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*Original article*

# Effect of bamboo vinegar powder on the expression of the immune-related genes *MyD88* and *CD14* in weaning piglets

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## Abstract

The aim was to explore the feasibility of using bamboo vinegar powder as an antibiotics substitute in weaning piglets. Forty-five healthy Duroc × Landrance × Yorshire piglets (weight 6.74 ± 0.17 kg; age 31 days) were randomly divided into the control group (basic diet), ANT group (basic diet + 0.12% compound antibiotics), BV1 group (basic diet + 0.1% bamboo vinegar powder), BV5 group (basic diet + 0.5% bamboo vinegar powder) and BV10 group (basic diet + 1% bamboo vinegar powder). *MyD88* and *CD14* expression in immune tissues was examined using real-time PCR. *MyD88* expression in the control group were significantly lower than that in other groups in all tissues ($p<0.05$), while *CD14* expression showed the opposite trend. *MyD88* expression was significantly higher in the BV10 group than in other groups in lung tissue ($P<0.05$), significantly higher in the ANT group than in the BV1 group in the kidneys ($P<0.05$), significantly higher in the BV10 group than in the BV1 group in the thymus ($P<0.05$), and significantly higher in the BV1 group than in the BV10 group in the lymphatic tissue ($P<0.05$). These differences between experimental groups were not observed for the *CD14* gene ($P>0.05$). Thus, adding bamboo vinegar powder to the basic diet of weaning piglets had immune effects similar to antibiotics and the effect was dose-dependent. Moreover, the *MyD88* and *CD14* genes appear to play a role in these immune effects.

**Key words:** bamboo vinegar powder, pig, antibiotics, MyD88 gene, CD14 gene

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## Introduction

In pig production, feed antibiotics are widely used as an additive because they have an inhibitory effect on microorganisms and slow down and reduce the immune response of animals, as a result of which animals have a relatively high growth rate (Tong 2000). However, the long-term use of antibiotics is associated with several health issues, such as drug resistance in pathogenic bacteria, decline in immunity, susceptibility to endogenous infection and double infection in livestock. Moreover, antibiotics are not fully absorbed, and most of the unabsorbed drugs are discharged into the natural environment along with faecal matter, and ultimately become a source of drug-resistant bacteria (Martinez 2009). Antibiotic residues in animal products also pose a serious threat to human health. Given these concerns, Sweden firstly prohibited the use of some of the antibiotics in animal feeds in 1986 (Castanon 2007), and European Union (EU) member nations banned all antibiotic growth promoters in 2006. However, these bans have brought adverse effects on animal production industries in EU, such as the increase of infections in animals and the decrease of animal production, which resulted in the increasing usage of therapeutic antibiotics and disinfectants and hence to increase the total usage amount of antibiotics in animals (Cheng et al. 2014). For all these reasons, in the context of large-scale intensive pig breeding, there is increasing interest in the research and development of biological active additives that are non-toxic and do not have drug residues, among others (Wu et al. 2009, Shi et al. 2015).

Bamboo vinegar is a new green product that is currently used as a feed additive and a poultry house deodorant in the livestock and poultry industry. Bamboo vinegar has a low pH, so when it is added to feed, it can increase the growth of intestinal lactic acid bacteria, reduce abnormal fermentation in the intestinal tract, and promote digestion and absorption to enhance appetite and livestock growth (Yao et al. 2012). Yamauchi et al. (2010) found that a mixture of bamboo charcoal and bamboo vinegar could reduce the ammonia content in faeces. Moreover, it has been reported that the addition of bamboo charcoal and bamboo vinegar to deoxynivalenol-contaminated diet can alleviate the negative effects of deoxynivalenol (Jiang et al. 2012). Further, because of the complex composition of bamboo vinegar, it has multiple effects, including antibacterial, antioxidant, and anti-inflammatory effects, and it can also improve production, immunity, meat quality, intestinal function etc. (Pu et al. 2015). In our previous study, we found that supplementing the diet of weaning piglets diets with bamboo vinegar powder could significantly decrease the feed-to-gain ratio and the incidence of diarrhoea (Liu et al. 2015). Moreover, the effect of bamboo powder on improving performance was comparable to that of antibiotics, and bamboo vinegar powder could significantly improve the biochemical, antioxidant and immune indexes of piglets, as well as significantly reduce the pH of gastric juices and improve the intestinal environment. In agreement with our earlier study, other studies have also reported that bamboo products can promote growth, enhance immunity, and destroy bacteria or inhibit their growth in the pig (Jiang et al. 2012, Chu et al. 2013, Ho et al. 2013, Wang et al. 2013). However, there is not enough information on the immunoregulatory effects of bamboo products in the pig.

