

Red Algae Sulfated Polysaccharides Effervescent Tablets Attenuated Ovalbumin-induced Anaphylaxis by Up-regulating Regulatory T cells in Mouse Models

Jing Han, Bo Liu, Qing-Mei Liu, Yafen Zhang, Yixiang Liu, Hong Liu, Min-Jie Cao, and Guang-Ming Liu

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.9b03132 • Publication Date (Web): 01 Sep 2019

Downloaded from pubs.acs.org on September 6, 2019

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.

HIGHLIGHTS

1. Red algae sulfated polysaccharide effervescent tablets (RASPET) were developed based on the anti-allergic activities of RASP.
2. RASP and RASPET attenuated intestinal villi injury in OVA-induced allergic mice.
3. RASP and RASPET up-regulated regulatory T cell populations and related immune-suppressive cytokines.

1 **Red Algae Sulfated Polysaccharides Effervescent Tablets Attenuated**
2 **Ovalbumin-induced Anaphylaxis by Up-regulating Regulatory T cells in Mouse**
3 **Models**

4

5 Jing Han[#], Bo Liu[#], Qing-Mei Liu, Ya-Fen Zhang, Yi-Xiang Liu, Hong Liu, Min-Jie Cao,
6 Guang-Ming Liu*

7

8 *College of Food and Biological Engineering, Xiamen Key Laboratory of Marine Functional Food,*
9 *Fujian Provincial Engineering Technology Research Center of Marine Functional Food, Fujian*
10 *Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources,*
11 *Jimei University, 43 Yindou Road, Xiamen 361021, Fujian, P. R. China*

12 # These authors contributed equally.

13

14 Running title: The anti-allergic activity of red algae sulfated polysaccharides effervescent tablets

15

16 * Corresponding author:

17 Guang-Ming Liu

18 College of Food and Biological Engineering, Jimei University, Xiamen, 361021, P.R. China

19 Tel: +86-592-6180378

20 Fax: +86-592-6180470

21 Email: gmliu@jmu.edu.cn

ABSTRACT

Red algae sulfated polysaccharides (RASP) were extracted from *Porphyra haitanensis* and *Gracilaria lemaneiformis*. RASP were applied to effervescent tablets to develop a type of functional food, termed red algae sulfated polysaccharide effervescent tablets (RASPET), based on the anti-allergic activities of RASP. The anti-allergic activities and the mechanisms of RASPET were investigated in an ovalbumin (OVA)-induced mouse model of food allergy. The results revealed that RASPET alleviated intestinal villi injury by scanning electron microscopy and anaphylactic symptoms, reduced OVA-specific immunoglobulin E, histamine, and mast cell protease-1 levels in the serum, reduced the level of serum interleukin-4, and increased serum interferon- γ level, decreased B cell and mast cell populations. Remarkably, RASPET increased the levels of serum interleukin-10 and transforming growth factor- β and up-regulated splenic CD4⁺foxp3⁺ T cell populations (15.28%, 16.82%, and 17.58%, respectively) compared to the OVA-group (13.17%). In conclusion, RASPET attenuated OVA-induced anaphylaxis via the up-regulation of regulatory T cells.

Keywords: red algae sulfated polysaccharides, effervescent tablets, anti-allergic activity, intestinal injury, regulatory T cells, mouse models

INTRODUCTION

Food allergy is defined as an adverse immune response to food proteins.¹ Approximately 8% of children and 2% – 3% of adults are suffering from food allergy, which has been a major global public health issue over the past several decades due to its increasing prevalence and severity.² Since it is impossible to avoid allergens completely, several studies and related drugs have been developed to pretreat or treat food allergy, including antihistamines, immunosuppressants, and calcium channel blockers.^{3, 4} However, in addition to drug resistance, these drugs may cause long-term side effects.⁵ Thus, products made from natural substances with more diverse targets and fewer side effects may provide new options for patients suffering from food allergy.⁶

Porphyra haitanensis (*P. haitanensis*) and *Gracilaria lemaneiformis* (*G. lemaneiformis*), an economical type of red algae, are widely grown and consumed in the coastal regions of China. Many studies have reported that sulfated polysaccharides in red algae have varieties of biological activities (e.g., anti-allergic,⁷ anti-tumor,⁸ and anti-inflammatory properties⁹). *Porphyra haitanensis* sulfated polysaccharides by the treatment of high temperature and pressure (PHSP_(hp)) and *Gracilaria lemaneiformis* sulfated polysaccharides by the treatment of high temperature and pressure (GLSP_(hp)), as natural bioactive substances are suitable for applications to functional products or medicine in related fields. Studies suggest that biomacromolecules with stretched structures and a lower molecular weight by degradation might lead to higher bioactivity and absorption both *in vitro* and *in vivo*.¹⁰ The results of our previous study

63 suggest that PHSP_(hp) and GLSP_(hp) combined with high temperature and pressure
64 treatment attenuated *E. coli*-induced diarrhea and inflammatory responses.¹¹ However,
65 the anti-allergic activities and mechanisms of PHSP_(hp) and GLSP_(hp) remain poorly
66 understood and require further investigation.

67 Allergic reactions induced by food allergens are often accompanied by a
68 reduction in body temperature and gastrointestinal symptoms such as diarrhea and
69 intestinal injury.¹² The small intestine plays an important role in digestion and
70 absorption as well as immune function.¹³ When the intestinal barrier is impaired, food
71 allergens can cross the intestinal barrier into the mucosa and lymphoid tissue to
72 promote the differentiation of type-2 helper T (Th2) cells, release Th2 cytokines, and
73 promote the production of food allergen-specific immunoglobulin (Ig)E, which leads
74 to anaphylaxis.¹⁴ Small intestinal injury can be clearly observed by scanning electron
75 microscopy (SEM) with disordered arrangements of small intestine villi, rupture of
76 the villi surface, and increased secretions.¹⁵

77 Food allergy is an immune disorder dominated by Th2 immune responses with
78 an increase in Th2 cytokine production, including interleukin (IL)-4, IL-5, IL-9, and
79 IL-13.¹⁶ IL-4 can promote the B cell differentiation and IgE production, induce the
80 differentiation of mast cells (MCs) as the key effector cells accompanied by an
81 increase in anaphylactic mediators (e.g., histamine and mast cell protease-1
82 [mMCP-1]), and inhibit the secretion of interferon (IFN)- γ as a Th1 cytokine, which
83 modulates allergic responses by activating the functions of immunosuppressive cells
84 (e.g., regulatory T [Treg] cells).^{17, 18} The conversion from a Th2 response to a Th1

85 response has been proposed as a strategy to modulate aberrant T cell immune
86 responses associated with food allergies.¹⁹ As previously reported, IL-10, and
87 transforming growth factor (TGF)- β , as the immunosuppressive cytokines, could
88 induce the differentiation and maintenance of Treg cells, which are critical for
89 immune tolerance and strengthening of the immune response.^{20, 21}

90 As key immunosuppressive cells, Tregs play an important role in modulating the
91 immune response by releasing IL-10 and TGF- β . Moreover, Shin et al. found that
92 turmeric promoted an increase in CD4⁺foxp3⁺ T cell populations and Treg-related
93 TGF- β production to modulate the Th1/Th2 immune response.²² Moreover, the
94 elevated expression of IL-10 and TGF- β can maintain immune tolerance and
95 alleviate anaphylaxis.²³ In addition, MCs are the key effector cells on which IgE
96 crosslinks high-affinity receptors (Fc ϵ RI). The crosslinking of food allergens with
97 IgE-Fc ϵ RI further triggers the production and release of anaphylactic mediators,
98 which results in an allergic reaction.^{24, 25}

99 In this study, RASP was applied into a practical product to develop a type of
100 functional anti-allergic food. Effervescent tablets are a new type of tablet with the
101 characteristics of rapid onset, high availability, and are less harmful to the stomach,
102 which are especially suitable for patients who have difficulty swallowing
103 conventional tablets or capsules.^{26, 27} Typically, effervescent tablets consist of active
104 drugs and excipients, including acids and alkalis, which leads to rapid disintegration.
105 The application of RASP into effervescent tablets will be suitable for a variety of
106 patients suffering from food allergies, with the advantage of having a rapid onset,

mildness, and high availability. Therefore, red algae sulfated polysaccharide effervescent tablets (RASPET) can provide an alternative to related functional foods. However, whether the bioactivity of RASP will be influenced by RASPET requires further exploration.

MATERIALS AND METHODS

Reagents

The red algae, *P. haitanensis* and *G. lemaneiformis*, were purchased from the local market on Dadeng Island (Xiamen, Fujian, China). RPMI 1640 was obtained from HyClone Co. (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Imject Alum and ovalbumin (OVA) were purchased from Thermo Fisher Scientific Inc (Waltham, MA, USA) and Sigma (Louis, MO, USA), respectively. Goat anti-mouse IgG1 and IgG2a antibodies were obtained from Abcam (Cambridge, UK). Enzyme-linked immunosorbent assay (ELISA) kits for anti-OVA-IgE and histamine were obtained from Cayman (Ann Arbor, MI, USA) and IBL (Hamburg, Germany), respectively. IFN- γ , IL-4, IL-10, TGF- β , and mMCP-1 were purchased from eBioscience (San Diego, CA, USA). All antibodies used for flow cytometry analysis were purchased from Biolegend (San Diego, CA, USA). Other chemicals were purchased from Sigma (Louis, MO, USA) and were all of analytical grade.

