https://doi.org/10.17221/44/2020-VETMED

# Evaluation of red clover isoflavone extract as a vaccine adjuvant for piglets against *Haemophilus parasuis*

Xiaohua Li[1,2], Longxin Qiu[1,2], Guo-Hua Qiu[1,2], Xiaoyan Yang[1,2], Xintian Zheng[1,2]*

[1]College of Life Science, Longyan University, Longyan, Fujian, P.R. China
[2]Fujian Provincial Key Laboratory of Preventive Veterinary Medicine and Veterinary Biotechnology, Fujian, P.R. China

*Corresponding author: zh-xintian@163.com

**Citation:** Li X, Qiu L, Qiu GH, Yang X, Zheng X (2020): Evaluation of red clover isoflavone extract as a vaccine adjuvant for piglets against *Haemophilus parasuis*. Vet Med-Czech 65, 387–393.

**Abstract:** Glässer's disease of swine caused by *Haemophilus parasuis* (*H. parasuis*) is one of the major bacterial diseases affecting pig farms worldwide. Vaccination is a crucial measure for controlling the *H. parasuis* infection. Adjuvants are employed to enhance the immunity effects of inactivated vaccines or subunit vaccines. In the present study, a red clover isoflavone extract (RCIE) was investigated as an adjuvant for the *H. parasuis* inactivated vaccine. Thirty colostrum-deprived (CD) piglets (mixed-breed: Large White × Landrace) aged 15 days were vaccinated on days 0 and 14 with an inactivated *H. parasuis* vaccine with or without an adjuvant. The adjuvant groups' vaccines were mixed with a high-dose RCIE (20 mg/ml), a middle-dose RCIE (10 mg/ml), a low-dose RCIE (5 mg/ml), or with Montanide Gel 01 (10%, v/v). Phosphate buffer saline (PBS) was also given as a blank control. Fourteen days after the booster immunisation, the piglets were challenged with *H. parasuis* LY02 (serotype 5). The IgG antibody, cytokines, T lymphocyte subpopulations, and clinical and pathological signs of the piglets were evaluated. The results showed that the RCIE enhanced the *H. parasuis* vaccine and elicited strong antibody levels as well as the cytokines IL-2, IL-4, and IFN-γ in serum, and the levels depended on the RCIE dose. Moreover, the piglets vaccinated with the inactivated LY02 containing the Middle-dose RCIE had a higher survival rate in the challenge experiments. In conclusion, RCIE can enhance the *H. parasuis* vaccine immunity by promoting titres of IgG antibody and by improving the Th1-type cellular immunity.

**Keywords:** adjuvant; *Haemophilus parasuis*; red clover isoflavone extract; vaccine; immunity

*Haemophilus parasuis (H. parasuis)*, a coloniser of the upper respiratory tract of pigs, is a small Gram-negative bacterium belonging to the family *Pasteurellaceae*. The bacterium is a pathogen causing swine Glässer's disease, which is prevalent in pig farms worldwide, including China (Zhao et al. 2018a). There are at least fifteen serotypes that have been identified using the heat-stable

Supported by the Production-College-Research Cooperation Program of Science and Technology Office of Fujian Province, P.R. China (Grant No. 2018N5012), Natural Science Foundation of Fujian Province, P.R. China (Grant No. 2017J01639), Special Projects for Local Science and Technology Development Guided by the Central Government (Grant No. 2019L3011), Longyan Xinluo District Chi Mai Innovation Fund Project (Grant No. XLQM006).

