Effects of casein glycomacropeptide supplementation on growth performance, intestinal morphology, intestinal barrier permeability and inflammatory responses in *Escherichia coli* K88 challenged piglets

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**A B S T R A C T**

Casein glycomacropeptide (CGMP) is a bioactive peptide derived from milk with multiple functions. This study was aimed at evaluating the effects of CGMP as a potential feed additive on growth performance, intestinal morphology, intestinal barrier permeability and inflammatory responses of *Escherichia coli* K88 (*E. coli* K88) challenged piglets. Eighteen weaning piglets were randomly assigned to three groups. Control group and K88 challenged group received a basal diet, and CGMP treated group received the basal diet supplemented with 1% of CGMP powder. The trial lasted for 12 days, K88 was orally administered to the piglets of K88 challenged group and CGMP treated group on days 8–10. The results showed that the diet containing 1% CGMP significantly alleviated the decrease in average daily gain (*P* < 0.05), increase in pathogenic bacteria amounts in intestinal contents (*P* < 0.05), intestinal morphology (*P* > 0.05) and barrier permeability damage (*P* < 0.05), and acute inflammatory response (*P* < 0.05) induced by *E. coli* K88 infection. In conclusion, CGMP supplementation in the diet protected the weaning piglets against *E. coli* K88 infection.

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1. Introduction

In the modern swine industry, mortality of piglets in weaning stage accounts for about 70% of the whole breeding period (Zhen et al., 2006). The traditional way to reduce the loss of piglets is to include high dose of antibiotics in feed, however the overuse of antibiotics has caused drug resistance, environmental pollution, residues in human food and many other problems, thus alternatives to antibiotics are desperately needed.

Casein glycomacropeptide (CGMP), derived from κ-casein, is a glycosylated phosphate peptide. During the process of cheese making, chymosin hydrolyzes phenylalanine–methionine bond of κ-casein into an insoluble para-κ-casein (residues 1–105) and a soluble polypeptide CGMP (residues 106–169) (Silva-Hernandez et al., 2002). Para-κ-casein is left in the curd, while CGMP is separated from whey and becomes by-products. It has been reported that many bioactive peptides with high nutritional values are located in whey protein such as immunoglobulins, lactoferrin and β-globulin; however CGMP is probably the least known cheese whey proteins despite its content in total whey protein being 15–20% (Saito et al., 1991).

The most important functional group of CGMP is polysaccharide sialic acid (SA) (N-acetyleneuraminic acid). It plays an important role in defense mechanism in vivo. SA is widely distributed in the key position of sugar chains, secreted glycoproteins and glycolipids (Schauer, 2004), and from the rule of the evolution of species indicates that higher species have abundant SA. Human breast milk contains high concentration of SA, which is closely related to infants' development and immune system maturation under infection conditions (Wang et al., 2001). More than 75% of the SA in milk...
is detected in the CGMP, indicating that CGMP’s physiological function is very important.

Studies have shown that CGMP can neutralize enterotoxin (Oh et al., 2000), inhibit virus or bacterial adhesion to cells (Bruck et al., 2006), inhibit gastrointestinal secretions (Brody, 2000), promote proliferation of beneficial bacteria (Janer et al., 2004) and exert immune regulation function (Otani and Hata, 1995). With these advantages, CGMP attracted widespread attention in the fields of functional food research, medicine, and health care. However, there are few reports about its potential as a feed additive or substitution of antibiotics.

In this study, we used the enterotoxigenic *Escherichia coli* K88 (*E. coli* K88) challenged weaned piglet model (Gao et al., 2013) to investigate the effects of CGMP on protecting piglets against post weaning diarrhea, and to lay experimental foundation for the potential application of CGMP as feed additives.

2. Materials and methods

The animal protocols for the present study were according to the guidelines stated in the guide for the care and use of agricultural animals in research and teaching and were approved by the animal care committee of Zhejiang University.

2.1. Casein glycomacropeptide source

This study used a commercial product (LACPRODAN® CGMP-10, Arla Foods) as a source of CGMP with the following declared composition: protein content 82–85%, CGMP content (of protein) 80 ± 5% and SA content approximated to 4.2%.

