Available online at www.sciencedirect.com

ScienceDirect

Procedia in Vaccinology 9 (2015) 66 – 79

Procedia in Vaccinology

www.elsevier.com/locate/procedia

8th Vaccine & ISV Congress, Philadelphia, USA, 2014

Construction and synergistic effect of recombinant yeast co-expressing pig IL-2/4/6 on immunity of piglets to PRRS vaccination

Gongming Luo[1]*, Luyi Yang[1*],Ge Liang[2]*, Xiaoping Wan[1]*, Chi Chen[1], Bing Wang[2], Jianlin Chen[1], Kai Zeng[2], Suqiong Zhan[2], Xiaohui Chen[2], Sancheng Ying[2], Jianglin Li[2], Xuebin Lu[2]**, Zezhou Wang[3]**, Rong Gao[1]**

1:Key Laboratory for Bio-Resource and Eco-Environment of Ministry Education, Key Laboratory for Animal Disease Prevention and Food Safety of Sichuan Province, Life Science College, Sichuan University, Chengdu，610064，China;

2:: Sichuan Academy of Animal Science, Chengdu, 610066, China

3: Center for Animal Disease Control of Sichuan Province, Chengdu, 610035, China

**: These authors contributed equally to the research. **:corresponding authors.*

Abstract

In order to develop cost-effective immunomodulator, the recombinant Pichia pastoris were firstly constructed to co-express porcine IL-2/4/6 genes, and then fermented to feed 45-days Tibetan piglets at different doses to evaluate its effects on immunity of piglets to PRRS vaccination, which simultaneously received intramuscular injection of inactivated PRRS vaccine. The results were found that the leukocytes, IgG and specific antibody to PRRSV, Th and Tc cells increased significantly in the blood of treated piglets in comparison with those of the control ($P<0.05$); the mRNA expression of TLRs (TLR-2, 3, 4, 7, 9), IFN-γ, IL-2, IL-4, IL-6, IL-7, IL-12 and IL-15 genes were elevated significantly in the immune cells from the blood of treated piglets ($P<0.05$). Moreover, the growth of the treated piglets also markedly improved whose average net weight gain was significantly higher than the control on 58 days post inoculation ($P<0.05$). These results suggest that the recombinant yeast can effectively enhance the systematic innate and adaptive immunity of piglets as well as promote the growth of piglet, which could be further developed as cost-effective promising immunomodulator to improve the control of pig PRRS disease.

Selection and peer-review under responsibility of the 8th Vaccine Conference Organizing Committee.

Corresponding authors, tel: +86-28-85411033; fax: +86 28 85471599.

Email address: gaorong96@gmail.com, lake96@qq.com;

Selection and peer-review under responsibility of the 8th Vaccine Conference Organizing Committee.
doi:10.1016/j.provac.2015.05.011

Keywords: piglet; pig interleukin genes; recombinant *Pichia pastoris;* immunity; growth.

1. Introduction

Nowadays pigs in intensive feeding farms are susceptible to various pathogens which could not be controlled only through vaccination. Viral and bacterial infectious diseases not only bring huge economical loss to pig industry but also result in severe public health problems in China. Although vaccines are very important for prevention of pig infectious diseases, immune failures of animal frequently happen worldwide due to inadequate immune responses or immunosuppression of animals caused by many reasons[1, 2]. Therefore, development of effective immunomodulator to enhance overall immunity of animals is one of practicable and economical choices to prevent animal diseases.

Cytokines have potent effects on the immune system, regulation haematopoietic cells differentiation, proliferation and/or activation. Since the early 1990s, many groups have examined to use cytokines as vaccine adjuvant [3], and have evidenced that most cytokines have the ability to modify and re-direct the immune response[4]. IL-2, IL-4 and IL-6 are very important cytokines that play key roles in regulation of innate and adaptive immunity, produced by various cells in animal. IL-2 is involved in T-cell proliferation and the induction of T regulatory responses, it not only can enhance cellular immune response, but also stimulate proliferation of activated B lymphocytes and induce immunoglobulin secretion[5]; IL-4 can influence humoral and cellular immune responses, such as the production, class switching and secretion of immunoglobulin[6]; IL-6 also has many biological activities that to promote interleukin gene expression, B cell differentiation, T cell activation, and play a important role in acute phase reaction[7, 8].

Our previous studies had demonstrated that pig IL-2 or IL-6 with CpG immunostimulatory sequences shuffled gene [9,10], or the fusion pig IL-4/6 gene could effectively enhance the resistance of animals against pathogen infection[11, 12]. The oral administration can elicit both local and systemic immune responses[13]. Yeast expression systems have a number of properties such as high expression levels, adjuvant properties, post-translational modifications, and "generally recognized as safe" status, these properties make them have potential advantages as live delivery systems for oral inoculation[14, 15]. In addition, yeast can be internalized by DCs and macrophages and promote presentation of antigens[16]. Moreover, yeast is a good probiotic that can resist the local stresses in gastrointestine, showing high survival rate in stomach and small intestine, and can carry out the bioconservation reaction throughout the digestive tract[17,18,19]. Therefore, we suppose that recombinant yeast could be utilized to express cytokine genes to effectively promote animal immunity via oral inoculation.

For sake of developing novel cost-effective immunomodulator for pig, and based on our previous reports, the present experiment was conducted to construct recombinant yeast to co-express pig IL-2 gene and fused IL-4/6 gene by 2A self-cleavage technique use[20], and to evaluate its effect on the immune response and growth of piglets at different dose.

2. Materials and methods

2.1 The recombinant *Pichia pastoris* and its construction

The cDNA of fused pig IL-4/6 gene and IL-2 gene were cloned respectively from recombinant VR1020 plasmids containing fused pig IL-4/6 gene or pig IL-2 gene, named VRIL4/6 and VRIL2 preserved in our lab.

