

## Effects of Feeding Chinese Herbal Medicine Additives to Newly Weaned Piglets on Mucosal Immune Function of the Small Intestine

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**Abstract:** This study investigated the influence of the inclusion of Chinese Herbal Medicine additive (CHMD) in diets for weaned piglets on parameters of mucosal immune function of the small intestine in a 3 weeks trial, focused on immune cell subsets and mucosal concentrations of TNF- $\alpha$ , IL-2 and IL-4. Total of 144 crossbred (Duroc x Landrace x Yorkshire) weaning piglets at 21 days of age were selected and divided randomly into 4 groups with 3 replicates of 12 piglets each. A corn soybean meal expanded soybean basal diet without antibiotics or probiotics was used as control and the other 3 groups were fed the control diet supplemented with the CHMD at ratios of 0.5, 1.0 and 1.5% (wt/wt). The data showed that compared to the control group, dietary supplementation with CHMD at the 1% dose: reduced the counts of IEL/100 enterocytes in the duodenum, jejunum and ileum ( $p < 0.05$ ); increased the number of goblet cells in the duodenum ( $p < 0.01$ ) in the jejunum and ileum ( $p < 0.05$ ); decreased the number of mast cells in the mucosa and submucosa in the duodenum, jejunum and ileum ( $p < 0.05$ ), respectively had higher number of cells per villus positive for sIgA in the duodenum, jejunum ( $p < 0.01$ ) and in the ileum ( $p < 0.05$ ); decreased mucosal concentrations of TNF- $\alpha$  in the duodenum, jejunum ( $p < 0.01$ ) in the ileum ( $p < 0.05$ ), increased mucosal concentrations of IL-2 in the duodenum and jejunum ( $p < 0.05$ ), increased mucosal concentrations of IL-4 in the duodenum, jejunum ( $p < 0.01$ ). All these results suggested that the use of CHMD as an additive to positively affect on the parameters of gastrointestinal health and mucosal immune function of the small intestine in weaning pigs would be a valid alternative to antibiotic growth promoters of defending weaned pigs from infections and weaning stress, additionally imply that the dose of 1% CHMD supplement is the most ideal concentration to achieve the most beneficial effects.

**Key words:** Weaned piglets, chinese herbal medicine additive, immune cell subsets, cytokine, jejunum

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### INTRODUCTION

After birth and the neonatal period, weaning is a critical period of pig growth because of increased susceptibility to gastrointestinal tract disorders, infections and diarrhoea due to psychological, social, environmental and dietary stresses interfering with gastrointestinal tract development and adaptation (Boudry *et al.*, 2007). This time is associated with changes in the intestinal microbial ecosystem, morphology, epithelial barrier function, increased pro-inflammatory cytokine expression and a change in the activity of digestive enzymes (Martin *et al.*, 2012; Montagne *et al.*, 2007; Pieper *et al.*, 2008). The weaning period in young pigs is also associated with inflammation of the gut, involving alteration in intestinal immunity and in the intestinal immune responses to dietary and bacterial antigens (Hannant, 2002). As a consequence, the growth rate and feed conversion efficiency of pigs following

weaning is reduced relative to that observed during lactation and well below the pigs genetic potential (Moorea *et al.*, 2011).

These post weaning problems are currently managed by incorporating antibiotics into weaning diets (Jansman *et al.*, 2007). However, the continuous use and misuse of antibiotics have led to the emergence of drug and antibiotic residues in animal products (Monroe and Polk, 2000). Increased bacterial resistance to antibiotics and environmental problems led the European Union to implement a full ban on in feed antibiotics from January, 2006 (Kong *et al.*, 2007a). The ban on the use of growth promoting antibiotics in animal feeds has put pressure on the development of new feeding strategies and formulations to support animal performance and gastrointestinal tract health.

As potential alternatives to antimicrobial agents, traditional Chinese herbal medicines or their extracts may reduce the impact of the postweaning growth check by

impairing microbial growth in early weaned piglets (Kong *et al.*, 2007b) by modulating cellular and humoral immunity in weaned piglets (Kong *et al.*, 2007a) by regulating the microbiota composition and maintaining a normal morphology of gut mucosa in weanling piglets (Fang *et al.*, 2009) by regulating the digestive enzyme activities (Ren-Jun *et al.*, 2002).