https://doi.org/10.17221/44/2020-VETMED

antigen extract serotyping method (Angen et al. 2004), and the most prevalent serotypes in China are types 4 and 5 (Zhang et al. 2012; Wang et al. 2017; Zhao et al. 2018b). To control the *H. parasuis* infection, antibiotics are usually somewhat effective in killing the bacteria. However, antibiotic tolerance is difficult to be cleared from pigs, and this can lead to safety issues regarding food (Zhang et al. 2018; Zhao et al. 2018a). Therefore, vaccination is still important for controlling this disease. Several types of *H. parasuis* vaccines have been developed, including inactivated vaccines and subunit vaccines (Li et al. 2015; Guo et al. 2017; Zheng et al. 2017). These have been demonstrated to be safe and effective against homologous serovar strains in mice or pig model tests (Takahashi et al. 2001; Bak and Riising 2002; Martinez-Martinez et al. 2013; Liu et al. 2016). Exploiting better adjuvants is one of the most common approaches to enhancing the effect of *H. parasuis* vaccines against these strains. There are several types of adjuvants for the *H. parasuis* vaccine, including Freund's adjuvant, mineral oil, and microspheres (Li et al. 2017a; Zheng et al. 2017). Chinese herbal medicinal ingredients (CHMI), including plant lectins and saponins (Granell et al. 2010; Liu et al. 2012), have attracted recent research interest regarding the developmentof new adjuvants for medical and veterinary usage due to the advantages of their extensive availability, low cost, reliable efficacy, and low risk of side effects and toxicity (Kong et al. 2004; Deng 2006; Kong et al. 2006).

In the present study, the adjuvant activity of a red clover isoflavone extract (RCIE) was evaluated for the inactivated *H. parasuis* vaccine in piglets. We found that the RCIE can improve the immunity of the inactivated *H. parasuis* vaccine.

## MATERIAL AND METHODS

### Material

The strain *H. parasuis* LY02 (serotype 5) (Li et. al 2015) was grown on a tryptic soy broth (TSB) (Oxoid, Basingstoke, England) or on a tryptic soy agar (TSA) (Oxoid, Basingstoke, England) with 10 μg/ml nicotinamide adenine dinucleotide (Oxoid, England) and 5% bovine serum (HyClone, Beijing, P.R. China) at 37 °C. The RCIE, whose isoflavones consisted of 10.2% formononetin, 9.6% biochanin A, 0.32% genistein, and 0.08% daidzein, was purchased from Naturalin Bio-Resources Co., Ltd (NAT-177; Hunan, Changsha, P.R. China). The Montanide Gel 01 was purchased from Seppic Inc. (Paris, France).

### Preparation of the inoculum

To harvest the *H. parasuis* LY02, the bacteria were serially passaged in the TSB medium three times at 37 °C, 180 *g* for 18 hours. After centrifugation at 900 *g* for 10 min, the pellets were resuspended in PBS, and the concentration of the bacteria was adjusted to $5 \times 10^9$ colony-forming units (CFU) per ml. The suspension was added with 0.4% formaldehyde and inactivated at 37 °C for 24 hours. To prepare the inoculum, the inactivated *H. parasuis* was homogenised with the RCIE or Montanide Gel 01 (Table 1).

### Animal immunisation schedule

All the animal experiments were performed in strict accordance with the recommendations of the China Regulations for the Administration of Affairs Concerning Experimental Animals 1988. The protocol was approved by the Ethics Committee of Longyan College (Permit No. LY201802L). Thirty female colostrum-deprived (CD) piglets (Landrace × Large White) aged 15 days were purchased from Longyan, P.R. China. All the piglets were antibody negative against *H. parasuis*, *Pseudorabies* virus, hog cholera virus, and Porcine reproductive and respiratory syndrome virus. The piglets were randomly and evenly divided into six groups. The animals were given inoculations according to the list in Table 1. All the animals were inoculated twice with the same dose at intervals of 21 days. To ob-

Table 1. Vaccination list

| Group | Number of animals | Inoculation | Adjuvants | Concentration of adjuvants |
|---|---|---|---|---|
| A | 5 | *H. parasuis* | RCIE | 5 mg/ml |
| B | 5 | *H. parasuis* | RCIE | 10 mg/ml |
| C | 5 | *H. parasuis* | RCIE | 20 mg/ml |
| D | 5 | *H. parasuis* | Montanide Gel 01 | 10% (v/v) |
| E | 5 | *H. parasuis* | – | – |
| F | 5 | PBS | – | – |

https://doi.org/10.17221/44/2020-VETMED

tain the sera, precaval vein blood samples were collected on days 0, 21, and 35, centrifuged at 600 *g* for 20 min, and then stored at −80 °C until analysis.