The IL-4/6 gene was firstly cloned into pGAPZαA (Invitrogen) under the control of GAP promoter with an N-terminal α-factor of *Saccharomyces cerevisiae* as secretion signal. Then, IL-2 gene was linked by 2A-α fragment that contained gene of FMDV 2A peptide and α-factor gene to guarantee the secreted expression of IL-2. Meanwhile, the termination codon of fused pig IL-4/6 gene was removed. Afterwards, the recombinant plasmid (pGAPZαAJMPIL4/6-PIL2) was transformed into *Escherichia coli (E. coli)* DH5α and positive transformants were screened out on low salt Lurea Bertani (LLB) plates containing 25μg/ml zeocin, and further identified by direct PCR and sequencing.

Eighty microliters of electrocompetent cells of *Pichia pastoris* SMD1168 was transformed with 10μg (in 10μl sterile water) of AVR□-linearized pGAPZαAJMPIL4/6-PIL2 or AVR□-linearized pGAPZαA vector by electroporation according to Invitrogen manual. After transformation, 200μl transformed cell was incubated in 1 ml of ice-cold 1 M sorbitol, and selected on YPDS plates with 100μg/ml zeocin. The positive transformant screened by genome analysis and PCR was cultured in 10 ml test tube with 3ml YPD (1% yeast extract, 2% peptone, 2% D-glucose) medium, 220 rpm shaken for 24h at 30°C, and the culture was preserved in 15% glycerol stored at -20°C until use.

2.2 Expanding culture of the recombinant *Pichia pastoris*

All fermentations were carried out in a 10 L seed fermenter and a 100 L fermenter, seed culture for the fermentation was started from the preserved recombinant *Pichia pastoris* and blank *Pichia pastoris* were thawed and then 30μl of these strains were respectively inoculated into 10 ml test tube with 3ml YPD medium and 100μg/ml zeocin, and incubated at 30°C, 220 rpm for 16h in a incubator shaker. Then, the culture of the two *Pichia pastoris* were respectively inoculated in 600 ml YPD medium in 2L flask for overnight at 30°C and 220 rpm.

The 600 ml culture of recombinant yeast was inoculated into 5.4 liters YPD medium in seed fermenter, 30°C, stirrer speed 150 rpm, dissolved oxygen (DO) was set at 30%, when the OD_{600} was to about 12, inoculated into 100L fermenter, consisting of 60L YPD medium and pH 5.8 phosphate buffer, the culture was carried out as the following conditions: 30°C, stirrer speed 150 rpm, DO was set at 30%, pH maintained at 5.5 by addition of NH_4OH (28%), foaming was controlled by using polyether defoamer GPE. Growth was monitored by measuring the OD at 600nm at different time points. After the fermentation, 0.3 g/L chitosan solution (food grade, MW: 50000) was added into the fermentation broth to flocculate yeast.

The two *Pichia postrois* were fermented two batches respectively, and the fermentation broth volume of control *Pichia pastoris* was 40L in each batch. All fermentations in this study were cultured in the same fermentation condition as the above-mentioned.

2.3 Piglets experiment

Forty healthy 30-day Tibetan piglets were used in this experiment, the body weight of the piglets about 10kg, they were randomly divided into four groups: three treated groups A1, A2, A3 and one control group (group C), ten piglets per group. All groups had the same forage treatment during the whole experimental period, and the treated groups were directly fed with recombinant *Pichia pastoris* fermentation broth preparation according to the different doses, continuous feeding 4 weeks, group C was directly fed with the fermentation broth of control *Pichia pastoris*, continuously for 4 weeks, the specific process as shown in table 1. All piglets were vaccinated with inactivated pig respiratory and reproduction syndrome (PRRS) vaccine (produced by Chengdu pharmaceutical company, Chengdu, China) as the routine protocol of manufacturer. On days 0, 7, 14, 28, 35, 45 and 58 of the experiment, three to four milliliters EDTA-stabilized precaval venous blood sample was collected from each piglet to evaluate the immune regulating effect of recombinant *Pichia pastoris* preparation on piglets**.**

Table 1 Animal experiment grouping and feeding scheme

group	preparation	piglet Numbers	feeding regime and dose
A1	Recombinant yeast	10	200ml per piglet; 1 time/day
A2	Recombinant yeast	10	200ml per piglet; 1 time/2 days
A3	Recombinant yeast	10	200ml per piglet; 1 time/3 days
C	Control yeast	10	200ml per piglet; 1 time/day

Note: The OD_{600} of the recombinant yeast and control yeast fermentation broth preparation were about 20, the preparations were directly

fed by mixing in forage, and continuously fed four weeks.

2.4 Piglet body weight

Body weight of each piglet was measured on days 0, 14, 28, and 58 post inoculation, average weight and net weight gain of each group were calculated to evaluate the effect of the recombinant yeast preparation on growth performance of piglets.

2.5 Assay of immune cells quantity in peripheral blood

The blood samples collected from each piglet 100μl per group were used to assay the number of immune cells by a routine method. The number of immune cells, platelets and hemoglobin content in precaval venous blood were assayed by MIND-RAY BC-3000 blood automatic counting instrument.

2.6 Measurement of antibody and IgG

Antibody to PRRS and pig IgG measurement ELISA kits were respectively purchased from Keqian Company of Wuhan, China and Bethyl Laboratory, Inc., America. The sandwich ELISA was conducted to detect level of specific antibody to PRRSV and IgG in the sera of piglets according to the manufacturer's protocols.

2.7 Assay of Th and Tc cells by flow cytometry

Mouse against pig CD4 and CD8 mAbs, labeled with phycoerythrin (PE) and spectral red (SPRD) respectively, were purchased from Southern Biotech (USA). Fifty μl of pig venous blood were mixed with 50μl normal saline, and then incubated with 2μl PE-conjugated anti-CD4 and 2μl SPRD labeled anti-CD8 for 20min in the dark. Two ml (10% v/v) lysing solution (Becton Dickinson, USA) was then added for 10 min to ensure complete lysis of erythrocytes, and surviving cells were washed twice with PBS, with centrifugation between each step for 5min at 500g. Finally, the cells were resuspended in 150μl PBS and analyzed in a FACScan flow cytometer (Becton Dickinson).

