peanut-induced allergic symptoms (Wagenaar et al., 2018). Several reports confirm that the degradation of macromolecular polysaccharides into low molecular weight oligosaccharides can increase the original biological activity of polysaccharides (Yan et al., 2016; Yao et al., 2016). As demonstrated in our previous report, the working concentration of GLSP (250 mg/kg) for anti-food allergic activity remains substantially high, and methods of improving the anti-allergic activity of GLSP were worthy of further investigation. Thus, GLSP degradation is expected to improve its anti-allergic activity; however, suitable degradation methods for GLSP have not been reported.

FA is typically an IgE-mediated immunoreaction which is induced by an allergen that is activated by anaphylactic mediators (Asero et al., 2007). Moreover, a FA response is often accompanied by intestinal symptoms, and recent research suggests that the gut microbiome plays a central role in regulating food-mediated anaphylaxis (Chua et al., 2018). Thus, dyspeptic fecal microbiota in allergic patients fails to protect against FA (Yamashiro, 2018). Previous reports have found that germ-free mice cannot be orally tolerated to innocuous antigens and have reduced gut IgA and decreased IL-10-producing regulatory T cells (Tregs) (Yamashiro, 2018). Moreover, Treg-mediated secretion of IL-10 and TGF- β can regulate the immune response and play an important role in avoiding an excessive immune response and damaging the body (Johnson et al., 2007). In addition, Tregs play an important role in the immunosuppression of FA responses, and allergen-specific Tregs are thought to control allergic responses by secreting IL-10 and TGF- β , which suppresses IgE production by B cells and decreasing T helper (Th)2 cytokine production; thus, indirectly inhibiting the effector function of mast cells (MCs) and basophils (Fontenot et al., 2003). Previous research has found that GLSP can accelerate IL-10 and TGF- β production (Liu et al., 2016); however, the effects of GLSP and its degradation production on Treg differentiation and function, as well as the gut microbiome, remains unknown.

In the present study, based on the reduced sugar content, the most suitable degradation method for GLSP of the different polysaccharide degradation methods was ensured. The properties and composition of the degradation products were further analyzed. Using an IgE-mediated MCs model and an OVA-induced FA murine model, the anti-allergic activity of the degraded product was investigated and compared to GLSP. Additionally, the potential immunological mechanism of the degradation product was explored by analyzing the gut microbial distribution, measuring the subpopulation of immune cells and related cytokines levels, and co-culturing the target cells with effector cells.

2. Materials and methods

2.1. Materials

The preparation and property analysis of the GLSP degradation products is provided in the Supplementary data section. GLSP degradation production was achieved by treatment with high temperature and pressure treatment (HPT) combined with Vitamin C treatment (VT) and termed sulfated oligosaccharide of *Gracilaria lemaneiformis* (GLSO).

2.2. Nuclear magnetic resonance (NMR) spectroscopy analysis

The 22 mg GLSO sample was exchanged with 0.5 mL deuterioxide. The ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance II 400 MHz spectrometer (Bruker, Billerica, MA, USA) at 25 °C.

2.3. Mast cell models

The rat basophil line (RBL)-2H3 cell model and a mouse bone marrow-derived MC (BMMC) assay were performed as described

previously (Liu et al., 2018). The degranulation inhibition rates were calculated as $100 \times [(\text{DNP-BSA release rate} - \text{Sample release rate}) / (\text{DNP-BSA release rate} - \text{PBS release rate})]$. And the histamine inhibition rates were calculated as $100 \times [(\text{DNP-BSA concentration} - \text{Sample concentration}) / (\text{DNP-BSA concentration} - \text{PBS concentration})]$. The release rate in the formula reflected the data of degranulation, and the concentration was the value from ELISA assay of histamine.

2.4. OVA-induced FA BALB/c model

Female BALB/c mice (6–8 weeks) were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). They were maintained in an SPF environment with a relatively stable temperature ($22 \pm 1^\circ\text{C}$) and humidity ($55 \pm 10\%$) for one week before experimentation. This study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985). Protocol was approved by the Institutional Animal Care and Use Committee of Animal Laboratory Center of Jimei University (Xiamen, China, No. SCXK 2016-0006).

The FA model was induced in BALB/c mice and monitored as previously described (Wang et al., 2016). Briefly, the mice were immunized with 100 μg ovalbumin (OVA, Sigma Aldrich Co., St. Louis, MO, USA) in phosphate-buffered saline (PBS) emulsified with Inject Alum (Thermo Fisher Scientific Inc., Waltham, MA, USA). Mice were challenged starting on day 28 via an oral administration of 50 mg OVA every three days. Anaphylactic symptoms and diarrhea were scored for 1 h after the last challenge (Yamaki & Yoshino, 2012), rectal temperatures were measured for 1 h after last challenge, and sera was collected. In the prevention protocol, 200 μL of the suspension containing GLSP (50, 100, and 250 mg/kg) or GLSO (25, 50, and 100 mg/kg) was intragastrically administered daily from day 27 to day 40. In the therapy protocol, GLSP (50, 100, and 250 mg/kg) or GLSO (25, 50, and 100 mg/kg) was gavaged daily from the fifth to eighth challenges (day 41 to day 49) in mice that had been immunized and challenged five times (Fig. 1). As a native control, the mice were immunized and gavaged with the same volume of PBS. In the murine FA model, six mice were included in each group.

2.5. Hematoxylin and eosin staining

A histological assessment of hematoxylin and eosin sections was performed in a blinded fashion by a pathologist using a previously described scoring system (Nakamura et al., 2012).

2.6. Scanning electron microscopic (SEM) analysis

The integrity of the jejunum villi was observed by SEM according to previous methods (Azumi et al., 2018).

2.7. Gut microbiota analysis

Stool samples from the four groups mice in the therapy protocol were snap-frozen in liquid nitrogen before storage at -80°C . The 16S rRNA gene comprising V3-V4 regions was detected by Shanghai hanyu biotechnology company (Shanghai, China) and amplified using a common primer pair. Then, a microbial diversity analysis was performed as described previously (Sanchez et al., 2017). After removing the unqualified sequences, a total of 1,154,568 raw reads and an average of $48,107 \pm 3010$ reads per sample were obtained. After selecting the effective reads, a total of 823,848 effective reads were generated and each fecal sample produced an average of $34,327 \pm 1515$ effective reads. No samples with a low number of effective reads (< 200) or continuous repeat bases > 8 or fuzzy bases > 0 were observed. High-quality reads were clustered into operational taxonomic units.

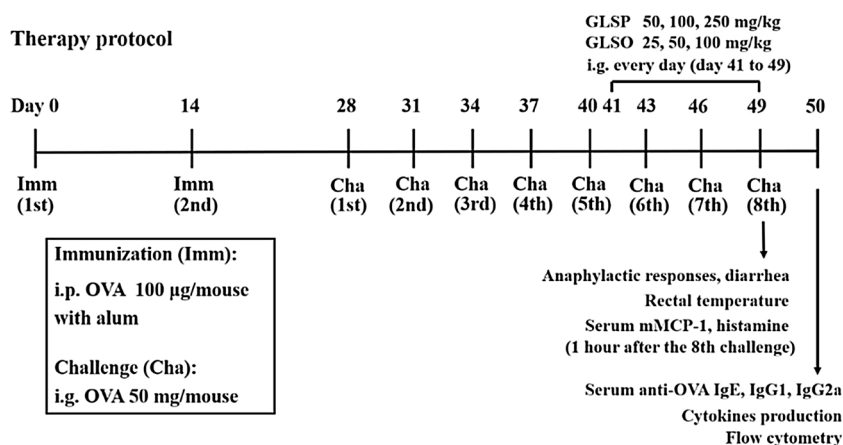


Fig. 1. The therapy protocol of FA mice model.

2.8. ELISA assay

The ELISA methods used for OVA-specific IgE, IgG1, and IgG2a, histamine, mouse mast cell proteinase (mMCP)-1, and cytokines are provided in the Supplementary data section in detail.

2.9. Flow cytometry analysis

The flow cytometry detection and analysis are described in the Supplementary data.

2.10. Cell isolation

According the description in the magnetic activated cell sorting (MACS) system instructions (Miltenyi, Germany), the CD4⁺ T cells and CD4⁺CD25⁺ T cells were sorted and obtained from the mouse splenic lymphocytes.

