

# Effect of the extract made from *Rhizoma Atractylodis Macrocephalae* (RAM) on the immune responses of mice to a commercial foot-and-mouth disease vaccine

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## Abstract

**Objectives:** Foot-and-mouth disease (FMD) is an economically important animal disease because of the speed of its transmission. Routine vaccination may not be effective; RAM can be considered as a potential facilitator for this. Present study was designed to evaluate the effects of feeding different treatment of the RAM in different days on the immune responses in mice immunised with FMDV type O vaccine.

**Material and methods:** In experiment 1, 50 ICR mice were randomly divided into five groups with 10 animals in each group, and the basic diet containing 1% Crush of RAM for 1-week ad libitum feeding period, 1% Crush of RAM for 6-week ad libitum feeding period, 1% Decoction of RAM for a 1-week ad libitum feeding period, 1% Decoction of RAM for a 6-week ad libitum feeding period, respectively. Blood samples were collected 2 weeks after boosting for measurement of FMDV-specific IgG level and the IgG subclasses, lymphocyte proliferation as well as production IL-5 and IFN- $\gamma$ . In experiment 2, four groups mice were fed basic diet and basic diet containing 5% Decoction of RAM for 2-, 4- and 6-day ad libitum feeding periods, respectively. Then we collected blood samples for detecting IgG and IgG subclasses, splenocytes for lymphocyte proliferation as well as production IL-5 and IFN- $\gamma$ , and tissue samples of small intestine for sIgA.

**Results:** The results indicated that 1% Decoction of RAM for a 1-week ad libitum feeding period group and 5% Decoction of RAM for 2-, 4- and 6-day ad libitum feeding period group enhance the FMDV-specific immune responses significantly.

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**Conclusions:** Taken together, the results demonstrate that doses and feeding time of RAM are important to affect the immune responses.

**KEYWORDS**

decoction, foot-and-mouth disease, *Rhizoma Atractylodis Macrocephalae*, vaccine

## 1 | INTRODUCTION

The rhizome of *Atractylodis macrocephalae* Koidz (RAM) as a traditional Chinese medicine has been utilised for at least 2000 years (Ying et al., 2017). The sliced RAM is often cooked together with other herbs and the soup is used for oral administration in the treatment of diseases such as diarrhoea and infections in humans and animals (e.g., foot-and-mouth disease, severe acute respiratory syndrome, *Helicobacter pylori*). In the theories of traditional Chinese medicine (TCM), RAM possesses significantly different therapeutic effects before and after processing (Shan et al., 2014). The active ingredients of RAM include volatile components, polysaccharides, amino acids etc., which have a variety of pharmacological effects, including regulating immunity, improving gastrointestinal function, anti-inflammatory and antibacterial (Bailly, 2021; Zhu et al., 2018). Our previous study has shown that oral administration of a crude aqueous extract of the rhizome of RAM has significantly increased immune responses to FMDV type O vaccine (Li et al., 2009; Xie et al., 2012). Foot-and-mouth disease (FMD) is an economically important animal disease because of the speed of its transmission. Vaccines for FMD prevention have existed for over 70 years and been widely used, yet the technical limitations of current vaccines have caused FMD to still exist for a long time. Strategies to improve the immune response to vaccination have included the use of higher vaccine dose or increasing number of doses, use of different route of administration (e.g., intradermal versus intramuscular administration), accelerating dosing schedule and use of adjuvants such as antigen delivery systems and various immunomodulators. There is growing evidence that medicinal herbs and the ingredients enhance the immune response to vaccines direct against infectious agents (Chavalp & Campbell, 1987; Hu et al., 2011). Medicinal herbs have a variety of effects on immune responses and co-administration with herbal extracts has been shown to increase the antibody response and to enhance the proliferative response of T cells (Yang et al., 2008). The efficacy of herbal extracts as adjuvant to vaccines has been an object of several clinical trials conducted in both humans and animals (Fan et al., 2015; Su et al., 2014). However, information is scant regarding the immunological effects, particularly with the respect to the mucosae, of different processing of RAM administered by the oral route.

The aim of the present study was to further analyse the immune response developed in mice fed different treatments of RAM to vaccination against foot-and-mouth disease.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

Female ICR mice were purchased from Shanghai Laboratory Animal Center (SLAC) Co. Ltd. (Shanghai, China) and housed in polypropylene cages with sawdust bedding in hygienically controlled environment. Feed and water were supplied ad libitum. Animal experiments were approved by the experimental animal welfare and ethics review committee of Zhejiang University and were performed in compliance with the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the government of China.

