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Influence of Cu^{2+}-loaded silicate on the growth performance and microflora of crucian carp *Carassius auratus*

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ABSTRACT: We investigated the effects of Cu^{2+}-loaded silicate (CLS) on the growth performance, microflora of skin, gill and intestine, and intestinal morphology of crucian carp *Carassius auratus*. A total of 225 native wild crucian carp, with an average initial body weight of 20 g, were randomly divided into 5 treatment groups using 3 replicate tanks of 15 fish per group. The dietary treatments were (1) basal diet, (2) basal diet + $CuSO_4$, (3) basal diet + silicate, (4) basal diet + 0.5% CLS and (5) basal diet + 50 mg kg^{-1} chlortetracycline (CTC, purity 98.8%). The trial lasted for 60 d. We found that body weight increased slightly while feed conversion ratio decreased in the CLS-treated group compared with the control groups. The total number of aerobic bacteria counted in the intestine of carp fed the diet supplemented with the CLS (i.e. *Vibrio* sp. and *E. coli*), was significantly lower ($p = 0.05$) compared with the control groups and the CTC-treated fish, while lactobacillus counts were significantly higher ($p = 0.05$). *Lactobacilli* counts of the intestine increased significantly ($p = 0.05$). However, the microflora of the skin and gill was not affected by the addition of $CuSO_4$, silicate, CLS or CTC. The height of the villi in the proximal, mid and distal intestine mucosa of the silicate- and CLS-treated groups was found to be longer ($p = 0.05$) compared with the villi of the control or the CTC-treated fish. Supplementation with $CuSO_4$ had no effect on the microflora and the intestinal morphology ($p = 0.05$). These results indicate that CLS had an antibacterial activity *in vivo,* which may help protect the intestinal mucosa from invasion of pathogenic bacteria and their toxins.

KEY WORDS: Cu^{2+}-loaded silicate · CLS · Growth performance · Microflora · Intestinal morphology · Crucian carp

INTRODUCTION

Aquatic diseases have increased rapidly in recent years with the development of aquaculture, and bacteriosis has become the most common and serious of these diseases. In order to control bacterial infections in aquaculture systems, a great deal of research has been carried out on microbial ecological agents and various antibiotics such as chloramphenicol, oxytetracycline, chlortetracycline (CTC), flavophospholipol and virginiamycin. These antibiotics have been widely used in aquaculture (Austin & Austin 1993, Bogut et al. 1998, Ringoe & Gatesoupe 1998). However, long-term application and misuse of these have resulted in the development of drug resistant bacteria (Aoki et al. 1972), immunosuppression (Rijkers et al. 1980) and drug residue in aquatic products (Schwarz et al. 2001), and can potentially have a detrimental effect on the environment. These can pose a direct or indirect threat to human health. Therefore, there is an urgent need to develop new antibacterial agents to replace the use of antibiotics in aquaculture.

The use of expandable layered silicates as adsorbents (e.g. bentonite, with its main ingredient mont-

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morillonite) has recently received attention (Rytwo et al. 2000, Wang et al. 2004). Montmorillonite is an aluminum silicate with a 2:1 tetrahedral:octahedral layer structure. Within this structure, cations are easily exchanged with other cations or compounds between the structural sheets (Brindley & Brown 1980). At the same time, motmorillonite has specific physical-chemical properties, such as large surface area, good absorbent properties, structural stability and a strong capacity to form stable suspensions (Brindley & Brown 1980). Montmorillonite is thought to be useful as an antimicrobial carrier and the adsorption of metal ions onto montmorillonite minerals has been studied extensively (Liu et al. 2006, Zarkesh et al. 2006, Abollino et al. 2008). Copper has many functions in biochemical and biological cycles (Kaim & Schwederski 1994) and is an essential element for animal growth (Anke & Groppel 1986). It also displays antimicrobial activity. In the present study, Cu^{2+}-loaded silicate (CLS) was prepared by an exchange reaction. The aim of the study was to investigate the effects of CLS on the growth performance, microflora and intestinal morphology in crucian carp *Carassius auratus*.