2.8 Analysis of immune gene expression by quantitative real-time polymerase chain reaction (QRT-PCR)

The 100μl blood samples were mixed with 1ml RNAiso plus (TAKATA), total RNA was extracted and reverse-transcribed at 42°C for 30 min as the guide (TransGen, TransScript™ One-Step gDNA Removal and cDNA Synthesis SuperMix). And 15 primers for PCR of immune genes were designed and synthesized as shown in table 2 according to the related gene sequences in GeneBank.

PCR was carried out with an initial denaturation for 3 min at 95°C, followed by 42 cycles of 6s at 95°C, 9s at optimal annealing temperature, 10s at 72°C, and in each run, the negative control was carried. The PPIA was used as the reference gene, and the mRNA levels of immune related genes were calculated in the four groups piglets using the geometric means method and the following formula: relative level = $2^{-\Delta\Delta Ct}$ [21].

Table 2 The primers for QRT-PCR

Gene	Oligonucleotide sequences（5'-3'）	Annealing temperature (°C)
PPIA -F	AGACAGCAGAAAACTTCCGTG	51.5
PPIA -R	ACTTGCCACCAGTGCCATTA	
IFN-γ-F	GAAGAATTGGAAAGAGGAGAGTGAC	61.5
IFN-γ-R	TCCATGCTCCTTTGAATGGC	
IL-2-F	AGCTCTGGAGGGAGTGCTAA	62.0

IL-2-R	TGTTTCAGATCCCTTTAGTTCCA	
IL-4-F	GCTGCCCCAGAGAACACGAC	61.0
IL-4-R	AGGTTCCTGTCAAGTCCGCTC	
IL-6-F	TGCAATGAGAAAGGAGATGTGTG	56.5
IL-6-R	CCCAGATTGGAAGCATCCGT	
IL-7-F	TCTGTTGCCAGTAGCATCATCT	56.5
IL-7-R	CTATCATCCTGTCCAAGTCATCG	
IL-10-F	GCTGGAGGACTTTAAGGGTTAC	60.6
IL-10-R	GAGTTCACGTGCTCCTTGATAT	
IL-12B(p40)-F	GAGGTCGTGCTGGAAGCTGTT	62.5
IL-12B(p40)-R	TCTTGGGAGGGTCTGGTTTGA	
IL-15-F	GCGATGAAGTGCTTTCTCCTG	57.0
IL-15-R	ACTCAATGGACGATAAACTGCTG	
TLR2-F	TGCTGCAAGGTCAACTCTCT	55.7
TLR2-R	CAGCAGGGTCACAAGACAGA	
TLR-3-F	GCATTGCCTGGTTTGTTAGTTG	56.0
TLR-3-R	TGTATCAAAAAGAATCACTGGGAG	
TLR-4-F	ATATGGCAGAGGTGAAAGCAC	60.2
TLR-4-R	GAAGGCAGAGATGAAAAGGGG	
TLR-7-F	ATAGCGAGCATCACTCCAGCC	54.0
TLR-7-R	TAATCTGCTGCCTTCTGGTGC	
TLR-9-F	ACAATGACATCCATAGCCGAGT	58.5
TLR-9-R	CAGATCGTTGCCGCTAAAGT	

F: forward. R: reverse

2.9 Statistical analysis

Data of the experimental groups were statistically evaluated by the statistical software program Systat 10 (SPSS). Differences between the groups were analyzed by two way ANOVA and Tukey multiple comparison. Data among the groups were considered to be significantly different when $P<0.05$.

3. Results

3.1 Identification of recombinant *Pichia pastoris*

The recombinant yeast was screened out via YPD medium containing 100 μg/ml Zeocin, and then its RNA was extracted and RT-PCR was conducted and analyzed through electrophoresis. The RT-PCR product of the recombinant yeast was shown in Fig.1, indicating that fusion pig IL4/6 and IL-2 gene was successfully expressed in the recombinant yeast (Fig.1).

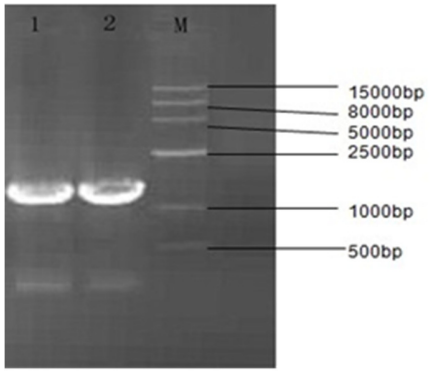


Figure 1. Electrophoresis of RT-PCR of PIL-4/6-PIL2 recombinant yeast (1% agarose gel)

Lane 1, 2: fragment of RT-PCR of the recombinant; Lane M: Wide Range DNA Marker.

3.2 Body weight change of the piglets

The effect of recombinant yeast preparation on piglet growth was shown in Fig.2 and table 3. Weight gain of A1 and A2 groups piglets were obviously higher than group C on days 28 and 58 post inoculation ($P< 0.05$) (Fig.2). On 58 days, the increase of average net weight gain of the two groups piglets were 3.88kg and 5.97kg respectively compared to group C (table 3). This result demonstrates that recombinant yeast can efficiently improve the growth of piglets in dosage dependent way.

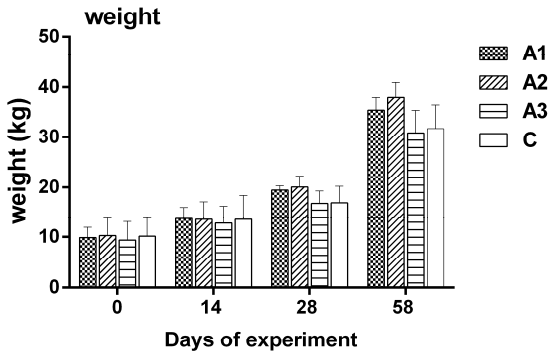


Figure 2. The average weight change of pigs. Weight gain of A1 and A2 groups piglets were obviously higher than group C on days 28 and 58 post inoculation ($P< 0.05$).

