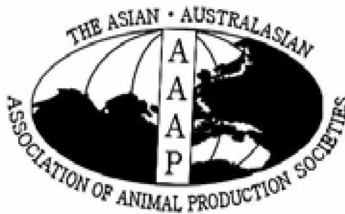

Asian-Aust. J. Anim. Sci.
Vol. 20, No. 4 : 569 - 576
April 2007

www.ajas.info

Anti-adherence of Antibacterial Peptides and Oligosaccharides and Promotion of Growth and Disease Resistance in Tilapia

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ABSTRACT: Four hundred and fifty tilapias (6.77±0.23 g) were assigned randomly to six groups to evaluate the feasibility of the tested antibacterial peptides (ABPs) and oligosaccharides as substitutes for antibiotics. The control group was fed with a commercial tilapia diet; other five groups were fed with the same commercial diet supplemented with konjac glucomannan (KGLM), cluster bean galactomannan (CBGAM), and three animal intestinal ABPs derived from chicken, pig and rabbit at 100 mg/kg respectively. After 21 days of feeding, growth, disease resistance, and *in vivo* anti-adherence were determined. Furthermore, the inhibitory effect of tested agents on adhesion of *Aeromonas veronii* biovar *sobria* (*A.vbs*) strain BJCP-5 to tilapia enteric epithelia *in vitro* was assessed by cell-ELISA system. As a result, the tested agents supplemented at 100 mg/kg show significant benefit to tilapia growth and disease resistance ($p<0.05$), and the benefit may be correlated with their interfering in the contact of bacteria with host mucosal surface. Although none of the tested agents did inhibit the growth of BJCP-5 in tryptic soy broth at 100 μg/ml, all of them did inhibit the adhesion of *A.vbs* to tilapia enteric epithelia *in vivo* and *in vitro*. *In vitro* mimic assays show that three ABPs at low concentrations of 25 μg/ml and 2.5 μg/ml have the reciprocal dose-dependent anti-adherence effect. The inhibition of ABPs may be correlated with a cation bridging and/or receptor-ligand binding, but not with hydrophobicity. The KGLM and CBGAM inhibited the adherence of BJCP-5 to tilapia enteric epithelia with dose-dependent manner *in vitro*, and this may be through altering bacterial hydrophobicity and interfering with receptor-ligand binding. Our results indicate that the anti-adherence of the tested ABPs and oligosaccharides may be one of the mechanisms in promoting tilapia growth and resistance to *A.vbs*. (**Key Words :** Antibacterial Peptides, Oligosaccharides, Anti-adhesion, Growth, Disease Resistance, Tilapia)

INTRODUCTION

China has been leading the world tilapia culture production since 1998. Today, tilapia is one of the best potential species to export for China aquaculture. However, this species is susceptible to *Aeromonas* infections, especially for tilapia fry under high-density cultural conditions (Austin and Austin, 1999). In this genus, *Aeromonas veronii* biovar *sobria* (*A.vbs*) is one of the most important pathogen of numerous animal and human diseases. The abuse of antibiotics against *Aeromonas* results in that water resources have become the depository of antibiotic resistance genes (Hatha et al., 2005), which not only increases the production costs in pisciculture, but also transfers the resistant genes to bacterial pathogens of animal and human origins (Lee et al., 2001).

The alarming increase of drug-resistant bacteria and banning of antimicrobial growth promoters world wide make the search for novel means of fighting bacterial infections and promoting animal growth imperative (Van den Bogaard and Stobberingh, 2000). One of attractive approaches is anti-adhesion strategy because the majority of infectious bacterial diseases are initiated by adhesion of pathogenic organisms to the tissues of the host (Ofek et al., 2003). Furthermore, the anti-adherence potential of antibiotics in sub-lethal concentrations to bacteria on intestinal epithelia is one of the mechanisms of antibiotic growth promoter and preventing bacterial infection (Shibl, 1985; Schifferli and Beachey, 1988).