MATERIALS AND METHODS

Materials. The main ingredient of the silicate sample used in this study is bentonite (Ningcheng Mining). The raw material was dried overnight at 80°C and then milled to less than 300 mesh. The sample was ground and washed in deionized water at a ratio of 10 g silicate:100 ml water for 24 h with stirring. Particles >1 µm were separated out by sedimentation while the suspension was centrifuged at 8000 × *g* for 15 min to obtain refined silicate. The resulting silicate clay suspension was centrifuged at 8000 × *g* and the water was discarded. The clay was rehydrated with 100 ml water and 0.025 mol l^{-1} Cu^{2+} ($CuSO_4 \cdot H_2O$, analytical grade; Shanghai Sangon) was added. The resulting mixture was then agitated for 24 h. The CLS was then separated by centrifugation and washed with water under agitation. The material was dried at 60°C, and ground to a size <300 mesh. The amount of copper in CLS, measured by atomic absorption spectroscopy, was 38 mmol 100 g^{-1} calculated as the difference between the Cu^{2+} concentration in a control sample sampled without this silicate and the sample supernatant content.

Feeding trial. A total of 225 native wild crucian carp at an average initial body weight of 20 g, provided by Hangzhou Yueteng Fishery, PR China, were randomly divided into 5 treatment groups with 3 replicates (i.e. tanks) of 15 fish each. The dietary treatments were (1) basal diet, (2) basal diet + $CuSO_4$ (40 mg kg^{-1} copper as $CuSO_4$), (3) basal diet + 0.5% silicate, (4) basal diet + 0.5% CLS, (5) basal diet + 50 mg kg^{-1} CTC (purity 98.8%; Wuhan Hezhongs). Diets were formulated to meet nutrient requirements suggested by the National Research Council (1993) for crucian carp. No antibiotic was included in basal diets (Table 1). The trial lasted for 60 d after 2 wk of adaptation to laboratory conditions.

Experimental fish were cultured in a closed recirculation system consisting of 15 self-cleaning aquaria (1 m long × 0.5 m wide × 0.5 m high). The experimental system was equipped with sedimentation tanks, UV filter and biological filter to prevent cross contamination of micro- organisms between treatments, and an environment-controlled laboratory maintained at 25°C, with a photoperiod of 12 h light:12 h dark was installed. The culture system was also provided with continuous aeration through an air compressor and heaters to keep water temperature at 22 to 25°C. Temperature and dissolved oxygen were measured daily, and weekly analyses were done of total ammonium, nitrite, nitrate and pH levels. The following values were used for carp cultivation: temperature, 22 to 25°C; dissolved oxygen, 5.5 to 7 mg l^{-1}; pH, 7.0 to 7.5; ammonia, 0.04 to 0.06 mg l^{-1}; nitrite, 0.03 to 0.05 mg

Table 1. *Carassius auratus.* Ingredients and nutrient composition of diets. Nutrient composition values are measured values, with the exception of gross energy, which is a calculated value

Composition	% of total
Ingredients	
Soybean meal	40
Wheat middlings	25.4
Cotton meal	10
Rapeseed meal	8.0
Fish meal	6.0
Corn oil	3.0
Dicalcium phosphate	2.5
Choline	0.3
Salt	0.3
Bond	0.5
Premix[a]	1.0
Nutrition composition	
Gross energy (kJ g^{-1})	15.3
Crude protein	36.2
Calcium	0.6
Phosphorous	0.9

[a]Premix (mg kg^{-1} of diet): $MgSO_4$, 3750; KH_2PO_4, 8000; NaCl, 250; $Ca(H_2PO_4)_2$, 5000; $FeSO_4$, 720; $Ca(CH_2CHCOO)_2 \cdot 5H_20$, 880; $ZnSO_4 \cdot 7H_2O$, 88; $MnSO_4 \cdot 4H_2O$, 40; $CuSO_4 \cdot 5H_2O$, 8; $CoCl_2 \cdot 6H_2O$, 0.25; $KIO_3 \cdot 6H_2O$, 0.75; riboflavin, 20; pyridoxine, 10; thiamine, 10; retinal, 4; cobalamin, 2; cholecalciferol, 0.4; phylloquinone, 80; calcium pantotheniate, 40; folic acid, 5; niacin, 150; inositol, 400; choline, 6000; ascorbic acid, 500; tocopherol, 60

l^{-1}; nitrate, 5 to 7 mg l^{-1}. The fish were fed at a rate of 4% (wet weight basis) of their total biomass per day. The daily ration was divided into 2 equal portions which were fed at 8:00 and 17:00 h respectively. Growth performance indicators measured were average daily weight gain (ADWG) and feed conversion ratio (FCR).