Table 3 Average net weight gain of pigs (kg)

Group	14d	28d	58d
A1	3.82	9.57	25.45
A2	3.27	9.80	27.54
A3	3.36	7.34	21.40
C	3.37	6.73	21.57

3.3 Change of immune cells in peripheral blood

From 7 to 45 days of the experiment, leukocytes of groups A1 and A2 significantly raised than group C ($P<0.05$), and from 28 to 45 days, the numbers of leukocytes in group A1 were significantly higher ($P<0.05$) than other groups (Fig.3 A). The erythrocytes, platelets and hemoglobin content did not show any obvious differences among the four groups ($P>0.05$), although there were some fluctuations during the experimental period (Fig.3 B, C, D).

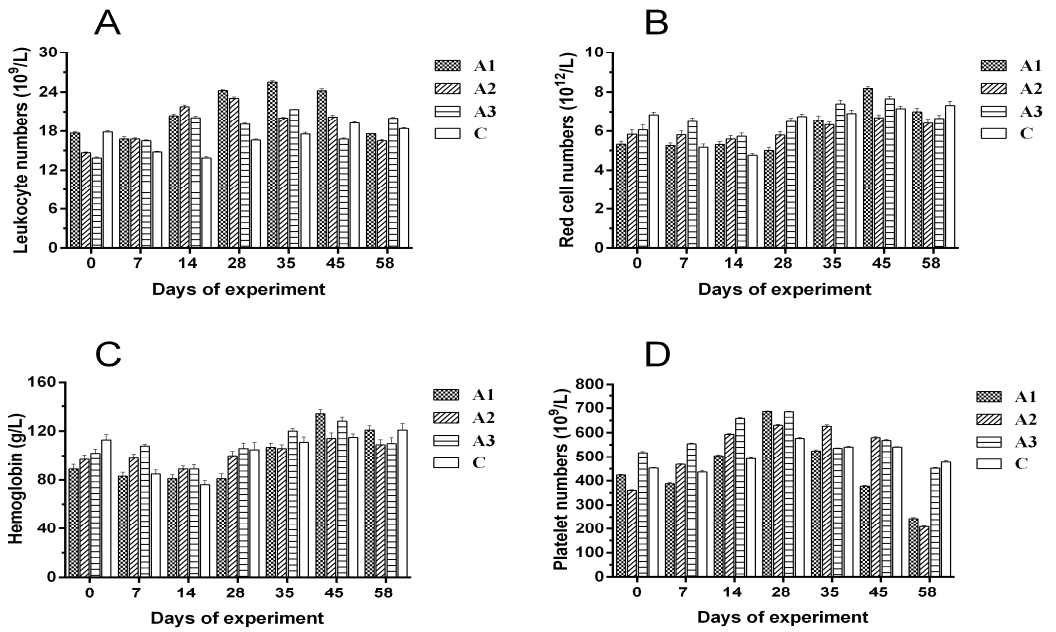


Figure 3. Change of the blood cells in the peripheral blood of experimental pigs. A, Leukocytes of group A1 and A2 significantly raised than group C from 7 to 45 days (P<0.05), and from 28 to 45 days, the numbers of leukocytes in group A1 were significantly higher (P<0.05) than other groups. B, C, D, The erythrocytes, platelets and hemoglobin content were some fluctuations during the experimental period, but did not show any obvious differences among the four groups (P>0.05).

3.4. Change of Th and Tc

The $CD4^+$ and $CD8^+$ T cells significantly increased in the blood of the A1 and A2 groups on 7, 14 and 28 days post inoculation compared to the control (P<0.05), and the $CD8^+$ T cells of group A1 seemed to be the highest among the three treated groups although their difference were not significant (P>0.05) (Fig.4).

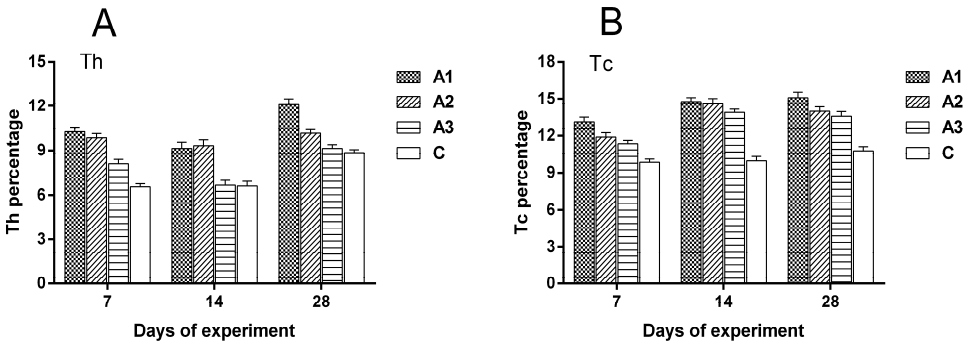


Figure 4. Th and Tc cells quantity in peripheral blood of experimental pigs. A, B, The $CD4^+$ and $CD8^+$ T cells significantly increased in the blood of the A1 and A2 groups on 7, 14 and 28 days post inoculation compared to the control (P<0.05).

3.5 Antibody and IgG

Figure 5 showed that the specific PRRSV antibody and IgG levels in the sera of the treated piglets significantly increased than the control group (P <0.05); The difference among the three treated group was not significant (P<0.05)(Fig. 5, A,B).