Previous studies have reported that the Toll-like inflammatory pathway plays an important role in the regulation of immune response against *Escherichia coli* F18 infection in weaned piglets, and have indicated that the *MyD88* and *CD14* genes may play a critical role in *E. coli* F18 infection in piglets (Wu et al. 2016). MyD88 is a key protein in the Toll-like receptor (TLR)/IL-1R signalling pathway; it plays a role in transmitting inflammatory signals, enhancing the intensity of the inflammatory response. Additionally, it triggers the release of intestinal inflammatory mediators (Moses et al. 2009). CD14 is a glycosyl-phosphatidyl inositol-anchored protein that is also a potential pattern recognition receptor, it plays an important role in the process of pathogen infection (Dobrovolskaia et al. 2002). Both of them play a key role in the animal's innate immune system and are important immune genes. So far, very few studies have been conducted on the regulation of immune mechanisms by bamboo vinegar. This study is aimed to understand these mechanisms further by studying the expression of *MyD88* and *CD14* in weaning piglets that were fed a bamboo vinegar powder-supplemented diet.

In this experiment, antibiotics and three different doses of bamboo vinegar powder (0.1%, 0.5% and 1.0%) were added to the basal diet of weaning piglets, and the expression of the *MyD88* and *CD14* genes was analysed in the spleen, lung, kidney, thymus and lymph nodes by real-time quantitative PCR. Based on the findings, we discuss the effects of bamboo powder on immune function regulation in weaning piglets and explore the underlying mechanisms.

## Materials and Methods

### Ethics statement

The animal study proposal was approved by the Institutional Animal Care and Use Committee (IACUC) of the Yangzhou University Animal Experiments

Table S1. Composition and nutrient levels of basal diet (air dry basis).

| Item | |
|---|---|
| Ingredients | Content |
| Corn | 62 |
| Soybean meal | 25 |
| Wheat bran | 8 |
| Pre-mix[1] | 5 |
| Total | 100 |
| Nutrient Level[2] | |
| DE (MJ/kg) | 13.05 |
| CP | 17.41 |
| Ash | 4.51 |
| Ca | 0.61 |
| TP | 0.68 |
| AP | 0.35 |
| Lys | 0.99 |
| Met | 0.39 |

[1)] Per kg of premix containing VA 1125000IU, $VD_3$ 250000IU, VE 2000 mg, $VK_3$ 204 mg, $VB_1$ 207 mg, $VB_2$ 600 mg, $VB_6$ 246 mg, $VB_{12}$ 2.5 mg, nicotinic acid 2475 mg, calcium pantothenate 1350 mg, folic acid 120 mg, biotin 5 mg. Per kg of premix containing copper sulfate 19500 mg, ferrous sulfate 22500 mg, zinc sulfate 14145 mg, manganese sulfate 4800 mg, calcium iodate (5%) 100 mg, sodium selenite 33 mg, cobalt chloride 5 mg.

[2)] Nutritional levels were the values obtained after calculation.

Ethics Committee (permit number: SYXK (Su) IACUC 2012-0029). All experimental procedures involving piglets were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China.

## Materials and instruments

The animal experiment was conducted at a pig breeding farm in Taicang Jinzhu Agricultural Development Co. Ltd. (Jiangsu China). Bamboo vinegar powder was supplied by Jiangyin Zhongli Co. Ltd. (Jiangsu China); trizol lysate was purchased from Invitrogen (USA); reverse transcription kits and teal-time PCR kits were purchased from Vazyme Biotech Co. Ltd. (Jiangsu China); NanoDrop-1000 Trace Nucleic Acid Analyzer was purchased from General Electric Co. Ltd.; Veriti™ 96-Well Thermal Cycler was purchased from Thermo Fisher Scientific Co. Ltd.; and 7500 Rapid Real-Time PCR was purchased from Applied Biosystems Co. Ltd.