### 2.2 | The experimental diets (feed) of *Rhizoma Atractylodis Macrocephalae* (RAM)

RAM was supplied by Huqingyutang Co., Hangzhou, China. Four hundred gram herbal medicines were decocted 2 h after saturated in 2000 ml water 1 h, respectively, in our laboratory. The herbal medicine was extracted to the extractive rates 2 g/ml by a R502B rotary evaporator (Shenko Tech Co Ltd, Shanghai, China), then mixed with 200 g substituting corn starch and dried at 40°C for 48 h. The experimental diets was consisted of 1% or 5% the decocted RAM. Two hundred grams of RAM were crushed and passed through a 1-mm screen, mixed into the feed.

### 2.3 | Experimental design

#### 2.3.1 | Experiment 1

Fifty ICR mice (5 weeks old) were allocated randomly to basic diet only (Control) or to the basic diet containing 1% Crush of RAM for a 1-week ad libitum feeding period (C-1 wk), 1% Crush of RAM for a 6-week ad libitum feeding period (C-6 wk), 1% Decoction of RAM for a 1-week ad libitum feeding period (D-1 wk) and 1% Decoction of RAM for a 6-week ad libitum feeding period (D-6 wk). Then the animals were subcutaneously injected twice with 200  $\mu$ l of FMDV type O vaccine within 2-week intervals. The active ingredient of the vaccine is composed of FC polypeptide of porcine FMDV type O. The potency was 5.2 PD<sub>50</sub> per dose when using 500 ID<sub>50</sub> viral dosage. The ID<sub>50</sub> was 10<sup>-7.0</sup> per 2 ml and the MID, which is the minimal infecting dose

was  $10^{-6.0}$  per 2 ml. One day before each immunisation, the mice had already been orally administered daily with the different treatment RAM. Blood samples were collected 2 weeks after the boost for detection of IgG level and the subclasses. Splenocytes were collected for determination of lymphocyte proliferation and production of IFN- $\gamma$  and IL-5.

### 2.3.2 | Experiment 2

Forty ICR mice were randomly divided into four groups with 10 mice in each. The animals were subcutaneously injected twice with 200  $\mu$ l of FMDV type O vaccine within 2-week intervals. One day before each immunisation, the mice had already been feed basic diet containing 5% decocted RAM for different days ad libitum feeding period (D-2d, D-4d, D-6d). Blood samples from each mouse were collected 2 weeks before the boost and 1, 2 and 3 weeks after the boost for detection of IgG level. The blood samples of 2 weeks after the boost were used for the detection of IgG subclasses were detected IgG subclasses. Five mice per group were euthanised 14 days after the last immunisation. Splenocytes were collected for determination of lymphocyte proliferation and production of IFN- $\gamma$  and IL-5. And tissue samples of small intestine (at least three pieces, 1 cm away from each other) were fixed in formalin solution for detecting sIgA.

### 2.4 | FMDV type O specific IgG titre and the IgG subclasses

Serum samples were analysed for measurement of serum IgG titre and the isotypes by an indirect double antibody sandwich enzyme-linked immunosorbent assay. The wells of polyvinyl 96-well microtitre plates were coated with 50  $\mu$ l rabbit anti-FMDV serotype O antibody (LVRI, China) diluted in 0.05 M carbonate/bicarbonate buffer (1:800), pH 9.6 and incubated overnight at 4°C. After five washes with phosphate buffer saline containing 0.05% Tween-20 (PBST), the wells were blocked with 5% skimmed milk and incubated at 37°C for 2 h. Thereafter, 50  $\mu$ l FMDV type O antigen (LVRI) (1:3 dilution) was added and incubated at 4°C for 2 h. After five washes, 50  $\mu$ l of serum (diluted serially for IgG titre analysis or diluted 1:50 in PBS 5% skimmed milk for isotype analysis) was added to each well and incubated at 37°C for 1 h. Plates were then washed five times in PBST. For IgG titre detection, 50  $\mu$ l of goat anti-mouse IgG (1:500) (Kirkegaard, Perry Lab., Maryland, USA) was added to the wells and incubated at 37°C for 1 h. Plates were washed again with PBST. Fifty microlitres of 3,3',5,5'-tetramethyl benzidine solution (100  $\mu$ g/ml of 0.1 M citrate-phosphate, pH 5.0) was added to each well and incubated for 15 min at 37°C. The reaction was stopped by adding 50  $\mu$ l of 2 M H<sub>2</sub>SO<sub>4</sub> to each well. The optical density of the plate was read by an automatic ELISA plate reader at 450 nm. Values above the cut-off background level (mean value of sera from unimmunised mice multiplied by a factor of 2.1) were considered positive. Titres were depicted as reciprocal end-dilutions. For subclasses, 50  $\mu$ l of biotin conjugated goat anti-mouse IgG1 or IgG2a or

IgG2b or IgG3 (1:600) (Santa Cruz Biotechnology Inc., California, USA) was added to corresponding plate and then incubated for 1 h at 37°C. After washing, 50  $\mu$ l of horseradish peroxidase conjugated anti-biotin (BD Biosciences, Pharmingen, USA) diluted 1:4000 in PBST was added to each well and incubated for 1 h at 37°C. Incubations, washing and development were as described above for detection of FMDV-specific IgG titre. The optical density of the plate was read at 450 nm.