Extraction of PHSP_(hp) and GLSP_(hp)

The extraction of PHSP_(hp) and GLSP_(hp) was performed based on previous methods.⁷

Preparation of red algae sulfated polysaccharide effervescent tablets

The RASPET consisted of 10% RASP (PHSP_(hp):GLSP_(hp) = 1:1) and 90% excipients (30% lactose, 13% mannitol, 10% tartaric acid, 10% citric acid, 20% sodium bicarbonate, 3% stevioside, and 4% Polyvinylpyrrolidone-K30). ET consisted of 100% excipients of equal proportions without RASP. RASPET or ET were pressed in a single punch machine (Saihao Machinery, Guangzhou, Guangdong, China) after the powder was evenly mixed.

OVA-induced mouse models of food allergy

Six-week-old female BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments were approved according to the guidelines of the Institutional Animal Care and Use Committee of the Jimei University (Xiamen, Fujian, China). All procedures were conducted in accordance with the Institutional Animal Care and Use Committee of Animal Laboratory Center of Jimei University (Xiamen, China, No. SCXK 2016-0006). The mice were housed under controlled temperature (23°C ± 2°C) with a

relative humidity of 40% – 60% and a 12h-light/dark cycle. Food and water were provided ad libitum.

OVA-induced mouse models of food allergy are described in Figure 1 and Figure 5 to investigate the anti-allergic activities of RASP or RASPET. Mice (six mice per group) were intraperitoneally (i.p.) injected twice with 100 µg of OVA plus 2 mg of Alum in 200 µL PBS on days 0 and 14 for sensitization. Starting on day 28, the mice were intragastrically (i.g.) challenged with 50 mg in 200 µL PBS at 9 a.m. on days 28, 31, 34, 37, and 40. From day 27, mice were orally administered with PHSP_(hp) and GLSP_(hp) at a dose of 5 mg/mouse or ET, RASPET-1, RASPET-2, and RASPET-3 at a dose of 200 mg/kg/day.

Anaphylactic symptoms and rate of diarrhea (proportions of mice with diarrhea in each group) were evaluated 1 h following each challenge, as previously reported.^{37, 38} Anaphylactic symptom scores were assessed as follows: 0, no symptoms; 1, scratching around nose and head; 2, puffiness around eyes and mouth; 3, wheezing, labored respiration, cyanosis around mouth and tail; 4, no activity after prodding or tremor and convulsion; and 5, death. In addition, the change in the rectal temperature was measured for 1 h following the final challenge by a digital thermometer (Prosper, Shanghai, China) and the serum collected at 1 h following the last challenge was stored at -80°C for measurements of the levels of histamine and mMCP-1. On day 41, the serum, spleen, and jejunum tissues were collected after the mice were sacrificed.

Scanning electron microscopic analysis of the jejunum tissue

The jejunum villi were observed by SEM. Briefly, the jejunum tissue was fixed in 2.5% glutaraldehyde overnight, followed by 1% OsO₄ for 1 h. The jejunum specimens were then dehydrated in a graded series of ethanol (30%, 50%, 70%, 80%, 90%, and 100%) for 20 min at each step and transferred into a mixture of alcohol and iso-amyl acetate (1:1) for 30 min and iso-amyl acetate for 1 h. After dehydration, the jejunum specimens were coated with gold-palladium and visualized using Phenom Pro Model desktop SEM (Hillsboro, OR, USA).

Measurements of OVA-specific immunoglobulins and anaphylactic mediators in the serum

The levels of OVA-specific IgE, histamine and mMCP-1 were determined using a commercial ELISA kit (Cat.No. EKC37529, RE59221, EMC113.96) purchased from Cayman (Ann Arbor, Mi, USA), IBL (Hamburg, Germany), eBioscience (San Diego, CA, USA) according to the manufacturer's instructions. The levels of IgG1 and IgG2a were determined using an indirect ELISA according to the method described by Shi et al., with slight modification.²⁸ Briefly, 96-well plates were coated with 100 µL OVA solution and maintained overnight at 4°C. The following day, the plate was washed twice with wash buffer (20 mM Tris, 0.145 M NaCl, 0.05% Tween 20, pH = 7.5) and incubated for 2 h in 5% skim milk in PBS at 37°C. After washing, the serum samples were added to the plate and incubated overnight at 4°C. Then, 100

190 μL at 1:5000 dilution for goat anti-mouse IgG1 secondary antibody or 1:1000 dilution
191 for the goat polyclonal secondary antibody to mouse IgG2a was added to the plates
192 and incubated at 37°C for 2 h. Next, 100 μL TMB substrate solution was added to
193 each well of the plates (Tiangen, Beijing, China). The wells were incubated at 37°C
194 for 20 min. The color reactions were stopped with 100 μL stop solution (2 M H_2SO_4).
195 The absorbance at 450 nm was read with an ELISA plate reader of Bio-Rad
196 (Hercules, CA, USA).

198 **Cytokines measurements in the serum**

199 The levels of IL-4, IFN- γ , IL-10, and TGF- β in the serum collected from the
200 orbital venous plexus 24 h after the last challenge was measured using a commercial
201 ELISA purchased from eBioscience (Cat.No EMC003.96, EMC101g.96, EMC005.96,
202 EMC107b.9) according to the manufacturer's instructions.

204 **Flow cytometry**

205 The suspension of splenic single cells was obtained from the intermediate
206 mononuclear cell layer by the density gradient centrifugation using the mouse
207 lymphocyte separation solution following the grinding of the spleens as much as
208 possible. Splenic lymphocytes were labeled with anti-CD4, anti-CD19, anti-Foxp3,
209 anti-c-kit, and anti-Fc ϵ RI α . Data was acquired with a Guava easyCyte 6-2L system

and analyzed with Guava Soft 3.1.1 software of Millipore (Billerica, MA, USA).

Statistical analysis

Statistical analysis was performed using SPSS v12.0 software (Chicago, IL, USA). Data were presented as the mean $\pm$ standard error of mean from three independent experiments. Statistical differences were determined using a Student's *t*-test and the threshold *P* value for significance was set at 0.05.

RESULTS

PHSP_(hp) and GLSP_(hp) alleviate anaphylactic symptoms in OVA-challenged mice

Successive OVA challenges by gavage resulted in a high anaphylactic symptom score, hypothermia, and severe diarrhea in OVA-sensitized mice as shown in Figure 1. However, pretreatment with PHSP_(hp) or GLSP_(hp) led to significant alleviation in anaphylactic symptoms accompanied by the reduction in the anaphylactic symptom scores (Figure 1B), recovery of rectal temperature (Figure 1C), and diarrhea relief (Figure 1D).

PHSP_(hp) and GLSP_(hp) protect jejunum tissues from injury in OVA-challenged mice

Evidence demonstrates that OVA challenges induced allergic diarrhea due to

intestinal tissue damage.²⁹ To determine the effect of PHSP_(hp) or GLSP_(hp) on anaphylaxis in OVA-induced mice, the microstructures of the jejunum villi were observed by SEM under 500×, 1000×, and 3000× magnification as shown in Figure 2. Compared with the PBS group, the jejunum villi in OVA-sensitized mice were injured, displaying a ruptured surface and increased secretion. However, jejunum villi injury was attenuated by both PHSP_(hp) and GLSP_(hp). These results suggested that PHSP_(hp) and GLSP_(hp) effectively protected the intestinal morphology and integrity in OVA-treated allergic mice.

Effects of PHSP_(hp) and GLSP_(hp) on the levels of serum OVA-specific immunoglobulins, mMCP-1, histamine, and cytokines

The levels of serum OVA-specific immunoglobulins, mMCP-1, histamine, and cytokines were determined to further investigate the effects of PHSP_(hp) or GLSP_(hp) on the anaphylaxis in OVA-induced allergic mice. As shown in Figure 3A-3C, successive OVA challenges in OVA-sensitized mice resulted in significant increases of OVA-specific IgE, IgG1, and IgG2a levels. There were significant decreases of the levels of OVA-specific IgE, IgG1 and IgG2a following the oral administration of GLSP_(hp). PHSP_(hp) decreased the levels of OVA-specific IgG1 and IgG2a, and IgE was decreased by PHSP_(hp) but the difference was no significant compared to the OVA group. In addition, the levels of the anaphylactic mediators, histamine, and mMCP-1, were measured. As shown in Figure 3D and 3E, the oral administration of

both PHSP_(hp) and GLSP_(hp) significantly decreased the levels of serum histamine and mMCP-1 compared to the OVA group. Moreover, serum cytokines were measured to investigate the effects of PHSP_(hp) and GLSP_(hp) on the cytokine patterns in the immune responses of allergic mice. These findings showed that the level of serum IL-4 was significantly decreased and IFN- γ was significantly increased following the pretreatment with either PHSP_(hp) or GLSP_(hp) as shown in Figure 3F and 3G. Remarkably, the levels of IL-10 and TGF- β related to Treg cells were increased following the oral administration of both PHSP_(hp) and GLSP_(hp).

258

259 **Effects of PHSP_(hp) and GLSP_(hp) on Treg cells in the spleen**

Considering the increase in the levels of IL-10 and TGF- β induced by both PHSP_(hp) and GLSP_(hp), the effects of RASP on Treg cells requires further investigation. Firstly, B cells associated with IgE production in the spleen were counted and labeled with anti-CD19 and analyzed by flow cytometry. As shown in Figure 4A, the CD19⁺ B cell populations in the OVA group were significantly increased (71.25%) compared to the PBS group (59.32%). However, neither PHSP_(hp) nor GLSP_(hp) had significant effects on the B cell subpopulations. Next, MCs were counted as the key effector cells involved in the immune response to investigate the effects of RASP on splenic MCs. As shown in Figure 4B, pretreatments with both PHSP_(hp) or GLSP_(hp) led to significant decreases on the splenic MC populations (0.31% and 0.13%) compared to the OVA group (1.21%). Based on the increased

levels of Treg-related IL-10 and TGF- β in the serum induced by RASP, Treg cells were labeled with anti-CD4 and anti-Foxp3 and analyzed by flow cytometry. Significant decrease was observed in the number of CD4⁺ foxp3⁺ T cells (11.09%) by successive OVA challenges compared to the PBS group (15.16%). However, pretreatment with either PHSP_(hp) or GLSP_(hp) increased the splenic Treg cell populations (13.70% and 11.90%) in allergic mice as shown in Figure 4C.