2.2. Bacterial strains

The bacterial strain used in this study (*Enterotoxigenic E. coli* K88 C83907) was purchased from the Chinese Institute of Veterinary Drugs Control (Beijing, China) and preserved by the National Engineering Laboratory of Bio-feed Safety and Pollution Prevention (Hangzhou, China). The challenge strain harbored the genes for fimbria F4ac as described by Gao (2014).

2.3. Animals and experimental design

A total of 18 crossbred (Duroc × Landrace × Yorkshire) weaning piglets (weaned on d 23, initial body weight 7.42 ± 0.19 kg) were randomly assigned to 3 experimental groups (6 pigs per treatment, half males and half females): control group, K88 challenged group and CGMP treated group. Control group and K88 challenged group were fed a corn–soybean basal diet, and CGMP treated group was fed the basal diets supplemented with 1% CGMP. All piglets had ad libitum access to feed and water throughout the 12-day trial. All weaning pigs were fed the basal diet for 3 days as pretreatment. In days 8–10, 30 mL of 10% (w/v) NaCHO3 and 20% (w/v) sucrose solution was orally administered to all of the piglets, and 30 min later, 30 mL of LB broth containing 10^9 CFU/mL of *E. coli* K88 was inoculated to the K88 challenged group and CGMP treated group, while 30 mL of sterile LB broth was inoculated to the control group. The average daily gain (ADG), average daily feed intake (ADFI), and feed/gain (F/G) of each pig were monitored throughout the experimental period. The fecal scores were graded by Castillo et al. (2008) (0 = normally shaped feces, 1 = shapeless feces, 2 = soft feces, and 3 = thin, liquid feces).

The corn–soybean meal basal diet was formulated to meet the nutrient requirements according to NRC (2012), (Table 1).

### Table 1

| Ingredients                      | Content, % | Calculated composition | Content, % |
|----------------------------------|------------|-------------------------|------------|
| Corn                             | 23.00      | Digestible energy, MJ/kg | 3.35       |
| Extruded corn                    | 20.00      | Crude protein           | 19.01      |
| Soybean meal                     | 6.50       | Crude fat               | 4.99       |
| Fermented soybean meal           | 6.50       | Crude fiber             | 1.77       |
| Oats meal                        | 15.00      | Calcium                 | 3.06       |
| Fish meal                        | 6.00       | Available phosphorus    | 0.59       |
| Whey permeate                    | 15.00      | SID-lysine              | 1.26       |
| Soybean oil                      | 1.00       | SID-methionine          | 0.46       |
| Limestone                        | 1.20       | SID-threonine           | 0.79       |
| Calcium bicarbonate              | 0.80       | SID-tryptophan          | 0.27       |
| Premix<sup>1</sup>               | 4.00       |                         |            |

**SID = standardized ideal digestible.**

*Provided per kilogram of diet: vitamin A 13,000 IU, vitamin D<sub>3</sub> 1,800 IU, vitamin E 60 mg, vitamin K<sub>1</sub> 3.0 mg, vitamin B<sub>1</sub> 2.0 mg, vitamin B<sub>2</sub> 6.0 mg, vitamin B<sub>6</sub> 10.0 mg, vitamin B<sub>12</sub> 0.02 mg, niacin 35.0 mg, calcium pantothenic 15.0 mg, biotin 0.12 mg, folic acid 1.0 mg; Fe 150.0 mg, Cu 120.0 mg, Zn 150.0 mg, Mn 45.0 mg, Se 0.30 mg, Co 1 mg, I 0.30 mg.

2.4. Sampling and processing

At the end of the experiment (day 13), blood samples were taken from the jugular vein and coagulated at room temperature for 60 min. Serum was separated by centrifugation (3,000 rpm, 10 min, 4 °C) and stored at −20 °C until biochemical analysis and ELISA. After blood sampling, all of the piglets were euthanized. The abdomen was immediately opened and intestinal contents of colon and cecum were collected for bacterial counts. Tissues from terminal ileum were removed and immediately frozen in liquid nitrogen. The samples were stored at −80 °C until analysis. Tissues from the jejunum were collected and fixed in 4% paraformaldehyde solution for analysis.

2.5. Biochemical determinations

Serum levels of pig major acute-phase protein (Pig-MAP), D-lactate and diamine oxidase (DAO) were determined using commercial ELISA kits purchased from Beijing Luyuan Byrd biological technology Co., Ltd. (Beijing, China), following the standard procedures described by the manufacturer. Serum levels of SA were measured using a commercial SA assay kit purchased from Nanjing Jiancheng Institute of Bioengineering (Jiangsu, China) according to the manufacturer's protocols.