### 2.5 | Splenocyte proliferation assay

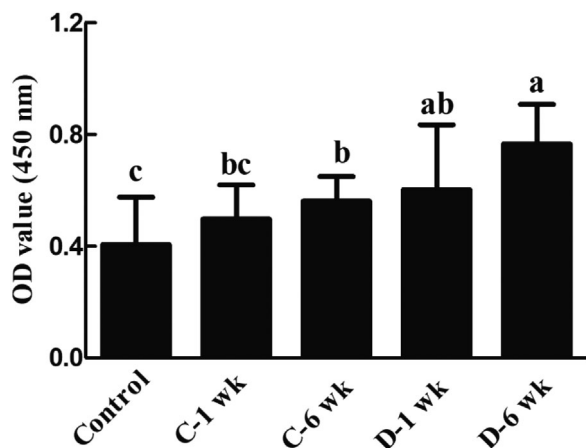
Spleen collected from the FMDV-immune ICR mice under aseptic conditions, in Hank's balanced salt solution (HBSS, Sigma), was minced and passed through a fine steel mesh to obtain a homogeneous cell suspension. After centrifugation (380  $\times$  g at 4°C for 10 min), the pelleted cells were washed three times in HBSS and resuspended in complete medium [RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin and 10% heat inactivated foetal calf serum (FCS)]. Cell numbers were counted with a haemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocyte proliferation was assayed as described previously (Xiao et al., 2007). The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen cultures divided by the absorbance value for non-stimulated cultures.

### 2.6 | IFN- $\gamma$ and IL-5 production by splenocytes in vitro

Single cell suspensions were adjusted to a concentration of  $2.5 \times 10^6$  cells/ml in complete medium. To a 96-well flat-bottom microtitre plate (Nunc), 100  $\mu$ l of the cell suspension and equal volume of Con A solution (Final concentration 5  $\mu$ g/ml) were added. The plates were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 48 h. After that, the culture supernatants were collected for cytokine assay. The concentrations of IFN- $\gamma$  and IL-5 were determined by a commercial capture ELISA kit (R & D Systems Inc., Minneapolis, USA). Concentrations of cytokines were calculated from interpolation of the cytokine standard curve.

### 2.7 | Immunohistochemical examination for IgA secreting cells

Frozen sections of small intestine were serially cut at 12  $\mu$ m and placed on poly-lysine coated glass slides. Then the slides were fixed at 4% paraformaldehyde in PBS buffer (0.01 M, pH 7.2). Twenty sections were prepared for each animal sample which size was at least 100  $\mu$ m, neutralised by 0.6% H<sub>2</sub>O<sub>2</sub> in PBS for 30 min, rinsed in PBS for 5 min. The sections were treated with 5% normal goat serum in PBS for 20 min to block non-specific binding and then stained separately with rabbit-anti-mouse IgA antibody at 4°C overnight; the sections were rinsed three times with 0.2% Triton-PBS for 15 min; then incubated with SPA-HRP for 1 h at 37°C. After the sections were rinsed with



**FIGURE 1** FMDV-specific IgG. Mice ( $n = 10$ ) were immunised s.c. twice with  $200 \mu\text{l}$  of FMDV vaccine at 2-week intervals. Serum FMDV-specific IgG was measured by an indirect ELISA. Values above the cut-off background level (the mean from unimmunised mice as negative control multiplied by a factor of 2.1) were considered positive. The values are presented as mean  $\pm$  SD. Bars with different letters are  $p < 0.05$

Tris-HCl for 5 min. The reactions were made visible with metal-enhanced diaminobenzidine (DAB). All incubations were performed in a moist chamber. Control staining was carried out simultaneously in which the first antibody was replaced with PBS. No specific staining was found in the control.

## 2.8 | Statistical analysis

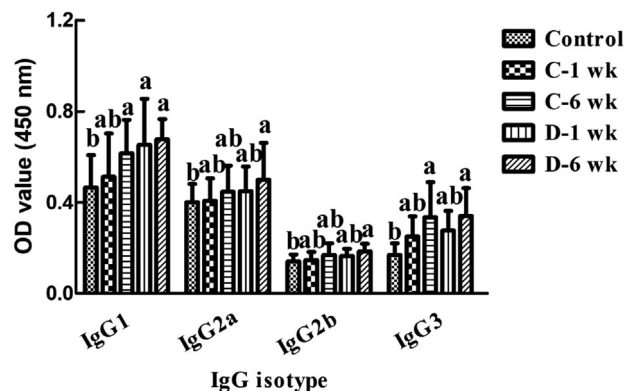
Data analysis was performed with SPSS software (SPSS, Version 16.0, SPSS Inc., Chicago, IL). ANOVA with Bonferroni post hoc test was used for multiple comparisons between groups. Values were expressed as the mean  $\pm$  standard deviation (SD).  $p$  Values of less than 0.05 were considered statistically significant.