On the basis of the recent finding that dietary supplementation with one complex additive of Chinese herbal medicines enhanced both indicators of gastrointestinal health, growth performance and antioxidant status (Ding *et al.*, 2011a), digestive enzymatic activities (Ding *et al.*, 2011b) in early weaned piglets, researchers hypothesized that such a treatment will improve gastrointestinal tract mucosal immune function and maturation in weaned piglets.

## MATERIALS AND METHODS

The study was carried out in accordance with the Chinese guidelines for animal welfare and experimental protocol (Yin *et al.*, 2004).

### **Composition of the Chinese herbal medicines additive:**

The Chinese Herbal Medicines additive (CHMD) used in this study is consisted of seven dried Chinese herbs including *Astragalus Membranaceus*, *Scutellaria*, *malt*, *Glycyrrhiza uralensis*, *Codonopsis pilosula*, *Poria cocos*, *Atractylodes macrocephala* and the ratio is 2:1:1:1:2:2:2. Before inclusion in the feed, they were mixed according to the aforesaid ratio and crumbled to an ultra fine powder with an average granule diameter of 30  $\mu\text{m}$ . All the medicinal herbs were purchased from WeiMing pharmacy in Hefei city of China.

**Animals, diets and experimental design:** At weaning, at 21 days of age, 144 crossbred (Duroc x Landrace x Yorkshire) piglets (72 females and 72 males) with a body weight of  $5.86 \pm 0.24$  kg were selected from 18 L (among the original 21 L) that were healthy were divided randomly into 4 groups balanced for sex, weight and litter origin. In each group, the piglets were divided randomly into 3 pens (12 animals per pen) and each group was fed one of 4 diets for 3 weeks. A basal diet without antibiotics or probiotics was fed to the control group. The other 3 groups were fed the control diet supplemented with 0.5, 1 and 1.5% (wt/wt) CHMD (T1-T3). The basal diet mainly contained maize, soyabean meal, expanded soybean, milk replacer, whey powder, soybean oil and a premix of vitamins and minerals and the nutrient contents met or exceeded nutrient requirements recommended by NRC (1998).

### **Histological measurements and lymphocyte subsets**

**analysis:** About 10 cm segments of duodenum, jejunum and ileum were taken and stored in 10% formalin. Briefly, 6 cross sections were obtained from each formalin fixed segment and processed for histological examination. Duodenum sample was taken at 30 cm away from the stomach. Jejunum sample was taken at 2 m before the ileal cecal junction. Ileum was sampled at 30 cm before the ileal cecal junction.

Intraepithelial Lymphocytes (IELs) and Goblet Cells (GCs) per 100 epithelial cells along an intact villous were counted and each count was repeated five times for each section in each piglet, the number of mast cells in the mucosa and submucosa was quantified by numbering mast cells in eight defined  $1 \text{ mm}^2$  areas of one segment histological slide with a Microcheck Grid (Beijing KeYiOptical Ltd., Beijing, China) containing 400 microchecks ( $1 \text{ mm}^2$ ) under a  $\times 20$  power. The mast cell number was then calculated by overall cell number divided by square millimeters. Periodic Acid Schiff (PAS) stain for GCs count and hematoxylin-eosin stain for IELs count, mast cells were visible by toluidine blue staining under a microscope (B $\times 50$ , Olympus Co., Japan). Each sample was represented by three independent histological slides.

