Effects of Copper-bearing Montmorillonite (Cu-MMT) on *Escherichia coli* and Diarrhea on Weanling Pigs*

M. S. Xia, C. H. Hu, Z. R. Xu, Y. Ye[1], Y. H. Zhou[1] and L. Xiong**
Animal Science College, Zhejiang University, Hangzhou 310029, P. R. China

ABSTRACT : Copper-bearing montmorillonite (Cu-MMT) was produced by Cu^{2+} cation exchange reaction. X-ray diffraction analysis showed that that the (001) basal spacing of the MMT crystal lattice increased from 1.544 to 1.588 nm after Cu^{2+} exchange. This indicated that Cu^{2+} entered into interlayer position of MMT as a hydrated cation or composite cation. *In vitro* results indicated that Cu-MMT had antibacterial activity on *Escherichia coli* K_{88}. Cu-MMT had unbalanced positive charge after cation exchange. Its antibacterial activity resulted from two aspects, one was electrostatic attraction which made *E. coli* K_{88} being adhered on the montmorillonite surface, the other was the Cu^{2+} slowly released, which could kill bacteria. In an *in vivo* study, four replicates of eight weanling pigs were assigned to each of two dietary treatments to study the effects of Cu-MMT on diarrhea, *E. coli* in the lumen of the jejunum and morphology of jejunal mucosa. As compared to the control, supplementation of the diet with 0.2% Cu-MMT improved average daily gain by 12.50% ($p<0.05$) and decreased F/G by 9.42% ($p<0.05$). The mean diarrhrea incidence was decreased by 71.80% ($p<0.05$). The viable counts of *Escherichia coli* in jejunal contents were significantly reduced ($p<0.05$). Villus height and the villus height to crypt depth ratio at the jejunal mucosa were increased by 19.09% ($p<0.05$) and 37.10% ($p<0.05$), respectively. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 12 : 1712-1716)***

Key Words : Copper-bearing Montmorillonite, *Escherichia coil,* Diarrhea, Intestinal Morphology, Weanling Pigs

INTRODUCTION

Diarrhoea in weaning pigs has become a serious problem due to the trend towards large intensive herds and early weaning. The major causes of diarrohea in piglets are enterotoxigenic *Escherichia coli* (ETEC) strains that bear the K_{88} and K_{99} fimbrial appendages. Among different ETEC, those expressing the K_{88} fimbrial antigen are the most prevalent forms of *E. coli* infection found world-wide (Song and Wang, 1997).

Nonmetallic minerals have been used as antimicrobial carriers for years (Hu et al., 2000; Wang et al., 2000). Ag^{+} carried on zeolite, montmorillonite (MMT) and other clays has been reported as effective antibacterial materials (Rivera-Garza et al., 2000; Onodera et al., 2001). Copper sulfate is one of the traditional inorganic antibacterial materials with wide usage. High level of Copper sulfate supplementation (125-250 mg/kg of Cu) has been shown to promote the growth. In order to decrease the Cu supplementation, we consider to carry the Cu^{2+} in/on clays with large specific surface area which, after dispersed in water, can adsorb bacterium rapidly. Montmorillonite (MMT) is a sort of aluminum silicate with 2:1 layer structure of tetrahedral and octahedral layers. Between the structural sheets, there are exchangeable cations ready being replaced by other cations or compounds. Taking into account that the nice disperse ability of fine MMT particles in water, it might be suitable for the use as an antimicrobial carrier. In this work, MMT supported Cu^{2+} (Cu-MMT) is produced via cation exchange reaction according to the method described by Xu et al. (2002b). An experiment was carried out to investigate its antibacterial abilities *in vitro* and the effects of Cu-MMT on diarrhrea incidence, intestinal *Escherichia coli*, intestinal morphology of weanling pigs *in vivo*.

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** Corresponding Author: Meisheng Xia. Tel: +86-571-86985607, Fax: +86-571-86985607, E-mail: msxia@zju.edu.cn
[1] Department of Earth Science, Zhejiang University, Hangzhou 310027, P. R. China.