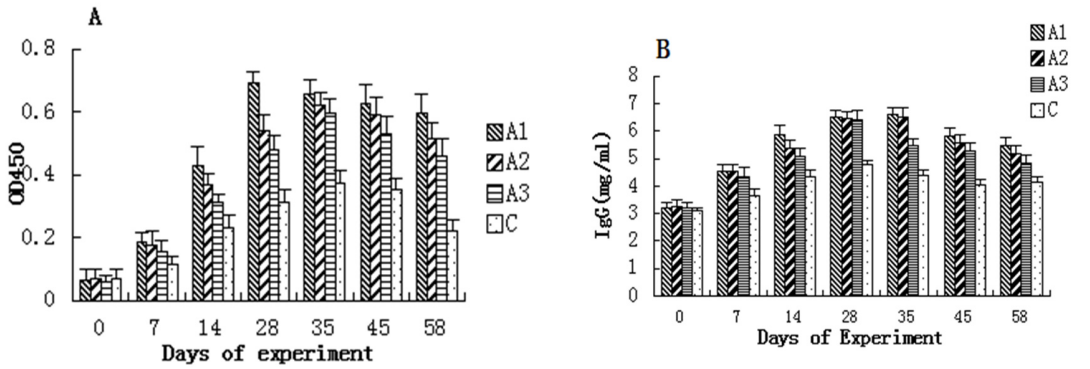
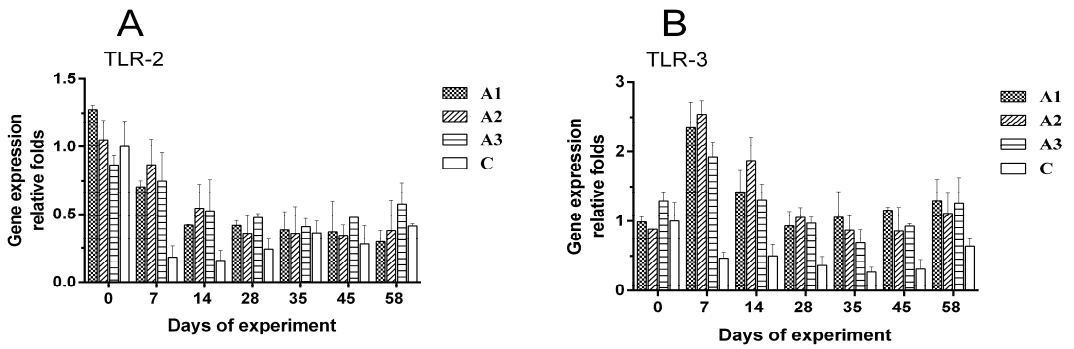


Figure 5. Changes of specific antibody to PRRSV and IgG content in the sera of experimental pigs. The level of antibody and IgG significantly increased in the A1 and A2 groups from 14 to 58 days post inoculation compared to the control (P<0.05).

3.6 Change of the immune gene expression

3.6.1 Change of TLR genes

To evaluate the effect on pig innate immune system, five TLRs were selected to detect dynamic expression change. The results were shown in the Fig.4. The expression levels of TLR-2, TLR-4 and TLR-9 were obviously higher in the treated groups compared with group C from 7 to 28 days post inoculation (P<0.05) (Fig.4 A, C, E); TLR-3 and TLR-7 also significantly increased during the whole period (P<0.05), and from 7 to 14 days, their levels of group A2 were significantly higher than group A1 and A3 (P<0.05) (Fig.4 B, D).



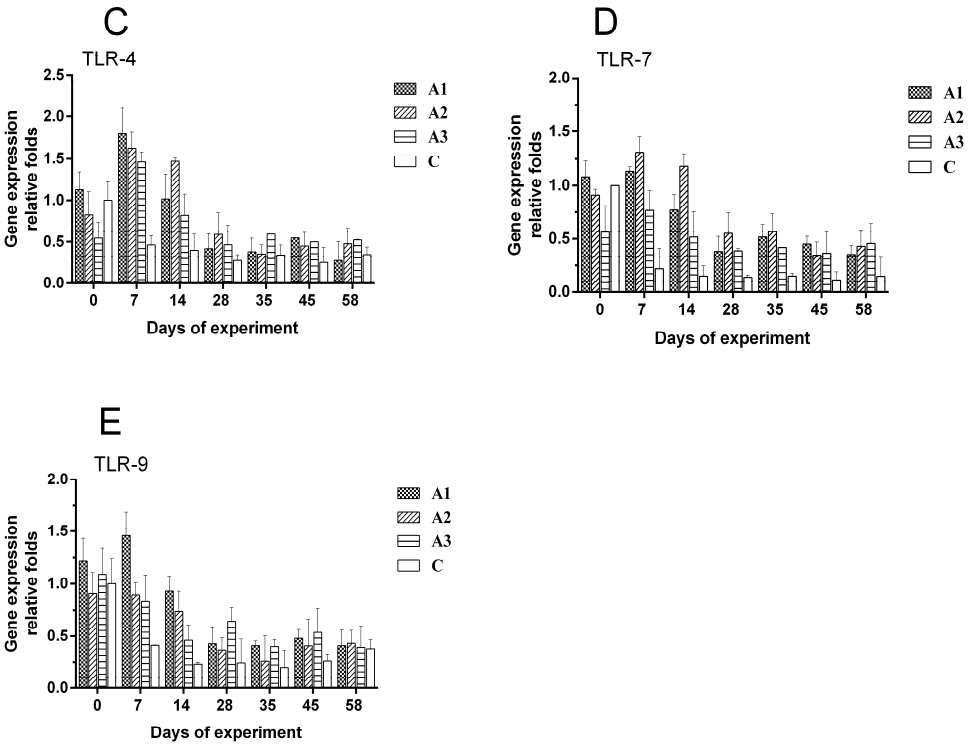


Figure 6. **E**xpression levels of TLR-2, 3, 4, 7 and 9 genes of the experimental pigs. A, C, E, The expression levels of TLR-2, TLR-4 and TLR-9 were obviously higher in the treated groups compared with group C from 7 to 28 days ($P<0.05$). B, D, The mRNA levels of TLR-3 and TLR-7 also significantly increased during the whole period ($P<0.05$), and from 7 to 14 days, their levels of group A2 were significantly higher than group A1 and A3 ($P<0.05$).