The ideal substitutes for bactericidal agents and

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Received March 23, 2006; Accepted September 6, 2006

Table 1. Effect of antibacterial peptides and oligosaccharides on growth, disease resistance and *in vivo* anti-adhesion in tilapia

	Growth		Disease resistance			*In vivo*
	Weight gain (%)	Feed conversion ratio (FCR)	Relative percent survival (RPS) (%)			anti-adhesion (cells/400 μm²)
			Injection	Immersion	Oral	
Control	285.71±24.75 b	1.1700±0.0400 a	0	0	0	15.200±1.643 a
ABP of chicken	382.54±52.45 a	1.0220±0.0727 b	38.46	40	75	7.800±0.837 b
ABP of pig	371.43±24.75 a	1.0040±0.0410 b	23.08	30	66.67	7.800±2.049 b
ABP of rabbit	361.11±28.80 a	1.0153±0.0133 b	30.77	40	58.33	8.400±0.894 b
KGLM	372.22±39.15 a	1.0483±0.0595 b	15.38	20	75	7.600±1.140 b
CBGAM	366.67±35.95 a	1.0333±0.0752 b	23.08	30	83.33	6.800±1.483 b

The small letters following the mean±SD indicate a significant difference ($p<0.05$). The values with the same letter are not significantly different.

antimicrobial growth promoters may include oligosaccharides (Suk, 2004; Cao et al., 2005; Tang et al., 2005) and ABPs (Hancock and Lehrer, 1998; Ma et al., 2004). The biological function of oligosaccharides was previously focused on immunostimulant effect (Cheng et al., 2004; Li et al., 2005), modification of the intestinal flora (Cao et al., 2005), anti-adhesion effect (Sharon, 2006), and antioxidant effect (Dvorska and Surai, 2004). The reported biological activities of ABPs included anti-microbial, antiviral, anti-tumor and immunomodulation effects, and chemotactic activity for immunocytes (Selsted et al., 1992; Ma et al., 2004). To our knowledge, it has been mentioned rarely that anti-adhesion of oligosaccharides from plants and ABPs of the animal intestine may be the mechanism of animal growth promoter and disease resistance (Ofek et al., 2003; Sharon, 2006).

Our purpose in this study was to assess the effect of ABPs of animal origin and hetero-oligosaccharides from plants on tilapia growth, disease resistance and the adhesion of *A.vbs* strain BJCP-5 to tilapia enteric epithelia, and to clarify the probable mechanisms of the tested agents promoting tilapia growth and resistance to *Aeromonas*.

MATERIALS AND METHODS

Bacterial strain and preparation of rabbit polyclonal anti-serum

A.vbs strain BJCP-5 was isolated from diseased red tilapia (*Oreochromis mossambicus*×*Oreochromis niloticus* hybrid) with motile *Aeromonas* septicemia in National Beijing Tilapia Seed Farm, and defined based on morphology, physiology, biochemistry and 16 S rDNA sequences (GenBank accession no. AY909606).

Except for the inactivation of the bacteria with 0.5% formaldehyde in PBS (4°C, 72 h), the preparation of rabbit polyclonal anti-serum against the strain BJCP-5 followed the previous description by Alwan et al. (1998).

Minimum inhibitory concentrations (MIC) of tested substances

The tested agents used in our study are shown in Table 1. Three tested ABPs with the molecular weights of 8.0 KD, 6.0 KD, and 7.0 KD derived from enteric mucus of chicken, enteric mucous membrane of pig, and *racculus rotundus* of rabbit respectively, were purified and identified following the methods of Ma et al. (2004). Konjac glucomannan (KGLM) with an average molecular weight of 2.7 KD derived from the konjac tuber. It is composed of β-(1→4) linked D-glucopyranose and D-mannopyranose as the main chain with branches through β-(1→3)-glucosyl units. Degree of branching is about 8%, the molar ratio of terminal mannosyl units to glucosyl units is 2:1, and the polymerization degree is 15. Cluster bean galactomannan (CBGAM) with an average molecule weight of 1.8 KD is purified from cluster bean. It has a typical monomer structure with the (1→4) linked β-D-mannopiranosyl backbone substituted in various degrees at O-6 with single unit α-D-galactopyranosyl residues in a molar ratio of 1:1.6. Its polymerization degree of CBGAM is 10.