## 3 | RESULTS

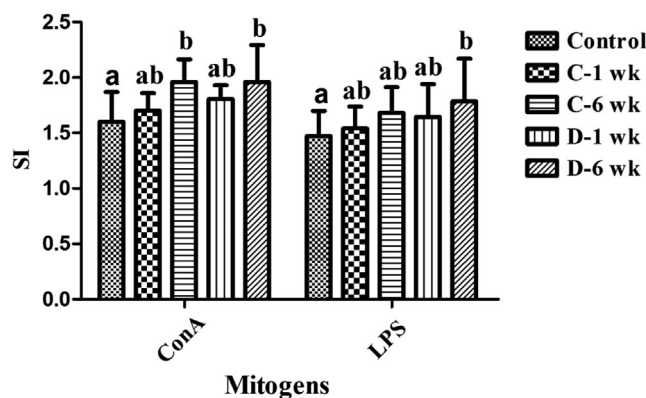
### 3.1 | Experiment 1

#### 3.1.1 | FMDV-specific IgG and the IgG isotypes

FMDV-specific IgG levels were significantly higher in mice feed Crush of RAM for 6 weeks, Decoction of RAM for 1 week, Decoction of RAM for 6 weeks at a dose of 1% than the basic diet only ( $p < 0.05$ ) (Figure 1). No significant differences between mice fed Crush of RAM for 1 week and the daily feed only were observed ( $p > 0.05$ ). D-6 wk group was significantly higher than other groups ( $p < 0.05$ ). Figure 2 indicated that some IgG isotypes tended to be higher in C-6 wk group and D-1 wk group than in the other group, but significantly increased IgG isotypes were found in D-6 wk group ( $p < 0.05$ ).



**FIGURE 2** FMDV-specific IgG isotypes. Mice were immunised s.c. twice with  $200 \mu\text{l}$  of FMDV vaccine at 2-week intervals. Serum (1:50 dilution) FMDV-specific isotypes were measured by an indirect ELISA. The values are presented as mean  $\pm$  SD ( $n = 10$ ). Bars with different letters are  $p < 0.05$



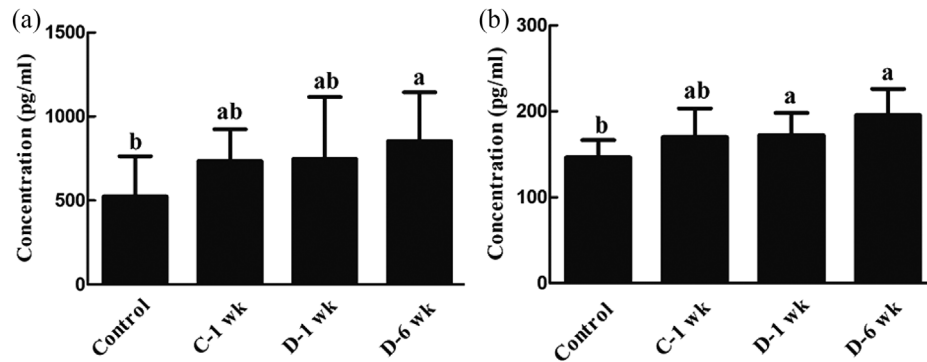
**FIGURE 3** Splenocyte proliferative responses to Con A and LPS. Mice were immunised s.c. with  $200 \mu\text{l}$  of FMDV vaccine. The control mice were injected with physiological saline solution ( $200 \mu\text{l}$ ) in the same manner. Splenocytes were prepared 2 weeks after the last immunisation and cultured with Con A ( $5 \mu\text{g}/\text{ml}$ ) or LPS ( $5 \mu\text{g}/\text{ml}$ ) or RPMI 1640. Splenocyte proliferation was measured by the MTT method as described in the text and shown as a stimulation index. The values are represented mean  $\pm$  SD ( $n = 10$ ). Bars with different letters are  $p < 0.05$

#### 3.1.2 | Proliferation of splenocytes isolated from FMDV-immunised mice

The effects of RAM on splenocyte proliferative responses to Con A and LPS are shown in Figure 3. SI for the proliferation in response to Con A and LPS were numerically increased in feed RAM group but significantly increased in D-6 wk group when compared with the control ( $p < 0.05$ ).