2.11. Cell co-culture

To isolate Tregs, 3×10^5 CD4⁺CD25⁺ T cells were sorted from the splenic lymphocytes of GLSO-treated mice (100 mg/kg) and co-cultured with 1×10^6 splenic CD4⁺ T cells (pre-stained with CFSE) from either the PBS or OVA mouse spleens in medium supplemented with 1 mg/mL purified mouse anti-CD3 (no azide; low endotoxin; eBioscience) and 100 µg/mL OVA peptide for three days. The percentage of CFSE was tested for the proliferation of CD4⁺ T cells by flow cytometry.

Sensitized RBL-2H3 of 9×10^5 cells were co-cultured with a total of 3×10^5 CD4⁺CD25⁺ T cells for 1 h, then stimulated with DNP-BSA. The β -hexosaminidase release rates were measured to investigate the level of Treg-mediated suppression on MCs.

2.12. Statistical analysis

All experiments were repeated at least three times. The results are presented as the means $\pm$ SD. Differences between the groups were analyzed with a one-way analysis of variance (one-way ANOVA). Differences between the OVA group compared to the other groups were measured using a Duncan's multiple range test. The threshold *p* value for significance was set at 0.05.

3. Results

3.1. GLSO preparation and characterization

Our previous study had reported that GLSP was a polysaccharide which showed 152 kDa of average molecular weight, 98.47 % of total

sugar content, 87.49 % of galactose component, 8.23 % of anhydrogalactose, and 11.07 % of sulfate content (Liu et al., 2016). In the present study, HPT and VT were found to degrade GLSP, the degradation products had lower viscosity and average molecular weight (Table S1). Thus, when HPT was combined with VT, GLSP was successfully degraded to oligosaccharides that had 97.95 % total sugar, 46.52 % reducing sugar, and 13.03 % sulfate radicals (Table S1). The average molecular weight of this degradation product was only 2031 Da (Table S1).

SEM analysis was performed to observe the GLSP microstructures and its degradation product. As shown in Figure S1A, GLSP presented a continuous smooth and flat sheet structure, while the product degraded by HPT combined with VT exhibited a fractured loose structure. In addition, as shown in the Fourier transform infrared spectroscopy (Figure S1B), the GLSP organic functional groups remained largely unchanged by PT combined with VT. Furthermore, the oligosaccharide analysis of the GLSP degradation product derived from HPT combined with VT was performed by ion chromatography. The degradation product was found to contain 64.57 % neoagarobiose, 15.74 % neoagarotetraose, 9.21 % neoagarohexaose, and 7.23 % neoagaroctaose (Figure S1C). The degradation product included a 96.75 % oligosaccharide component and was termed GLSO. Finally, the GLSO transport rate was significantly greater than that of GLSP (45.05 % vs 20.13 %) in the caco-2 cell transport model (Figure S1D). Thus, it was shown that the method of HPT combined with VT could degrade GLSP into GLSO, which had a low viscosity, low molecular weight, and was easily transported.

The ¹H and ¹³C NMR spectra of GLSO were demonstrated in Fig. 2. The chemical shifts from 4.56 ppm to 5.28 ppm in ¹H NMR spectrum (Fig. 2A) were corresponded to anomeric protons that were related with glycosidic bonds, and it suggested that GLSO contains the α -configuration of the pyranose units (Yang et al., 2016). The small shoulder on the signal at 5.28 ppm to the ¹H of AnGalp-2-sulphate linked to Galp (Stephanie et al., 2010). Signal at 4.56 ppm could be due to galactopyranosyl (Anand et al., 2018). The 3,6- α -L-AnGal displayed C-1/H-1 at 4.96/97.94 ppm (Zhao et al., 2017). As shown in the ¹³C NMR spectrum (Fig. 2B), the intense resonance at 71.84 ppm downfield of the unsubstituted-C6 peak of the 3-linked residue, was assigned to C6 of 3-linked 6-MeGal-4-sulphate (Chiovitti et al., 1996). The signal which was observed in the anomeric region at 102.08 ppm was assigned the former to the C1 carbons of 3-linked 6-MeGal-4-sulphate, Gal-4-sulphate, and Gal residues (Chiovitti et al., 1996). The ¹³C NMR spectrum of GLSO had characteristic signal at low field from 160 to 180 ppm, which illustrated it contained uronic acid.

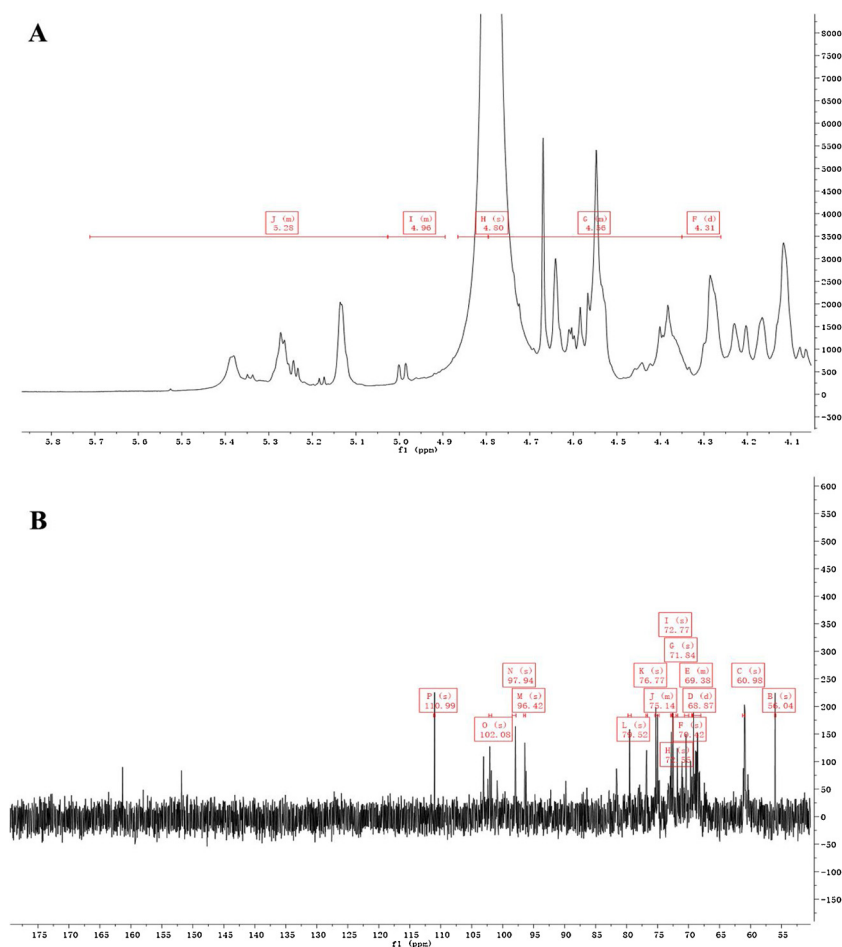


Fig. 2. NMR spectra of GLSO.

A. ^1H NMR spectrum;
B. ^{13}C NMR spectrum.

3.2. Inhibitory effect of GLSO on MCs activation

As an indicator of mast cell activation, the release of β -hexosaminidase and histamine were measured in IgE-mediated RBL-2H3 cells and BMMCs. Consistent with a previous report (Liu et al., 2016), GLSP could inhibit both RBL-2H3 cell and BMMC activation (Table 1). Following treatment with HPT combined with VT, GLSO (100 $\mu\text{g}/\text{mL}$) obtained from GLSP retained this activity, and the inhibitory rates of GLSO on the β -hexosaminidase release was significantly higher than that of GLSP (35.64 % vs 26.53 % in RBL-2H3 cells; 24.98 % vs 18.23 % in BMMCs) (Table 1). The inhibitory rate of GLSO on the histamine level was also greater than GLSP in RBL-2H3 cells (29.68 % vs 21.96 %) (Table 1). It was observed that GLSO obtained after GLSP degradation may have a higher inhibitory activity on MC activation.

3.3. Prevention and therapeutic effect of GLSO on an OVA-induced FA model

Daily doses of GLSP (250 mg/kg) in the prevention protocol (Fig. 3A) strongly decreased the occurrence rate and severity score of the anaphylactic response (Fig. 3B), diarrhea (Fig. 3C), and hypothermia (Fig. 3D). GLSO exhibited similar preventive activity at a concentration of 50 mg/kg (Fig. 3A-3D) in an OVA-induced FA model. However, there was a lack of a remission effect of GLSP in the therapy protocol, and a concentration of 50 mg/kg or 100 mg/kg GLSO could significantly inhibit OVA-induced food anaphylaxis (Fig. 4A-4C). Compared with GLSP, GLSO showed a higher anti-allergic activity *in vivo*, particularly in the therapeutic model.