3.6.2 Change of the immune memory related genes expression

To evaluate the immune memory of piglets, the dynamic change of IL-7 and IL-15 expression levels were detected, the results were shown in Fig.7 A, B and C. IL-7 and IL-15 expression levels of the treated groups were significantly higher than group C from 7 to 58 days ($P<0.05$).

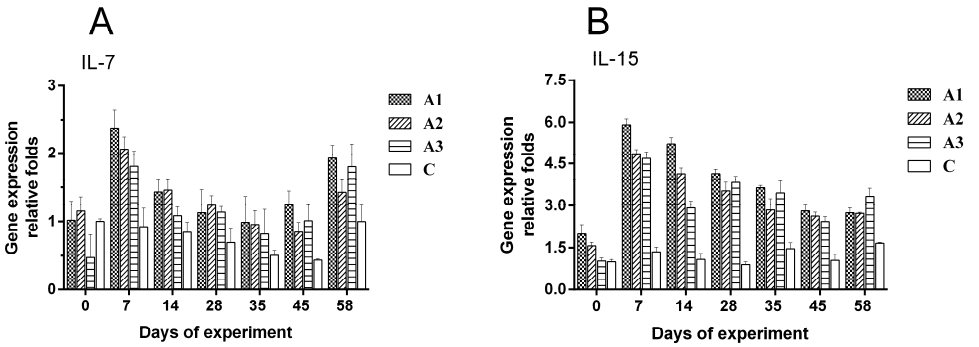
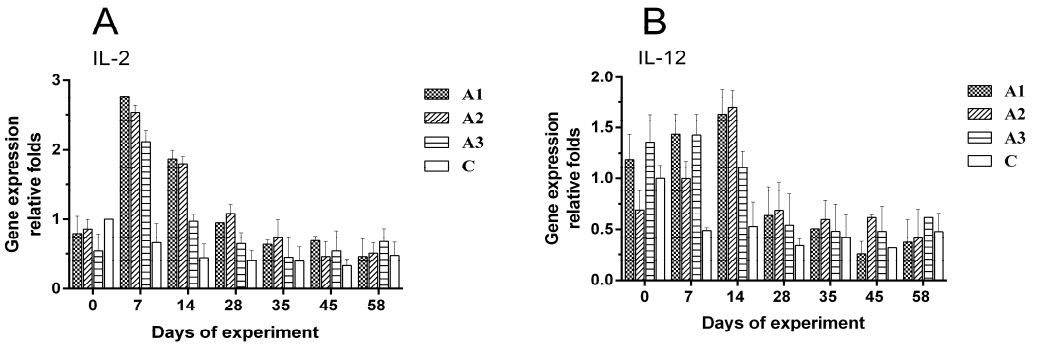


Fig.7 Expression levels of IL-7 and IL-15 genes of the experimental pigs. A, B, The expression levels of IL-7 and IL-15 were significantly higher than group C from 7 to 58 days in treated groups (P<0.05).

3.6.3 Change of cytokine genes expression

IL-2, IL-4, IL-6, IL-12 and IFN-γ from various immune cells, are pivotal in determining the effectiveness of pig immune responses. As shown in Fig.8 A, B, C, D, E, the expression of IL-2, IL-4 and IFN-γ genes of treated groups significantly increased from 7 to 45 days compared to those of the control (P<0.05), and the expression of IL-2 and IFN-γ respectively reached the maximum on day 7 and day 14. Meanwhile, the IL-6 and IL-12 expression levels of treated groups were obviously higher than group C from 7 to 35 days, and the IL-6 expression level of group A1 was significantly higher than the other groups (P<0.05). In addition, the IL-10 expression levels of group A1 was significantly higher than group C from 7 to 14 days and so was the group A2 on 7 days (P<0.05) (Fig.8 F).