The minimum inhibitory concentrations (MICs) of each agent for *A.vbs* strain BJCP-5 were determined by the broth micro-dilution method of Atkinson (1980).

Preparation of feed and growth trials

Puhui tilapia diet (Puhui feedstuff Inc., Beijing, China) was used as a basal diet. Each tested agent was added to the basal diet at 100 mg/kg respectively. The diet was prepared by thoroughly mixing the dry basal diet and the tested agent with cold water until a stiff dough resulted. The dough was then passed through a mincer with die and the resulting spaghetti-like strings were dried. The dried diets were broken up and sieved into 4 mm mesh and stored at 4°C. Tilapia were fed 5% of body weight daily. The daily ration was divided into three equal feeding and fed once every four hours. Fish were weighed weekly and the daily ration was adjusted accordingly.

Four hundred and fifty healthy tilapias from the same pond in National Beijing Tilapia Seed Farm with an average weight of 6.77±0.23 g were assigned randomly to one of six groups, with 3 cages/group (25 tilapias/cage). All cages (L100×W50×D40 cm) were placed in a 36 m^3 tank supplied with flow-through water at 0.6 L/min. The daily dissolved

Table 2. Dose of test substance for *Aeromonas veronii* biovar *sobria* strain BJCP-5 growth and adherence assay *in vitro*

		Antibacterial peptides				Oligosaccharides			
		H	M	L	C	H	M	L	C
Exp. I	Bacterial growth	250	25	2.5	0	500	50	5	0
	Adherence assay	0	0	0	0	0	0	0	0
Exp. II	Bacterial growth	0	0	0	0	0	0	0	0
	Adherence assay	250	25	2.5	0	500	50	5	0
Exp. III	Bacterial growth	250	25	2.5	0	500	50	5	0
	Adherence assay	250	25	2.5	0	500	50	5	0

oxygen was 5.5±0.7 mg/L and water temperature was 27±1°C. A control group was fed with the basal diet without tested agents supplement; the other test groups were fed with the basal diet with different tested agents supplement, respectively.

On the 21st day of feeding trial, the growth was monitored by collectively weighing each group of fish. Weight gain (%) and feed conversion ratio (FCR) were determined for each group using the following equations: weight gain (%) = [(final weight-initial weight)/initial weight]×100; FCR = Feed given (dry weight)/Body weight gain (wet weight).

Challenge experiments

BJCP-5 were grown on TSB for 24 h at 30°C and harvested by centrifuging the culture at 4,000 rpm for 20 min at 4°C. The cells were then washed three times in sterile PBS (pH 7.4) and final concentration was adjusted to 10^8 CFU/ml. Three challenged methods, namely intraperitoneal injection, immersion, and oral administration, were used to determine the disease resistance of tilapia to BJCP-5.

After the feeding trial, all fish were survived. Seventy-five fish of each group were assigned randomly to four subgroups: 20 fish for intraperitoneal injection, 20 fish for immersion, 25 fish for oral administration, and 10 fish for non-challenge. Each fish for intraperitoneal injection was injected with 0.1 ml (10^7 CFU/ml) of the BJCP-5 suspension. The fish for immersion were immersed into the BJCP-5 suspension with the concentration of 10^8 CFU/ml for 2 h. The fish for oral administration were fed with the former diet in feeding trial supplemented additionally BJCP-5 at 10^5 CFU/g for three days. After the challenge, the challenged subgroups were transferred to separate tank with the same density and water quality as the unchallenged subgroups. During the challenged trial, all fish of each group were fed with the same diet as that in feeding trial.

The mortality was observed for 14 days. Tissues were removed from the freshly dead fish for bacteriological culture to confirm *A.vbs* strain BJCP-5 as the cause of death. The relative percent survival (RPS) in different treatment groups were calculated by the following formula: relative percent survival (%) = 100-[(test mortality/control mortality)×100].