### Determination titres of antibody and cytokines

The specific antibodies against *H. parasuis* were determined using an ELISA (enzyme-linked immunosorbent assay) kit (FEIKAI; Biotech Co., Ltd., Beijing, P.R. China) following the manufacturer's instructions (Li et al. 2015). The cytokine levels of the IFN-γ, TNF-α, IL-2, and IL-4 in sera were determined using ELISA kits (Bogoo Biotech Co., Ltd., Shanghai, P.R. China). All the data were representative of three independent experiments.

### Flow cytometric analysis of T lymphocyte subpopulations

To analyse the effects of the RCIE on the cellular immune response, the proportions of $CD4^-$ and CD8 T lymphocyte subsets were assessed by flow cytometry. A red blood cell (RBC) lysis solution (BD Biosciences, San Diego, USA) was used to lyse the red blood cells, and the red supernatant was removed by centrifugation at 500 × *g* for 10 min and washing with PBS three times. The lymphocytes were subsequently treated with Alexa Fluor-(AF-)labelled CD3 MAb (clone BB23-8E6-8C8, BD Biosciences, San Diego, USA), phycoerythrin-(PE-)labelled CD4 MAb (clone 74-12-4, BD Biosciences, San Diego, USA), and fluorescein isothiocyanate-(FITC-)labelled CD8 Mab (clone 76-2-11, BD Biosciences, San Diego, USA) antibodies in the dark for 30 min. After washing with PBS, the cells were fixed by incubation with a 5% paraformaldehyde solution in PBS containing 1% BSA and 0.1% sodium azide. All the samples were analysed by fluorescence profiles on a FACScan flow cytometer (BD Biosciences, San Diego, USA) using the SYSTEM II software (Coulter).

### Challenge test

Fourteen days after the second immunisation, the piglets from each group were challenged intraperitoneally with a lethal dose of $7.5 \times 10^9$ CFU (2 × LD50) *H. parasuis* strain LY02 (serotype 5). The rectal temperatures and other clinical signs of piglets were monitored, and the morbidity and mortality were recorded for seven days post-challenge.

### Clinicopathological evaluation

The temperatures of the piglets were measured rectally using a calibrated thermometer at the timepoints of 4 h and 24 h post challenge and then once a day during the monitoring period. The lesion scores were evaluated as described in a previous report (Olvera et al. 2011) and were calculated as the sum of the individual lesions/signs (lack of lesion = 0; presence of lesion = 1), catarrhal rhinitis, pulmonary consolidation, fibrin in the abdomen and/or ascites, fibrin in the thorax and/or hydrothorax, fluid and/or fibrin in the right elbow, in the left elbow, in the right knee, or in the left knee, and meningitis (ranging from 0 to 9). To confirm the association between the mortality and the *H. parasuis* infection, the bacterial isolates from the lesions were used in a 16s rDNA-based PCR (Polymerase chain reaction) as described previously (Zheng et al. 2017).

### Statistical analysis

The experimental data were expressed as the means ± standard deviation (SD). The differences between the groups were analysed using a two-way analysis of variance (ANOVA) by the GraphPad Prism statistical software, v8.0. *P*-values of < 0.05 were considered statistically significant.

## RESULTS

### Evaluation of humoral immune responses

The specific IgG antibodies against *H. parasuis* were evaluated by ELISA. As shown in Figure 1, on days 21 and 35 post-inoculation, the high-dose and middle-dose RCIE groups and the Montanide Gel 01 group (groups A, B, and D) produced significantly higher titres of IgG than the RCIE-free groups (groups E and F), and there were significant differences ($P < 0.01$) between groups B and E. Moreover, the levels of IgG in groups A and B were

https://doi.org/10.17221/44/2020-VETMED

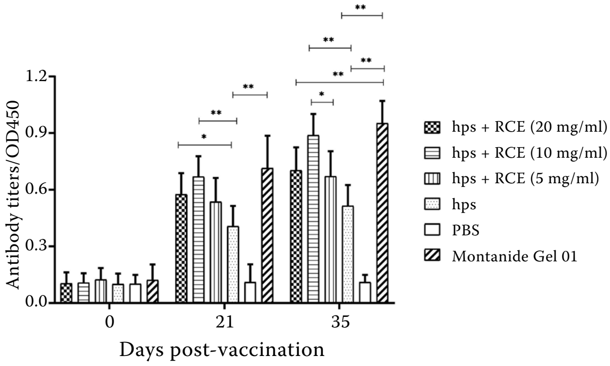


Figure 1. IgG titres of the piglets stimulated by the inactivated *H. parasuis* vaccines with different concentrations of RCIE. *$P < 0.05$, **$P < 0.01$, compared to group E

significantly higher than those in group C. However, there was no difference in the IgG levels between groups A and B ($P > 0.05$).