RASPET alleviates anaphylactic symptoms in OVA-treated mice

After determining the anti-allergic activities of RASP, PHSP_(hp) and GLSP_(hp), we established another mouse model of food allergy to confirm the anti-allergic effects of RASPET. We investigated the effects of RASPET with 10%, 20%, and 40% RASP, respectively, upon OVA-induced allergic mice with ET as a control. As shown in Figure 5B-5D, an oral administration of RASPET significantly attenuated the allergic symptoms in OVA-sensitized mice as assessed by the reduced anaphylactic symptom scores (Figure 5B), recovery of the rectal temperature (Figure 5C), and reduced diarrhea rates (Figure 5D) in contrast to the OVA group. There was a significant difference in all RASPET groups compared to the ET group for rectal temperature and diarrhea rates, except RASPET-1, for the evaluation of the anaphylactic symptom score.

RASPET protected the jejunum tissues from injury in OVA-challenged mice

As shown in Figure 6, the jejunum villi of the mice in the PBS group displayed a regular arrangement without surface ruptures. In contrast, severe damage to the jejunum villi was observed in the mice in the OVA group. Following an oral administration of RASPET, fewer broken jejunum villi were clearly observed in the jejunum tissue of all RASPET groups compared with either the OVA or ET groups.

Effects of RASPET on the levels of OVA-specific immunoglobulins, mMCP-1, histamine, and cytokines in the serum

As shown in Figure 7, RASPET pretreatment resulted in a significant decrease in the levels of OVA-specific IgE (Figure 7A), histamine (Figure 7D), and mMCP-1 (Figure 7E) compared to the OVA group. However, no significant effects were detected for the levels of OVA-specific IgG1 and IgG2a following the RASPET pretreatment in OVA-induced allergic mice (Figures 7B and 7C). As a representative Th2-related cytokine, IL-4 was decreased following the oral administration of RASPET, which was dependent on the concentrations of RASP (Figure 7F), and there is significant difference between RASPET-2 or RASPET-3 group and ET group. As a representative Th1-related cytokine, the level of IFN- γ was significantly increased following the oral administration of RASPET (Figure 7G); however, none of the RASPET groups were significantly different compared to the ET group. In addition, RASPET led to the excessive expression of TGF- β and IL-10 in all RASPET groups (Figure 7H and 7I), similar to that of RASP. In contrast, ET containing no RASP did

not have significant effects on the levels of Treg-related cytokines compared to the OVA group. Thus, these findings suggested that RASPET might increase the levels of TGF- β and IL-10 via the active components of RASP.

Effect of RASPET on Tregs in the spleen

To further investigate the mechanisms of the effects of RASPET on the OVA-induced allergic reactions in mice, isolated splenocytes from all of the groups were measured by flow cytometry. As shown in Figure 8A, successive OVA challenges resulted in significant increases in CD19⁺ B cell populations in the spleens of the mice in the OVA group (72.48%) compared to the PBS group (56.64%). Compared to the OVA group, the CD19⁺ B cell populations in the spleen of OVA-induced allergic mice significantly decreased (66.24%, 58.96%, and 59.68%) following RASPET pretreatment, among which there was significant difference between RASPET-2 or RASPET-3 and ET group.

As previously described, RASPET significantly inhibited the levels of histamine and mMCP-1 in the serum, and we further counted the populations of c-kit⁺ Fc ϵ R I α ⁺ MCs in the spleen. As shown in Figure 8B, there was significant increase in the c-kit⁺ Fc ϵ R I α ⁺ MC populations in the spleen of the OVA group (1.38%) compared to the PBS group (0.53%). In addition, splenic MC populations were significantly decreased (0.85%, 0.62%, and 0.90%) following pretreatment with RASPET compared to the OVA group (1.38%); and only the RASPET-2 group exhibited significant differences

compared to the ET group.

Moreover, based on the levels of serum IL-10 and TGF- β , the numbers of CD4⁺foxp3⁺ T cells in the spleen were counted and analyzed by flow cytometry. As shown in Figure 8C, there was significant decrease in the CD4⁺foxp3⁺ T cell populations in the spleen of the OVA group (13.17%) compared to the PBS group (16.18%). Oral administration with ET containing no RASP did not significantly alter the percentages of CD4⁺foxp3⁺ T cells compared to the OVA group. However, significant increases in the CD4⁺foxp3⁺ T cell population was observed in the OVA-induced mice pretreated with RASPET in all of the RASPET groups (15.28%, 16.82%, and 17.58%), compared to either OVA group (13.17%) or ET group (13.97%).

DISCUSSION

Evidence indicates that bioactive activities of sulfated polysaccharides by proper degradation might be improved with a lower molecular weight and increasing the stretched structure.^{33, 34} In our previous study, we extracted PHSP_(hp) and GLSP_(hp) by high temperature and pressure treatment with the characteristics of total sugar contents (99.12% and 98.85%), protein contents (0.64% and 0.39%) and sulfate contents (10.94% and 11.26%), as well as a lower molecular weight (165.45 kDa and 71.87 kDa) and viscosity (0.17 dL/g and 0.10 dL/g).¹¹ The red algae-derived sulfated polysaccharides through the high temperature and pressure method led to their wider

application ranges with the advantages of lower molecular weight and viscosity.

In the present study, PHSP_(hp) and GLSP_(hp) were applied to effervescent tablets that intended to provide an alternative for patients having difficulty swallowing. We previously carried out a mouse model as pre-experiment with a low dose of 200 mg/kg and high dose of 800 mg/kg for RASPET, results showed that the low dose of 200 mg/kg for RASPET played a significant difference compared to OVA group in serological and cytological detections. Based on the pre-experiment results, the dose of 200 mg/kg for RASPET was carried out in the final experiment and RASP concentrations in RASPET were set as 10%, 20% and 40%, with ET as a control for RASPET. Our results demonstrate that both RASP and RASPET significantly alleviated the OVA-induced anaphylactic symptoms. It indicated that the product form of effervescent tablets didn't destroy the activities of RASP. Moreover, intestinal injury was also attenuated as exhibited by fewer surface ruptures and neater arrangement of the intestinal villi observed. This confirmed that both RASP and RASPET protected the intestinal villi from injury and further enhanced the intestinal barrier functionality, which was dependent on the integrity of the intestinal tissue. Moreover, both RASP and RASPET significantly decreased histamine and mMCP-1 as the key anaphylactic mediators which induced the expansion of capillary vessels, increased vascular permeability, and enhanced smooth muscle contraction in allergic reactions.^{35, 36}

In addition to the anaphylactic mediators which acted directly on related target organs, other mediators (e.g., cytokines) also play an important role in allergic

377 reactions.³⁷ Food allergies are dominated by a Th2 immune response and presents
378 with an imbalance of the Th1/Th2 immune responses.³⁸ As the most important Th2
379 cytokine, IL-4 stimulates B cells to release IgE, which activates the degranulation of
380 MCs, aggravates the sensitivity of tissues to anaphylactic mediators produced by
381 effector cells, and inhibits the differentiation and functions of immunosuppressive
382 cells (e.g., Th1 and Treg cells).³⁹ In the present study, the levels of IL-4 and IgE were
383 decreased by treating with GLSP_(hp) and RASPET, but there was no significant
384 difference on the subpopulation of B cells and the production of IgE between OVA
385 group with PHSP_(hp) group while the IL-4 level was inhibited. IFN- γ is a critical
386 cytokine for IgG2a isotype switching, and the IgG2a antibody were released by B
387 cells. In the present study, it was found that RASPET could significantly increase the
388 production of IFN- γ and reduce the subpopulation of CD19⁺ B cells. Thus, the
389 increased IFN- γ level and steady IgG2a production were showed in our present paper.
390 Additionally, our results showed that the suppressive effects of Treg cells in in
391 RASPET-treated mice this study mainly lied on the mast cells and Th2 cells, as well
392 as the production of OVA-specific IgE, but not on the production of IgG1 and IgG2a.
393 Similar with the previous research,^{40,41} the present study also found that the
394 OVA-specific IgG1 and IgG2a production had little changes while Treg cells and
395 related cytokines were up-regulated by RASPET.

396 Moreover, as a Th1 cytokine, IFN- γ can promote the differentiation of Th1 cells by
397 up-regulating the expression level of the transcription factor, T-bet.⁴² And studies
398 have shown that the binding of TGF- β and IFN- γ exosomes can effectively promoted

the transformation of mononuclear cells to Tregs.⁴³ Our study shows that the level of IL-4 was significantly decreased and IFN- γ was significantly increased by both RASP and RASPET. Thus, this data suggests that both RASP and RASPET modulated the imbalanced Th1/Th2 immune response induced by anaphylaxis.