## Experimental design

A total of 45 Duroc × Landrance × Yorshire healthy pigs (31 days old) with an average initial body weight of 6.74 ± 0.17 kg were randomly allocated to one of five experimental groups, with three replicates per group and three pigs per replicate. The five experimental groups were as follows: (1) control group (CON, basal diet without antibiotics or bamboo powder), (2) antibiotic group (ANT, basic diet + 0.12% compound antibiotics [bacitracin zinc, colistin, and roxarsone]), (3) 0.1% bamboo vinegar powder group (BV1, basic diet + 0.1% bamboo vinegar powder), (4) 0.5% bamboo vinegar powder group (BV5, basic diet + 0.5% bamboo vinegar powder), (5) 1% bamboo vinegar powder group (BV10, basic diet + 1% bamboo vinegar powder). The basic diet was formulated according to NRC (1998) nutritional requirements of weaning piglets. The composition and nutrient levels of the basal diets of the weaning piglets are provided in Table S1. For producing bamboo vinegar powder, a solution of refined bamboo vinegar and edible dextrin is spray dried at a low temperature. We have earlier analysed and detected the main chemical components of bamboo vinegar powder (Liu et al. 2014), which mainly include phenols, aldehydes, organic acids, ketones and heterocyclic compounds (Table S2). The feeding trial lasted for 35 days, including 6 days of pre-feeding and 29 days of feeding. Throughout the feeding trial, the animals had ad libitum access to feed and water, and the piggeries were sterilized routinely. At the end of the trial, two pigs were randomly selected from each replicate and slaughtered; that is, six pigs per group were selected. We collected spleen, lung, kidney, thymus,

Table S2. Main chemical components of bamboo vinegar powders.

| No. | RT / min | Name | Molecular Formula | Molecular weight | Relative contents / % |
|---|---|---|---|---|---|
| 1 | 1.40 | Ethanol | $C_2H_6O$ | 46 | 14.53 |
| 2 | 2.89 | Acetic acid | $C_2H_4O_2$ | 60 | 23.08 |
| 3 | 3.09 | 2-propanone, 1-hydroxy | $C_3H_6O_2$ | 74 | 7.86 |
| 4 | 3.19 | Propanoic acid | $C_3H_6O_2$ | 74 | |
| 5 | 3.72 | Pyridine | $C_5H_5N$ | 79 | 0.32 |
| 6 | 3.86 | 1-hydroxy-2-butanone | $C_4H_8O_2$ | 88 | 1.14 |
| 7 | 4.02 | 3-cyclopentene-1, 2-diol-cis | $C_5H_8O_2$ | 100 | 1.39 |
| 8 | 4.44 | Pyridine, 2- methyl | $C_6H_7N$ | 93 | 0.48 |
| 9 | 4.58 | Tetrahydrofuran, 2-propyl | $C_7H_{14}O$ | 114 | 0.39 |
| 10 | 5.62 | Butyrolactone | $C_4H_6O_2$ | 86 | 1.81 |
| 11 | 5.84 | 2, 5-Hexanedione | $C_6H_{10}O_2$ | 114 | 0.26 |
| 12 | 5.99 | 2( 5H) furanone, 5-methyl | $C_5H_6O_2$ | 98 | 0.2 |
| 13 | 6.24 | 2-Cyclopenten-1-one, 3-methyl | $C_6H_8O$ | 96 | 0.38 |
| 14 | 6.41 | Phenol | $C_6H_6O$ | 94 | 3.64 |
| 15 | 7.31 | 2-Cyclopenten-1-one, 2-hydroxy-3-methyl | $C_6H_8O_2$ | 112 | 2.51 |
| 16 | 7.63 | Phenol, 2-methyl | $C_7H_8O$ | 108 | 0.54 |
| 17 | 7.98 | Phenol, 3-methyl | $C_7H_8O$ | 108 | 1.26 |
| 18 | 8.18 | Phenol, 2-methoxy | $C_7H_8O_2$ | 124 | 1.08 |
| 19 | 8.72 | 1, 3-Cyclopentanedione, 2, 4-dimethyl-2 | $C_7H_{10}O_2$ | 126 | 0.63 |
| 20 | 9.40 | Phenol, 4-ethyl | $C_8H_{10}O$ | 122 | 0.73 |
| 21 | 9.79 | Phenol, 2-methoxy-4-methyl | $C_8H_{10}O_2$ | 138 | 0.28 |
| 22 | 9.95 | 1, 2-benzenediol | $C_6H_6O_2$ | 110 | 0.25 |
| 23 | 10.84 | 2-Methoxyresorcinol | $C_7H_8O_3$ | 140 | 0.6 |
| 24 | 11.09 | Acetophenone, 2, 5-dihydroxy | $C_8H_8O_3$ | 152 | 0.22 |
| 25 | 12.04 | Phenol, 2, 6-dimethoxy | $C_{10}H_8O_3$ | 154 | 3.82 |
| 26 | 13.29 | 1, 2, 4-Trimethoxybenzene | $C_9H_{12}O_3$ | 168 | 1.2 |
| 27 | 13.99 | 1, 6-anhydro-D-glucopyranose | $C_6H_{10}O_5$ | 162 | 1.21 |
| 28 | 14.26 | 5-tert-Butylpyrogallol | $C_{10}H_{14}O_3$ | 182 | 0.46 |
| 29 | 23.57 | Tetraacetyl-d-xylonic nitrile | $C_{14}H_{17}NO_3$ | 343 | 0.2 |