## Cytokine production

As shown in Figure 2, the cytokines IL-2, IL-4, IFN-γ, and TNF-α were detected in the sera. On day 21 post-inoculation (PI), the RCIE-added *H. parasuis* vaccine stimulated significantly higher titres of IL-2, IL-4, and IFN-γ compared with those in the adjuvant-free group (group E) and blank control (group F) ($P < 0.05$). However, on day 35 PI, there were no significant differences ($P > 0.05$) in these four cytokines between groups A, B, C, D, and E. Furthermore, the level of TNF-α was not significantly increased with the successive immunisations compared to the controls ($P > 0.05$).

## T-cell subpopulation analysis

To evaluate the T-cell subset changes after inoculating the vaccine, the $CD4^+$ and $CD8^+$ T-cells were analysed by flow cytometry. As shown in Table 2, group B had the highest percentage of $CD4^+$ and $CD8^+$ lymphocyte subsets compared with the other groups. Moreover, the $CD4^+/CD8^+$ ratio in group F was the highest among the six groups, and group B had the highest ratio among the RCIE groups (groups A, B, and C). However, there were no significant differences between the groups A, B and C ($P > 0.05$).

## Clinical and pathological evaluation

The clinical and pathological features are summarised in Table 3. Clinical symptoms including

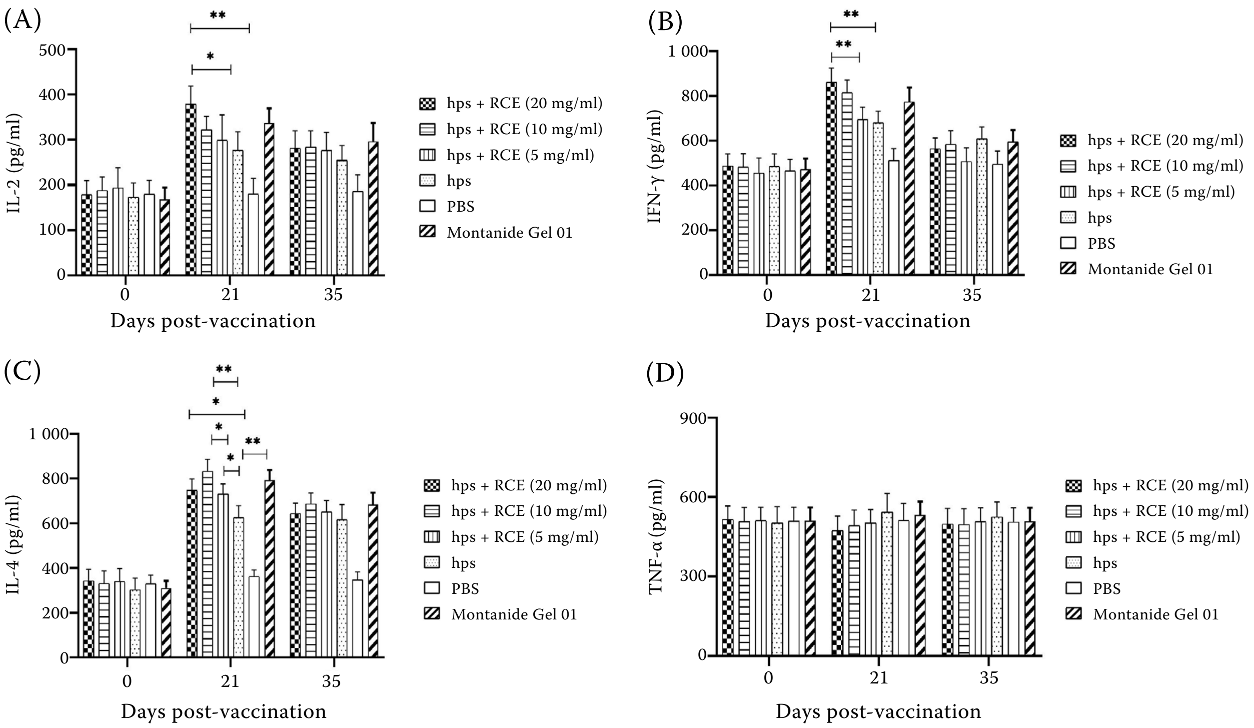


Figure 2. Cytokines in the serum samples of the piglets after inoculation with the *H. parasuis* vaccine containing the RCIE. *$P < 0.05$, **$P < 0.01$, compared to group E

https://doi.org/10.17221/44/2020-VETMED

Table 2. Comparison of T lymphocyte subset percentage of the piglets two weeks post booster immunisation

| Group | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| $CD4^+$ lymphocyte subset % | 24.90 ± 1.80[b] | 27.11± 1.90[a] | 24.62 ± 1.37[b] | 25.78 ± 1.64[b] | 24.54 ± 2.03[ab] | 22.20 ± 1.85[b] |
| $CD8^+$ lymphocyte subset % | 19.34 ± 1.63[b] | 21.86 ± 1.26[a] | 18.52 ± 0.76[b] | 20.48 ± 1.53[b] | 17.46 ± 1.58[b] | 17.42 ± 1.58[b] |
| $CD4^+/CD8^+$ ratio | 1.29 ± 0.11 | 1.24 ± 0.07 | 1.33 ± 0.08 | 1.26 ± 0.06 | 1.41 ± 0.06 | 1.27 ± 0.05 |