Histological measurements of jejunum were analyzed according to H&E staining described by Moeser et al. (2012). Villus height and crypt depth were measured under a Leica microscope (DM3000; Leica, Wetzlar, Germany). A minimum of 10 villi from each pig were measured.

Intestinal segments were removed from distal ileum of each piglet, rinsed with 1× PBS to remove excess blood, homogenized in 1 mL of 1× PBS, and stored overnight at −20 °C. After freeze−thaw cycles to break up the cell membranes, the homogenate was centrifuged at 5,000 × g for 5 min at 4 °C, and the supernatant was collected, aliquoted in a 1.5 mL tube, and stored at −20 °C until use (Gao et al., 2013). The ileal protein amount was determined using BCA Protein Assay Kit (KEYGEN, Nanjing, China) following the manufacturer’s procedures. The ileal secretory immunoglobulin A (SIgA) levels (pg/mL) were measured using a commercially available ELISA kit (Luyuan Byrd biological technology Co., Ltd., Beijing, China) according to the manufacturer’s protocols. The final results were calculated in the form of mg SIgA per g protein.

The intestinal content samples were diluted in ten-fold serial dilutions. The 10 µL of each serial dilution was spread on bacteriological media to allow enumeration of specific bacterial types.
(Broom et al., 2006). E. coli was determined by growth on Eosin-Methylene Blue Agar (Hopebio, Qingdao, China), following incubation at 37 °C for 18–24 h in air.

2.6. Statistical analysis

All data are presented as means and SEM. The statistical analyses were performed in SPSS 16.0 (SPSS Inc., Chicago, IL). The significance of the differences between all of the treatment groups was analyzed by one-way ANOVA and means separation using Duncan’s significant difference test except for the data of fecal scores and E. coli counts of cecum and colon, which were analyzed by rank sum test. A significance level of 0.05 was used as default and each row with different small letter superscripts meant significant difference.

3. Results

3.1. Serum sialic acid concentration

Results for SA concentration in serum are shown in Table 2. The results showed that no significant differences were observed in the serum SA concentrations of control group and K88 challenged group, but the serum SA concentration of CGMP treated group was higher than those of control group and K88 challenged group (P = 0.027).

3.2. Growth performance and fecal scores

Results for initial and final weight, ADG, ADFI, F/G and fecal scores are shown in Table 3. There were no significant differences in ADG, ADFI, and F/G among all groups in days 1–7 (before K88 challenge). However, after challenged with K88 strains, ADG of K88 challenged group was strongly affected compared with control group (P = 0.009), but CGMP treated group had the same performance parameters as control group.

On days 8–12, E. coli K88 challenge sharply increased fecal scores of K88 challenged group compared with control group (P = 0.048), while CGMP inclusion kept the piglets from a significant increase in diarrhea. CGMP treated group even maintained lower fecal scores than control group during the experimental period.

3.3. E. coli counts in intestinal contents

Counts of E. coli in cecum and colon contents were presented in Table 4. A significant increase in E. coli counts was found in cecum content of K88 challenged group compared with control group (P = 0.018), but CGMP inclusion kept the pathogenic bacteria amounts the same level as that of control group. The same pattern was also found in colon contents.

3.4. Intestinal morphology

The jejunal morphological data, including villus height, crypt depth, and their ratios are presented in Table 5. K88 challenged piglets exhibited severe villous atrophy and decrease of villi-to-crypt ratio, but administration of CGMP had the trend to reduce the influence of K88 challenging on jejunal morphology (P > 0.05), which showed no significant differences with control group (P > 0.05).

3.5. The level of diamine oxidase and D-lactate in serum

Intestinal permeability of weaning piglets was evaluated by serum levels of D-lactate and DAO presented in Table 2. Compared with control group, K88 challenging significantly increased serum DAO and D-lactate levels (P < 0.05), whereas, compared with K88 challenged group, dietary supplement with CGMP considerably lowered intestinal permeability (P < 0.05).