It is important to note that the levels of Treg-related IL-10 and TGF- β was up-regulated by the oral administration of either RASP or RASPET. Evidence indicates that the production of IgE and Th2 cytokines was inhibited by IL-10, and TGF- β could modulate the expression of the transcription factors, T-bet and GATA-3, to balance the Th1/Th2 immune response.^{44, 45} In addition, the CD4⁺foxp3⁺ T cell populations in the spleen were significantly up-regulated by both RASP and RASPET. Treg cells are immunosuppressive cells that are important to induce immune tolerance and maintain immune balance via the secretion of IL-10 and TGF- β .⁴⁶ Our findings indicated that RASP and RASPET modulated the Th1/Th2 immune response by increasing the levels of IL-10 and TGF- β (Figure 7F-7I). Moreover, RASP and RASPET suppressed anaphylaxis in OVA-induced mice by up-regulating the number of Treg cells (Figure 8C). In addition, c-kit⁺Fc ϵ R I α ⁺ mast cell populations were decreased by pretreatment with either RASP or RASPET (Figure 4A). The CD19⁺ B cell populations were also decreased by RASPET but not influenced by RASP although the IgE levels were suppressed by both RASP and RASPET, and we speculated that the immunosuppressive effects of Treg cells induced by RASP and RASPET were mainly reflected in the inhibition of mast cells and Th2 cells, which suppressed the levels of related allergic mediators subsequently.

It is important to note that although RASPET containing either 20% or 30% RASP was significantly different compared to ET containing no RASP, ET alone had an immunosuppressive effect that was observed to modulate the Th1/Th2 immune response and inhibit the splenic B cell and MC differentiation in OVA-induced mice (Figure 7F, 7G, 8A and 8B). We predict that the components in addition to RASP in RASPET also exhibited anti-allergic activities. It has been previously reported that lactose possesses probiotic activity, which increases *Lactobacillus* and *Bifidobacteria* populations in the intestine, and decreased the populations of *Bacteroides* and *Clostridia*.^{47, 48} Evidence indicates that the maintenance of a normal intestinal flora could promote the integrity of intestinal barrier, which play an important role on the growth and function of the immune system by preventing food allergens from entering the mucosa and lymphoid tissue.⁴⁹⁻⁵¹ however, RASPET had the ability to induce CD4⁺foxp3⁺ T cells, which ET could not; such ability was derived from RASP as supported by our reported findings. These results indirectly demonstrate that algae sulfated polysaccharides, which are biomacromolecules with more complex structures, might possess various bioactive targets compared to saccharides with simple structures (e.g., lactose that are used widely and at high-doses in tablets).

In summary, RASPET attenuated anaphylaxis by up-regulating Treg cells in a mouse model and such ability was derived from RASP. RASPET are also highly economical since as little as 10% RASP could up-regulate Treg cells in a mouse model. The present research provides the foundation and novel insight into the development of related functional food or medicine in the field of food allergy.

443 **ABBREVIATIONS**

444 ELISA, enzyme-linked immunosorbent assay

445 ET, effervescent tablets

446 GLSP, *Gracilaria lemaneiformis* sulfated polysaccharides447 GLSP_(hp), GLSP by the treatment of high temperature and pressure448 IFN- γ , interferon- γ

449 Ig, immunoglobulin

450 IL, interleukin

451 mMCP-1, mouse mast cell protease-1

452 OVA, ovalbumin

453 PHSP, *Porphyra haitanensis* sulfated polysaccharides454 PHSP_(hp), PHSP by the treatment of high temperature and pressure

455 RASP, the red algae sulfated polysaccharides

456 RASPET, the red algae sulfated polysaccharides effervescent tablets

457 TGF- β , transforming growth factor- β

458 Treg, regulatory T

459 ACKNOWLEDGEMENTS

460 This work was supported by the grants from the National Natural Scientific
461 Foundation of China (31871720), the Science and Technology Program of Fujian
462 province (2018N5009, 2018R0071), and the Marine Scientific Research Special
463 Foundation for Public Sector Program (DY135-B2-07, 201505026-03).

464

465 CONFLICT OF INTEREST

466 There are no conflicts to declare.

REFERENCES

- (1) Burks, A. W.; Tang, M.; Sicherer, S.; Muraro, A.; Eigenmann, P. A.; Ebisawa, M.; Fiocchi, A.; Chiang, W.; Beyer, K.; Wood, R.; Hourihane, J.; Jones, S. M.; Lack, G.; Sampson, H. A. ICON: Food Allergy. *J. Allergy Clin. Immunol.* **2012**, 129, 906-920.
- (2) Sicherer, S. H.; Sampson, H. A. Food Allergy: Epidemiology, Pathogenesis, Diagnosis, and Treatment. *J. Allergy Clin. Immunol.* **2014**, 133, 291-307.
- (3) Pajno G. B.; Fernandez-Rivas, M.; Arasi, S.; Roberts, G.; Akdis, C. A.; Alvaro-Lozano, M. EAACI Guidelines on Allergen Immunotherapy: IgE-Mediated Food Allergy. *Allergy*. **2017**, 73, 799-815.
- (4) Tong, P.; Chen, S. G.; Gao, J. Y.; Li, X.; Wu Z. H.; Yang, A. S. Yuan, J. L.; Chen, H. B. Caffeic acid-assisted cross-linking catalyzed by polyphenol oxidase decreases the allergenicity of ovalbumin in a Balb/c mouse model, *Food Chem. Toxicol.* **2018**, 111, 275-283.
- (5) Oyoshi, M. K.; Oettgen, H. C.; Chatila, T. A.; Geha, R. S.; Bryce, P. J. Food Allergy: Insights into Etiology, Prevention, and Treatment Provided by Murine Models. *J. Allergy Clin. Immunol.* **2014**, 133, 309-317.
- (6) Morikawa, T. Search for Bioactive Constituents from Several Medicinal Foods: Hepatoprotective, Antidiabetic, and Antiallergic Activities. *J. Nat. Med.* **2007**, 61, 112-126.

- 485 (7) Vo, T. S.; Ngo, D. H.; Kang, K. H.; Jung, W. K.; Kim, S. K. The Beneficial Properties of
486 Marine Polysaccharides in Alleviation of Allergic Responses. *Mol. Nutr. Food Res.* **2015**, 59,
487 129-138.
- 488 (8) Liu, Q. M.; Wang, Y. Z.; Cao, M. J.; Pan, T.; Yang, Y.; Mao, H. Y.; Sun, L. C.; Liu, G. M.
489 Anti-allergic Activity of R-phycoerythrin from *Porphyra Haitanensis* in Antigen-sensitized
490 Mice and Mast cells. *Int. Immunopharmacol.* **2015**, 25, 465-473.
- 491 (9) Pereira, J. G.; Mesquita, J. X.; Aragao, K. S.; Franco, A. X.; Souza, M. H. L. P.; Brito, T. V.;
492 Dias, J. M.; Silva, R. O.; Medeiros, J. R.; Oliveira, J. S.; Abreu, C. M. W. S.; de Paula, R. C.
493 M.; Barbosa, A. L. R.; Freitas, A. L. P. Polysaccharides Isolated from *Digenea Simplex* Inhibit
494 Inflammatory and Nociceptive Responses. *Carbohydr. Polym.* **2014**, 108, 17-25.
- 495 (10) Saravana, P. S.; Cho, Y. N.; Patil, M. P.; Cho, Y. J.; Kim, G.; Park, Y.; Woo, H.; Chun, B.
496 Hydrothermal Degradation of Seaweed Polysaccharide: Characterization and Biological
497 Activities. *Food. Chem.* **2018**, 268, 179-187.
- 498 (11) Liu, B.; Liu, Q. M.; Li, G. L.; Sun, L. C.; Gao, Y. Y.; Zhang, Y. F.; Liu, H.; Cao, M. J.; Liu,
499 G. M. The Anti-diarrhea Activity of Red Algae-originated Sulphated Polysaccharides on
500 ETEC-K88 Infected Mice. *RSC Advances.* **2019**, 9, 2360-2370.
- 501 (12) Moneret-Vautrin, D. A.; Morisset, M.; Flabbee, J.; Beaudouin, E.; Kanny, G. Epidemiology
502 of Life-threatening and Lethal Anaphylaxis: a Review. *Allergy.* **2005**, 60, 443-451.

- 503 (13) König, J.; Wells, J.; Cani, P. D.; García-Ródenas, C.L.; MacDonald, T.; Mercenier, A.;
504 Whyte, J.; Troost, F.; Brumme, R. Human Intestinal Barrier Function in Health and Disease.
505 *Clin. Transl Gastroen.* **2016**, 7, e196.
- 506 (14) Groschwitz, K. R.; Hogan, S. P. Intestinal Barrier Function: Molecular Regulation and
507 Disease Pathogenesis. *J. Allergy Clin. Immunol.* **2009**, 124, 3-20.
- 508 (15) Earley, Z. M.; Suhail, A.; Green, S. J.; Naqib, A.; Khan, O.; Cannon, A. R.; Hammer, A. M.;
509 Morris, N. L.; Li, X.; Eberhardt, J. M.; Gamelli, R. M.; Kennedy, R. H.; Choudhry, M. A.
510 Burn Injury Alters the Intestinal Microbiome and Increases Gut Permeability and Bacterial
511 Translocation. *PLoS One.* **2015**, 10, e0129996.
- 512 (16) Lee, D.; Kim, H. S.; Shin, E.; Dob, S.; Lee, C.; Kim, Y. M.; Lee, M. B.; Min K. Y.; Koo, J.;
513 Kim, S. J.; Nam, S. T.; Park, Y. H.; Choi, W. S. Polysaccharide Isolated from Aloe Vera Gel
514 Suppresses Ovalbumin-induced Food Allergy through Inhibition of Th2 Immunity in Mice.
515 *Biomed. Pharmacother.* **2018**, 101, 201-210.
- 516 (17) Zhang, D.; Yang, L.; Cohn, L.; Parkyn, L.; Homer, R.; Ray, P.; Ray, A. Inhibition of Allergic
517 Inflammation in a Murine Model of Asthma by Expression of a Dominant-negative Mutant of
518 GATA-3. *Immunity.* **1999**, 11, 473-482.
- 519 (18) Zhu, J.; Yamane, H.; Paul, W. E. Differentiation of Effector CD4 T Cell Populations. *Annu.*
520 *Rev. Immunol.* **2010**, 28, 445-489.