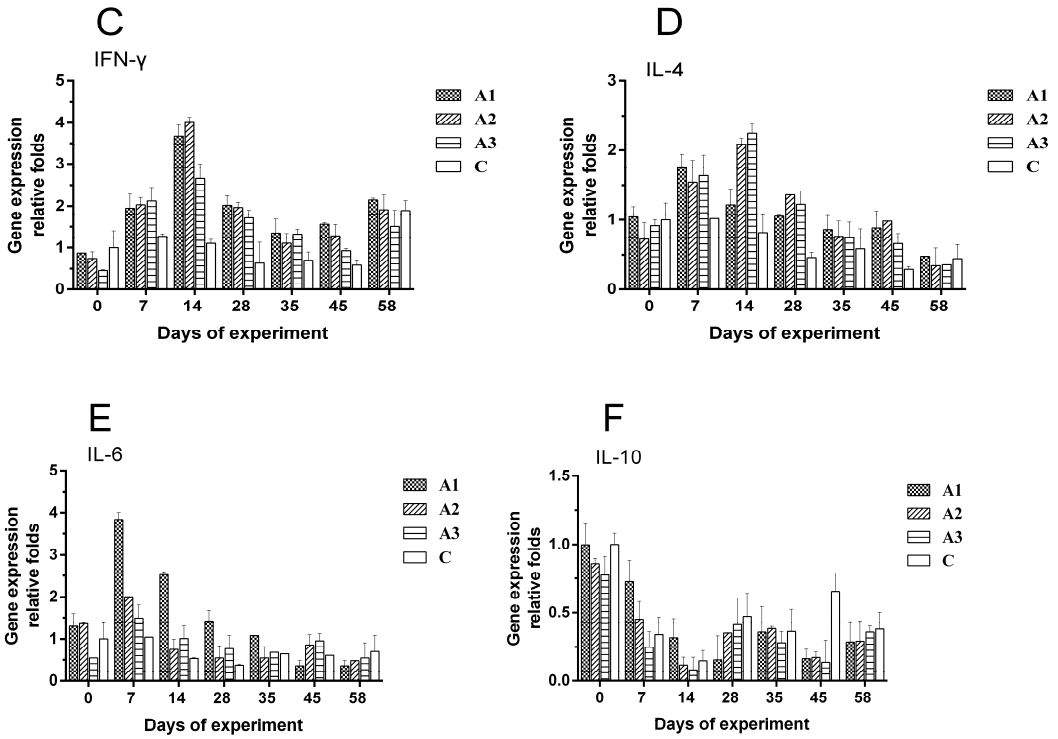


Figure 8. Expression levels of IL-2, IL-4, IL-6, IL-10, IL-12 and IFN-γ genes of the experimental pigs. A, C, D, the expression of IL-2, IL-4 and IFN-γ genes of treated groups significantly increased from 7 to 45 days compared with those of the control (P<0.05). B, E, the IL-6 and IL-12 expression levels of treated groups were obviously higher than group C from 7 to 35 days, and the IL-6 expression level of group A1 was significantly higher than the other groups (P<0.05). F, the IL-10 expression levels of group A1 was significantly higher than group C from 7 to 14 days and so was the group A2 on 7 days (P<0.05).

4. Discussion

Cytokines are vital regulators of immune functions, and mainly produced by various immune system can bind to specific receptors on the surface of their target cells, and induce intracellular signaling pathways that trigger a series of biological processes, such as cell proliferation, activation and differentiation. At present, many cytokines have been used as adjuvants to enhance primary and memory immune responses[23]. IL-2, majorly secreted by Th0 cells, serves as a key cytokine to support the growth and proliferation of T cells and NK cells[24], and also to stimulate proliferation of activated B-lymphocytes and to induce immunoglobulin secretion[5]. IL-4 is primarily produced by Th2 cells, it is able to promote B cell responses and influence immunoglobulin class switching in B cells[6]. IL-6 is a multifunctional cytokine with a wide range of biological activities in immune regulation, hematopoiesis and inflammation. Our previous study demonstrated that co-expression of pIL4/6 fused gene and IL-2 shuffled gene *in vivo* could elicit significant enhancement of mice immunity compared with the individual genes (data in our lab).

Therefore, in this study, the recombinant *Pichia pastoris* co-expressing porcine interleukin-2 and interleukin-4/6 was employed to evaluate whether it could promote the immunity and growth of piglets through oral inoculation. Interestingly, we observed that the recombinant yeast efficiently improved the growth of A1 and A2 piglets in suitable dosage (P<0.05). Early works had recognized that cytokines not only regulate immune function but also modify the growth performance, and adipose tissue can respond to cytokines signals and produce a number of metabolic

regulators to regulate growth[25, 26, 27]. Therefore, this may be correlated with the "reprogramming" of metabolism mediated by cytokines increments.

Moreover, feeding the recombinant yeast significantly elevated the expression levels of the five TLRs and the important immune genes such as IL-2, IL-4, IL-6, IL-7, IL-12 and IL-15 in comparison with those of group C ($P<0.05$); Meanwhile, the feedings also elicited obvious increase of Th and Tc cells as well as specific antibody and IgG content in the blood of the treated piglets ($P<0.05$), which maybe resulted from the enhancement of cytokines production and better cellular and humoral immunity to vaccination with PRRSV. Furthermore, the immunity of group A1 and A2 are better than the other groups, and it is probably related with the stimulation increment of recombinant yeast.

TLRs are first line of immune system to detect pathogen infection, they play a central roles in the innate immune response by recognizing conserved molecules patterns of diverse pathogens, and also participate in activation the pathogen-specific humoral and cellular adaptive immune responses [22, 28]. IL-7 and IL-15 serve as critical regulators that mediate pleiotropic effects on multiple cell types, they are required for the development, proliferation, differentiation and survival of specific immune cells[29], especially for immunological memory cells[30, 31]. Furthermore, IL-10, IL-12 and IFN-γ also play an important role in animal immune response. Therefore, significant increase of these genes expression and leukocytes in the blood indicate that the recombinant yeast can effectively strengthen innate and adaptive immune response of piglets. Besides, these results also provide experimental support for the possible application of the recombinant *Pichia pastoris* in oral feeding way[19].

Briefly, our results confirm that the recombinant yeast co-expressing porcine IL-2 and IL-4/6 is a novel cost-effective immunomodulator that can not only markedly enhance the innate and adaptive immunity of piglet but also improve piglet growth, which can facilitate the development of an easy and cost-effective immunomodulator for better control and prevention of pig infectious diseases in the future.

Acknowledgement

This research was supported by the grants from National Natural Science Foundation of China (grant no.30571359 and 30871855), National International Cooperation Program (No.2011DFA10101103) and a breeding project from Sichuan Province of China. We sincerely thank Dr. Gang Wang (National Engineering Research Center for Biomaterials) for technical support.

References

1. Pastoret PP. Veterinary vaccinology. Comptes Rendus de l'Académie des Sciences-Series III-Sciences de la Vie , 1999;322(11):967-972.

2. Shin MK, Kang ML, Jung MH, Cha SB, Lee WJ, Kim JM, Kim DH, Yoo HS. Induction of protective immune responses against challenge of Actinobacillus pleuropneumoniae by oral administration with Saccharomyces cerevisiae expressing Apx toxins in pigs. Veterinary Immunology and Immunopathology 2013; 151(1-2):132-139.

3. Thompson AL, Staats HF. Cytokines: the future of intranasal vaccine adjuvants. Clinical & Developmental Immunology 2011; 2011:17.

4. Vajdy M, Srivastava I, Polo J, Donnelly J, O'Hagan D, Singh M. Mucosal adjuvants and delivery systems for protein-, DNA- and RNA-based vaccines. Immunology and Cell Biology 2004; 82(6):617-627.