***In vivo* anti-adhesion assays**

After 3 days of challenge, five fish per subgroup taken *A.vbs* strain BJCP-5 orally at 10^5 CFU/g diet were anaesthetized with 2-phenoxyethanol and dissected. The anterior intestines were removed and washed six times with cold PBS containing 0.05% Tween-20 (PBS-T, pH 7.4), and subsequently cut into pieces, about 0.3 cm×1.0 cm, and fixed in 2% glutaraldehyde in PBS overnight at 4°C. After washing with PBS buffer, the specimens were postfixed with 2% osmium tetroxide for 90 min. After three washings with PBS, the specimens were dehydrated in a graded series of ethanol to 100%, then were critical point dried using liquid carbon dioxide, and coated with gold. The resulting sections were examined with a HITACH S-570 scanning electron microscope (SEM). To analyze the bacterial adherence on foregut by SEM quantitatively, the numbers of adhered bacteria of each different visual field (6,000×) were calculated. Results of adherence of BJCP-5 to foregut were expressed in the average values of ten different visual fields per 400 μm^2.

Culture conditions of bacteria for hydrophobicity and adherence assays

Tryptic soy broth (TSB), prepared according to the manufacturer's instructions, was chosen for the basal medium of bacterial growth. BJCP-5, stored in TSB with 50% sterile glycerol at -20°C, was sub-cultured in TSB at 30°C for 16 h, and diluted to 10^6 CFU/ml with sterile PBS. One hundred microlitre of the cell suspension was transferred to 20 ml of fresh TSB with or without various concentrations of agents (Table 2), and incubated for 16 h at 30°C with shaking at 150 rpm.

Bacterial hydrophobicity analysis

The ability of the organisms to adhere to hydrocarbons as a measure of their hydrophobicity was determined according to the method of Pérez et al. (1998). Cultures of BJCP-5 were harvested in the exponential phase by centrifugation at 12,000 rpm for 5 min at 4°C, washed twice in PBS and finally resuspended in the same buffer. The cell suspension was adjusted to an A_{560nm} value of approximately 1.0 with the buffer and 2 ml of the bacterial suspensions were put in contact with 0.4 ml of xylene and

vortexed for 120 s. The two phases were allowed to separate for 10 min at 25°C. The aqueous phase was carefully removed and the A_{560nm} was measured. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H %), which was calculated with the formula. $H\% = [(A_0-A)/A_0]\times 100$, where A_0 and A are the absorbance before and after extraction with xylene, respectively.

In vitro adherence assays

Preparation of bacteria for adherence assays: The cells were harvested from cultures at the exponential phase of growth by centrifugation (4,000 rpm at 4°C for 30 min), washed twice with PBS and resuspended in PBS (pH 7.4) containing 1 mM $MgCl_2$ at a density of 10^9 cells/ml, and used immediately for the adhesion assays.

Preparation of tilapia enteric epithelia: The following technique was modified from previous descriptions (Alwan et al., 1998). Healthy young tilapia (approximate 50 g) raised in 24-26°C from National Beijing Tilapia Seed Farm were anaesthetized with 2-phenoxyethanol. Fish were dissected, and the anterior intestines were removed and transferred into chilled PBS (pH 7.4) kept on ice. The tissue were washed six times with cold PBS containing 0.05% Tween-20 (PBS-T, pH 7.4), then scoured with cold PBS containing penicillin (200 IU/ml) and streptomycin (200 μg/ml). Tissue preparations were dipped in 0.1% sodium hypochlorite for 3 min. After four times washing with sterile cold PBS, tissue preparations were incubated for 1 min in 70% ethanol, and finally tissues were washed three times with sterile glacial PBS.

The tissue preparations were cut into pieces of approximate 0.1 mm×0.1 mm, after washing twice with cold PBS, and then incubated in PBS containing 10 mM EDTA for 20 min at 25°C with shaking at 50 rpm. After incubation, sections were washed four times with PBS to remove the EDTA. Epithelia were dislodged by rubbing the intestinal samples with a sterile syringe plunger. The suspension enriched with epithelia was drained into another tube and washed twice in PBS. The released cells were isolated by filtering the suspension through a 75-μm cell mesh. Cell pellets were collected following a 10 min centrifugation at 1,500 rpm, washed twice and resuspended in PBS.