Food allergic reactions, especially with the occurrence of diarrhea, are often accompanied by inflammation in the intestines (Ballmer-Weber & Beyer, 2018). In the present study, HE staining of the mouse

Table 1
Inhibitory rates of GLSO and GLSP on the MCs activation.

Sample (100 $\mu\text{g}/\text{mL}$)	Inhibitory rates of β -hexosaminidase (%)		Inhibitory rates of histamine release (%)	
	RBL-2H3	BMMCs	RBL-2H3	BMMCs
GLSP	26.53 $\pm$ 2.28	18.23 $\pm$ 1.92	21.94 $\pm$ 3.05	23.83 $\pm$ 0.72
GLSO	35.64 $\pm$ 5.79**	24.98 $\pm$ 2.83*	29.68 $\pm$ 2.73*	24.57 $\pm$ 2.25

Data are shown as mean $\pm$ SD. The *p* value referred to comparisons of GLSO and GLSP group (*: *p* < 0.05, **: *p* < 0.01).

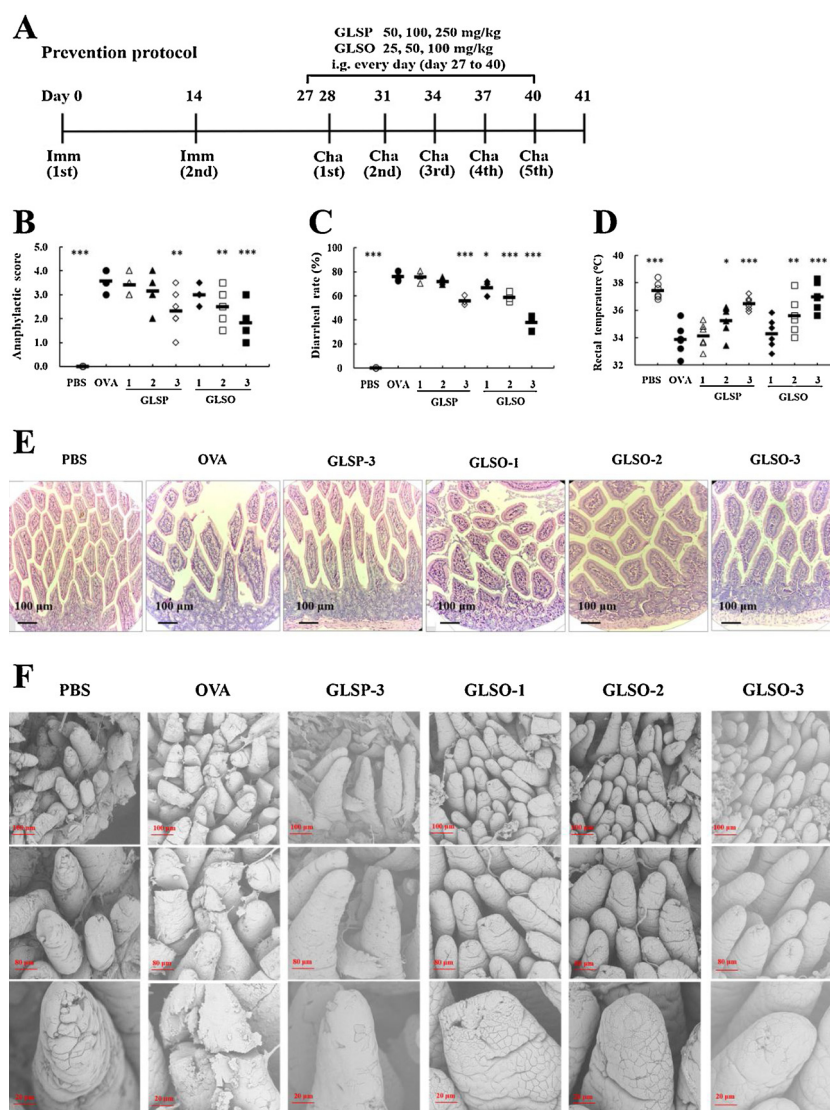


Fig. 3. Prevention activities of GLSO in OVA-induced FA mice.

A: The prevention protocol of FA mice model;

B: Effects of GLSO on the anaphylactic score;

C: Effects of GLSO on the diarrhea rate;

D: Effects of GLSO on rectal temperature;

E: The representative of liver sections in which inflammation was assessed using hematoxylin and eosin staining;

F: Effects of GLSO on jejunal tissue injury by SEM.

GLSP-1, 50 mg/kg; GLSP-2, 150 mg/kg; GLSP-3, 250 mg/kg; GLSO-1, 25 mg/kg; GLSO-2, 50 mg/kg; GLSO-3, 100 mg/kg. The *p* values in B–D referred to comparisons of other groups and OVA groups (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

jejunum tissues and an SEM scan of the jejunal villi were performed to examine the degree of intestinal inflammation in OVA-induced FA mice. HE staining of the jejunum in mice treated with 50 mg/kg or 100 mg/kg GLSO revealed no pathology in the prevention (Fig. 3E) and therapy protocol (Fig. 4D). Compared with the PBS group, the jejunum villi in OVA-sensitized mice were injured, displaying a ruptured surface and increased local damage. However, jejunum villi injury was attenuated by GLSO, in both the prevention (Fig. 3F) and therapy protocols (Fig. 4E). These results suggested that GLSO could effectively protect the intestinal morphology and integrity in OVA-induced FA mice.

3.4. GLSO beneficially alters the FA-type gut microbiota

It has been reported that changes in the composition of the gut microbiota are associated with the development of FA (Abdel-Gadir et al., 2019). To assess whether the gut microbiota was involved in the

GLSO-mediated anti-food allergic effects, we performed a high-throughput sequencing-based assay of bacterial 16S rRNA in the fecal samples from different groups of mice in the therapy protocol. The composition of the gut microbiota using a phylum-level analysis is presented in Fig. 4F. In the PBS group, the most abundant and largest phylum was *Bacteroidetes* and *Firmicutes*, comprising 52.69 % and 44.87 % of the average sequences, respectively. After eight gavages with OVA, although the dominant bacterial community remained unchanged, the abundance of the bacterial community exhibited a significant difference with increased *Bacteroidetes* and reduced *Firmicutes*, and the number of *Proteobacteria* increased to 1.96 %. Compared with the OVA group, the flora of the GLSP-treated mice did not exhibit substantial changes at the phylum-level, but the effects of GLSO intervention were obvious as decreased *Bacteroides* and significant increased *Firmicutes* (Fig. 4F).

At the family-level, the dominant bacteria in the PBS group consisted of *S24-7*, *Clostridiales*, *Lachnospiraceae*, *Rikenellaceae*, and