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MATERIALS AND METHODS

Materials

MMT ore used in this work was a hydrothermal product of volcano sedimentary rocks from Chifeng, the Inner Mongolia Autonomous Region, China. Besides MMT, there were minor amounts of quartz and volcanic glass in the ore. To get rid of the impurities, the raw material was dried in oven over night at 80°C and then milled to less than 300 mesh. The milled material was dispersed in water to form a 10% suspension that was churned up in a stirrer for about 10 min. Particles larger than 2 μm were separated out by sedimentation while the suspension was centrifuged to get refined MMT. The refined MMT was dried at 80°C followed by another milling to less than 300 mesh for use. The formula of the purified MMT is $[Na_{0.158}K_{0.082}Ca_{0.256}Mg_{0.063}][Mg_{0.376}Fe^{2+}_{0.014}Fe^{3+}_{0.136}Al_{1.474}][Si_{3.87}Al_{0.13}]O_{10}(OH)_2 \cdot nH_2O$ with the cation exchange capability (CEC) of 139.9 mmol/100 g.

Table 1. Ingredient and chemical composition of the basal diet

Ingredients (%)	
Corn	58.5
Soybean meal	17.0
Wheat middling	6.5
Expanded soybean	7.0
Fish meal	4.5
Whey, dehydrated	3.0
Dicalcium phosphate	1.0
Limestone	1.1
Sodium chloride	0.25
L-lysine	0.15
Vitamin-mineral premix[1]	1.0
Analyzed chemical composition (% as feed)	
DE (kcal/kg)	3,309
Crude protein	19.2
Lysine	1.11
Met.+Cys.	0.63
Calcium	0.85
Phosphorus	0.70

[1] The vitamin/mineral premix provided (per kg feed): 1,500 IU vitamin A, 200 IU vitamin D_3, 10 IU vitamin E, 0.5 mg vitamin K_3, 0.05 mg biotin, 0.3 mg folic acid, 10 mg niacin, 10 mg d-pantothenic acid, 3.6 mg riboflavin, 1.0 mg thiamine, 1.5 mg pyridoxine, 15 mg cobalamin, 3 mg Mn, 80 mg Zn, 80 mg Fe, 5.0 mg Cu, 0.14 mg I and 0.15 mg Se.
[2] DE was based on calculated values.

Cu bearing montmorillonite (Cu-MMT) was prepared by Cu^{2+} cation exchange reaction according to the method described by Xu et al. (2002b). 5 g of the refined MMT was mixed with 100 ml of 0.1 mol/L $CuSO_4$ solution to form suspension by churning. The pH value of the suspension was adjusted to 5.0. The suspension was placed at 60°C for about 6 h to accelerate the cation exchange. The product was centrifuged at a speed of 8,000 rpm for about 15 min. The clear liquid was pored out and was replaced by another 100 ml of solution. The product was washed with distilled water and centrifuged for 3 times. The product was dried at 80°C over night, and ground in agate mortar to a size less than 300 mesh. Cu content in the product is found to be 24.5 g/kg on the basis of atomic absorption spectrum analysis.

Antibacterial experiment *in vitro*

Methods used in the antibacterial activity assay were modifications of procedures from a previously published study (Rivera-Garza et al., 2000). *E. Coli* K_{88} were grown aerobically in a flask with 150 ml of LB broth and shaken at 37°C for 12±2 h. From this cultured fluid, bacteria was separated and collected by centrifuge (3,000 rpm for 5 min at 4°C), and then washed twice with sterilized physiological salt solution. After washing, the bacteria was diluted to achieve a bacterial concentration of 10^4 cells ml^{-1}. *E. coli* K_{88} suspended in 100 ml of physiological salt solution was put into contact with 1, 2.5, 5, 10 and 20 mg of Cu-MMT, and shaken at 37°C for 24 h. 0.1 ml was taken from samples of the above mixture at 0, 2, 4, 6 and 24 h. These aliquots were 10-fold diluted in physiological salt solution. 0.1 ml of each diluted samples were spread on LB agar plates and incubated at 37°C for 24 h. Then, bacterial colonies were counted. Each growth assay was performed with three replicated samples.

Animals and experimental diets

All procedures were approved by the University of Zhejing Institutional Animal Care and Use Committee. A total of 64 weaning pigs (Duroc×Landrace×Yorkshire) at an average BW of 8.3 kg were allocated to 2 treatments for 24 days, each of which was replicated four times with eight pigs per replicate. The pigs received the same basal diet and Cu-MMT was added to the basal diet at 0 and 0.2%, respectively. Diets were formulated to meet or exceed nutrient requirements suggested by the NRC (1998) for 10- to 20 kg pigs. Antibiotic was excluded from all diets (Table 1). All pigs were given *ad libitum* access to feed and water. Average daily gain (ADG), average daily feed intake (ADFI), and feed/gain (F/G) were collected. The daily diarrhrea incidence was recorded. At the 24th day of the feeding trial, eight pigs from each treatment (two pigs per pen) were slaughtered under general anaesthesia. The pigs were then immediately eviscerated in order to collect jejunal contents and specimens (0.5×0.5 cm) of the mid-jejunum.