- 521 (19) Morafo, V.; Srivastava, K.; Huang, C. K.; Kleiner, G.; Lee, S.; Sampson, H. A.; Li, X.
522 Genetic Susceptibility to Food Allergy is Linked to Differential TH2-TH1 Responses in
523 C3H/HeJ and BALB/c Mice. *J. Allergy Clin. Immunol.* **2003**, 111, 1122-1128.
- 524 (20) Sakaguchi, S.; Yamaguchi, T.; Nomura, T.; Ono, M. Regulatory T Cells and Immune
525 Tolerance. *Cell.* **2008**, 133, 775-787.
- 526 (21) Nagalakshmi, M. L.; Murphy, E.; McClanahan, T.; Malefyt, R. W. Expression Patterns of
527 IL-10 Ligand and Receptor Gene Families Provide Leads for Biological Characterization. *Int.*
528 *Immunopharmacol.* **2004**, 4, 577-592.
- 529 (22) Shin, H. S.; See, H.; Jung, S. Y.; Choi, D. W.; Kwon, A.; Bae, M.; Sung, K.; Shon, D.
530 Turmeric (*Curcuma longa*) Attenuates Food Allergy Symptoms by Regulating Type 1/Type 2
531 Helper T Cells (Th1/Th2) Balance in a Mouse Model of Food Allergy. *J. Ethnopharmacol.*
532 **2015**, 175, 21-29.
- 533 (23) Okamoto, A.; Kawamura, T.; Kanbe, K.; Kanamaru, Y.; Ogawa, H.; Okumura, K.; Nakao, A.
534 Suppression of Serum IgE Response and Systemic Anaphylaxis in a Food Allergy Model by
535 Orally Administered High-dose TGF-beta. *Int. Immunol.* **2005**, 17, 705-712.
- 536 (24) Moñino-Romero, S.; Erkert, L.; Schmidthaler, K.; Diesner, S. C.; Sallis, B. F.; Pennington,
537 L.; Jardetzky, T.; Oettgen, H. C.; Bohle, B.; Fiebiger, E.; Szepefalusi, Z. The Soluble Isoform
538 of Human FcεRI is an Endogenous Inhibitor of IgE-mediated Mast Cell Responses. *Allergy.*
539 **2019**, 74, 236-245.

- 540 (25) Liu, Q. M.; Xie, C. L.; Gao, Y. Y.; Liu, B.; Lin, W. X.; Liu, H.; Cao, M. J.; Su, W. J.; Yang,
541 X. W.; Liu, G. M. Deep-Sea-Derived Butyrolactone I Suppresses Ovalbumin-Induced
542 Anaphylaxis by Regulating Mast Cell Function in a Murine Model. *J. Agric. Food Chem.*
543 **2018**, 66, 5581-5592.
- 544 (26) Fu, W.; Dai, Y.; Ma, T.; Wei, J.; Chen, H.; Xu, S. Tongluo Xingnao Effervescent Tablet
545 Reverses Memory Deficit and Reduces Plaque Load in APPswe/PS1dE9 Mice. *Exp. Ther.*
546 *Med.* **2018**, 15, 4005-4013.
- 547 (27) Munoz, H.; Castan, H.; Clares, B.; Ruiz, M. A. Obtaining Fast Dissolving Disintegrating
548 Tablets with Different Doses of Melatonin. *Int. J. Pharm.* **2014**, 467, 84-89.
- 549 (28) Shi, C.; Pan, T.; Cao, M.; Liu, Q.; Zhang, L.; Liu, G. Suppression of Th2 Immune Responses
550 by the Sulfated Polysaccharide from *Porphyra Haitanensis* in Tropomyosin-sensitized Mice.
551 *Int. Immunopharmacol.* **2015**, 24, 211-218.
- 552 (29) Monti, G.; Muratore, M. C.; Peltran, A.; Bonfante, G.; Silvestro, L.; Oggero, R.; Mussa, G.
553 C. High Incidence of Adverse Reactions to Egg Challenge on First Known Exposure in
554 Young Atopic Dermatitis Children: Predictive Value of Skin Prick Test and
555 Radioallergosorbent Test to Egg Proteins. *Clin. Exp. Allergy.* **2002**, 32, 1515-1519.
- 556 (30) Wijesekara, I.; Pangestuti, R.; Kim, S. Biological Activities and Potential Health Benefits of
557 Sulfated Polysaccharides Derived from Marine Algae. *Carbohydr. Polym.* **2011**, 84, 14-21.

- 558 (31) Pérez-Recalde, M.; Matulewicz, M. C.; Pujol, C. A.; Carlucci, M. J. In Vitro and in Vivo
559 Immunomodulatory Activity of Sulfated Polysaccharides from Red Seaweed *Nemalion*
560 *helminthoides*. *Int. J. Biol. Macromol.* **2014**, 63, 38-42.
- 561 (32) Liu, Q. M.; Yang, Y.; Maleki, S. J.; Alcocer, M.; Xu, S. S.; Shi, C. L.; Cao, M. J.; Liu, G. M.
562 Anti-Food Allergic Activity of Sulfated Polysaccharide from *Gracilaria Lemaneiformis* is
563 Dependent on Immunosuppression and Inhibition of p38 MAPK. *J. Agric. Food Chem.* **2016**,
564 64, 4536-4544.
- 565 (33) Bhadja, P.; Tan, C.; Ouyang, J.; Yu, K. Repair Effect of Seaweed Sulfated Polysaccharides
566 with Different Contents of Sulfate Group and Molecular Weights on Damaged HK-2 Cells.
567 *Polymers.* **2016**, 8, 188.
- 568 (34) Yu, X.; Zhou, C.; Yang, H.; Huang, X.; Ma, H.; Qin, X.; Hu, J. Effect of Ultrasonic
569 Treatment on the Degradation and Inhibition Cancer Cell Lines of Sulfated Polysaccharides
570 from *Porphyra Yezoensis*. *Carbohydr. Polym.* **2015**, 117, 650-656.
- 571 (35) Feldweg, A. M. Food Dependent, Exercise Induced Anaphylaxis: Diagnosis and Management
572 in the Outpatient Setting. *J. Allergy Clin. Immunol. Pract.* **2017**, 5, 283-288.
- 573 (36) Karasuyama, H.; Miyake, K.; Yoshikawa, S.; Kawano, Y.; Yamanishi, Y. How do Basophils
574 Contribute to Th2 Cell Differentiation and Allergic Responses? *Int. Immunol.* **2018**, 30,
575 391-396.

- (37) Shuilian, F.; Saihong, N.; Danni, W.; Meng, F.; Tie, H. Berberine Suppresses Mast Cell-mediated Allergic Responses via Regulating FcεRI-mediated and MAPK Signaling. *Int. Immunopharmacol.* **2019**, 71, 1-6.
- (38) Ji, N.; Xie, Y.; Zhang, M.; Zhao, X.; Cheng, H.; Wang, H.; Yin, K.; Huang, M. Ligustrazine Corrects Th1/Th2 and Treg/Th17 Imbalance in a Mouse Asthma Model. *Int. Immunopharmacol.* **2014**, 21, 76-81.
- (39) Gupta, R. K.; Kumar, S.; Gupta, K.; Sharma, A.; Roy, R.; Verma, A. K. Chaudhari, B. P.; Das, M.; Ansari, I. A.; Dwivedi, P. D. Cutaneous Exposure to Clinically-relevant Pigeon Pea (*Cajanus cajan*) Proteins Promote TH2-dependent Sensitization and IgE-mediated Anaphylaxis in Balb/c Mice. *J. Immunotoxicol.* **2016**, 13, 827-841.
- (40) Fu, L.; Peng, J.; Zhao, S.; Zhang, Y.; Su, X.; Wang, Y. Lactic Acid Bacteria-specific Induction of CD4⁺Foxp3⁺T Cells Ameliorates Shrimp Tropomyosin-induced Allergic Response in Mice via Suppression of mTOR Signaling. *Sci. Rep.* **2017**, 7, 1987.
- (41) Xu, S. S.; Liu, Q. M.; Xiao, A. F.; Maleki, S. J.; Alcocer, M.; Gao, Y. Y.; Liu, G. M. *Eucheuma Cottonii* Sulfated Oligosaccharides Decrease Food Allergic Responses in Animal Models by Up-regulating Treg Cells. *J. Agric. Food Chem.* **2017**, 65, 3212-3222.
- (42) Sicherer, S. H.; Sampson, H. A. Food Allergy. *J. Allergy Clin. Immunol.* **2010**, 125, 116-125.
- (43) Zhang, Q.; Fu, L.; Liang, Y.; Guo, Z.; Wang, L.; Ma, C.; Wang, H. Exosomes Originating from MSCs Stimulated with TGF-β and IFN-γ Promote Treg Differentiation: Exosomes Promote the Differentiation of Tregs. *J. Cell Physiol.* **2018**, 233, 6832-6840.