and lymph node tissue and immediately froze them in liquid nitrogen, after which they were stored at -80° until analysis.

### RNA extraction and reverse transcription

Total RNA was extracted from various tissues (50-100 mg) using the Trizol reagent according to the manufacturer's instructions. Precipitated RNA was suspended in 20 $\mu$L RNase-free $H_2O$, diluted to 2 ng/$\mu$L and stored at -80°C. RNA quality was assessed by formaldehyde denaturing gel electrophoresis, and the concentration and purity of RNA were determined using the Nanodrop ND-1000 spectrophotometer.

The reaction mixture for cDNA synthesis contained 2 $\mu$L of 5× qRT SuperMix II and 500 ng total RNA, to which RNase-free $H_2O$ was added to make up a final volume of 10 $\mu$L. The reaction was carried out at 25° for 10 min, 50° for 5 min, and 85° for 5 min, and the reaction mixture was stored at 4°.

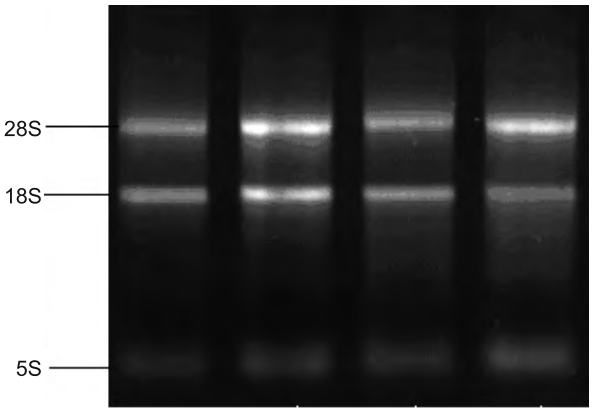


Fig. 1. The results of total RNA extraction.

Table 1. Real-time PCR primer sequences.

| Gene | Accession No. | Sequence (5′→3′) | Length |
|---|---|---|---|
| *MyD88* | NM_001099923.1 | F: GCTGGAACAGACCAACTAT | 153 |
| | | R: TCCTTGCTTTGCAGGTAAT | |
| *CD14* | NM_001097445.2 | F: CCTCAGACTCCGTAATGTG | 180 |
| | | R: CCGGGATTGTCAGATAGG | |
| GAPDH | NM_001206359.1 | F: ACATCATCCCTGCTTCTACTGG | 188 |
| | | R: CTCGGACGCCTGCTTCAC | |

### Primer design and real-time PCR conditions

Using the Primer-BLAST software, *MyD88*, *CD14* and *GAPDH* primers were designed based on the deposited sequences in GenBank, and the synthesized primer sequences are listed in Table 1. Real-time PCR amplification was performed using a PCR kit in a 25-$\mu$L reaction mixture containing 2 $\mu$L cDNA, 0.5 $\mu$L of 10 $\mu$M of each forward and reverse primer, 0.5 $\mu$L of 50× ROX Reference Dye II, and 10 L of the 2× SYBR Green real-time PCR Master Mix, to which dd$H_2O$ was added to make up the final volume of 25 $\mu$L. The PCR conditions were as follows: 95° for 5 min, which was followed by 40 cycles of 95° for 5 s and 60° for 34 s. The dissociation curve was analysed after amplification.

### Statistical analysis

Statistical analyses were carried out using the SPSS 17.0 software, and SPSS 18.0 was used to perform one-way ANOVA for analysis of variance and Duncan's test for multiple comparisons. The significance level was set at a p value of 0.05.