#### 3.1.3 | IL-5 and IFN- $\gamma$ production by mouse splenocytes

Cytokines secreted in the supernatants are shown in Figure 4. IFN- $\gamma$  and IL-5 production were significantly higher in the cultures of cells



**FIGURE 4** IFN- $\gamma$  (a) and IL-5 (b) production by ICR mouse splenocytes stimulated with Con A. Mice were immunised s.c. with 200  $\mu$ l of FMDV vaccine at 2-week intervals. The control mice were injected with physiological saline solution (200  $\mu$ l) in the same manner. Splenocytes were prepared 2 weeks after the last immunisation and cultured with Con A (5  $\mu$ g/ml). The culture supernatants were harvested after 48 h incubation of the splenocytes with Con A. The values are presented as mean  $\pm$  SD ( $n = 10$ ). Bars with different letters are  $p < 0.05$

**TABLE 1** Effects of feed RAM on the mean body weight (mean  $\pm$  SD gram) of mice ( $n = 10$ ) injected with FMDV vaccine (200  $\mu$ l)

Group	Before immunisation	Before booster	2 weeks after booster
Control	27.17 $\pm$ 1.71	30.76 $\pm$ 2.11	34.45 $\pm$ 2.63
C-1 wk	27.35 $\pm$ 0.96	30.78 $\pm$ 1.36	34.32 $\pm$ 1.85
C-6 wk	27.49 $\pm$ 1.19	30.59 $\pm$ 1.53	33.60 $\pm$ 1.96
D-1 wk	27.01 $\pm$ 1.04	30.55 $\pm$ 1.79	34.80 $\pm$ 2.01
D-6 wk	27.26 $\pm$ 0.83	30.43 $\pm$ 1.47	33.97 $\pm$ 2.01

Note: No statistical difference between groups at the same time ( $p < 0.05$ ).

isolated from the mice feed basic diet containing Decoction of RAM for 6 weeks than from the mice basic diet only ( $p < 0.05$ ).

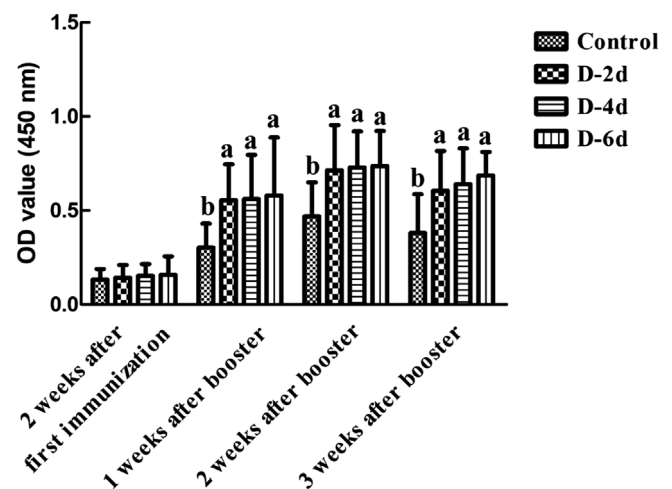
### 3.1.4 | Effects of feed RAM on the body weight of mice

No abnormal behaviour and side effects were found in mice throughout the experiment. There was no significant difference for the body weight between the mice feed basic diet with RAM and the control mice feed basic diet only as indicated in Table 1 ( $p > 0.05$ ).

## 3.2 | Experiment 2

### 3.2.1 | FMDV-specific IgG and the IgG isotypes

FMDV-specific IgG levels in different immune period were measured (Figure 5). There were no significant differences among the experiment groups and the control group at 2 weeks after first immunisation, but after booster IgG levels of D-2d, D-4d and D-6d groups were significantly higher than control group ( $p < 0.05$ ) during the whole immune period. Similar results were obtained in IgG isotypes (Figure 6); that is D-2d, D-4d and D-6d groups increased significantly than the basic diet group ( $p < 0.05$ ).



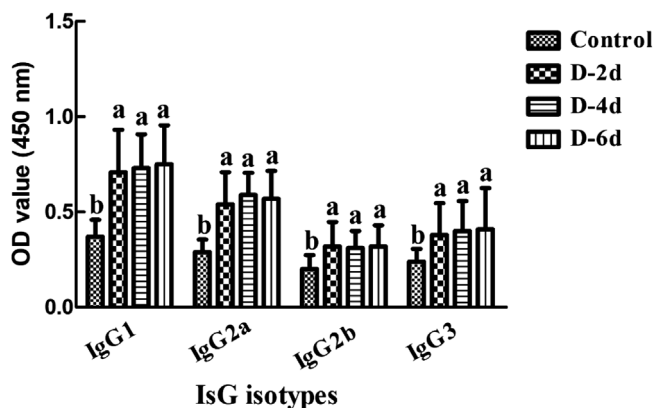
**FIGURE 5** FMDV-specific IgG. Mice ( $n = 10$ ) were immunised s.c. twice with 200  $\mu$ l of FMDV vaccine at 2-week intervals. Serum FMDV-specific IgG was measured by an indirect ELISA. Values above the cut-off background level (the mean from unimmunised mice as negative control multiplied by a factor of 2.1) were considered positive. The values are presented as mean  $\pm$  SD. Bars with different letters are  $p < 0.05$

### 3.2.2 | Proliferation of splenocytes isolated from FMDV-immunised mice

The effects of RAM on splenocyte proliferative responses to Con A and LPS are shown in Figure 7. SI for the proliferation in response to Con A and LPS were significantly increased in feed RAM group in different days when compared with the control ( $p < 0.05$ ).