Coating of microtitre plates with epithelia : The following process was modified from Alwan et al. (1998). Microtitre plates (96-well) (BIOFIL, Canada JET Biochemical Int., Inc.) were coated with 100 μl of 1 M lysine solution and incubated at 25°C for 30 min. The plates were washed three times with distilled water, and 100 μl of a 1.25% glutaraldehyde solution was immediately added, and incubated at 25°C for 5 min, and then washed twice with distilled water. Epithelia (in PBS) were added to each well. Inverted microscopy was used to determine the optimum number of cells for full coverage of each well, and aliquots varying from 10^3-10^7 cells/well were tested. Epithelium suspensions of 10^5 cells fully coated each well. The plates were dried overnight incubation at 30°C, sealed with cling film and stored at room temperature for up to three days. Epithelia bound to microtitre wells were fixed by incubation with 0.25% glutaraldehyde for 10 min. Wells were then washed three times with PBS-T. Non-specific binding of bacteria to the plastic of microtitre wells was blocked by incubation of the plates with 100 μl PBS containing 0.5% (w/v) bovine serum albumin (BSA) for 1 h at 25°C. Wells were then washed three times with PBS-T.

Experimental design and quantitative analysis of adherence using ELISA : To determine the effect of each tested substance on adhesive course, we conducted three experiments shown in Table 2. In experiment I, BJCP-5 was pre-incubated in TSB with the tested agents, and the *in vitro* adhesive assay without the tested agents was done. In experiment II, BJCP-5 was pre-incubated in TSB without the tested agents, and the *in vitro* adhesive assay with the tested agents was performed. The experiment III mimicked *in vivo* adhesion, both pre-incubation of BJCP-5 and then *in vitro* adhesive assay were performed with the tested agents supplemented. There are four groups in each experiment, namely high dose group (H), middle dose group (M), low dose group (L), and control group (C) in which the tested agents were not used both in pre-incubation of BJCP-5 and *in vitro* adhesive assay.

According to the experimental design shown in Table 2. One hundred microlitre of washed bacterial suspensions (10^9 cells/ml) and/or corresponding agents were added in quadruplet to wells coated with epithelia. The plates were incubated at 30°C for 1.5 h. The adherent bacteria were fixed by overnight incubation with 0.3% formaldehyde in PBS following three times washes with PBS-T. Rabbit anti-sera against BJCP-5 (100 μl) diluted at 1/500 in PBS with 1% BSA, was added to each well, and incubated for 1 h at 37°C following three times washes with PBS-T. Horseradish peroxidase conjugated goat-anti- rabbit IgG (100 μl) diluted at 1/1,000 in PBS with 1% BSAwas added to each well and reacted for 1 h at 37°C. After a final wash, 100 μl of ortho-phenylenediamine substrate was added to each well and the plates were incubated for an additional 15 min at 30°C in the dark. The enzyme reaction was stopped by the addition of 50 μl of 2 M H_2SO_4. ELISA optical density (OD) at 492 nm was determined using a microtitre plate reader (MULTISKAN MK3, Thermo Electron Corporation). Adhesion of BJCP-5 to tilapia epithelia was expressed in OD units.

Table 3. Effect of antibacterial peptides on hydrophobicity and adherence of *Aeromonas veronii* biovar *sobria* strain BJCP-5