**Immunohistochemistry:** Tissue samples from duodenum, jejunum and ileum were taken immediately and snap frozen in liquid nitrogen after being embedded in freezing media and stored at  $-70^\circ\text{C}$ . Immunohistochemistry procedures were adapted from the methods of Vega-Lopez *et al.* (1993). About 4-6  $\mu\text{m}$  of frozen cross sections were obtained, mounted on poly-L-lysine slides, fixed in cold acetone for 15 min and placed in 1 M Phosphate Buffered Saline (PBS) overnight. Sections were placed in a methanol bath consisting of 0.05% hydrogen peroxide for 20 min to quench endogenous peroxidase activity. Sections were then washed with PBS and placed in PBS containing 2.5% heat inactivated horse serum for 30 min at room temperature in a humid chamber. Commercially available cell molecule specific mouse monoclonal antibody was used to identify pig secretory immunoglobulin A (sIgA) (Sigma Chemical Co., St. Louis, MO). A 75 mL volume of predetermined optimal dilution of monoclonal antibody was placed over the tissue and tissues incubated for 2 h at room temperature in a humid chamber. Affinity purified, biotinylated, horse antimouse immunoglobulin (Vector Laboratories, Burlingame, CA) was used as secondary antibody at a dilution of 1:100 for 1 h at room temperature in a humid chamber. Subsequently, tissues were incubated with a complex of streptavidin horse radish peroxidase conjugated biotin

(Vector Laboratories) and the reaction was visualized using a 0.05% solution of diaminobenzidine in 1M PBS (pH 7.6). Between steps, slides were thoroughly washed in PBS. After visualization, slides were counterstained in Harris hematoxylin (Sigma Chemical Co.) placed through a dehydration bath, mounted with aquamount and examined under a bright field light microscope (B× 50, Olympus Co., Japan). Positively stained cells were counted within 10 randomly selected villi per section.

**Measurement of cytokines in intestine mucosa:** Mucosal samples were used to determine TNF- $\alpha$ , IL-2, IL-4 levels. After homogenization of duodenum, jejunum and ileum mucosa samples in saline solution (1:10, w:v) and centrifugation at 1500×g for 20 min, the supernatants were analyzed for TNF- $\alpha$ , IL-2, IL-4 levels by using the commercially available pig enzyme-linked immunosorbent assay kits (ADL, USA) according to the manufacturer's instructions.

**Statistical analyses:** Data are presented as arithmetic means with standard deviation of the mean (Mean±SD). Differences among groups were compared by SPSS18.0 statistics software using one-way ANOVA and LSD method test. Here,  $p < 0.05$  was selected as significant standard,  $p < 0.01$  was selected as remarkably significant standard.

**RESULTS**

**Immune cell subsets in epithelium and mucosa of the small intestine:** There were differences associated with the treatment in immune cell numbers in epithelium and mucosa (Table 1).

Counts of IEL/100 enterocytes were reduced in duodenum, jejunum and ileum of 1% CHMD fed pigs ( $p < 0.05$ ) in duodenum of 1.5% CHMD fed pigs ( $p < 0.05$ ) than basal diet fed pigs. Number of goblet cells were increased in duodenum ( $p < 0.01$ ), jejunum and ileum ( $p < 0.05$ ) of 1% CHMD fed pigs in ileum ( $p < 0.05$ ) of 1.5% CHMD fed pigs than basal diet fed pigs. Compared to the control in the 1% CHMD fed group the number of mast cells in the mucosa and submucosa was decreased in duodenum, jejunum and ileum ( $p < 0.05$ ), respectively. Compared to the basal diet fed group in the 1% CHMD fed group, the number of cells per villus positive for sIgA were higher in duodenum, jejunum ( $p < 0.01$ ) and in ileum ( $p < 0.05$ ) in the case of the 1.5% CHMD fed group, the number of sIgA secreting cells were higher in duodenum, jejunum ( $p < 0.05$ ), respectively.

**Mucosal concentrations of TNF- $\alpha$ , IL-2 and IL-4:** Mucosal TNF- $\alpha$ , IL-2 and IL-4 concentrations of weaned piglets were shown in Table 2. Compared to the control in the case of the 1% CHMD fed group, mucosal

Table 1: Effect of dietary CHMD level on immune cell subsets in epithelium and mucosa of the small intestine in weaned piglets