### 3.2.3 | IL-5 and IFN- $\gamma$ production by mouse splenocytes

Cytokines secreted in the supernatants are shown in Figure 8. IFN- $\gamma$  and IL-5 productions were significantly higher in the cultures of cells



**FIGURE 6** FMDV-specific IgG isotypes. Mice were immunised s.c. twice with 200  $\mu$ l of FMDV vaccine at 2-week intervals. Serum (1:50 dilution) FMDV-specific isotypes were measured by an indirect ELISA. The values are presented as mean  $\pm$  SD ( $n = 10$ ). Bars with different letters are  $p < 0.05$

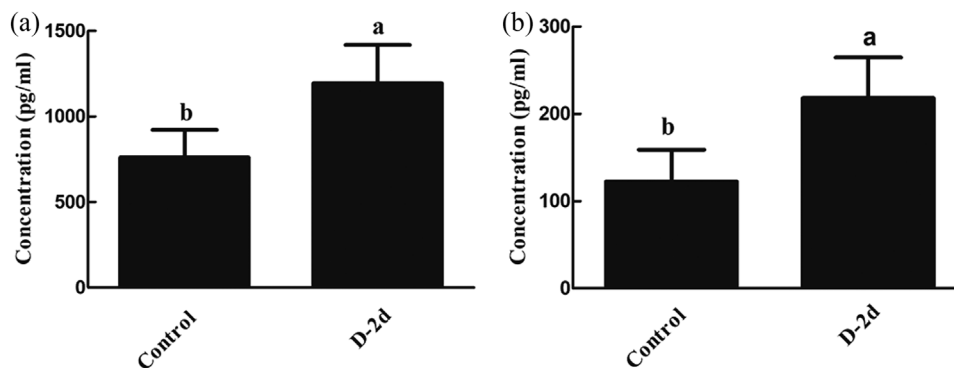
isolated from the mice feed basic diet containing 5% Decoction of RAM for 2 days than from the mice basic diet only ( $p < 0.05$ ).

### 3.2.4 | On the body weight of mice

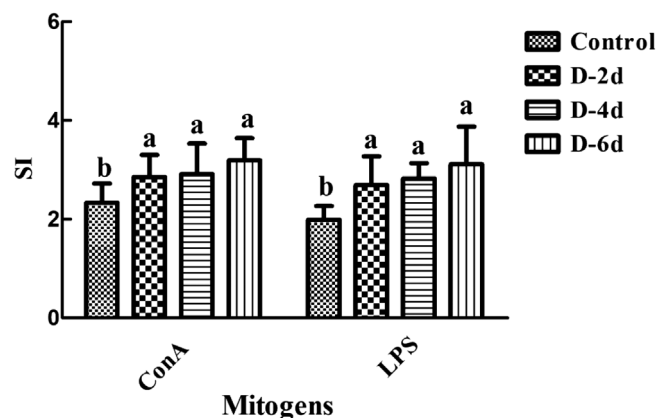
No abnormal behaviour and side effects were found in mice throughout the experiment. There was no significant difference for the body weight between the mice feed basic diet with RAM and the control mice as indicated in Table 2 ( $p > 0.05$ ).

### 3.2.5 | The area changes of IgA secreting cells in small intestine

In each fragment of intestine samples, IgA secreting cells were recognised as lymphocytes by their characteristic morphology: rounded, with a nucleus surrounded by a ring of yellow-brown cytoplasm



**FIGURE 8** IFN- $\gamma$  (a) and IL-5 (b) production by ICR mouse splenocytes stimulated with Con A. Mice were immunised s.c. with 200  $\mu$ l of FMDV vaccine at 2-week intervals. The control mice were injected with physiological saline solution (200  $\mu$ l) in the same manner. Splenocytes were prepared 2 weeks after the last immunisation and cultured with Con A (5  $\mu$ g/ml). The culture supernatants were harvested after 48 h incubation of the splenocytes with Con A. The values are presented as mean  $\pm$  SD ( $n = 10$ ). Bars with different letters are  $p < 0.05$



**FIGURE 7** Splenocyte proliferative responses to Con A and LPS. Mice were immunised s.c. with 200  $\mu$ l of FMDV vaccine. The control mice were injected with physiological saline solution (200  $\mu$ l) in the same manner. Splenocytes were prepared 2 weeks after the last immunisation and cultured with Con A (5  $\mu$ g/ml) or LPS (5  $\mu$ g/ml) or RPMI 1640. Splenocyte proliferation was measured by the MTT method as described in the text, shown as a stimulation index. The values are represented mean  $\pm$  SD ( $n = 10$ ). Bars with different letters are  $p < 0.05$