5. Collins R, Oldham G. Recombinant human interleukin 2 induces proliferation and immunoglobulin secretion by bovine B-cells: tissue differences and preferential enhancement of immunoglobulin A. Veterinary Immunology and Immunopathology 1993 ; 36(1):31-43.

6. Pasquini S, Xiang Z, Wang Y, He Z, Deng H, Blaszczyk-Thurin M, Ertl H. Cytokines and costimulatory molecules as genetic adjuvants. Immunology and Cell Biology 1997; 75(4):397-401.

7. Kishimoto T. IL-6, from its discovery to clinical applications. International Immunology 2010; 22(5):347-352.

8. Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, Zinkernagel R, Bluethmann H, Köhler G.Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature 1994; 368:4.

9. Yang X, Sun W-K, Chen W-L, Chen J-L, Wan X-P, Zhang H, Cai L, Wang Z-Z, Lv X-B, Wang H-N et al: Promotion of the immunity of piglets to hog cholera vaccine induced by shuffled pig interleukin-2 gene and CpG immunostimulatory sequences encapsulated in chitosan nanoparticles. Procedia in Vaccinology 2010;2(1):51-59.

10. Li D, Chen JL, Zhang H, Yang X, Wan XP, Cheng C, Li Y, Wang ZZ, Lv XB, Wang HN et al. Improvement of the immunity of pig to hog cholera vaccine by recombinant plasmid with porcine interleukin-6 gene and CpG motifs. Vaccine 2011;29(22):3888-3894.

11. Yang X, Xiao Y-L, Chen J-L, Chen C, Zhang H, Wan X-P, Lv X-B, Li J-L, Wang Z-Z, Gao R. Potentiation of immunity of piglets to mycoplasma hyopneumoniae vaccine with fused gene for pig IL-4 and IL-6 embodied in chitosan nanoparticles. Procedia in Vaccinology 2013; 7:15-22.

12. Zhang H, Cheng C, Zheng M, Chen JL, Meng MJ, Zhao ZZ, Chen Q, Xie Z, Li JL, Yang Y et al. Enhancement of immunity to an Escherichia coli vaccine in mice orally inoculated with a fusion gene encoding porcine interleukin 4 and 6. Vaccine 2007; 25(41):7094-7101.

13. Azizi A, Kumar A, Diaz-Mitoma F, Mestecky J. Enhancing oral vaccine potency by targeting intestinal M Cells. PLoS Pathogens 2010; 6:7.

14. Shin MK, Yoo HS. Animal vaccines based on orally presented yeast recombinants. Vaccine 2013; 31(40):4287-4292.

15. Blanquet S, Marol-Bonnin S, Beyssac E, Pompon D, Renaud M, Alric M. The 'biodrug' concept: an innovative approach to therapy. Trends in Biotechnology 2001;19(10):393-400.

16. Stubbs AC, Martin KS, Coeshott C, Skaates SV, Kuritzkes DR, Bellgrau D, Franzusoff A, Duke RC, Wilson CC. Whole recombinant yeast vaccine activates dendritic cells and elicits protective cell-mediated immunity. Nature Medicine 2001;7(5):625-629.

17. Blanquet S, Meunier JP, Minekus M, Marol-Bonnin S, Alric M. Recombinant Saccharomyces cerevisiae Expressing P450 in artificial digestive systems: a model for biodetoxication in the human digestive environment. Applied and Environmental Microbiology 2003; 69(5):2884-2892.

18. Garrait G, Jarrige JF, Blanquet-Diot S, Alric M. Genetically engineered yeasts as a new delivery vehicle of active compounds to the digestive tract: in vivo validation of the concept in the rat. Metabolic Engineering 2009; 11(3):148-154.

19. Kiflmariam MG, Yang H, Zhang Z: Gene delivery to dendritic cells by orally administered recombinant Saccharomyces cerevisiae in mice. Vaccine 2013; 31(10):1360-1363.

20. de Felipe P, Hughes LE, Ryan MD, Brown JD: Co-translational, intraribosomal cleavage of polypeptides by the foot-and-mouth disease virus 2A peptide. The Journal of Biological Chemistry 2003; 278(13):11441-11448.

21. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods 2001, 25(4):402-408.

22. Kumar H, Kawai T, Akira S: Toll-like receptors and innate immunity. Biochemical and BiophysicalResearch Communications 2009; 388(4):621-625.

23. Kayamuro H, Yoshioka Y, Abe Y, Arita S, Katayama K, Nomura T, Yoshikawa T, Kubota-Koketsu R, Ikuta K, Okamoto S et al: Interleukin-1 family cytokines as mucosal vaccine adjuvants for induction of protective immunity against influenza virus. Journal of Virology 2010;84(24):12703-12712.

24. Smith KA: Interleukin-2: inception, impact, and implications. Science 1988; 240(4856):1169-1176.

25. Spurlock M: Regulation of metabolism and growth during immune challenge: an overview of cytokine function. Journal of Animal Science 1997; 75(7):1773-1783.

26. Schaffler A, Scholmerich J: Innate immunity and adipose tissue biology. Trends in Immunology 2010;31(6):228-235.27.

27. Gabler NK, Spurlock ME: Integrating the immune system with the regulation of growth and efficiency. Journal of Animal Science 2008; 86(14 Suppl):E64-74.

28. Kawai T, Akira S: The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature Immunology 2010; 11(5):373-384.

29. Stevceva L, Moniuszko M, Ferrari MG: Utilizing IL-12, IL-15 and IL-7 as mucosal vaccine adjuvants. Lett Drug Des Discov 2006; 3(8):12.

30. Kondrack RM, Harbertson J, Tan JT, McBreen ME, Surh CD, Bradley LM: Interleukin 7 regulates the survival and generation of memory CD4 cells. The Journal of Experimental Medicine 2003; 198(12):1797-1806.

31. Tan JT: Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. Journal of Experimental Medicine 2002;195(12):1523-1532.