- 596 (44) Gorelik, L.; Flavell, R. A. Transforming Growth Factor-beta in T-cell Biology. *Nat. Rev.*
597 *Immunol.* **2002**, 2, 46-53.
- 598 (45) Sakaguchi, S.; Setoguchi, R.; Yagi, H.; Hori, S.; Fehervari, Z.; Shimizu, J.; Takahashi, T.;
599 Nomura, T. Foxp3(+)CD25(+)CD4(+) Natural Regulatory T Cells in Dominant Self-tolerance
600 and Autoimmune Disease. *Immunol. Rev.* **2006**, 212, 8-27.
- 601 (46) Veldhoen, M.; Hocking, R. J.; Atkins, C. J.; Locksley, R. M.; Stockinger, B. TGF Beta in the
602 Context of an Inflammatory Cytokine Milieu Supports De Novo Differentiation of
603 IL-17-producing T Cells. *Immunity.* **2006**, 24, 179-189.
- 604 (47) Daly, K.; Darby, A. C.; Hall, N.; Nau, A.; Bravo, D.; Shirazi-Beechey, S. P. Dietary
605 Supplementation with Lactose or Artificial Sweetener Enhances Swine Gut *Lactobacillus*
606 Population Abundance. *Br. J. Nutr.* **2014**, 111, S30-S35.
- 607 (48) Francavilla, R.; Calasso, M.; Calace, L.; Siragusa, S.; Ndagijimana, M.; Vernocchi, P.;
608 Brunetti, L.; Mancino, G.; Tedeschi, G.; Guerzoni, E.; Indrio, F.; Laghi, L.; Miniello, V. L.;
609 Gobbetti, M.; Angelis, M. D. Effect of Lactose on Gut Microbiota and Metabolome of Infants
610 with Cow's Milk Allergy. *Pediatr. Allergy Immunol.* **2012**, 23, 420-427.
- 611 (49) Kukkonen, K.; Savilahti, E.; Haahtela, T.; Juntunen-Backman, K.; Korpela, R.; Poussa, T.;
612 Tuure, T.; Kuitunen, M. Probiotics and Prebiotic Galacto-oligosaccharides in the Prevention
613 of Allergic Diseases: A Randomized, Double-blind, Placebo-controlled Trial. *J. Allergy Clin.*
614 *Immunol.* **2007**, 119, 192-198.

- 615 (50) Huang, C.; Lin, Y.; Jan, T. *Lactobacillus Reuteri* Induces Intestinal Immune Tolerance
616 Against Food Allergy in Mice. *J. Funct. Foods.* **2017**, 31, 44-51.
- 617 (51) Ashraf, R.; Shah, N. P. Immune System Stimulation by Probiotic Microorganisms. *Crit. Rev.*
618 *Food Sci.* **2014**, 54, 938-956.

FIGURE CAPTIONS**Figure 1. OVA-induced mouse model of food allergy and the effects of PHSP_(hp) and GLSP_(hp) on the allergic response**

A. Protocol for the mouse model of food allergy. Mice were sensitized twice by intraperitoneal injection (100 µg/mouse OVA) and challenged by gavage (50 mg/mouse OVA). The mice were pretreated with PHSP_(hp) or GLSP_(hp) (5 mg/mouse) by gavage daily starting from day 27.

B. The anaphylactic symptom scores evaluated for 1 h following each challenge

C. The rectal temperature in 1 h measured for 5 times (0 min, 15 min, 30 min, 45 min, 60 min) following the last challenge

D. The diarrhea rates for 1 h following each challenge

The data is presented as the average value of six mice per group and represented as the mean ± standard deviation from three independent experiments. Statistical differences were assessed by *P* values (one-way ANOVA). * *P* < 0.05; ** *P* < 0.01, compared to the OVA group.

Figure 2. Effects of PHSP_(hp) and GLSP_(hp) on jejunum tissue injury by SEM

The SEM results shown at 500×, 1000×, and 3000× magnification.

A. The jejunum villi of the mice in the PBS group displayed regular arrangements without surface ruptures.

B. The jejunum villi in OVA-sensitized mice showed ruptured surface and increased secretion.

C. Regular arrangements and few broken jejunum villi were observed in PHSP_(hp) group.

D. Regular arrangements and few broken jejunum villi were observed in GLSP_(hp) group.

Figure 3. Effects of PHSP_(hp) and GLSP_(hp) on the levels of OVA-specific immunoglobulins, histamine, mMCP-1, and cytokines

A–C. Effects of PHSP_(hp) and GLSP_(hp) on serum OVA-specific IgE, IgG1 and IgG2a, respectively.

D–E. Effects of PHSP_(hp) and GLSP_(hp) on serum histamine and mMCP-1, respectively.

F–I. Effects of PHSP_(hp) and GLSP_(hp) on serum IL-4, IFN- γ , IL-10, and TGF- β , respectively.

The data is presented as the average value of six mice per group and represented as the mean $\pm$ standard deviation from three independent experiments. Statistical differences were assessed by *P* values (one-way ANOVA). * *P* < 0.05, ** *P* < 0.01, compared to the OVA group.

Figure 4. Effects of PHSP_(hp) and GLSP_(hp) on the populations of B cells, MCs, and Treg cells in the spleen

A. Histograms of B cells by flow cytometry analysis. Splenic lymphocytes were labeled with anti-CD3 and anti-CD4.

B. The scatter plots and the corresponding histogram of MCs by flow cytometry analysis. Splenic lymphocytes were labeled with anti-c-kit and anti-Fc ϵ R 1 α .

657 C. The scatter plots and the corresponding histogram of Treg cells by flow cytometry analysis.
658 Splenic lymphocytes were labeled with anti-CD4 and anti-Foxp3.
659 The data is presented as the average value of six mice per group and represented as the mean $\pm$
660 standard deviation of three independent experiments. Statistical differences were assessed by P
661 values (one-way ANOVA). * $P < 0.05$, ** $P < 0.01$, compared to the OVA group.

662

663 **Figure 5. OVA-induced mouse model of food allergy and the effects of RASPET on the**
664 **allergic response**

665 A. Protocol for the mouse model of food allergy. Mice were sensitized twice by intraperitoneal
666 injection (100 μ g/mouse OVA) and challenged by gavage (50 mg/mouse OVA). Mice were
667 pretreated with RASPET containing 10%, 20%, or 40% RASP at a dose of 200 mg/kg, with the
668 ET control containing no RASP, delivered by gavage daily starting on day 27.

669 B. The anaphylactic symptom scores evaluated for 1 h following each challenge

670 C. The rectal temperature at 1 h following the last challenge

671 D. The diarrhea rates for 1 h following each challenge

672 The data is presented as the average value of six mice per group and represented as the mean $\pm$
673 standard deviation of three independent experiments. Statistical differences were assessed by P
674 values (one-way ANOVA). * $P < 0.05$, ** $P < 0.01$, compared to the OVA group; # $P < 0.05$, ##:
675 $P < 0.01$, for all RASPET groups compared to the ET group.

676

677 **Figure 6. Effects of RASPET on jejunum tissue injury by SEM**

678 The SEM results shown at magnifications of 500×, 1000×, and 3000×.

679 A. PBS group showed regular arrangements of jejunum villi without surface ruptures.

680 B. OVA group showed severe damage of jejunum villi with ruptured surface and increased
681 secretion.

682 C. Jejunum villi injury was attenuated slightly with a few broken jejunum villi in ET group.

683 D. Regular arrangements and few broken jejunum villi were observed in RASPET-1 group.

684 E. Regular arrangements and few broken jejunum villi were observed in RASPET-2 group.

685 F. Regular arrangements and few broken jejunum villi were observed in RASPET-3 group.

686

687 **Figure 7. Effects of RASPET on the levels of OVA-specific immunoglobulins, histamine,**
688 **mMCP-1, and cytokines**

689 A–C. Effects of RASPET on serum OVA-specific IgE, IgG1, and IgG2a, respectively.

690 D–E. Effects of RASPET on serum histamine and mMCP-1, respectively.

691 F–I. Effects of RASPET on serum IL-4, IFN- γ , IL-10, and TGF- β , respectively.

692 The data is presented as the average value of six mice per group and represented as the mean $\pm$
693 standard deviation from three independent experiments. Statistical differences were assessed by *P*

38

values (one-way ANOVA). * $P < 0.05$; ** $P < 0.01$, compared to the OVA group; # $P < 0.05$; ## $P < 0.01$, for all the RASPET groups compared to the ET group.

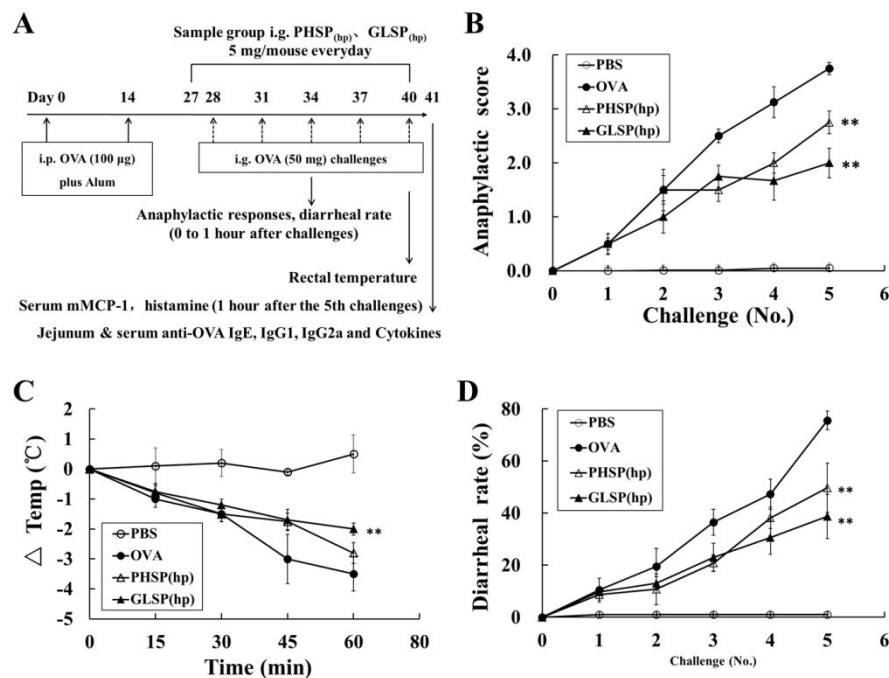
Figure 8. Effects of RASPET on the populations of B cells, MCs, and Treg cells in the spleen

A. Histograms of splenic B cells by flow cytometry analysis. Splenic lymphocytes were labeled with anti-CD3 and anti-CD4.