Analytical methods

XRD analyzing for mineral : Crystal structure of refined MMT and Cu-MMT were analyzed using XD98 automatic X-ray diffraction instrument with the Cu target Kα under the condition of 40 kV and 20 mA as well as a scan speed of 4°C min^{-1}.

E. coli in jejunal contents : Samples of the contents from the jejunum were immediately collected and transported to the lab for enumeration of *E. coli*. One gram of mixed contents was blended under CO_2 in 9 mL of anaerobic dilution (ADS, Bryant and Allison, 1961). The initial dilution in ADS was used as a source for serial dilutions in PBS for enumeration of *E. coli*. Triplicate plates were then inoculated with 0.1 ml samples and incubated at 37°C aerobically. Three dilutions were plated for each medium. Bacteria were enumerated on MacConkey's No.2 (Oxoid; *Escherichia coli*). Single colonies were removed from selective media plates and grown in peptone yeast glucose (PYG) broth (Holdeman et al., 1977). Subsequently, the bacteria were characterized to genus level on the basis of colonial appearance, Gram reaction, spore production, cell morphology and fermented end-product formation (Holdeman et al., 1977).

Histomorphometry : Samples of the mid-jejunum were excised, rinsed in physiological saline and preserved in 10%

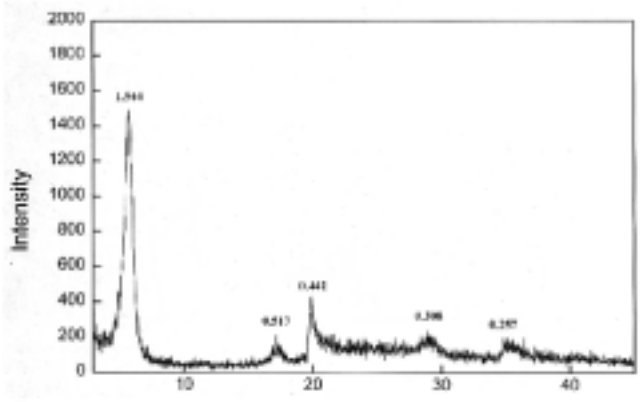

Figure 1. X-ray powder diffraction pattern of refined MMT.

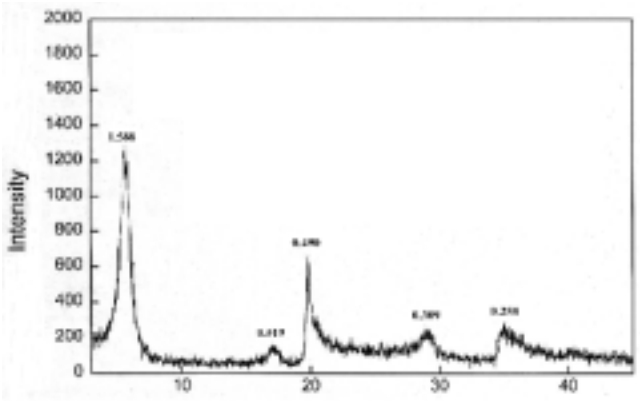

Figure 2. X-ray powder diffraction pattern of Cu-MMT.

formalin. Three cross-sections for each intestinal sample were then prepared after staining with hematoxylin and eosin using standard paraffin embedding procedures (Xu et al., 2003). A total of 10 intact, well-oriented crypt-villus units were selected in triplicate for each intestinal cross-section (30 measurements for each sample, total of 240 measurements per dietary treatment). Villus height was measured from the tip of the villi to the villus crypt junction and crypt depth was defined as the depth of the invagination between adjacent villi. Morphological indices were determined using image processing and analysis system (Version 1, Leica Imaging Systems Ltd., Cambridge, England).

Statistical analysis

Comparisons of means were performed using the student's t-test (Steel and Torrie, 1980). A significant level of 0.05 was used.