## Results

### Purity and integrity of total RNA

RNA quality was assessed by formaldehyde denaturing gel electrophoresis. As shown in Fig. 1, three bands-representing 28S, 18S, and 5S-were observed on the gel, no bands were indicative of DNA contamination or significant degradation. RNA purity and concentration were also examined, and the A260/A280 ratios of the samples ranged from 1.9 to 2.0. The integrity and purity of total RNA were both good, and they could be used in subsequent experiments.

### Differential expression of the *MyD88* gene in immune tissues

The real-time PCR results are shown in Table 2. In the five tissues that were examined, the expression of the *MyD88* gene in the CON group was significantly lower than that in other groups ($p<0.05$). With regard to other groups, with the exception of the CON group, the following findings were obtained: in the spleen tissue, the expression of the *MyD88* gene was not significantly different between the ANT, BV1, BV5 and BV10 group ($p>0.05$); in the lung tissue, *MyD88* expression was significantly higher in the BV10 group than in the

Table 2. The expression level of *MyD88* gene in 5 tissues under different treatments.

| Tissue | Group | | | | |
|---|---|---|---|---|---|
| | CON group | ANT group | BV1 group | BV5 group | BV10 group |
| Spleen | 5.07±1.34$^{b}$ | 61.19±19.15$^{a}$ | 51.88±16.93$^{a}$ | 51.93±21.99$^{a}$ | 44.39±9.57$^{a}$ |
| Lung | 4.58±1.05$^{c}$ | 22.01±6.03$^{b}$ | 22.13±9.18$^{b}$ | 32.58±8.21$^{b}$ | 52.56±22.92$^{a}$ |
| Kidney | 0.86±0.32$^{c}$ | 36.51±9.88$^{a}$ | 26.99±8.92$^{b}$ | 33.44±7.25$^{ab}$ | 29.17±2.95$^{ab}$ |
| Thymus | 0.83±0.24$^{c}$ | 21.38±4.88$^{ab}$ | 18.45±3.78$^{b}$ | 17.13±7.23$^{ab}$ | 23.86±3.31$^{a}$ |
| Lymph | 2.90±0.89$^{c}$ | 25.32±10.97$^{ab}$ | 33.88±15.37$^{a}$ | 24.34±6.64$^{ab}$ | 15.23±6.40$^{b}$ |

Note: In the same row, values with the same letter superscripts mean no significant difference ($p>0.05$), while with different small letter superscripts mean significant difference ($p<0.05$). The same as below.

Table 3. The expression level of *CD14* gene in 5 tissues under different treatments.

| Tissue | Group | | | | |
|---|---|---|---|---|---|
| | CON group | ANT group | BV1 group | BV5 group | BV10 group |
| Spleen | 186.30±43.66$^{a}$ | 11.08±2.81$^{b}$ | 9.58±2.36$^{b}$ | 15.60±5.31$^{b}$ | 15.66±4.83$^{b}$ |
| Lung | 104.50±45.91$^{a}$ | 5.57±2.15$^{b}$ | 7.62±3.07$^{b}$ | 21.07±6.23$^{b}$ | 26.69±4.55$^{b}$ |
| Kidney | 120.07±56.96$^{a}$ | 2.10±0.64$^{b}$ | 2.39±1.01$^{b}$ | 5.73±2.20$^{b}$ | 5.39±1.98$^{b}$ |
| Thymus | 97.22±30.42$^{a}$ | 2.80±0.54$^{b}$ | 1.69±0.83$^{b}$ | 2.00±0.96$^{b}$ | 7.80±1.10$^{b}$ |
| Lymph | 111.00±29.73$^{a}$ | 3.20±0.81$^{b}$ | 5.71±2.87$^{b}$ | 6.61±3.08$^{b}$ | 6.42±3.18$^{b}$ |

ANT group, BV1 group and BV5 group ($p<0.05$); in the renal tissue, *MyD88* expression in the ANT group was significantly higher than that in the group BV1 ($p<0.05$); in the lymph nodes, *MyD88* expression was significantly higher in the BV1 group than in the BV10 group ($p<0.05$).