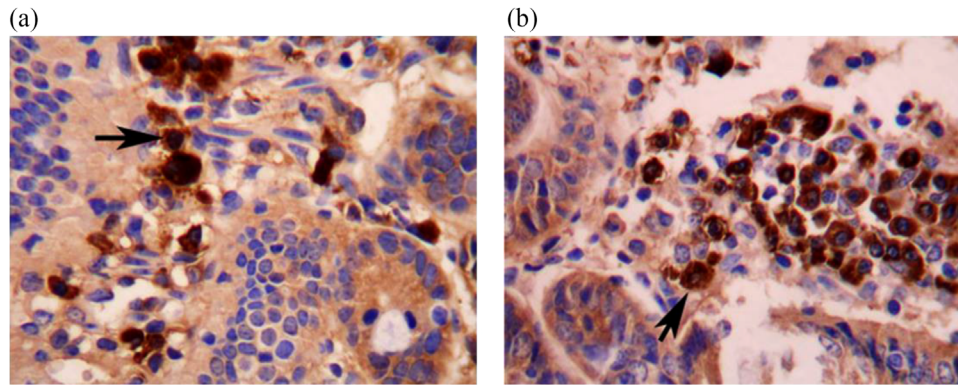
**TABLE 2** Effects of feed RAM on the mean body weight (mean  $\pm$  SD gram) of mice ( $n = 10$ ) injected with FMDV vaccine (200  $\mu$ l)

Group	Before immunisation	Before booster	2 weeks after booster
Control	23.93 $\pm$ 1.16	29.97 $\pm$ 1.65	33.90 $\pm$ 3.03
D-2d	23.79 $\pm$ 0.89	30.17 $\pm$ 2.08	32.89 $\pm$ 2.22
D-4d	23.90 $\pm$ 1.45	30.33 $\pm$ 2.39	33.40 $\pm$ 2.99
D-6d	23.71 $\pm$ 1.03	29.20 $\pm$ 1.46	32.51 $\pm$ 2.03

Note: No statistical difference between groups at the same time ( $p > 0.05$ ).

(Figure 9a and b). In our study, the number of IgA secreting cells of D-2d group was significantly higher than control group as indicated in Table 3 ( $p < 0.05$ ).





**FIGURE 9** The IgA secreting cells in small intestine in the control group (a) and the D-2d group (b). IgA secreting cells were recognised as lymphocytes by their characteristic morphology: rounded, with a nucleus surrounded by a ring of yellow-brown cytoplasm. (Immunohistochemical, 10 × 40)

**TABLE 3** Effects of feed RAM on the number of IgA secreting cells in small intestine

Group	The number of IgA secreting cells
Control	12.49 ± 0.40
D-2d	21.73 ± 0.84*

\*The difference of two groups was statistically significant ( $p < 0.05$ ).

#### 4 | DISCUSSION

Information relating to immunological effect of RAM has seldom been obtained in animal models involving feed. Many animal experiments have suggested that the immunological effects of RAM extracts by oral or using cell culture systems were different. In our research, immunomodulatory effect of feeding RAM has been demonstrated in mice.

The cellular immune response plays an important role in the host response against the intracellular pathogens via limiting their replication and accelerating clearance of the infected cells. Among T lymphocytes, the helper T cells induce B lymphocytes to secrete antibodies, and the cytotoxic T lymphocytes help phagocytes to destroy ingested microbes and to clear the intracellular microbes. However, the humoral immunity mediated by antibodies neutralise and eliminate the extracellular microbes and microbial toxins. The capacity to elicit the effective T- and B-lymphocyte immunity can be shown by the lymphocyte proliferation response (Feng et al., 2013). Studies have shown that RAM can effectively promote the transformation of splenic lymphocytes (Lin et al., 2015), change the immune status of CD4+ T cells, stimulate Th2 response (Kwak et al., 2018) and improve the body's immunity (Fan et al., 2016). In experiment 1, we added 1% RAM that treated in different processing methods (crush or decoction) to the basic diet, then fed to mice for 1- or 6-week ad libitum feeding period. Lymphocyte transformation rate is an important index to evaluate cellular immune function (Ling et al., 1990). The results of our experiment are consistent with previous research conclusions, which together confirm that RAM as a vaccine adjuvant can significantly improve the immune function of FMDV vaccine. The mice fed RAM for 6 weeks tend