RESULTS

XRD for MMT and Cu-MMT

Figure 1 and 2 shows the XRD curves of the MMT before and after Cu^{2+} cation exchange. The (001) basal spacing of the purified MMT is 1.544 nm (Figure 1), revealing a typical calcium MMT. Diffraction peaks of other minerals are not recognizable in the curve, suggesting the successful separation and purification. After Cu^{2+} exchange, the (001) basal spacing is shifted to 1.588 nm (Figure 2).

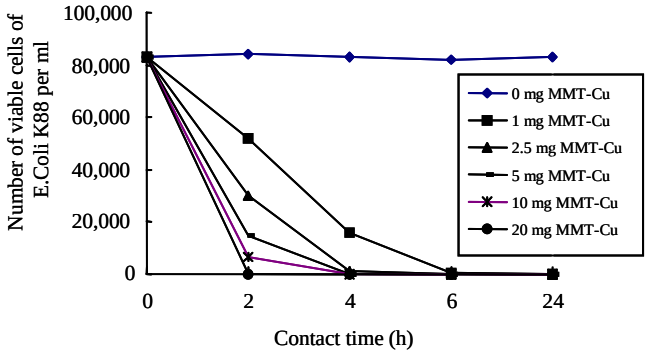

Figure 3. Antibacterial activity of Cu-MMT on E. *coli* K_{88} *in vitro*.

Table 2. Growth performance as affected by MMT-Cu in weaning pigs[1]

	Control	MMT-Cu
ADG(g)	328±18 [b]	369±20 [a]
ADFI(kg)	628±31	637±25
F/G	1.91±0.08 [b]	1.73±0.06 [a]

[1] Values are presented as means and standard deviations; n=32 for ADG, n=4 for ADFI and F/G per treatment.
Means in a row with different letters differ significantly ($p<0.05$).

Antibacterial activity of Cu-MMT on *E. coli* K_{88} *in vitro*

Figure 3 showed the number of viable cells of *E. coli* K_{88} after being in contact with 1, 2.5, 5, 10 and 20 mg of Cu-MMT at different contact times. The number of viable cells of *E. coli* K_{88} changed slightly during 24 h when Cu-MMT was not added in the medium. It was observed that as the amount of Cu-MMT increased, the number of viable cells of *E. coli* K_{88} decreased. When *E. coli* K_{88} was in contact with 20 mg Cu-MMT for 2 h, the number of viable cells of this microorganism reduced to zero. When 100 ml culture medium contained 1 mg Cu-MMT, *E. coli* K_{88} die out in 24 h. When Cu-MMT content increased to 5 mg, *E. coli* K_{88} was all killed in 6 h.

Growth performance of weaning pigs as affected by Cu-MMT

Growth performance of weaning pigs is presented in Table 2. As compared to control, supplementation with 0.2% MMT-Cu significantly improved ADG and feed conversion ratio. However, feed intake was unaffected by the dietary treatments.

Effect of MMT-Cu on diarrhrea of weaning pigs

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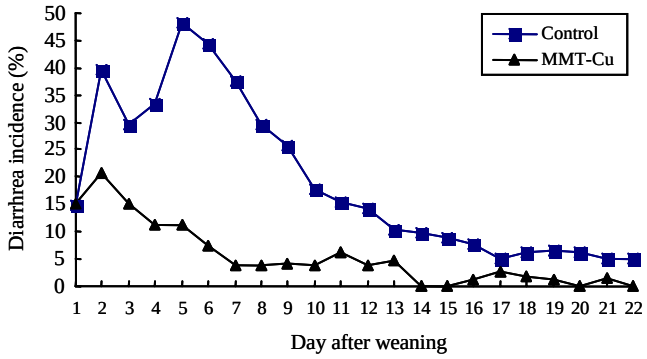

Figure 4. Effect of MMT-Cu on diarrhrea of weaning pigs.

presented in Figure 4. Supplementation with MMT-Cu significantly decreased the mean diarrhrea incidence from 19.15% to 5.40% ($p<0.01$).

Effects of MMT-Cu on *Escherichia coli* in jejunal contents of weanling pigs

As compared to control, supplementation with MMT-Cu significantly reduced the total viable counts of *Escherichia coli* in jejunal contents of weanling pigs (Table 3).

Morphological measurement of jejunal mucosa

Villus height and the villus height to crypt depth ratio at the jejunal mucosa were significantly higher in MMT-Cu supplementation to control (Table 4).