B. The scatter plots and the corresponding histogram of splenic MCs by flow cytometry analysis. The splenic lymphocytes were labeled with anti-c-kit and anti-FcεR 1α.

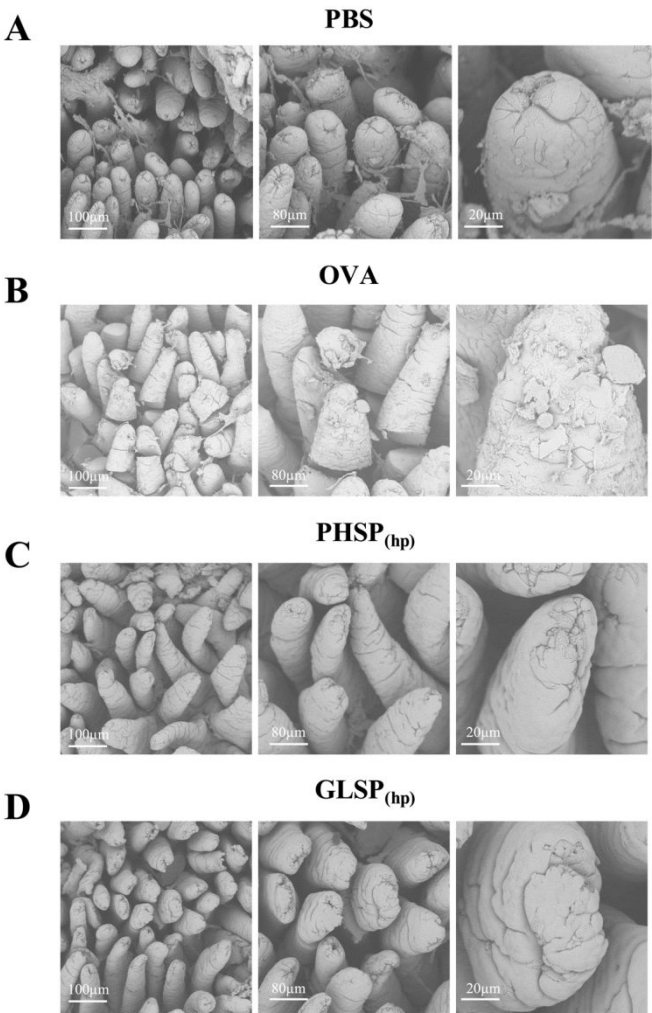
C. The scatter plots and the corresponding histogram of splenic Treg cells by flow cytometry analysis. Splenic lymphocytes labeled with anti-CD4 and anti-Foxp3.

The data was presented as the average value of six mice per group and represented as the mean $\pm$ standard deviation of three independent experiments. Statistical differences were assessed by P values (one-way ANOVA). * $P < 0.05$; ** $P < 0.01$, compared to the OVA group; # $P < 0.05$; ## $P < 0.01$, for all the RASPET groups compared to the ET group.

Figure 1

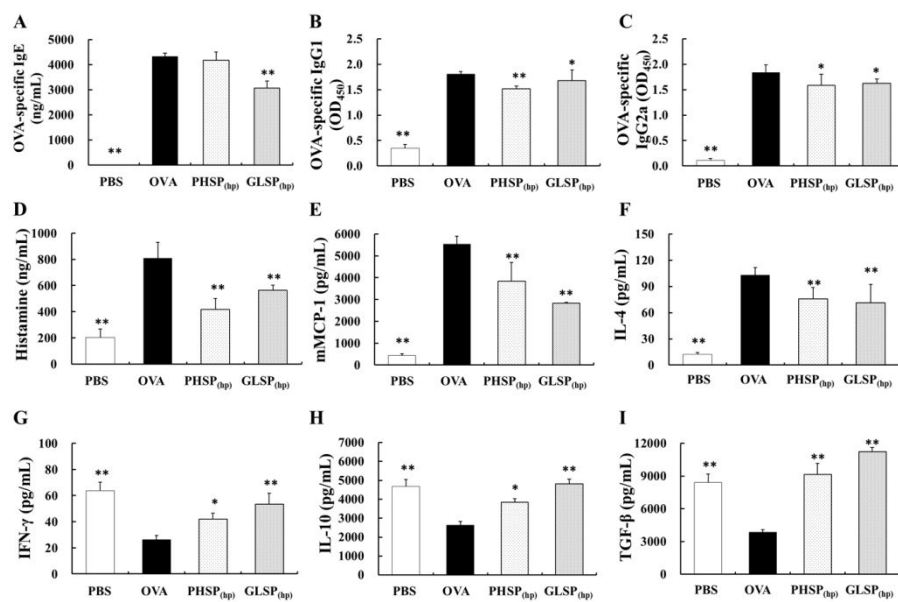
711

712 **Figure 2**



713

714

715 **Figure 3**

716

Figure 4

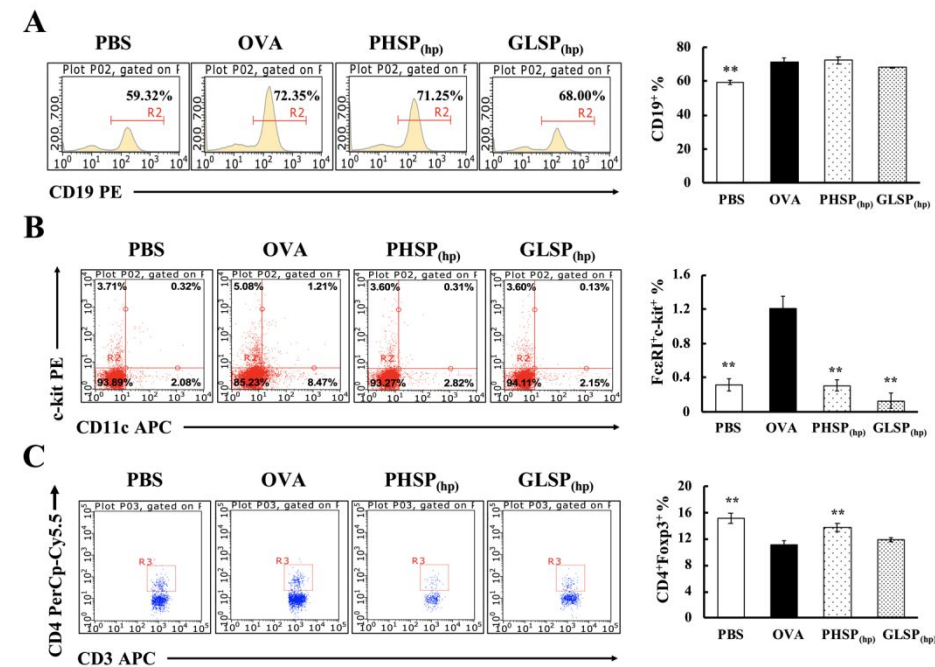
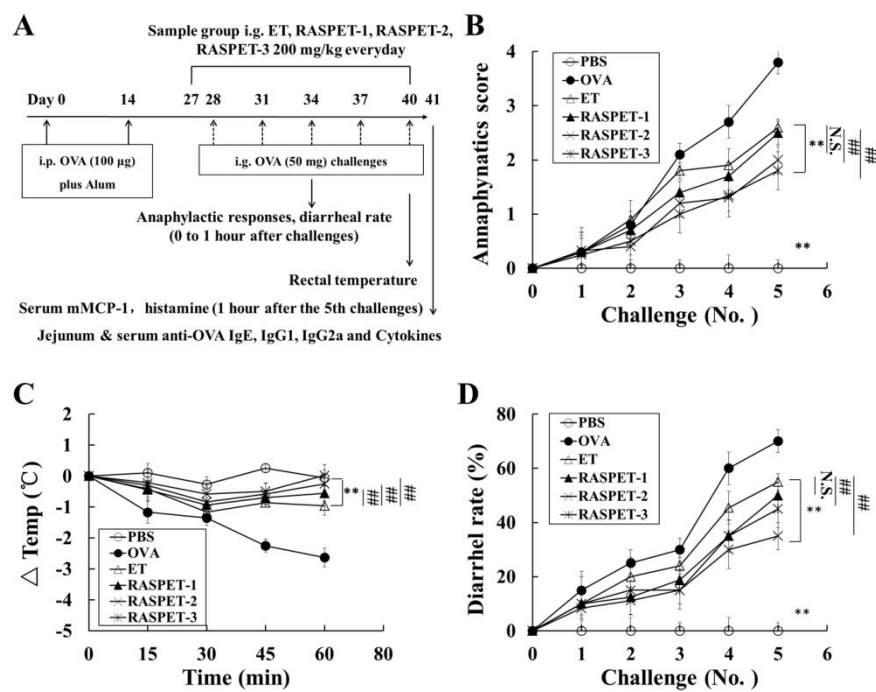
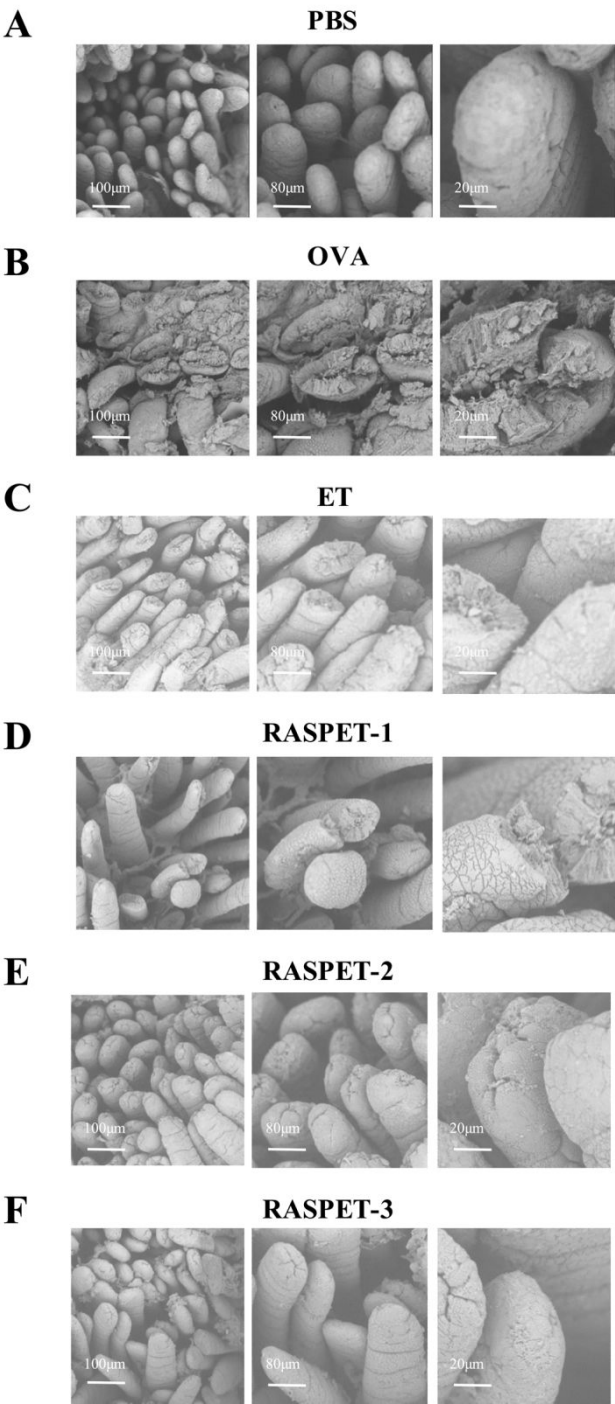