to have higher adjuvant immunity to FMDV than mice fed for 1 week. However, effects of feeding time of RAM on the immune responses were not evident. Humoral immune response against FMD infection has been well documented and the contribution of antibodies to the major immune defence against FMDV is clear (Meloan et al., 1979). Studies in animal models have shown that specific antibodies play an important role in the immune defence against FMD. Figure 1 showed that feeding different treatments of RAM significantly increased serum FMDV-specific IgG levels to a commercial FMD vaccine except feeding crush RAM for 1 week. Feeding Decoction of RAM 6 weeks significantly increased IgG1, IgG2a, IgG2b and IgG3 as indicated in Figure 2. In mice, Th2 lymphocyte cytokines such as IL-4, IL-5 and IL-10 augment the production of IgG1, while the IL-2, TNF- $\beta$  and IFN- $\gamma$  produced by Th1 lymphocytes improve the production of IgG2a. In terms of the body response against infectious diseases, the Th1 response primarily targets intracellular pathogens such as viruses and certain bacteria, while the Th2 response is mainly targeted to extracellular pathogens, such as most bacteria and certain parasites. Enhanced production of all IgG subclasses may be explained by increased release of both IL-5 and IFN- $\gamma$  as shown in Figure 4. Our results are similar to those in the following research studies. It has been demonstrated that the protein and polysaccharide samples of RAM led to greater lymphocyte proliferation and TNF- $\alpha$  and IL-6 production in cultured splenocytes than did the crude water extracts at the same concentrations tested (Son et al., 2017). Recently, it was reported that the polysaccharide of *Atractylodes macrocephala* Koidz enhances TNF- $\gamma$  secretion by stimulating the TLR4-MyD88-NF- $\kappa$ B signalling pathway in the mouse spleen (Li et al., 2019). All these suggested that both Th1 and Th2 immune responses were activated. Thus, when the purpose of a vaccination is to activate both Th1 and Th2 immune response, feeding the Decoction of RAM for a 6-week ad libitum feeding period is indicated.

The results of experiment 1 indicated that treatment of decoction was better than crush. Therefore, we then added 5% RAM decoction to the basal diet and fed the mice for 2, 4 and 6 days, respectively. Both humoral immune response and cellular immune response to FMDV improved significantly by feeding 5% Decoction of RAM in 2–6 days. The increased production of IgG and the IgG subclasses (as indicated in

Figures 5 and 6) may be due to an activation of specific lymphocytes by RAM. Figure 7 shows that both Con A- and LPS-induced proliferations were significantly enhanced, suggesting that T as well as B cells were activated (Ren et al., 2012). To induce antibody production, triggered B lymphocytes are required for clonal expansion. The enhanced lymphocyte responses to Con A or LPS stimulation paralleled the increased serum IgG responses detected in the mice fed RAM.

Most experimental animal studies have focused exclusively on systemic immunity. Thus, information is scant regarding the immunological effects, particularly with respect to the mucosae, of feeding RAM. The lack of information on the influence of RAM on the intestinal immune system cannot be ignored. In the present experiment, ingestion of RAM increased significantly mucosal IgA responses. sIgA plays an important role in host defence to prevent bacteria and toxins from invading the body through the wet mucosae. It has also been reported that *Atractylodes* I (AT-I) stimulates intestinal epithelial cell migration and proliferation via the polyamine-mediated Ca<sup>+</sup> signalling pathway and maintains the integrity of the intestinal mucosal epithelial barrier (Song et al., 2017). Researchers have previously indicated that RAM can improve the function of the gastrointestinal tract and regulate the intestinal flora (Wang et al., 2014). We speculated that it has a potential therapeutic effect on diseases such as enteritis and diarrhoea.

In summary, RAM could enhance the immune responses to FMDV and the treatment of RAM is important for its efficacy. The results show that feeding 1% Decoction of RAM 1 week or 6 weeks can significantly improve the antibody levels to FMDV. Feeding the decoction treatment of RAM can also improve the proliferation activity of spleen lymphocytes in mice stimulated by LPS and have a certain synergistic effect on the sIgA. And that no adverse reactions were observed in mice fed *atractylodes*, and no significant effects were observed on weight. Therefore, it is necessary to further understand the molecular mechanism and structure–function relationship of the effective ingredients of RAM, as well as their potential synergistic and antagonistic effects.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ETHICS STATEMENT

All animal experiments were conducted in accordance with institutional guidelines and Institutional Animal Care and Use committee at Zhejiang University. All efforts were made to minimise the suffering of experimental animals.

## AUTHOR CONTRIBUTIONS

Ruilu Li: data curation; formal analysis; writing-original draft; writing-review and editing. Ming Qin and Kedsirin Sakwivatkul: formal anal-

ysis; methodology; writing-review and editing. Han Yan and Xiaoyu Chang: conceptualisation; project administration; supervision; writing-review and editing. Jiewei Liu, Shengbo Chi and Yutao Li: conceptualisation; funding acquisition; methodology; project administration; supervision; writing-review and editing.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1002/vms3.871>.

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