Parameters	CHMD dose in wt/wt (%)			
	0	0.5	1	1.5
<b>Lymphocytes % (IELs, per 100 epithelial cells)</b>				
Duodenum	18.31±1.80 <sup>a</sup>	18.05±1.91 <sup>ab</sup>	15.73±1.11 <sup>c</sup>	16.08±1.07 <sup>bc</sup>
Jejunum	14.44±0.93 <sup>a</sup>	14.17±0.75 <sup>a</sup>	12.82±1.20 <sup>b</sup>	13.33±1.00 <sup>ab</sup>
Ileum	11.90±1.38 <sup>a</sup>	11.45±1.04 <sup>ab</sup>	10.28±1.33 <sup>b</sup>	10.72±0.88 <sup>ab</sup>
<b>Goblet cells % (GCs, per 100 epithelial cells)</b>				
Duodenum	4.62±0.31 <sup>bc</sup>	4.82±0.62 <sup>ABbc</sup>	5.81±0.29 <sup>AA</sup>	5.44±0.79 <sup>ABab</sup>
Jejunum	5.46±0.65 <sup>b</sup>	5.70±0.61 <sup>ab</sup>	6.67±0.78 <sup>a</sup>	6.44±0.89 <sup>ab</sup>
Ileum	9.63±0.72 <sup>c</sup>	9.91±0.57 <sup>bc</sup>	10.99±0.91 <sup>a</sup>	10.81±0.75 <sup>ab</sup>
<b>Mucosal mast cell numbers (No. mm<sup>-2</sup>)</b>				
Duodenum	33.23±1.29 <sup>a</sup>	33.04±1.67 <sup>a</sup>	30.79±1.45 <sup>b</sup>	31.60±1.36 <sup>b</sup>
Jejunum	27.81±1.82 <sup>a</sup>	27.79±1.25 <sup>ab</sup>	25.70±1.24 <sup>b</sup>	26.04±1.56 <sup>b</sup>
Ileum	20.12±1.29 <sup>a</sup>	20.03±1.58 <sup>a</sup>	17.85±1.14 <sup>b</sup>	18.57±1.15 <sup>ab</sup>
<b>sIgA secreting cells numbers % (per 100 histiocytes in lamina propria)</b>				
Duodenum	7.29±0.25 <sup>bc</sup>	7.51±0.56 <sup>Bbc</sup>	8.62±0.48 <sup>AA</sup>	8.16±0.79 <sup>ABab</sup>
Jejunum	5.86±0.63 <sup>bc</sup>	6.38±0.41 <sup>ABbc</sup>	7.41±0.87 <sup>AA</sup>	6.77±0.39 <sup>ABab</sup>
Ileum	6.63±0.65 <sup>b</sup>	6.68±0.35 <sup>b</sup>	7.54±0.43 <sup>a</sup>	7.28±0.82 <sup>ab</sup>

In the same row values with different small letter superscripts mean significant difference ( $p < 0.05$ ) and with different capital letter superscripts mean extremely significant difference ( $p < 0.01$ )

Table 2: Effect of dietary CHMD level on mucosal concentrations of TNF- $\alpha$ , IL-2 and IL-4 of the small intestine in weaned piglets

Parameters	CHMD dose in wt/wt (%)			
	0	0.5	1	1.5
<b>TNF-<math>\alpha</math> (ng mL<sup>-1</sup>)</b>				
Duodenum	4.09±0.75	3.07±0.78	2.62±0.49	2.95±0.70
Jejunum	4.85±0.67	4.37±0.33	3.41±0.79	3.62±0.62
Ileum	2.81±0.55	2.61±0.75	1.94±0.63	2.20±0.42
<b>IL-2 (ng mL<sup>-1</sup>)</b>				
Duodenum	2.13±0.24	2.43±0.42	2.99±0.69	2.79±0.67
Jejunum	1.93±0.42	2.03±0.54	2.67±0.35	2.38±0.44
Ileum	2.61±0.40	2.72±0.52	3.05±0.21	2.89±0.45
<b>IL-4 (ng mL<sup>-1</sup>)</b>				
Duodenum	0.74±0.33	0.91±0.47	1.43±0.36	1.33±0.39
Jejunum	0.97±0.22	1.13±0.27	1.48±0.35	1.37±0.28
Ileum	0.76±0.44	1.12±0.38	1.37±0.56	1.19±0.47