Isolation and identification of bacteria. At the end of the feeding trial, 9 fish were randomly selected from each treatment (n = 3 per tank), anesthetized with tricaine methanesulfonate (MS-222, Sigma) and then killed. Total counts of aerobic bacteria were measured from carp skin by putting a 5 × 5 cm sterile template on the fish surface (in the central region) and swabbing this area enclosed by the template. Gill and intestine samples were removed using a sterile knife. The samples of skin, gill and intestine were homogenized with sterilized 0.85% NaCl saline solution at room temperature (20°C). Poured plates were prepared and a 1.0 ml inoculum was placed on the bottom of the Petri dish; 20 ml molten agar was then poured onto this and mixed with the inoculum.

The plate media used were brain-heart infusion agar (Oxoid) for total aerobes, TCBS (Oxoid) for *Vibrio*, MacConkey's No. 2 agar (Oxoid) for *Escherichia coli*, and MRS agar (Difco) for *Lactobacilli*. Total counts of aerobic bacteria, *Vibrio* and *E. coli* were determined on nutrient agar after incubation at 30°C for 24 h, and 48 h for *Lactobacilli*. Thirty colonies were randomly chosen (to select many different phenotypes) from every sample and restreaked on nutrient agar 3 times to obtain pure cultures. Total numbers of bacterial colonies were counted at the end of each incubation period. Microorganisms isolated from the samples were identified using Bergey's Manual (Holt et al. 1993). The bacteria were characterized to genus level on the basis of colonial appearance, Gram reaction, cell morphology, spore production and fermentation end-product formation (Holdeman et al. 1977).

Examination of intestinal morphology. Specimens from the proximal, mid- and distal intestine segment were removed and rinsed with physiological saline. All samples were fixed in 10% formalin. A total of 9 samples for each of the 3 intestinal segments per dietary treatment were prepared. The fixed samples were embedded in paraffin. Transverse sections were cut into 5 µm samples, every 10th section was collected and stained with hematoxylin and eosin. Villus height was measured using image processing and analysis system (Version 1, Leica Imaging Systems). Ten intact villi were selected in triplicate for each intestinal cross-section (30 measurements for each sample). Finally, mean villus height (µm) was determined for each treatment.

Statistical analysis. All data measured in the study were analyzed by comparing means according to least significant difference test, using the general linear model procedure of SAS (version 6.12). Data are expressed as mean ± SD. The experimental unit for all data was 1 tank of fish. A significance level of 0.05 was used.

RESULTS

The growth performance of crucian carp is shown in Table 2. Compared with the control, ADWG of carp fed with the diet supplementation with CLS increased and FCR decreased slightly ($p > 0.05$). Similar results were also found in the silicate-treatment and CTC-treatment groups.

The effects of CLS treatments on microflora of carp skin, gill and intestine are shown in Fig. 1. Total counts of aerobic bacteria, *Vibrio*, *E. coli* and *Lactobacilli* on the skin and gill were not affected by the supplementation of $CuSO_4$, silicate, CLS or CTC. However, total aerobic bacterial, *Vibrio* and *E. coli* counts in the intestine of carp fed the diet supplemented with silicate or CLS decreased significantly ($p = 0.05$) as compared with the control or CTC treatment groups. *Lactobacilli* counts in the intestine increased significantly ($p = 0.05$). The same tendency was noted for the effects of CTC on intestinal microflora, but the value was lower than that of CLS. $CuSO_4$ had no effect on the microflora of the intestine.