3.6. Serum pig major acute-phase protein and ileum secretory immunoglobulin A concentration

The effects of K88 challenge on serum Pig-MAP and ileum SlgA concentration are showed in Table 2. The K88 challenge dramatically increased serum Pig-MAP and ileum SlgA concentration compared with control group (P < 0.05), but dietary supplement with CGMP significantly decreased serum Pig-MAP level (P < 0.05) and tended to lower the SlgA concentration (P = 0.162) caused by K88 challenge.

4. Discussion

CGMP, which is known as free of aromatic amino acid (van Calcar and Ney, 2012), can be an amino acid source in diets for phenylketonuria patients (they lack the ability to metabolize phenylalanine). It has also been widely applied in food additives field, especially in baby food. Due to the frequent use in human food, most researches focus on the production and extraction process of CGMP, but few studies have paid attention to its application on animal feed additive field. Whey powder is often used as a milk substitute in diets of weaning piglets, however, CGMP takes up about 15–20% in total whey protein. Therefore, the whey permeate was used in the basal diet instead, which contains only about 3% of whey protein. Calculated by the theoretical value, the basal diet would contain less than 0.1% of CGMP.

Post weaning diarrhea (PWD) problem normally happens in two weeks after weaning, which may cause 10–20% mortality rate. There are two kinds of PWD: one is Non Infectious Diarrhea commonly happening in 3–7 days after weaning, and caused by the change of food characteristics, reduced digestion, and absorption function, the other is Weaning Diarrhea Syndrome (WDS), which occurs 7–10 days after weaning, caused by intestinal flora change.

Table 2

| Item                  | Control group | K88 challenged group | CGMP treated group | P-value |
|-----------------------|---------------|----------------------|--------------------|---------|
| SA, mmol/L            | 3.80 ± 0.23a  | 3.69 ± 0.18a         | 4.70 ± 0.32b       | 0.027   |
| DAO, U/mL             | 13.84 ± 0.95a | 23.77 ± 0.84a        | 19.30 ± 0.90b      | 0.000   |
| D-Lactate, μmol/L     | 20.47 ± 0.74a | 32.46 ± 1.38b        | 24.64 ± 0.69b      | 0.000   |
| Pig-MAP, mg/L         | 23.07 ± 0.46a | 46.39 ± 1.86b        | 27.63 ± 0.79b      | 0.000   |
| SlgA, mg/g protein    | 1.39 ± 0.10a  | 7.15 ± 1.07a         | 5.35 ± 1.08b       | 0.001   |

Results are given as mean ± SEM. CGMP — casein glycomacropeptide; SA — sialic acid; DAO — diamin oxidase; Pig-MAP — pig major acute-phase protein; SlgA — secretory immunoglobulin A.

abc Different superscript letters within a row represent statistically significant differences among groups (P < 0.05).
pathogenic bacteria (like *E. coli*) infection, adhesion and producing toxins, which increased intestinal permeability (Bingzhao et al., 1996). The present study was based on CGMP’s biological function of inhibiting bacterial adhesion, which was to protect the weaning piglets from WDS induced by pathogenic bacteria.

SA is the main functional group in CGMP, the determination of SA content in CGMP is a common indirect way to detect the content of CGMP (Yali et al., 2010). Its concentration in serum may indicate the piglets’ absorption of CGMP. The results showed that piglets supplemented with CGMP had a higher SA concentration in serum than other groups, which suggested that CGMP in diets was absorbed by the piglets and participated in blood circulation.

In this study, K88 challenged piglets supplied with 1% CGMP showed a similar growth performance and fecal scores as control group, which suggested the ability of CGMP to alleviate the negative effect caused by K88 challenge. However, Hermes et al. (2013) reported that neither CGMP diet nor enterotoxigenic *E. coli* (ETEC) challenge had impact on growth performance or feed intake. The reason for this discrepancy may be caused by the lower dose and shorter time of ETEC challenge which led to unconspicuous diarrhea problem and other side effects.