concentrations of TNF- $\alpha$  was decreased by 35.94% in duodenum ( $p < 0.01$ ), by 29.69% in jejunum ( $p < 0.01$ ), by 30.96% in ileum ( $p < 0.05$ ), mucosal concentrations of IL-2 was increased in duodenum and jejunum ( $p < 0.05$ ), mucosal concentrations of IL-4 was increased in duodenum and jejunum ( $p < 0.01$ ). Compared to the control in the case of 1.5% CHMD-fed group, mucosal concentrations of TNF- $\alpha$  was decreased in duodenum, jejunum ( $p < 0.05$ ), mucosal concentrations of IL-4 was increased in duodenum ( $p < 0.05$ ). Compared to the control, in the case of 0.5% CHMD-fed group, mucosal concentrations of TNF- $\alpha$  was decreased in duodenum ( $p < 0.05$ ).

**DISCUSSION**

James (1993) reported that villous epithelium cells in the small intestinal mucosa of piglet were the first line of defence against dietary toxins and exogenous pathogens

and that they contained IEL, GC and mast cell with immune functions. Lillehoj (1989) found that IEL could participate in modulating intestinal immunity by both non-special immune mechanisms and special immune mechanisms and the number of IEL might be associated with the immune activating status. The more strongly the diet stimulated the small intestine, the greater the number of IELs found in the layer of intestinal epithelial cells which implied that the immune activating status of the small intestine was stronger (Gu and Li, 2004). In the current experiment, researchers demonstrated that CHMD reduced the number of IELs in the mucosa of the small intestine. This may imply that a role for CHMD in preventing the small intestine from possible activation in pigs. Then, it could be proposed that the mechanism of action of dietary CHMD on growth performance involved the immune system, because the degree of immune cell activation may limit the availability of food energy for growth (Perez-Bosque *et al.*, 2004).

Goblet cells can secrete mucus, participate in adjusting local intestinal immune function and prevent from bacterial and fungal invasion to villi (Iijima *et al.*, 2001). The greater the number of GCs in small intestinal mucosa, the stronger the preventive function (Liu *et al.*, 1997). In the experiment, as the dietary CHMD level increased to 1%, GCs in the mucosa of the small intestine increased remarkably which might indicate an improvement in the immune status of the small intestine. Weaning piglets onto solid stater diets is begun at between 3 and 4 weeks of age in modern pig industry. More than 80% of mortality during the month post weaning in pigs seems to be caused by digestive disorders and food hypersensitivity reactions in pigs (Madec *et al.*, 1998). Mast cells are located in the gut mucosa and submucosa to function in intestinal peristalsis, inflammatory process and related immune responses. Many studies involving food allergies suggest that mast cells are prominent in the development of hypersensitivity through an excessive release of large amounts of inflammatory factors including histamine and some cytokines after activation and degranulation (Van Wijk and Knippels, 2007).

Actually, food-allergic patients often have an increase in the number of mast cells in the intestinal mucosa. An important finding of the current study is that the number of mast cells in the mucosa and submucosa in the small intestine of pigs fed CHMD was lower than that of the control group which demonstrated that CHMD could reduced the intestinal mast cell numbers resulting in an reducing release of histamine which may contribute

to the change of intestinal motility and malabsorption of nutrients supporting the results of the increased performance and reduced diarrhea of pigs in this study.

Previous studies have demonstrated that sIgA was a principal mediator of mucosal immunity. It plays important roles in the immunity of the digestive and respiratory systems (Kudsk, 2002; Mantis *et al.*, 2011). During suckling, piglets ingest sowmilk which contains immunoglobulin IgA, IgA is absorbed into the mucus covering the villous surfaces and prevents *E. coli* and other organisms from attaching to the villi (Lu *et al.*, 2010). Ramos-Clamont *et al.* (2007) reported that IgA oligosaccharides partially inhibit adherence of *E. coli* K88 to porcine intestinal mucins. If the organisms are unable to attach, they are unable to cause disease. The sIgA also helps to destroy bacteria in addition, sIgA can remove translocated antigen by transporting it back across the epithelium (Kagnoff, 1993). After weaning however, no more maternal IgA is available the levels rapidly decline and bacteria damage the villi, decreases in mucosal sIgA levels and sIgA producing cells in animals resulted in increased bacterial translocation in the mucosal surface (Kuru *et al.*, 2004; Zhang *et al.*, 2010). In the current study, the number of mucosal sIgA producing cells in the duodenum, jejunum and ileum increased significantly in the animals administered the optimal dose of CHMD which is consistent with prior findings that some potential alternatives to antimicrobial agents such as zinc oxide antimicrobial peptide cecropin stimulates intestinal mucosal sIgA responses (Broom *et al.*, 2006; Wu *et al.*, 2004). Therefore, it is conceivable that CHMD was capable of enhancing the secretion of intestinal mucosal sIgA in early weaned piglets was able to improve intestinal mucosal resistance to *E. coli* and other organisms infection and thus alleviate diarrhoea.