[a,b]The data marked by the same superscripts in each row are not statistically significantly different ($P < 0.05$)

Table 3. Comparison of the clinical features of the piglets after the challenge with *H. parasuis*

| Group | Temperature (°C) | Lesion score | Survival rate |
|---|---|---|---|
| A | 40.5 ± 0.3[ab] | 2.6 ± 0.89[b] | 60% |
| B | 39.7 ± 0.3[b] | 1.8 ± 0.83[b] | 80% |
| C | 40.2 ± 0.5[ab] | 2.4 ± 0.55[b] | 60% |
| D | 39.6 ± 0.2[b] | 1.9 ± 0.89[b] | 100% |
| E | 40.7 ± 0.4[ab] | 2.8 ± 0.84[b] | 60% |
| F | 41.3 ± 0.2[a] | 7.2 ± 1.30[a] | 0 |

[a,b]The numbers marked by the same superscripts in each column are not statistically significantly different ($P < 0.05$)

prostration and anorexia in the piglets were observed in all the groups with varying degrees within 24 h PI. The temperatures of all the challenged pigs were between 39.6 °C and 41.8 °C. The temperatures in the piglets of groups B and F were normal and were significantly lower than those of group E. All the pigs in group F were dead at 52 h PI, having shown additional clinical symptoms of incoordination, ataxia, anorexia, severe dyspnoea, and coughing. The survival rates of the pigs in the other groups ranged from 60% to 100%. The piglets that were euthanised on day 7 post-challenge had minor or moderate lesions when examined, with focal pneumonia and mild peritonitis. The pathological scores of the vaccinated pigs were significantly lower than those of the blank control (group F) ($P < 0.05$).

### Protection of vaccinated pigs

To evaluate the protective immunity of this adjuvant, 14 days after the last immunisation, pigs from each group were challenged with a lethal dose of the LY02 strain. All the pigs in the blank control group (group F) were dead by about 52 h PI (Table 3). Group E had the highest rate of protection at 100%. Meanwhile, the pigs immunised with vaccines survived until the end of the observation period. The immunisation of the piglets with the LY02 strain antigen significantly increased the pigs' survival rate ($P < 0.05$). Group B had the highest rate of protection (80%) against *H. parasuis* LY02.