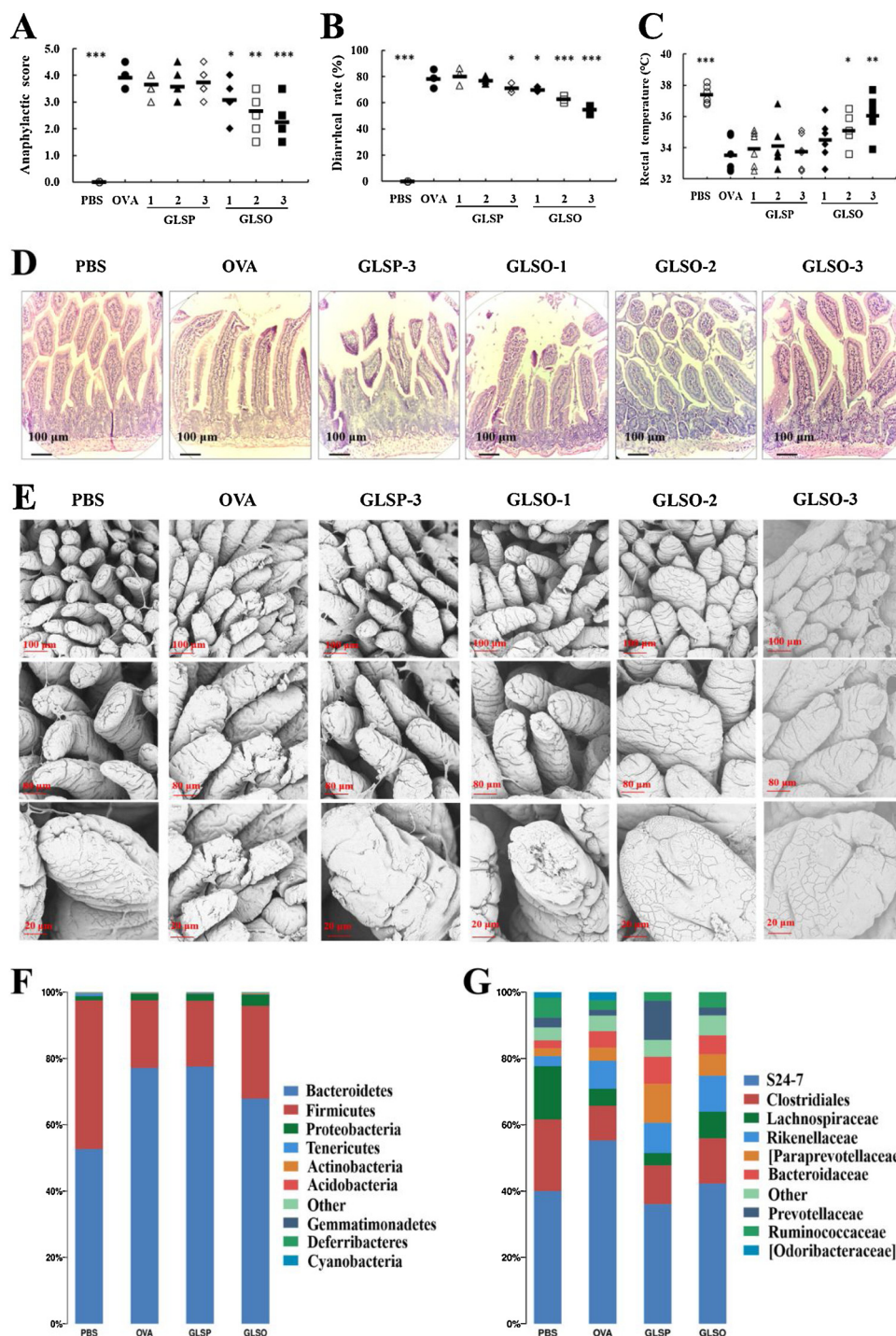


Fig. 4. Therapeutic activity of GLSO in OVA-induced FA mice.

A: Effects of GLSO on the anaphylactic score; B: Effects of GLSO on the diarrhea rate; C: Effects of GLSO on rectal temperature; D: Representative liver sections in which inflammation was assessed; E: Effects of GLSO on jejunum tissue injury by SEM; F: Relative abundance of gut microbiota at the phylum-level; G: Relative abundance of gut microbiota at the family-level. GLSP-1, 50 mg/kg; GLSP-2, 150 mg/kg; GLSP-3, 250 mg/kg; GLSO-1, 25 mg/kg; GLSO-2, 50 mg/kg; GLSO-3, 100 mg/kg. The *p* values in B–D refers to comparisons between the OVA group and the other treatment groups (**p* < 0.05; ***p* < 0.01; ****p* < 0.001).

Ruminococcaceae (Fig. 4G). In the OVA group, the percent of S24-7 flora was increased and the microflora in *Clostridiales*, *Lachnospiraceae*, and *Ruminococcaceae* was reduced. However, the GLSO-treated mice had a significantly different intestinal strain composition compared to the OVA mice but was highly similar to that of the PBS mice. As shown in Fig. 4G, the S24-7 family of microorganisms was similar to that of the PBS group, and there was a greater proportion of *Clostridiales*, *Lachnospiraceae*, and *Ruminococcaceae* microflora in the GLSO group compared to that of the OVA group. Thus, it appears that GLSO could regulate the OVA-induced intestinal microbial imbalance in the mice in the therapy model.

3.5. Effects of GLSO on OVA-specific antibodies, anaphylactic mediators, and cytokines

Mice challenged with OVA exhibited increased levels of OVA-specific antibodies (IgE, IgG1, and IgG2a), anaphylactic mediators (histamine and mMCP-1), inflammatory cytokines (TNF- α and IL-6), and Th2 cytokines (IL-4 and IL-13) (Fig. 5 and 6). The daily administration of GLSO significantly reduced the level of OVA-specific IgE and IgG2a, and decreased the production of histamine, mMCP-1, TNF- α , and IL-6 in both the prevention (Fig. 5) and therapy protocols (Fig. 6). The secretion of Th2 cytokines, IL-4 and IL-13, in the serum was markedly lower in the GLSO-administered mice compared to that of the OVA-induced mice, and the level of the Th1 cytokine, IFN- γ , was moderately

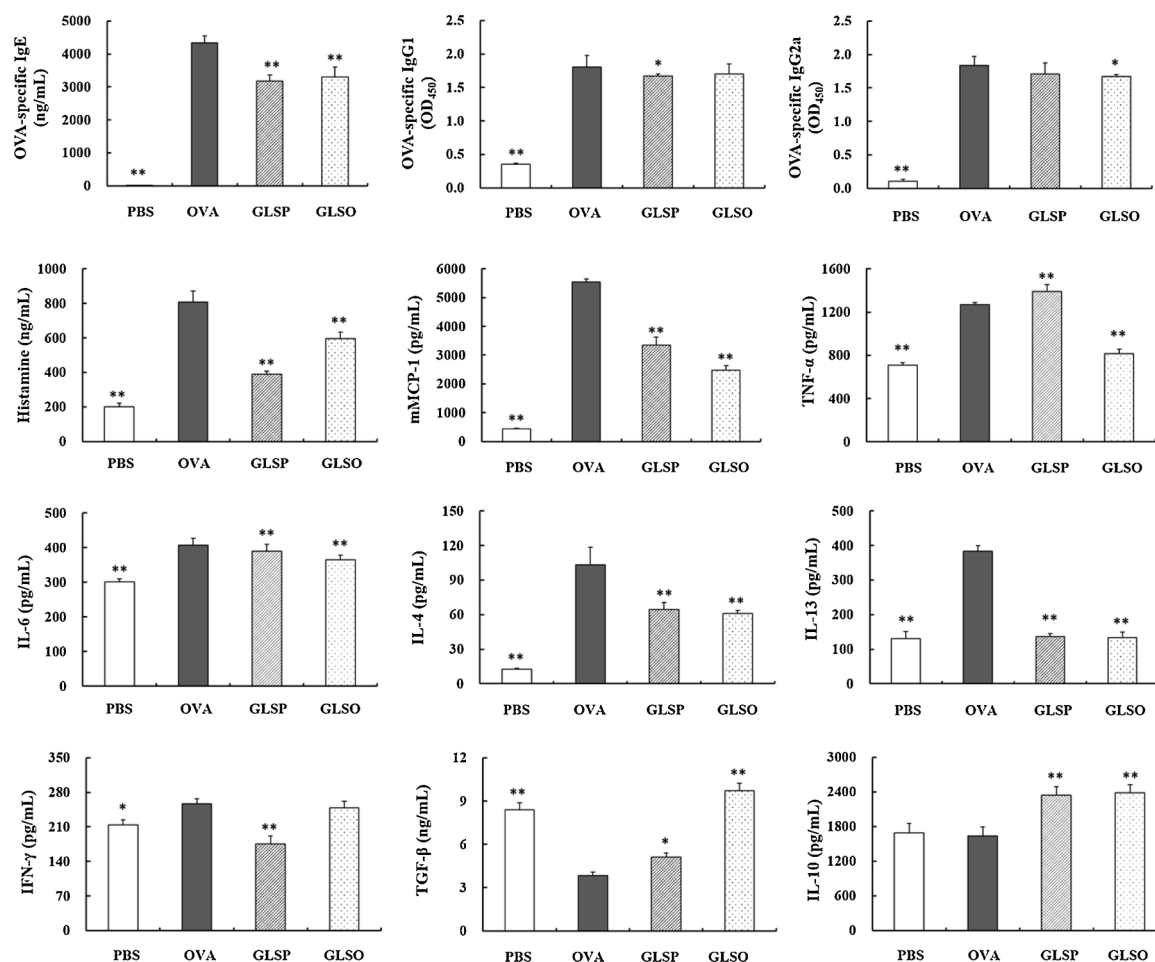


Fig. 5. Effects of GLSO on antibodies, mediators, and cytokines in prevention FA model.

diminished (Fig. 6). In contrast, GLSO could significantly promote the production of the Treg cytokines, IL-10 and TGF- β , in the prevention and therapy protocols (Fig. 5 and 6).

Mice sera were collected at day 40 or day 41 for measuring the levels of IgE, IgG1, IgG2a, histamine, mMCP-1, TNF- α , IL-6, IL-4, IL-13, IFN- γ , TGF- β , and IL-10. The p value referred to comparisons of other groups and OVA groups (*: $p < 0.05$, **: $p < 0.01$).