Figure 5

723

724 **Figure 6**



725

Figure 7

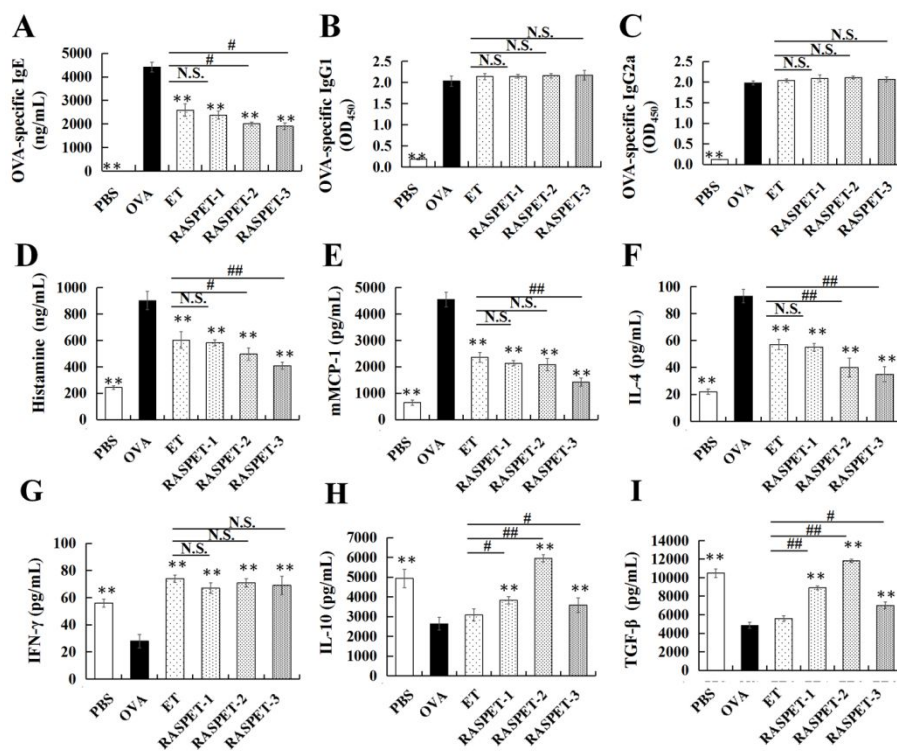
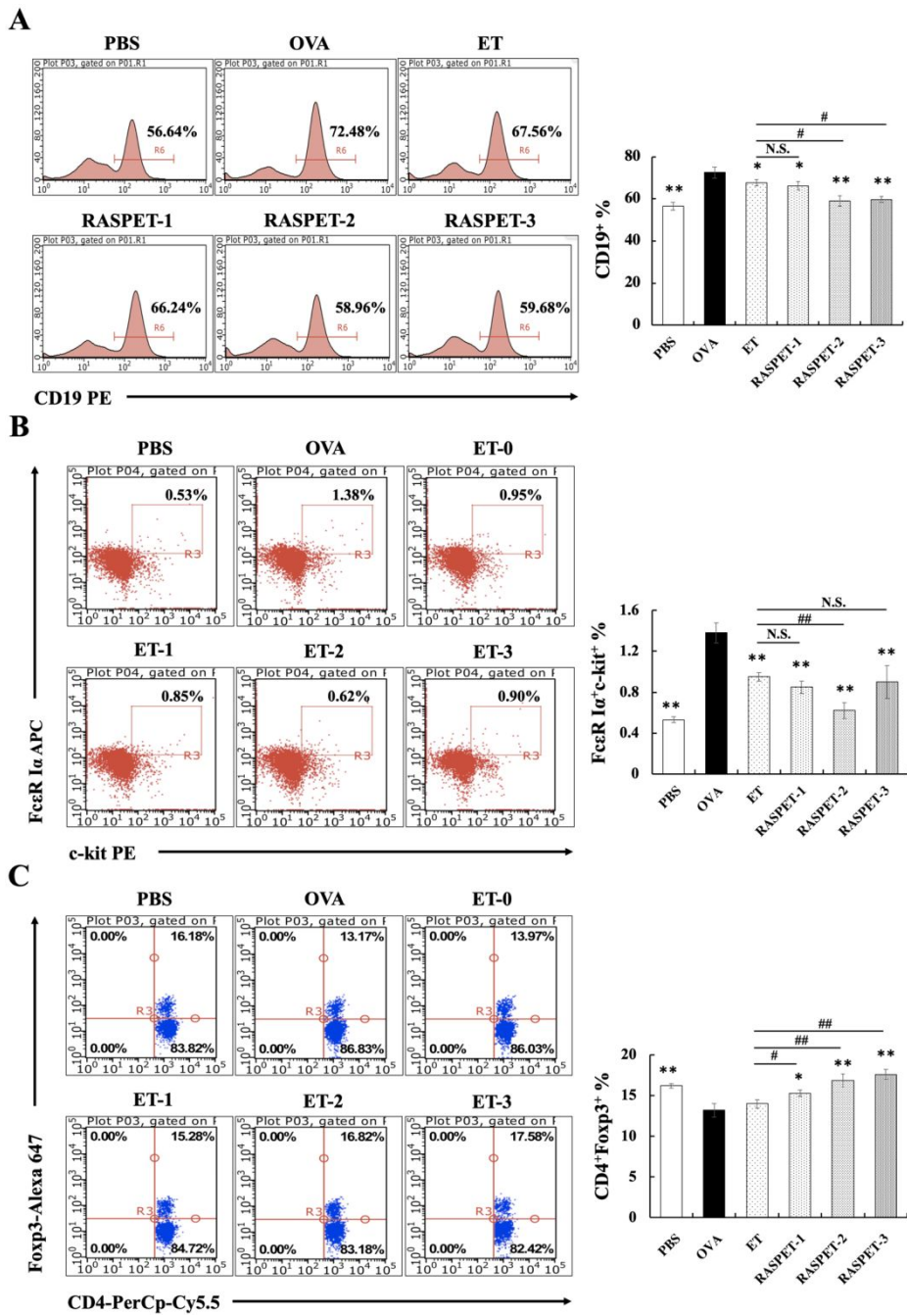
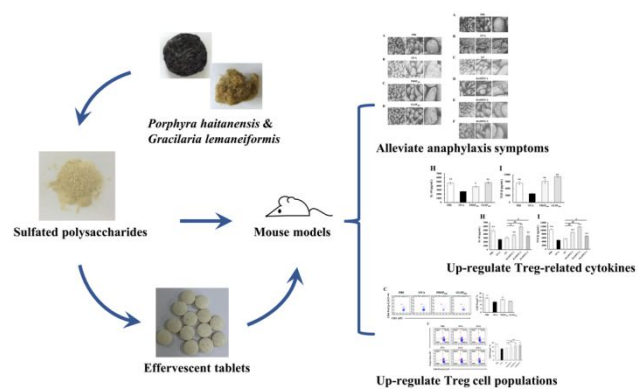


Figure 8



732

733 **TABLE OF CONTENTS GRAPHICS**

734