Intestinal villus height in each intestinal mucosa of carp is presented in Table 3. Villus height at the proximal, mid- and distal intestine mucosa of carp fed the CLS-supplemented diet was greater than in the control, $CuSO_4$ and CTC treatment groups ($p = 0.05$). Supplementation with silicate increased villus and microvillus height at the mid- and distal intes-

Table 2. *Carassius auratus*. Growth performance of crucian carp. Data are expressed as mean ± SD of 45 fish in each treatment. Percent survival was 100%. CLS: Cu^{2+}-loaded silicate; CTC: chlortetracycline; BW_i: initial body weight; BW_f: final body weight; ADWG: average daily weight gain; FCR: feed conversion ratio. Means within a row with different letters differ significantly ($p < 0.05$)

Growth parameter	Control	$CuSO_4$	Silicate	CLS	CTC
BW_i (g)	20.60 ± 1.02	20.85 ± 1.13	20.50 ± 1.02	20.93 ± 1.21	21.05 ± 1.08
BW_f (g)	27.40 ± 1.51	27.63 ± 1.57	28.45 ± 1.50	29.05 ± 1.84	28.59 ± 1.44
ADWG (g)	0.11 ± 0.01[a]	0.11 ± 0.01[a]	0.13 ± 0.01[a]	0.14 ± 0.01[a]	0.13 ± 0.01[a]
FCR	3.65 ± 0.18[a]	3.57 ± 0.16[a]	3.22 ± 0.12[a]	3.11 ± 0.18[a]	3.36 ± 0.13[a]

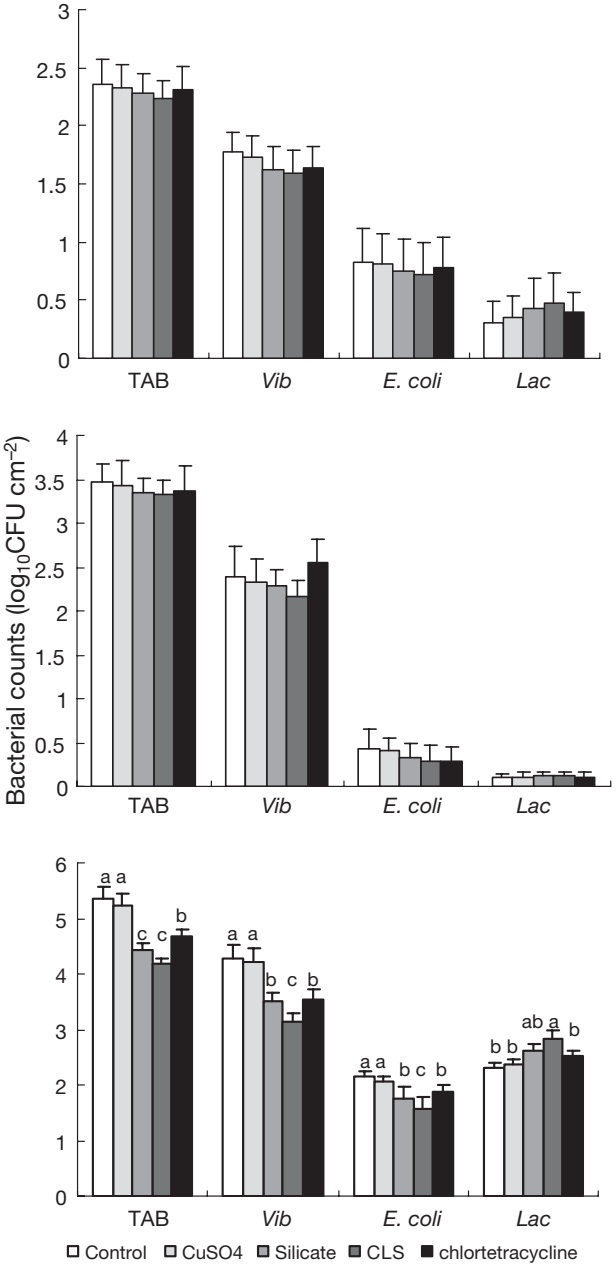

Fig 1. *Carassius auratus.* Effects of Cu^{2+}-loaded silicate (CLS) on microflora of (a) carp skin, (b) gill and (c) intestine. Data are expressed as mean ± SD of 9 fish in each treatment. TAB: total aerobes; *Vib*: *Vibrio*; *E. coli*: *Escherichia coli*; *Lac*: *Lactobacilli.* Different letters indicate significant differences ($p < 0.05$)

tine mucosa as compared with the control ($p = 0.05$). CTC supplementation also increased villus height of all intestinal parts as compared with the control. Addition of $CuSO_4$ had no effect on the intestinal morphology as compared with the control ($p > 0.05$).