Mice sera were collected on day 49 or day 50 to measure the levels of IgE, IgG1, IgG2a, histamine, mMCP-1, TNF- α , IL-6, IL-4, IL-13, IFN- γ , TGF- β , and IL-10. The p value refers to the comparisons between the OVA group with the other groups (* $p < 0.05$; ** $p < 0.01$).

3.6. Regulatory effect of GLSO on immune cell subpopulations

To investigate the effect of GLSO on the subpopulation of allergy-related immune cells, mouse splenic lymphocytes were obtained and analyzed for the number of dendritic cells (DCs), T helper (Th) cells, B cells, MCs, and Tregs by flow cytometry. In prevention model, both GLSP and GLSO could induce the percentage of Tregs (Fig. 7A) and regulate the subpopulation of Th cells and B cells (Fig. 7B). As shown in Fig. 8A, while the percentage of CD4⁺CD183⁺ Th1 cells was not affected by GLSO, the percentage of CD4⁺ST2⁺ Th2 cells was significantly reduced in the GLSO-administered mice (Fig. 8B). Additionally, the subpopulation of CD4⁺Foxp3⁺ Tregs was lower in OVA-induced FA mice compared to that in PBS mice (13.14 % vs 17.92 %), GLSO could markedly upregulate the percentage of Tregs (16.08 %) (Fig. 8C). In a general survey of the subpopulation of allergy-related immune cells, it was found that GLSO could inhibit the number of Th2 cells and B cells, and significantly increase the number of Tregs in the

prevention and therapy protocol (Fig. 7A; Fig. 8D). However, the DC and MC subpopulations remained unchanged between the OVA and GLSO groups (Fig. 7B; Fig. 8D).

3.7. Immunosuppression of Tregs from GLSO-treated mice

According to the cytokine measurements and flow cytometry analysis, it was found that GLSO could promote Treg polarization and related cytokine secretion. To further verify the immunosuppressive effect of the Tregs from GLSO-treated mice, CD4⁺ T cells from PBS or OVA mice were isolated by MACS. After culturing the cells for three days, the rates of CFSE in CD4⁺ T cells from PBS or OVA mice were 8.46 % and 55.42 %, respectively (Fig. 9A). However, the percentages decreased to 1.49 % and 5.93 % after co-culturing with Tregs from GLSO-treated mice (Fig. 9A). Three repeated experimental results are presented in Fig. 9B, and GLSO was found to significantly suppress CD4⁺ T cell proliferation. A previous report showed that CD4⁺CD25⁺ Tregs could suppress the activation of MCs (Gri et al., 2008). In the present study, we also investigated the effect of Tregs from GLSO-treated mice on MC activation. It was found that β -hexosaminidase release was inhibited in RBL-2H3 cells co-cultured with Tregs from GLSO-treated mice (Fig. 9C).

4. Discussion

Seaweed saccharides have been emphasized to have potential applications as bioactive food ingredients, as well as nutraceuticals for the prevention of allergic disorders (Vo et al., 2015). Our previous research also found that GLSP and sulfated polysaccharides from *porphyra han-tanensis* could both attenuate food anaphylaxis in mice (Liu et al., 2016;

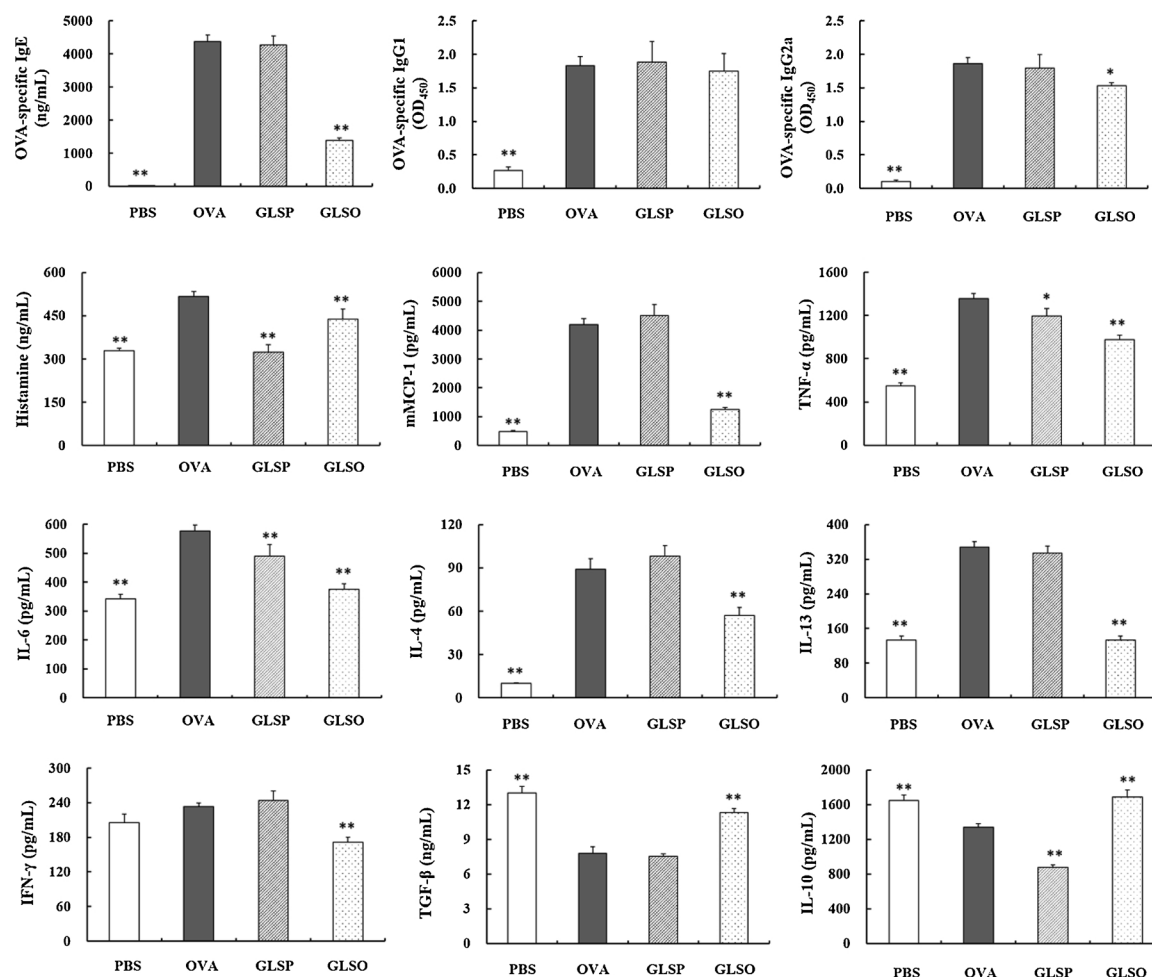


Fig. 6. Effects of GLSO on antibodies, inflammatory mediators, and cytokines in therapy FA model.

Shi et al., 2015). Several studies have shown that the degradation of macromolecular polysaccharides into low molecular weight oligosaccharides can increase the original biological activity of polysaccharides (Xu et al., 2019; Yan et al., 2016). After degrading the water-extractable polysaccharides from the seed of mung beans, the low molecular weight product exhibited a higher immunoregulatory activity (Yao et al., 2016). In the present study, by treatment with HPT combined with VT, which were the appropriate methods for cracking the glycosidic bonds and degrading free radicals by physical action and oxidation, GLSP could be degraded to GLSO. Compared with other degradation methods, HPT combined with VT had the advantages of simplicity, low cost, easy operation, and was suitable for the development of anti-allergic functional foods. By analyzing the suppression effects of MC activation *in vitro* between GLSP and GLSO, the more effective anti-allergic activity of GLSO was found.

FA is an immunologically adverse reaction, which induces a range of IgE-mediated disorders, including anaphylaxis, decreased body temperature, and adverse gastrointestinal reactions (Sampson et al., 2018). Jiang et al. found that chitosan oligosaccharides could exert anti-allergic activity in shrimp tropomyosin-induced FA mice (Jiang et al., 2019). Our previous report also demonstrated the inhibitory effect of *Eucommia cottonii* oligosaccharides on food anaphylaxis (Xu et al., 2017). In the present study, 50 mg/kg GLSO was able to attenuate the symptoms of FA, decrease diarrhea rates, and improve hypothermia in the mice in both the prevention and therapy models. The effective active GLSO concentration was only one-fifth that of GLSP (250 mg/kg), and unlike GLSP, GLSO could also play a role in treating FA. The higher transport ratio of GLSO indicated its higher bioavailability,

which may be a reason for its more effective activity. Therefore, it was shown that both *in vitro* and *in vivo*, the anti-allergic activity of GLSO was superior to that of GLSP.