Cytokines play a central role in the cell mediated immune response and they also participate in the maintenance of tissue integrity (Pie *et al.*, 2004). TNF- $\alpha$ , an inflammatory mediator is induced in response to infection which exerts a number of different biological effects in various cells thus initiating and regulating the immune response (Bemelmans *et al.*, 1996). The reduction in TNF- $\alpha$  secretion after weaning was seen at the initial stage of acute stress and changes in metabolic status (Carstensen *et al.*, 2005). As opposed to the initial reduction in the TNF- $\alpha$  response during the initial stage of acute stress, chronic stress increases the responsiveness in TNF- $\alpha$  (Kusnecov and Rossi-George, 2002). The weaning period in young pigs is also

associated with inflammation of the gut involving alteration in intestinal immunity and in the intestinal immune responses to dietary and bacterial antigens (Hannant, 2002). It is well known that inflammation is mediated by increased production of pro inflammatory cytokines among these, IL-8, TNF- $\alpha$  and IFN- $\gamma$  are markedly expressed in inflamed gut mucosa (Bosi *et al.*, 2004). The results showed that feeding the optimal dose of CHMD induced a marked decrease on mucosal concentrations of TNF- $\alpha$  in the duodenum, jejunum and ileum. Thus, all these data were in favor of a role of CHMD in decreasing intestinal inflammation by down regulating inflammatory cytokine expression.

The role of cytokines in the regulation and modulation of the immune response has been widely studied since the Th1/Th2 paradigm was first postulated (Mossman *et al.*, 1986). IL-2, IL-12 and IFN- $\gamma$  are considered to be key cytokines in the Th1 response and serve as indicators of a predominance of cell-mediated responses whereas IL-4 and IL-10 participate in Th2 polarisation and secretion and suggest a predominance of humoral responses (Raymond and Wilkie, 2004). In this study, researchers found that dietary supplementation with the CHMD increased mucosal concentrations of IL-2 in the duodenum and jejunum of weaned piglets throughout the experiment, the herb treatment also markedly enhanced mucosal concentrations of IL-4 in the in the duodenum and jejunum of weaned piglets. These results confirmed those of Boudry *et al.* (2007) and Kong *et al.* (2007a) and suggest an immunomodulatory effect of some potential alternatives to antimicrobial agents such as bovine colostrum, herbs targeting mainly the gut associated lymphoid tissues which responded by producing at different levels both Th1 pro inflammatory cytokines (IL-2, IFN- $\gamma$  and IL-12) and anti inflammatory Th2 cytokines (IL-4 and IL-10). This bipolarity is important in a context of exposure to a wide range of antigens associated with pathogens, commensal bacteria and food (Boudry *et al.*, 2007) and it includes the ability to generate tolerance to food and commensal bacterial antigens as well as to activate the immune response to pathogens.

## CONCLUSION

The data on parameters of mucosal immune function of the small intestine demonstrated that the diet supplementation with CHMD has noticeable positive effects on some immunological properties of the structures associated with the gastrointestinal tract in

weaning piglets by generating different types of responses protecting from infectious and allergic diseases. These findings indicate that the CHMD is safe and effective in preventing the weaning associated digestive disturbances and improving the growth performance in piglets, dietary supplementation with the CHMD may offer an effective alternative to antibiotics for early weaned piglets.

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