## DISCUSSION

The red clover isoflavone extract (RCIE) consists mainly of formononetin, biochanin A, genistein, and daidzein. The extract is an important bio-activator, with multiple biological and pharmacological effects in animals and humans (Ren et al. 2001; Setchell et al. 2001; Jiang et al. 2011). The RCIE enhanced the immune effect of *H. parasuis* vaccine in mice by increasing the phagocytosis by the macrophages (Zhu et al. 2008), and isoflavone can enhance the cellular and humoural immunity by promoting the *IGF-1R* gene expression in the thymus of pigs (Wang et al. 2002). Considering the low production cost and the adjuvant activity mentioned above, the RCIE was tested as an *H. parasuis* vaccine adjuvant in a piglet model in the present study.

The protective immunity against extracellular bacteria mainly depends on the antibodies (Aragon et al. 2019). Therefore, the promotion of the titres of IgG against *H. parasuis* is extremely important for the adjuvants. In this study, we found that the titres of IgG in the vaccine groups with the RCIE were significantly higher than those in the RCIE-free groups, and the titres peaked in the group at the concentration of 10 mg/ml of RCIE (Figure 1). These results indicated that the RCIE-added *H. parasuis* vaccine could significantly boost the humoural immunity of the piglets.

In contrast, several studies have indicated that cellular responses are often indispensable for eliminating an *H. parasuis* infection (Chase and Lunney 2019). As a result, adjuvants that can improve cellular immunity induced by vaccine are attractive. In the present study, the RCIE was shown to possess this function. As a component of isoflavone, daidzein has been shown to stimulate the proliferation of T lymphocytes in mice (Zhang

https://doi.org/10.17221/44/2020-VETMED

et al. 1997). Consistent with the latter report, we found that the RCIE also enhanced the proliferation of the T lymphocytes in the piglets (Table 2). Furthermore, high levels of cytokines, including IL-2 and IFN-γ, were detected in the sera of the piglets (Figure 2), suggesting that the Th1 response was boosted. We postulated that the RCIE may activate the cellular immunity of the piglets, since IL-2 is produced by the activated T lymphocytes and plays a central role in the activation and expansion of the T-cells (Dembic 2015). Similar findings of RCIE enhancing the IL-2 in the serum of chickens were reported by a previous study (Jiang et al. 2011). IFN-γ, a major pro-inflammatory cytokine, is essential for the control of an *H. parasuis* infection (de la Fuente et al. 2009). Currently, there are several IFN-γ-inducible molecules that have been implicated in a host control against an *H. parasuis* infection (de la Fuente et al. 2009; Martin de la Fuente et al. 2009). Moreover, these pro-inflammatory cytokines are secreted by the Th1 T-cells, which are associated with protective immunity and are critical for controlling an *H. parasuis* infection (Wang et al. 2012). Another pro-inflammatory cytokine, TNF-α, also plays multiple roles in activating the macrophages, including playing a crucial role in anti-infection against *H. parasuis* (de la Fuente et al. 2009; Martin de la Fuente et al. 2009), and TNF-αis reduced by the red clover extract and its compounds (Mueller et al. 2010). However, we found that there were no differences in the TNF-α levels in the serum samples among the five groups in the present study, compared with the reduced levels of TNF-α in the tissue samples as reported by Li et al. (2017b). This difference may be attributed to the different types of samples used to test the cytokines. Therefore, the reduced titres of TNF-α in the lungs or lymph nodes may be expected. However, IL-4 is an anti-inflammatory cytokine and is related to the Th2 response. In our study, the IL-4 levels were significantly higher in the RCIE groups than in the adjuvant-free group (Group E), consistent with a higher portion of the $CD4^+$ T-cells and titres of IgG in the RCIE groups.

Although the adjuvanticity of RCIE has been demonstrated by this study, there is much work to be done before a commercial application can begin. We will conduct experiments of cross protection for the RCIE-added *H. parasuis* vaccine, and the side effects of RCIE during the immune period will also be evaluated in future studies.

In conclusion, the RCIE enhanced the titres of the antibodies against *H. parasuis*, with higher titres of IgG and cytokines (IL-2, IFN-γ, and IL-4) in the RCIE-adjuvant groups.

## Conflict of interest

The authors declare no conflict of interest.

## REFERENCES


Angen O, Svensmark B, Mittal KR. Serological characterization of Danish Haemophilus parasuis isolates. Vet Microbiol. 2004 Nov 15;103(3-4):255-8.