	Tested groups	Dose (μg/ml)	Hydrophobicity (%)	Adherence (A_{492nm}) Exp. I	Exp. II	Exp. III
Antibacterial peptide of chicken	H	250	39.682±1.416 a	0.471±0.087 a	0.367±0.018 c	0.531±0.017 a
	M	25	26.185±2.125 b	0.341±0.047 b	0.461±0.034 b	0.403±0.050 b
	L	2.5	22.829±1.556 b	0.221±0.041 c	0.534±0.028 a	0.301±0.060 c
	C	0	22.786±1.884 b	0.529±0.033 a	0.529±0.033 a	0.529±0.033 a
Antibacterial peptide of pig	H	250	36.348±2.113 a	0.418±0.049 b	0.167±0.026 c	0.514±0.026 a
	M	25	22.973±2.279 b	0.380±0.022 b	0.425±0.029 b	0.425±0.019 b
	L	2.5	24.022±1.072 b	0.292±0.049 c	0.516±0.025 a	0.228±0.020 c
	C	0	22.786±1.884 b	0.529±0.033 a	0.529±0.033 a	0.529±0.033 a
Antibacterial peptide of rabbit	H	250	28.145±3.751 a	0.410±0.057 b	0.160±0.050 c	0.512±0.054 a
	M	25	24.219±0.999 ab	0.378±0.058 b	0.420±0.037 b	0.423±0.031 b
	L	2.5	22.027±1.408 b	0.287±0.066 c	0.521±0.073 a	0.233±0.065 c
	C	0	22.786±1.884 b	0.529±0.033 a	0.529±0.033 a	0.529±0.033 a

The experimental designs are shown in Table 2. The small letters following the mean±SD indicate a significant difference ($p<0.05$). The values with the same letter are not significantly different.

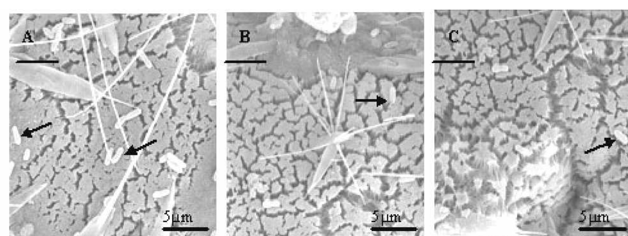

Figure 1. Effect of antibacterial peptides and oligosaccharides on adhesion of *Aeromonas veronii* biovar *sobria* strain BJCP-5 to tilapia foregut *in vivo*. A: control group; B: group supplemented with intestinal antibacterial peptide of rabbit, C. group supplemented with galactomannan. Scanning electron micrographs show the bacteria (arrow) adhering to the tilapia foregut.

The pre-immune rabbit sera were used for negative controls. Wells without epithelia were coated with bacteria only (in carbonate/bicarbonate buffer, pH 9.6) to determine the reactivity of the immunized sera and to be used as a positive control. Background values for the ELISA, or the amount of non-specific binding of the immunized sera to epithelia were determined by coating wells with epithelia but without bacteria added.

Statistical analyses

All experiments were performed at least in triplicate. Results were expressed as mean ± standard deviation (SD), and the calculations were performed using MINITAB release 10.1 package (Minitab Inc. USA.). The Duncan's Multiple Range Test of the data was analyzed by the ANOVA Procedure from SAS Software Release 8.1(SAS Institute Inc., Cary, NC, USA).

RESULTS

MIC value of the tested substances

MIC values of the two tested oligosaccharides were above 12,500 μg/ml, and they were generally far higher than that of the ABPs with MICs of 625 to 1,250 μg/ml. BJCP-5 had natural resistance to ampicillin with a MIC of 2,000 μg/ml, and the strain was susceptive to kanamycin with a MIC of 15 μg/ml (Peng et al., 2005). BJCP-5 was not susceptible to the tested agents.

Growth

The weight gain percent and feed conversion ratio of different experimental groups are shown in Table 1. Dietary supplementation with the tested agents enhanced the growth and decreased feed conversion ratio significantly ($p<0.05$).

Disease resistance

No death was observed in the unchallenged subgroups. All mortalities recorded during the trials were due to the challenge infection with BJCP-5, and observed from the third to tenth day post-challenge. The clinical signs observed in fish were hyperaemia on the ventral side of the body, swollen abdomen, reddish vent slightly protruding, opaque eyes and loss of equilibrium; all typical signs associated with *A.vbs* infection (Peng et al., 2005). The RPS of groups supplemented with ABP and oligosaccharide were higher than that of the control group. Furthermore, the RPS of groups supplemented the same tested agent by oral administration was the highest, followed by the immersion challenge, and the lowest was for injection challenge (Table 1).