OVA-induced food anaphylaxis is often accompanied by severe intestinal inflammation (Hussain et al., 2018). As observed in the present study, mice challenged with OVA exhibited inflammation in the jejunal tissue, atrophy and rupture of the jejunal villi, and increased levels of inflammatory factors. GLSO was able to reduce diarrhea rates, suppress intestinal inflammation, and inhibit the release of inflammatory factors in OVA-induced FA mice. Intestinal diseases and immune dysfunction in allergic diseases appear to be related to differences in the function and composition of the gut microbiome (Muir et al., 2016). Through comparing the 16S rRNA sequencing results between patients with intestinal inflammation and healthy subjects, Morgan et al. found that an abundance in *Firmicutes* and *Enterobacteriaceae* was associated with disease status, as expected (Morgan et al., 2012). We also found that the abundance of *Firmicutes* in OVA-induced FA mice was significantly decreased. *Firmicutes* generally account for 40%–60% in normal subjects, including some beneficial bacteria (e.g., *Lactobacillus* and *Lactococcus*). Interestingly, an intervention with GLSO could change the composition of the intestinal flora, particularly inducing an increase in *Firmicutes*. On the other hand, a study by Berni et al. demonstrated that the abundance of *Lachnospiraceae*, *Ruminococcaceae*, and *Rikenellaceae* were significantly increased in the intestinal tract of infants with milk allergies (Berni et al., 2016). In the intestines of OVA-induced mice in the present study, the abundance of *S24-7*, *Lachnospiraceae*, *Ruminococcaceae*, and *Rikenellaceae* were substantially different compared with the PBS-treated mice. However, the gut microbiota composition was

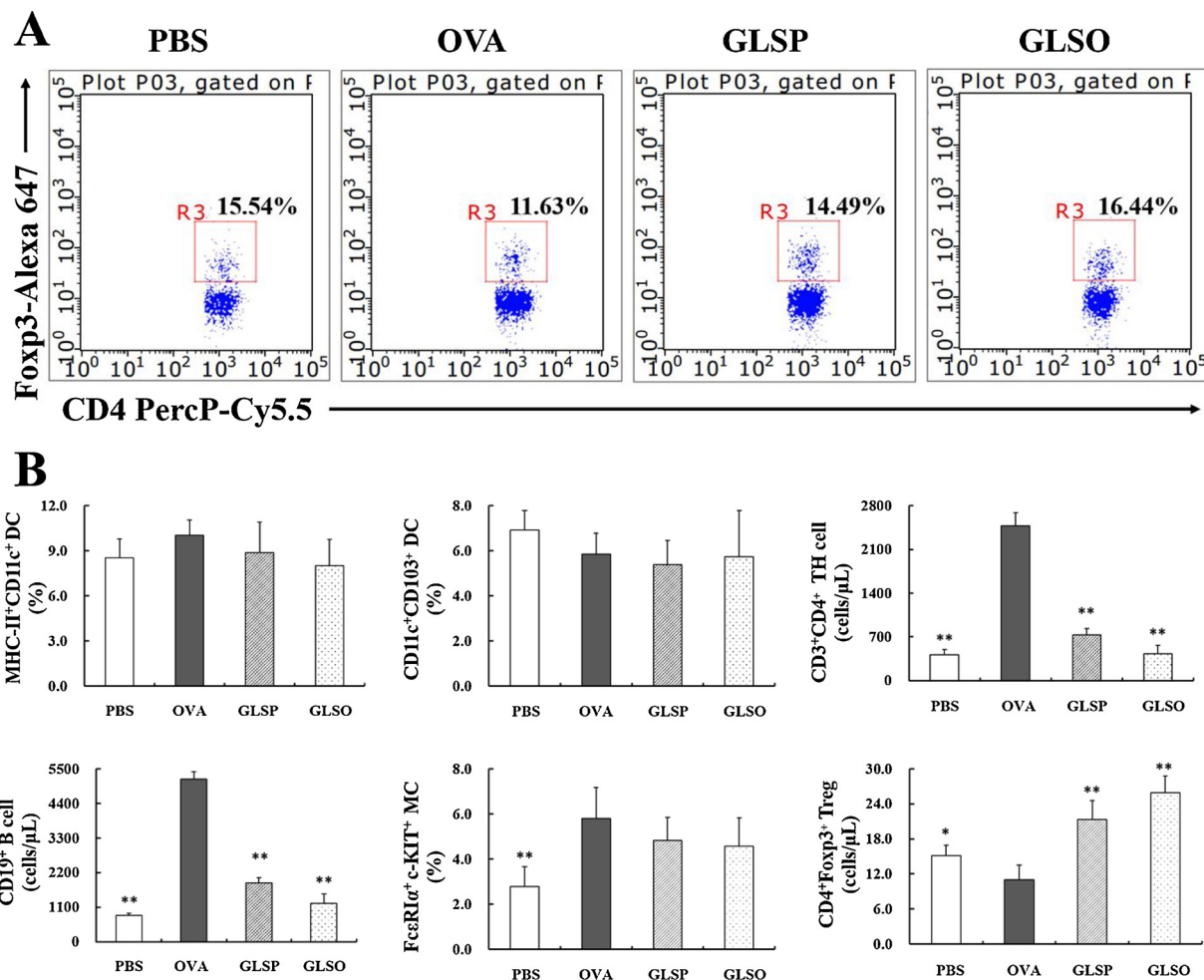


Fig. 7. Effects of GLSO on splenic lymphocytes in prevention FA model.

A: Effects of GLSO on Tregs subpopulation;

B: The statistical analysis of immune cells subpopulation.

The p value referred to comparisons of other groups and OVA groups (*: $p < 0.05$, **: $p < 0.01$).

regulated similar to that of normal levels in GLSO-treated FA mice. Thus, GLSO could promote the normalization of the intestinal flora, which indicated that GLSO could be used as a new nutrient component to prevent or remedy food allergies or other intestinal diseases.

Immune cell subpopulations and associated cytokine production are important foundations for the food allergic response (Satitsuksanoa et al., 2018). Several studies have reported that active oligosaccharides can inhibit allergic reactions by regulating the immune response. For example, Jiang et al. found that chitosan oligosaccharides displayed anti-allergic properties by regulating the level of Th1 and Th2 cytokines (Jiang et al., 2019). In our previous study, *Eucommia cottonii* oligosaccharides were found to reduce the levels of Th2 cytokines and increase the level of Th1 cytokine production in a FA mouse model (Xu et al., 2017). In the present study, cytokines, anaphylactic mediators, as well as allergen-specific antibodies were found to be regulated by GLSO. Moreover, IL-4 has been shown to be a critical cytokine for IgE isotype switching, and IgE is a Th2-dependent antibody (Hussain et al., 2018). Accordingly, GLSO was able to simultaneously suppress the release of OVA-specific IgE and Th2 cytokines (IL-4 and IL-13) in the present study. Further investigation was performed by flow cytometry analysis of mouse splenic lymphocytes. As shown in both the preventive and therapy mouse model, the polarization of B cells that could both produce antibodies and activate T cells, especially Th2 cells, could be significantly decreased via treatment with GLSO.

MCs function as the main effector cells that can release

inflammatory mediators (e.g., histamine and mMCP-1) and guide the development of the allergic response (Olivera et al., 2018). The effects of GLSO on MCs were reflected in the inhibition of MC activation and reduction of histamine and mMCP-1 levels; however, MC differentiation was not affected.

It is now well-accepted that the generation and maintenance of functional Tregs are essential for a healthy immune response to allergens. The effects of Tregs in allergic diseases are diverse, including the inhibition of the activation of effector T cells (Th1, Th2, and Th17), basophils, MCs, eosinophils, and inflammatory DCs, which promotes the generation of tolerated DCs, suppresses the production of IgE, induces IgG4, and inhibits the infiltration of T cells into inflamed tissues, as well as tissue remodeling (Novak Rivas & Chatila, 2016). It has been reported that galacto-oligosaccharides exhibit a protective effect in the prevention of allergic disease by supporting Treg function (Verheijden et al., 2016). A study by Castillo et al. also found that human milk oligosaccharides reduced the symptoms of OVA-induced FA through induction of IL-10⁺ Tregs and the indirect stabilization of MCs (Castillo Courtade et al., 2015). In our present study, the subpopulation of Tregs and related cytokines (IL-10 and TGF- β) were significantly promoted in the GLSO-treated mice. Moreover, Atarashi et al. reported that *Clostridia* species in both mice and humans were potent inducers of colonic Tregs capable of suppressing food allergy (Atarashi et al., 2013). It was speculated that an increase of *Clostridiales* in GLSO-treated mice may induce Treg polarization.