Aragon V, Segales J, Oliveira S. Glässer‘s disease. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz K, Stevenson GW, Zhang J, editors. Diseases of swine. 11th ed. New York: Wiley; 2019. p. 844-53.

Bak H, Riising HJ. Protection of vaccinated pigs against experimental infections with homologous and heterologous Haemophilus parasuis. Vet Rec. 2002 Oct;151(17):502-5.

Chase C, Lunney JK. Immune system. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz K, Stevenson GW, Zhang J, editors. Disease of swine. 11th ed. New York: Wiley; 2019. p. 264-91.

de la Fuente AJ, Ferri EF, Tejerina F, Frandoloso R, Martinez SM, Martin CB. Cytokine expression in colostrum-deprived pigs immunized and challenged with Haemophilus parasuis. Res Vet Sci. 2009 Aug;87(1):47-52.

Dembic Z. The cytokines of the immune system. London: Academic Press; 2015. 310 p.

Deng XH. [Research progress in Chinese traditional immunopoteniators]. J Chongqing Technol Business Univ (Nat Sci Ed). 2006;23:556-9. Chinese.

Granell A, Fernandez del-Carmen A, Orzaez D. In planta production of plant-derived and non-plant-derived adjuvants. Expert Rev Vaccines. 2010 Aug;9(8):843-58.

Guo L, Xu L, Wu T, Fu S, Qiu Y, Hu CA, Ren X, Liu R, Ye M. Evaluation of recombinant protein superoxide dismutase of Haemophilus parasuis strain SH0165 as vaccine candidate in a mouse model. Can J Microbiol. 2017 Apr;63 (4):312-20.

Jiang Y, Wang C, Yang Y. Effect of red clover isoflavone on antioxidant and immunity of chicken under heat stress. In: International Conference on Remote Sensing, Environment and Transportation Engineering. IEEE; 24–26 June 2011. P.R. China: Nanjing; 2011:6774-7.

Kong X, Hu Y, Rui R, Wang D, Li X. Effects of Chinese herbal medicinal ingredients on peripheral lymphocyte prolif-

https://doi.org/10.17221/44/2020-VETMED

eration and serum antibody titer after vaccination in chicken. Int Immunopharmacol. 2004 Jul;4(7):975-82.

Kong XF, Hu YL, Yin YL, Wu GY, Rui R, Wang DY, Yang CB. Chinese herbal ingredients are effective immune stimulators for chickens infected with the Newcastle disease virus. Poult Sci. 2006 Dec;85(12):2169-75.

Li XH, Zhao GZ, Qiu LX, Dai AL, Wu WW, Yang XY. Protective efficacy of an inactive vaccine based on the LY02 isolate against acute Haemophilus parasuis infection in piglets. Biomed Res Int. 2015;5:1-8.

Li M, Cai R, Song S, Jiang Z, Li Y, Gou H, Chu P, Li C, Qiu H. Evaluation of immunogenicity and protective efficacy of recombinant outer membrane proteins of Haemophilus parasuis serovar 5 in a murine model. Plos One. 2017a Apr 27;12(4):e176537.

Li L, Luo Q, Huang C, Chen X, Chen P, Li Y, Liu W, Chen Z. [Effects of soy isoflavones on expression of IL-2, IL-4, TNF-α and INF-γ in male rats' spleen]. Acta Agri Zhejiang Ensis. 2017b Sep;29(9):1458-64. Chinese.

Liu Y, Zhang S, Zhang F, Hu R. Adjuvant activity of Chinese herbal polysaccharides in inactivated veterinary rabies vaccines. Int J Biol Macromol. 2012 Apr 1;50(3):598-602.

Liu H, Xue Q, Zeng Q, Zhao Z. Haemophilus parasuis vaccines. Vet Immunol Immunopathol. 2016 Nov 1;180:53-8.

Martin de la Fuente AJ, Rodriguez-Ferri EF, Frandoloso R, Martinez S, Tejerina F, Gutierrez-Martin CB. Systemic antibody response in colostrum-deprived pigs experimentally infected with Haemophilus parasuis. Res Vet Sci. 2009 Apr;86(2):248-53.