Regarding to the result of intestinal *E. coli* counts, it is interesting to remark that the amount of significant enterobacteria *E. coli* both in cecum and colon contents of CGMP treated group was not increased after K88 challenge. Several researches reported that less adhesion of K88 on intestinal mucosa or Caco-2 cells after CGMP treated (Bruck et al., 2006; Gonzalez-Ortiz et al., 2014; Hermes et al., 2013). Hermes et al. (2013) reported that *E. coli* K88 specifically adhered to the villus enterocytes whereas non-ﬁmbriated *E. coli* had no adhesion ability. The *E. coli* strain K88 used in the present study was conﬁrmed to express fimbia F4ac, which is the most prevalent variant of ETEC that usually colonize in neonatal and weaned piglets’ intestine (Fairbrother et al., 2005). The initial phase of microbial infection is caused by the adhesion of bacteria to speciﬁc receptors on intestinal epithelial cells, and SA is found to be one of the speciﬁc receptors, so it is reasonable to deduce that the

### Table 3

| Item                              | Control group | K88 challenged group | CGMP treated group | P-value |
|-----------------------------------|---------------|----------------------|--------------------|---------|
| Body weight, kg                   |               |                      |                    |         |
| Day 1                             | 8.09 ± 0.39   | 7.59 ± 0.37          | 7.69 ± 0.42        | 0.620   |
| Day 7                             | 9.37 ± 0.42   | 9.13 ± 0.52          | 9.55 ± 0.64        | 0.854   |
| Day 12                            | 10.94 ± 0.74  | 8.79 ± 0.75          | 10.43 ± 0.52       | 0.098   |
| ADFI, g/d                         |               |                      |                    |         |
| Days 1–7                          | 307.14 ± 47.56| 342.86 ± 53.61       | 344.05 ± 18.48     | 0.793   |
| Days 8–12                         | 470.90 ± 60.60| 248.27 ± 63.91       | 285.52 ± 106.90    | 0.189   |
| Days 1–12                         | 375.38 ± 52.95| 303.44 ± 53.59       | 314.88 ± 29.75     | 0.519   |
| ADG, g/d                          |               |                      |                    |         |
| Days 1–7                          | 216.43 ± 41.26| 220.48 ± 28.97       | 266.57 ± 34.29     | 0.574   |
| Days 8–12                         | 267.67 ± 72.30| −68.50 ± 50.08       | 175.80 ± 85.96     | 0.009   |
| Days 1–12                         | 237.78 ± 53.60| 100.07 ± 36.92       | 228.75 ± 26.78     | 0.061   |
| F/G                              |               |                      |                    |         |
| Days 1–7                          | 1.45 ± 0.09   | 1.57 ± 0.18          | 1.26 ± 0.14        | 0.339   |
| Days 1–12                         | 1.64 ± 0.14   | 3.78 ± 1.11          | 1.46 ± 0.07        | 0.078   |
| Fecal scores                      |               |                      |                    |         |
| Days 1–7                          | 0.29 ± 0.09   | 0.14 ± 0.43          | 0.21 ± 0.09        | 0.483   |
| Days 8–12                         | 0.20 ± 0.06A  | 0.70 ± 0.17B         | 0.20 ± 0.03A       | 0.048   |
| Days 1–12                         | 0.25 ± 0.06   | 0.38 ± 0.11          | 0.21 ± 0.05        | 0.630   |

Results are given as mean ± SEM.

CGMP = casein glycomacropeptide; ADFI = average daily feed intake; ADG = average daily gain; F/G = feed/gain.

### Table 4

| Item                          | Control group | K88 challenged group | CGMP treated group | P-value |
|-------------------------------|---------------|----------------------|--------------------|---------|
| *E. coli* in cecum content    | 2.96 ± 0.69 × 10⁶ | 3.99 ± 1.45 × 10⁸   | 2.13 ± 2.10 × 10⁶ | 0.018   |
| *E. coli* in colon content    | 3.40 ± 1.11 × 10⁷ | 6.27 ± 4.72 × 10⁸   | 3.66 ± 2.42 × 10⁸ | 0.036   |

Results are given as mean ± SEM.

### Table 5

| Item                              | Control group | K88 challenged group | CGMP treated group | P-value |
|-----------------------------------|---------------|----------------------|--------------------|---------|
| Villus height, μm                  |               |                      |                    |         |
|                                 | 396.5 ± 53.6a | 266.0 ± 36.6b        | 480.9 ± 57.1ab     | 0.037   |
| Crypt depth, μm                   |               |                      |                    |         |
|                                 | 195.6 ± 21.9a | 302.0 ± 23.1b        | 364.7 ± 35.1b      | 0.040   |
| Villus height/crypt depth         |               | 2.28 ± 0.38a         | 0.94 ± 0.16b       | 1.47 ± 0.31b | 0.028   |

Results are given as mean ± SEM.