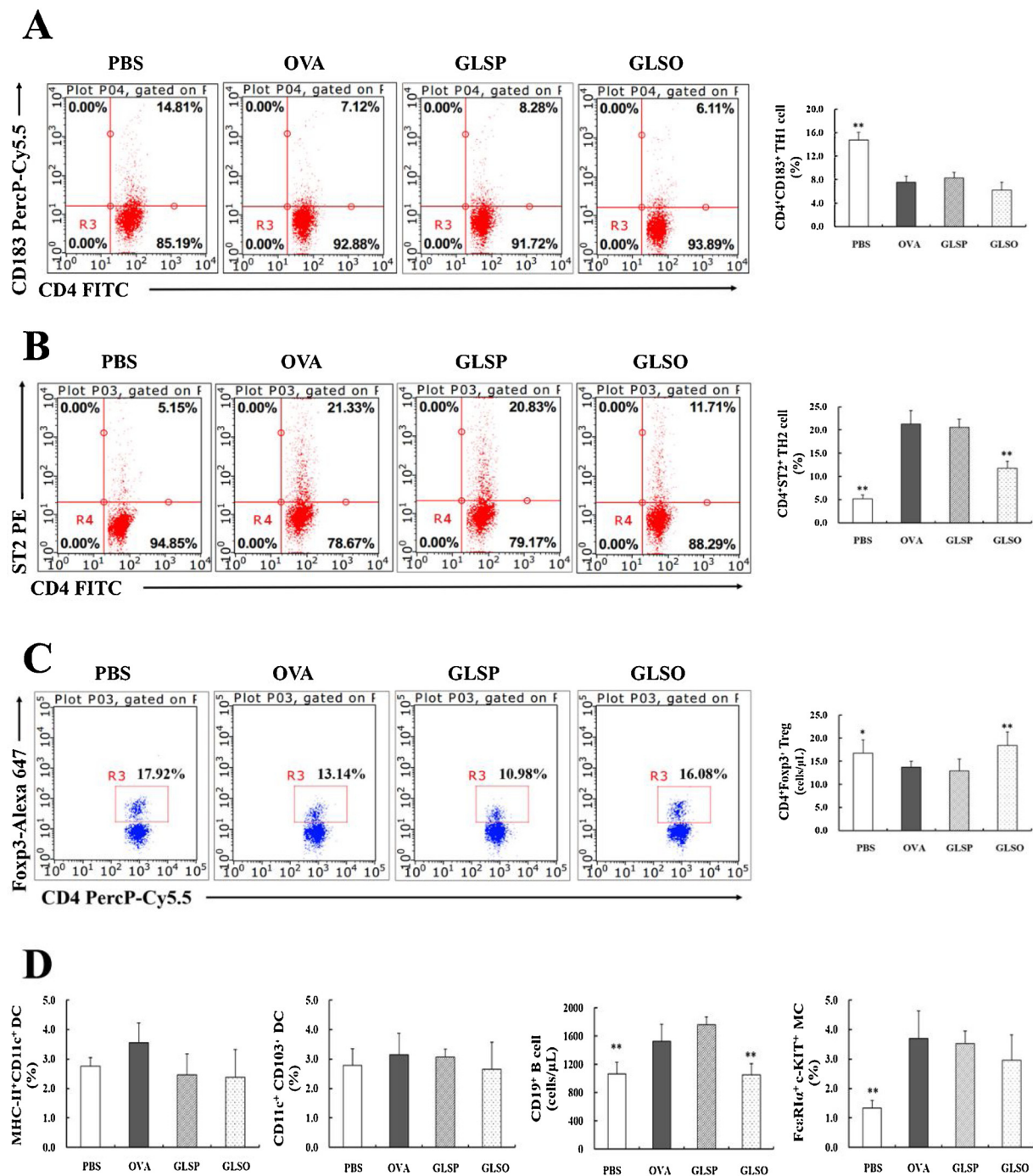


Fig. 8. Effects of GLSO on splenic lymphocytes in therapy FA model.

A: Effects of GLSO on Th1 cell subpopulation;

B: Effects of GLSO on the Th2 cell subpopulation;

C: Effects of GLSO on the Treg subpopulation;

D: The statistical analysis of the immune cell subpopulation.

The *p* value refers to comparisons between the OVA group and the other groups (**p* < 0.05; ***p* < 0.01).

Tregs were considered to be critical cells for the inhibitory activity of GLSO on OVA-induced FA. To further verify the immunosuppressive effect of GLSO, CD4⁺ T cells from the PBS or OVA groups of mice were sorted and co-cultured with the Tregs isolated from GLSO-treated mice. The results revealed that GLSO-induced Tregs could significantly induce the proliferation of CD4⁺ T cells, and corroborated that both the Th2 cell subpopulation and Th2 or Th1 cytokines were restrained by GLSO. Moreover, Su et al. had previously reported that CD4⁺Foxp3⁺ Tregs could inhibit MC function through TGF-β (Su et al., 2012). We also

found that Tregs isolated from GLSO-treated mice could decrease the degranulation of RBL-2H3 cells. The inhibition of GLSO-induced Tregs on the degranulation may explained why GLSO reduced the mediator release without affecting the subpopulation of MCs. In a word, the inhibiting effects of GLSO-induced Tregs on T cells and MCs could reflect the immunosuppression of GLSO *in vivo*. In the future, the anti-CD25 antibody may be able to use for verifying the down-regulation of GLSO *in vivo*.

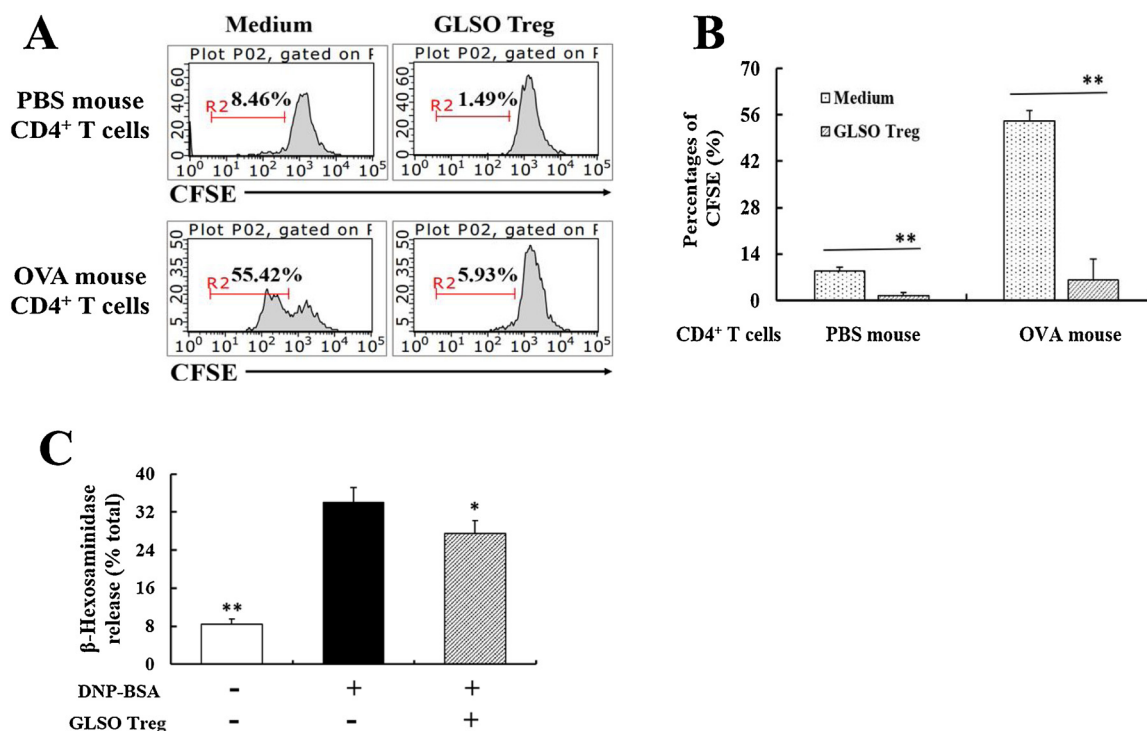


Fig. 9. Immunosuppression mediated by Tregs isolated from GLSO-treated FA mice.

A: CFSE analysis of CD4⁺ T cells by flow cytometry;

B: The statistical analysis of CD4⁺ T cell proliferation;

C: The degranulation measurement of RBL-2H3 cells co-cultured with or without Tregs.