Martinez-Martinez S, Frandoloso R, Rodriguez FE, Gil C, Hernandez-Haro C, Yubero S, Gutierrez MC. Immunoproteomic analysis of the protective response obtained with subunit and commercial vaccines against Glasser's disease in pigs. Vet Immunol Immunopathol. 2013 Feb 15; 151(3-4):235-47.

Mueller M, Hobiger S, Jungbauer A. Red clover extract: A source for substances that activate peroxisome proliferator-activated receptor alpha and ameliorate the cytokine secretion profile of lipopolysaccharide-stimulated macrophages. Menopause. 2010 Mar;17(2):379-87.

Olvera A, Pina S, Perez-Simo M, Aragon V, Segales J, Bensaid A. Immunogenicity and protection against Haemophilus parasuis infection after vaccination with recombinant virulence associated trimeric autotransporters (VtaA). Vaccine. 2011 Mar 24;29(15):2797-802.

Ren MQ, Kuhn G, Wegner J, Chen J. Isoflavones, substances with multi-biological and clinical properties. Eur J Nutr. 2001 Aug;40(4):135-46.

Setchell KD, Brown NM, Desai P, Zimmer-Nechemias L, Wolfe BE, Brashear WT, Kirschner AS, Cassidy A, Heubi JE. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. J Nutr. 2001 Apr;131(4 Suppl):1362-75.

Takahashi K, Naga S, Yagihashi T, Ikehata T, Nakano Y, Senna K, Maruyama T, Murofushi J. A cross-protection experiment in pigs vaccinated with Haemophilus parasuis serovars 2 and 5 bacterins, and evaluation of a bivalent vaccine under laboratory and field conditions. J Vet Med Sci. 2001 May;63(5):487-91.

Wang G, Zhang X, Han Z, Liu Z, Liu W. Effects of daidzein on body weight gain, serum IGF-I level and cellular immune function in intact male piglets. Asian Austral J Anim. 2002 Jul;15(7):1066-70.

Wang Y, Liu C, Fang Y, Liu X, Li W, Liu S, Liu Y, Liu Y, Charreyre C, Audonnet JC, Chen P, He Q. Transcription analysis on response of porcine alveolar macrophages to Haemophilus parasuis. BMC Genom. 2012;13(1):1-13.

Wang Z, Zhao Q, Wei H, Wen X, Cao S, Huang X, Wu R, Yan Q, Huang Y, Wen Y. Prevalence and seroepidemiology of Haemophilus parasuis in Sichuan province, China. PeerJ. 2017 May 30;5:e3379.

Zhang R, Li Y, Wang W. Enhancement of immune function in mice fed high doses of soy daidzein. Nutr Cancer. 1997;29(1):24-8.

Zhang J, Xu C, Guo L, Shen H, Deng X, Ke C, Ke B, Zhang B, Li A, Ren T, Liao M. Prevalence and characterization of genotypic diversity of Haemophilus parasuis isolates from southern China. Can J Vet Res. 2012 Jul;76(3):224-9.

Zhang J, Xia Y, Zheng R, Liang Z, Shen Y, Li Y, Nie M, Gu C, Wang H. Characterisation of a novel plasmid containing a florfenicol resistance gene in Haemophilus parasuis. Vet J. 2018 Apr;234:24-6.

Zhao Y, Guo L, Li J, Huang X, Fang B. Characterization of antimicrobial resistance genes in Haemophilus parasuis isolated from pigs in China. PeerJ. 2018a Apr 9;6:e4613.

Zhao Y, Wang Q, Li J, Lin X, Huang X, Fang B. Epidemiology of Haemophilus parasuis isolates from pigs in China using serotyping, antimicrobial susceptibility, biofilm formation and ERIC-PCR genotyping. PeerJ. 2018b Jun 13;6:e5040.

Zheng X, Yang X, Li X, Qiu G, Dai A, Huang C, Huang Q, Guo X. Omp16-based vaccine encapsulated by alginate-chitosan microspheres provides significant protection against Haemophilus parasuisin mice. Vaccine. 2017 Mar 7;35(10):1417-23.

Zhu Y, Ren H, Sun J, Wang R. [Effects of isoflavone extract from red clover on growth performance and immune function in mice]. J Shenyang Agric Univ. 2008 Feb;39 (1):104-7. Chinese.

Received: February 17, 2020
Accepted: August 22, 2020