DISCUSSION

In the present study, CLS was made from silicate, the main ingredient of which was bentonite. Bentonite is usually included in animal diet as a growth promoter. Other studies have shown that the addition of bentonite to animal feed (10 to 30 g kg^{-1}) increases growth performance and feed conversion efficiency in pigs and chickens (Venglovsky et al. 1999, Tauqir & Nawaz 2001). In our study, ADWG and FCR of carp fed the diet containing CLS improved slightly compared with the control, but the tendency was not significant. The difference between this and the studies on pigs or chickens may be due to the different dosage of CLS added to the diet.

In the present study, CLS supplementation significantly decreased the counts of total aerobic bacteria, *Vibrio* and *E. coli*, and increased *Lactobacilli* counts in the intestine of carp. However, dietary addition of CLS had no significant influence on microflora of carp skin or gill. The results indicate that CLS had a greater effect on intestinal microflora than CTC. Cik et al. (2001) found that zeolite, an aluminosilicate clay, following the ion-exchange reaction of Na^+ for Cu^{2+} ions, could effectively inhibit *E. coli* growth. Similar results were also found for humans; Wang & Fang (1995) showed that intestinal *Bifidobacteria* bacteria counts increased significantly in diarrhoeal children, while those of *E. coli* decreased after the children had been treated with montmorillonite (the main ingredient of bentonite) for 5 d. Thus, the changes in the composition of intestinal microflora caused by CLS in the present study may be related to the characteristics of silicate.

It is well known that bentonite can adhere to bacteria, and copper sulfate is a widely used, traditional inorganic antibacterial material. Thus, the effectiveness of CLS on intestinal microflora is probably due to the adsorption and the antimicrobial action of CLS. Copper ions tend to enter the interlayer position of clay predominantly as $[Cu(AlO)_n(H_2O)_{4n}]^{x+}$ or locate in the ditrigonal intra-crystal hole surrounded by an Si-O tetrahedron, or take a position in an Al-O octahedron in clay (Mosser et al. 1997). This gives the clay a net positive charge, while the cellular wall of the bacteria is negatively charged; CLS particles can adsorb bacteria, which is ascribed to the opposite static charge. An *in vitro* antibacterial trial showed that montmorillonite had no antibacterial activity while Cu- montmorillonite had strong antibacterial ability on the tested aquacultural pathogenic bacteria (Hu & Xia 2005). This indicated that the released Cu^{2+} would act directly on the bacteria adsorbed on the surface of CLS. In the present study supplementation with $CuSO_4$ had no significant effect on the intestinal microflora. Therefore the effect of CLS on microflora in the intestine may be due to the adsorption of silicate and the antibacterial ability of Cu^{2+}.

Table 3. *Carassius auratus.* Effect of CLS on villus height (µm) at different sites of carp intestine. Data are expressed as mean ± SD of 9 fish in each treatment. CLS: Cu^{2+}-loaded silicate; CTC: chlortetracycline. Different letters for means within a row indicate significant differences ($p < 0.05$)

Gut section	Control	$CuSO_4$	Silicate	CLS	CTC
Proximal intestine	109.81 ± 14.16^{b}	114.32 ± 13.20^{b}	123.41 ± 12.43^{ab}	130.60 ± 13.12^{a}	120.10 ± 10.19^{ab}
Mid intestine	51.54 ± 7.65^{c}	56.25 ± 7.54^{c}	72.13 ± 8.61^{ab}	77.44 ± 11.46^{a}	65.72 ± 7.26^{b}
Distal intestine	42.34 ± 6.79^{c}	43.37 ± 6.35^{c}	56.24 ± 6.23^{ab}	61.12 ± 6.16^{a}	50.74 ± 6.59^{b}

The structure of the intestinal mucosa provides some information on gut health. A shortening of the villus usually decreases the surface area for nutrient absorption, resulting in poor nutrient absorption. In the present study, an increase in villus height was observed in the intestinal mucosa of carp fed a diet containing silicate or CLS. This indicates that the dietary addition silicate or CLS may improve carp's intestinal mucosal morphology. Montmorillonite protects intestinal mucosa and can increase the intestinal mucosal barrier and help in the regeneration of the epithelium; it effectively acts by attaching to the mucus to preserve the mucosa from toxic effects of drugs and toxins (Albengres et al. 1985). Thus, the positive effect of silicate or CLS on intestinal mucosal morphology may be explained by the substances' antimicrobial activity and mucosa-enhancing function.

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