### Differential expression of the *CD14* gene in immune tissues

As shown in Table 3, the expression of the *CD14* gene in the five examined tissues showed a relatively consistent trend within each group. The expression of the *CD14* gene was not significantly different between the groups that were fed bamboo vinegar powder (that is, the BV1, BV5 and BV10 groups) ($p>0.05$), or between the bamboo vinegar powder groups and the ANT group ($p>0.05$). However, the expression of *CD14* was significantly higher in the CON group than in other treatment groups ($p<0.05$).

## Discussion

We previously found that bamboo vinegar powder could significantly improve the immune indexes of piglets, as well as significantly lower the incidence of diarrhoea (Liu et al. 2015). The bamboo vinegar powder is a complex product from bamboo, and organic acids are one of the main components. It has been showed that phenyllactic acid can decrease the number of *E. coli*, and this novel dietary acid may have potential to stimulate the immune system for both weaning and growing pigs (Wang et al. 2009). Organic acids appear to reduce the proliferation of coliform bacteria, and blends of organic acids can serve as an alternative to in-feed antibiotics during the first few weeks post-weaning in pigs (Namkung et al. 2004). These indicate that bamboo vinegar powder may reduce the diarrhea rate due to the organic acids, but its specific regulatory mechanism is not clear. On this basis, we have tried to explore the immune-related mechanisms of bamboo vinegar powder in weaning piglets by analysing the expression of the *CD14* and *MyD88* genes, which are known to play a role in pathogenic infections in piglets. The findings do indicate that these two genes, which are closely linked with TLR pathways, are involved in the immunoregulatory effects of bamboo vinegar powder in piglets.

*MyD88* is the key gene of innate immune and inflammatory response in the body, and the change of its expression may indicate the change of immune response, while the inhibition of CD14 gene expression can effectively reduce the proinflammatory cytokines,

chemokine release, and inhibit the early inflammatory response, thereby reducing the damage of tissues (Thorgersen et al. 2012). In this experiment, the expression level of the *MyD88* gene in immune tissues was significantly higher in the groups that were fed bamboo vinegar powder and antibiotics than in the control group, while expression of the *CD14* gene showed an opposite trend. These findings indicate that the immune system activation level was low in the piglets fed the antibiotic- and bamboo powder-supplemented diets. Studies have shown that when bacteria and other antigenic substances invade the body, antibiotics can remove or modify antigens before the body's immune response is activated, thereby reducing the degree of activation of the immune system of the animal, and preventing any reduction in animal growth and development as a result of the infection (Guo et al. 2007). In agreement with this, another study found that the level of antigens as well as the serum immunoglobulin level are low in piglets that are fed a 0.5% bamboo vinegar powder diet. The findings of these two studies seem to indicate that bamboo vinegar powder may have similar effects as antibiotics with regard to reducing the degree of activation of the immune system in weaning piglets. However, it is unclear whether the mechanism of action of bamboo vinegar powder and antibiotics is similar, and this is therefore a topic that should be further studied in the future. In addition, although organic acids are the main effective constituents mentioned in the literature on bamboo vinegar, other constituents of bamboo vinegar may also play an important role. Therefore, it remains to be further studied which factors in bamboo vinegar powder could enhance the immune ability of piglets.

Although similar differences in the expression of *MyD88* and *CD14* were observed between the control and treatment groups, the expression of *MyD88* differed between groups treated with different doses of bamboo vinegar powder in certain tissues. For example, in the lung, *MyD88* expression was significantly higher in the BV10 group than in the other two bamboo vinegar powder groups; in the thymus, *MyD88* expression was significantly higher in the BV10 group than in the BV1 group; and in the lymph nodes, *MyD88* expression was significantly higher in the BV1 group than in the BV10 group. Such differences were not observed in *CD14* expression between the bamboo vinegar groups, it might be due to differences in the role and regulatory mechanisms of *MyD88* and *CD14* in different immune tissues. These findings indicate that the dose plays an important role in the effect of bamboo vinegar powder. Thus, it is important to determine the optimal dose of bamboo vinegar powder that can be added to the diet of weaning piglets, probably based on certain economic indicators, and this should be the focus of future research on this topic. Moreover, it is necessary to investigate other immune-related genes that could be involved in the effects of bamboo vinegar powder, as it would help identify more genetic markers of immune function in piglets.

In conclusion, the findings of this study indicate that bamboo vinegar powder has similar immunological effects as antibiotics in weaning piglets, and that these effects are dose dependent. Moreover, the role of the *CD14* and *MyD88* genes, and therefore possibly TLR pathways, in the underlying mechanism of these effects is also indicated.

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