"Different superscript letters within a row represent statistically significant differences among groups (P < 0.05)."
CGMP used in this study which contains 4.2% of SA had an effective blocking activity against K88 attachment, which resulted in a lower amount of E. coli left in cecum and colon contents.

Changes in intestinal morphology such as shortening villus and deepening crypts have been associated with the presence of toxins. The shortened villi height decreases the surface area for nutrient absorption, and the deepened crypt depth indicates faster tissue turnover and much more demand for new tissue (Xu et al., 2003). In the present research, ETEC challenge caused a reduction in the ratio of villus height to crypt depth in jejunum, which suggested a deterioration of intestinal morphology. Supplement of 1% CGMP in diet had a higher ratio than ETEC challenged group, which suggested the ability of CGMP to relieve the intestinal damage caused by K88 challenge. According to the results of microbial population, we believed that CGMP’s ability to protect the small intestine morphology under bacteria attacking was based on its specific adhesion to K88 strain, which led to less bacteria invasion. Chen et al. (2014) established oxazolone induced ulcerative colitis mice model and treated with different dose of CGMP, the results showed that mice given 50 mg/kg per day of CGMP had better histopathological evaluation and less visible lesions in colon.

The intestinal barrier permeability would increase when the intestinal barrier function is impaired, which relates to the impaired epithelial cell function and the invasion of pathogenic bacteria, finally caused inflammation. DAO and D-lactate are recognized as sensitive markers for monitoring the alteration of intestinal barrier permeability (Brandt et al., 1980; Zhao et al., 2011). DAO is one of the diamine oxidases catalyzed by deaminases, mainly expressed in small intestine and rarely in serum under normal circumstances (Wolvekamp and Debruijn, 1994). When intestinal barrier integrity is damaged, tissue DAO levels decrease and serum DAO levels increase (Zhao et al., 2014), indicating that DAO in serum reflects the destruction of the intestinal epithelial cell layer and intestinal mucosa barricade indirectly. D-Lactate is the metabolic end product of intestinal bacteria. Mammals neither produce D-lactate nor have D-lactate dehydrogenase, thus the body retains a low level of D-lactate under health condition (Murray et al., 1993). When the balance of microbiota in intestinal are broken, pathogens multiply and produced excessive amount of D-lactate which released into the blood through the damaged mucosa (Zhao et al., 2014). This research showed that CGMP treated piglets had lower serum DAO and D-lactate levels than ETEC challenged piglets, indicating its ability to protect the barrier function of the intestinal mucosal.

When exposed to infection, inflammation or trauma, there will be a sharp rise in the concentration of a group of serum protein, causing acute phase reaction. Those proteins, called acute phase proteins (APPs), are synthesized and secreted by liver (Alava et al., 1997). Pro-inflammatory cytokines (mainly interleukin (IL)-1, IL-6 and tumor necrosis factor-α) combined to the receptors on liver cell surface can induce high expression of APPs. So it is of great importance that the measurement of the concentration of APPs in pig blood, which can be an indirect parameter of piglets with infection or inflammatory lesions (Murata et al., 2004). Pig-MAP is significantly important in APPs, the main function of Pig-MAP is binding with impaired cell and antagonize inflammation (Choi-Miura et al., 2000). In this study, we observed an increase in serum Pig-MAP in K88 challenged group, which indicated an acute immune response to the pathogen, while dietary supplement with CGMP significantly attenuated this response, suggesting that CGMP could reduce the inflammatory response caused by K88. SlgA is the main immune globulin, which can effectively block infection and invasion of pathogens (Huang et al., 2015). The present study showed that addition of CGMP could reduce the secretion of ileum SlgA in K88 challenged piglets.

5. Conclusion

From the above results, we concluded that dietary supplement with 1% CGMP significantly alleviated the decrease in growth performance caused by E. coli infection. The beneficial effects of CGMP may rely on the inhibition of pathogen overgrowth and specific adhesion to K88 strain, which prevented intestinal morphology from being damaged, inhibited the increase of intestinal permeability, and avoided the inflammatory responses induced by pathogen invasion. These results suggested that CGMP might be used to develop an effective feed additive to protect the weaning piglets from PWD.

Conflict of interest

The authors declare that there are no competing interests.

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