Table 3. Effects of MMT-Cu on *Escherichia coli* in jejunal contents of weanling pigs[1,2]

	Control	MMT-Cu
Escherichia coli	8.54±0.51 [a]	7.06±0.37 [b]

[1] Bacterial numbers are expressed as $\log_{10}$ cfu/g DM.
[2] Values are presented as means and standard deviations; n=8 per treatment.
Means in a row with different letters differ significantly ($p<0.05$).

Table 4. Effects of MMT-Cu on the morphology of the jejunal mucosa[1]

	Control	MMT-Cu
Villus height (μm)	440±30 [b]	524±42 [a]
Crypt depth (μm)	356±32	309±35
Villus height:crypt depth	1.24±0.13 [b]	1.70±0.24 [a]

[1] Values are presented as means and standard deviations; n=8 per treatment.
Means in a row with different letters differ significantly ($p<0.05$).

DISCUSSION

Antibacterial activity of Cu-MMT on *E. coli*

X-ray diffraction pattern of raw montmorillonite showed that the (001) basal spacing of the purified MMT was 1.544 nm (Figure 1), a typical calcium montmorillonite. After Cu^{2+} exchange, the (001) basal spacing shifted to 1.588 nm (Figure 2). For that the radius of Cu^{2+} (0.072 nm) was smaller than that of Ca^{2+} (0.099 nm), it seemed that Cu^{2+} entered into the interlayer position of MMT as hydrated cation or composite cation that made planar distance increase (Bahranowski et al., 1996). Stadler and Schindler (1993) found that in aqueous solution with pH>4.5, Cu^{2+} tended to enter interlayer position of MMT dominantly as $[Cu(AlO)_n(H_2O)_{4-n}]^{x+}$. Cu^{2+} may also locate in the ditrigonal intra-crystal hole surrounded by Si-O tetrahedron, or take a position in Al-O octahedron in MMT (Heller-Kallai et al., 1995; Mosser et al., 1997). For that the inter-layer position in MMT can only housing cations, when Ca^{2+} was replaced by $[Cu(AlO)_n(H_2O)_{4-n}]^{x+}$, or Cu^{2+} entered the tetrahedron and octahedron, MMT would lose its electrical balance. These made the mineral have surplus positive charge. On the other hand, *E. coli* cell wall had negative charge, so that Cu-MMT particles would attract bacteria, due to the opposite static charge. A similar phenomenon has been reported by Herrera et al. (2000). In their work, MMT was treated with cytylpyridinimu. The product CP-MMT was organic cation exchanged MMT, just like Cu-MMT, also with a surplus positive charge on surface. Under SEM they found that large amount of *Salmonella enteritidis* accumulated on CP-MMT surface, but untreated MMT was not attractive to *Salmonella enteritidis*. Surplus positive charge of Cu-MMT and CP-MMT was most probably an important factor for their antibacterial capability. In this case, the released Cu^{2+} would act directly on the attracted bacteria, instead of into the medium and indirectly on the bacteria. In other words, the active Cu^{2+} density on mineral surface was much higher than its concentration in the solution. Summary, Static attraction and the bactericidal effect of Cu^{2+} ion on *E. coli* are two ways of the antimicrobial action of Cu-MMT.

Effect of MMT-Cu on diarrhrea and intestinal morphology of weaning pigs

It was observed that supplementation with MMT-Cu significantly decreased the diarrhrea incidence of weaning pigs in the present study. It was obvious that the antibacterial activity of Cu-MMT on *E. coli* K_{88} might contribute much to the observed effects on the diarrhrea of weaning pigs. The structure of the intestinal mucosa can reveal some information on gut health. Stressors that are present in the digesta can lead relatively quickly to changes in the intestinal mucosa due to the close proximity of the mucosal surface and the intestinal content. Changes in intestinal morphology such as shorter villi and deeper crypts have been associated with the presence of toxins (Yason et al., 1987; Xu et al., 2002a, 2003). A shortening of the villi decreases the surface area for nutrient absorption. The crypt can be regarded as the villi factory, and a deep crypt indicates fast tissue turnover and a high demand for new tissue. Changes in intestinal morphology as descried

above can lead to poor nutrient absorption, increased secretion in the gastrointestinal tract, diarrhoea, reduced disease resistance and lower growth performance. In the present study, an increase in villus height and villus height: crypt depth ratio of the jejunal mucosa in MMT-Cu-fed pigs was found. It is likely that these changes are due to MMT-Cu's ability to improve the intestinal microflora.

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