The *p* value refers to comparisons of other groups and groups co-cultured without Tregs (**p* < 0.05; ***p* < 0.01).

5. Conclusions

In conclusion, the present study obtained a low molecular weight and low viscosity oligosaccharide mixture by degrading GLSP. The inhibitory rate of GLSO on MC activation was higher than GLSP *in vitro*. Moreover, GLSO could relieve OVA-induced food anaphylaxis, and inhibit the level of specific-IgE, inflammation, and Th2 cytokines in both the prevention and therapy FA mouse model. Moreover, the gut microbiota disorder observed in FA mice could be balanced by GLSO. Furthermore, GLSO was able to suppress the proliferation of effector T cells and the activation of MCs by inducing Treg differentiation. Collectively, the findings presented here support the potential use of GLSO as a nutritional component for the prevention or treatment of food allergies in humans.

Declaration of Competing Interest

The authors declare no competing financial interest.

Acknowledgements

The study was supported by the grants from the National Natural Scientific Foundation of China (31871720), the science and technology program of Fujian province (2018N5009, 2018R0071), the specific fund project of Xiamen Marine and fishery development (19CZP011HJ08).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.carbpol.2019.115567>.

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Supplementary data

Materials and methods

Preparation and Properties Analysis of GLSP Degradation Production

Sulfated polysaccharide from *Gracilaria lemaneiformis* was obtained according the previous methods (Liu et al., 2016). Using six polysaccharide degradation methods (ultrasonic treatment, high temperature and pressure treatment (HPT, 127 °C, 0.16 MPa, 3 h), H₂O₂ treatment, Vitamin C treatment (VT, 30 mM of Vitamin C), galactosidase treatment, and agarase treatment), the reducing sugar contents of degradation production which treatment with HPT or VT were significantly more than GLSP according 3,5-dinitrosalicylic acid assay (Teixeira, Da, Ferreira-Leitao & Da, 2012). In the present study, the properties analysis of GLSP degradation production included the contents of total sugar, reducing sugar, and sulfate radical, the viscosity, and average molecular weight, according the previous reports (Liu et al., 2016). The microstructure of GLSP and its degradation production was observed by scanning electron microscope, and the infrared spectra of them were collected in a Fourier transformed infrared spectrometer (VECTOR-22, BRUKER) in the wavenumber range of 400-500 cm⁻¹ using the KBr-disk method. The oligosaccharide analysis of GLSP degradation production with HPT combined with VT was performed by Ion Chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) via a Dionex CarboPac PA100 anion-exchange column (4 × 250 mm²) with the standards (mixture of neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagaroctaose (Aglyco, Beijing, China)), and named as sulfated oligosaccharide from *Gracilaria*

lemaniformis (GLSO). The transport of GLSO was measured by a Caco-2 cell model according to the previous methods (Devriese et al., 2017).

ELISA Assay

The sera from mice caudal vein at day 49 were collected to test the levels of histamine and mouse mast cell proteinase (mMCP)-1 by ELISA kit (IBL (Hamburg, Germany), R&D Systems (Minneapolis, MN, USA)). At day 50, 24 h after the last challenge, mice eyeball bloods were obtained and serum was used for detecting the below index. OVA-specific IgE levels were measured according to the instruction of the ELISA kit (Cayman, Ann Arbor, MI, USA). The levels of IgG1 and IgG2a were determined by indirect ELISA as previously reported (Liu et al., 2018). Based on the specification of ELISA kit (R&D Systems, Minneapolis, MN, USA), the cytokines (IL-4, IL-6, IL-10, IL-13, IFN- γ , TGF- β , TNF- α) production were tested.

Flow Cytometry Analysis

Single-cell suspensions were obtained from the spleens of the OVA-induced food allergic mice on Day 50. Cells were stained for surface markers including CD3, CD4, CD19, MHC-II, CD11c, CD103, CD183, ST2, c-KIT, and Fc ϵ RI α (Biolegend, San Diego, CA, USA). If required, cells were fixed and permeabilized with BD fixation/permeabilization reagent, and stained for Foxp3 (BD bioscience, Franklin lake, NJ, USA). Cells were absolute counted in flow cytometry analysis by microspheres (Millipore, MA, USA). Data were acquired with a Guava easyCyte 6-2L

45 system, and analyzed by ACS Paragon Plus Environment Guava Soft 3.1.1 software
46 (Millipore, MA, USA).

47 **Table and Figure Legends**

48 **Table S1 The properties analysis of GLSP degradation products**

49 HPT: High temperature and pressure treatment; VT: Vitamin C treatment

50

51 **Figure S1. The property analysis of the GLSP degradation products**

52 A: SEM analysis of the GLSP degradation products;

53 B: Fourier transform infrared spectroscopy analysis of the GLSP degradation products;

54 C: Ion chromatography analysis of GLSO;

55 D: Transport rates of GLSP and GLSO.

56 The p value in D refers to comparisons between the GLSO and GLSP groups ($^*p < 0.05$; $^{**}p <$

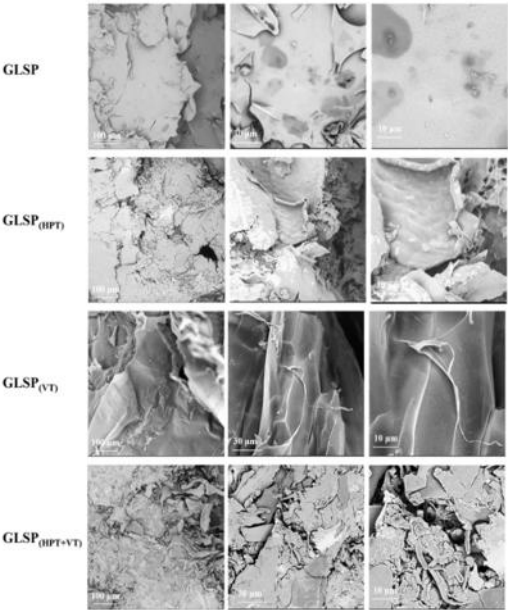
57 0.01).

58 **Table S1**

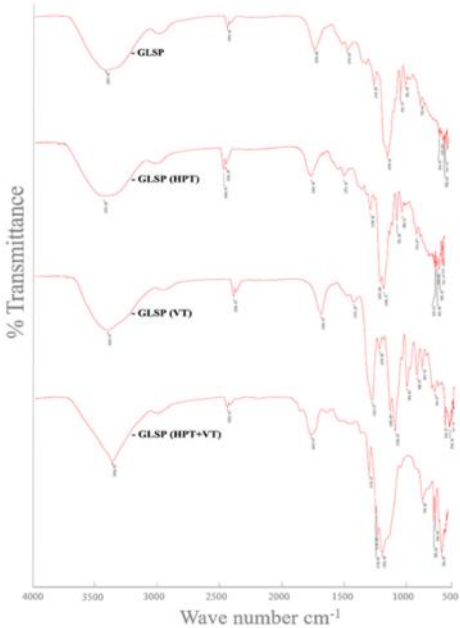
Samples	Total sugar (%)	Reducing sugar (%)	Viscosity η_{re} (dL/g)	Sulfate radical (%)	Average molecular weight (Da)
GLSP	98.47±1.27	0.25±0.09	0.630±0.053	11.07±2.35	152481
GLSP_(HPT)	98.65±2.04	28.95±4.71	0.009±0.002	12.68±1.82	63018
GLSP_(VT)	97.86±2.47	12.04±3.46	0.250±0.061	11.06±2.07	131293/6566
GLSP_(HPT+VT)	97.95±1.86	46.52±7.42	0.002±0.001	13.03±2.91	2031

59

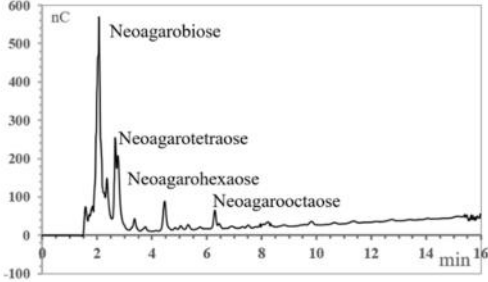
A



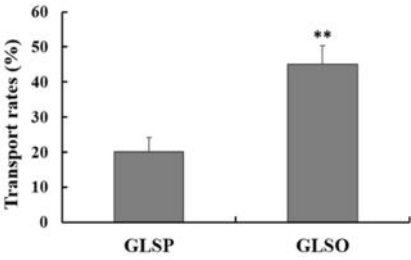
B



C



D



62 **References**

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