Effect of tested substance on *in vivo* anti-adhesion

The tested agents supplemented to diet can decrease the adhesion of bacteria to tilapia foregut in oral challenge subgroups with BJCP-5. The average numbers of bacteria per visual field of control group were 15.2 which were significantly higher than that of the tested agent supplement groups with 6.8 to 8.4 cells ($p<0.05$) (Table 1 and Figure 1).

Table 4. Effect of oligosaccharides on hydrophobicity and adherence of *Aeromonas veronii* biovar *sobria* strain BJCP-5

	Tested groups	Dose (μg/ml)	Hydrophobicity (%)	Adherence (A_{492nm})		
				Exp. I	Exp. II	Exp. III
KGLM	H	500	26.148±1.604 bc	0.053±0.061d	0.332±0.025 c	0.270±0.031 c
	M	50	29.537±3.792 ab	0.269±0.023 c	0.410±0.109 b	0.409±0.052 b
	L	5	31.363±2.770 a	0.348±0.041 b	0.403±0.080 b	0.420±0.026 b
	C	0	22.786±1.884 c	0.529±0.033 a	0.529±0.033 a	0.529±0.033 a
CBGAM	H	500	21.663±0.484 c	0.185±0.040 d	0.211±0.021 d	0.271±0.015 d
	M	50	24.479±0.767 b	0.285±0.022 c	0.350±0.041 c	0.344±0.010 c
	L	5	27.524±0.679 a	0.451±0.061 b	0.431±0.041 b	0.409±0.034 b
	C	0	22.786±1.884 bc	0.529±0.033 a	0.529±0.033 a	0.529±0.033 a

The experimental designs see Table 2. The small letters followed the mean±SD indicate a significant difference ($p<0.05$). The values with the same letter are not significantly different.

Effect of tested substance in bacterial growth media on Bacterial hydrophobicity

The hydrophobicity of BJCP-5 was enhanced gradually with increasing concentrations of ABPs, but only at the highest concentration of 250 μg/ml the hydrophobicity of bacteria was significantly higher than that in control ($p<0.05$) (Table 3). Although the hydrophobicity of BJCP-5 pre-incubated in KGLM and CBGAM with the lowest concentration of 5 μg/ml were significantly higher than that in control respectively ($p<0.05$), the hydrophobility decreased with the concentration of tested agents increased (Table 4).

Effect of test substance on bacterial adherence *in vitro*

In experiment I, the BJCP-5 co-incubated respectively with three ABPs and two oligosaccharides had lower adhesion than that of control. Furthermore, three ABPs showed the reciprocal dose-dependent anti-adherence effect (Experiment I in Table 3); KGLM and CBGAM showed the dose-dependent anti-adhesion (Experiment I in Table 4).

In the course of the contact between bacteria and epithelia, the influence of tested agents on adhesion was estimated in experiment II. Three ABPs, KGLM and CBGAM inhibited the adherence of BJCP-5 to tilapia enteric epithelia, and the dose-dependent inhibitory effect was observed (Experiment II in Tables 3 and 4).

The condition of *in vivo* adherence was mimicked in experiment III. Three ABPs at 25 μg/ml and 2.5 μg/ml inhibited attachment and showed a reciprocal dose-dependent inhibitory effect ($p<0.05$) (Experiment III in Table 3). The KGLM and CBGAM showed a dose-dependent inhibitory effect ($p<0.05$) (Experiment III in Table 4).

DISCUSSION

Growth promotion and disease resistance of tested agents

ABPs (Ma et al., 2004) and oligosaccharides (Tang et al., 2005) are promising agents to antagonize bacteria and promote animal growth. our results are consistent with those previous studies. In present study, the tested ABPs and oligosaccharides at 100 mg/kg in supplement, which was far lower than the MICs of tested agents to BJCP-5, promoted tilapia growth and resistance to BJCP-5 infection, but did not inhibit the growth of BJCP-5. In addition, the subgroups challenged by oral administration and immersion showed higher RPS than that challenged by injection. These results suggest that the mechanism of growth promotion and disease resistance is not related to interference in intestinal bacterial growth, but may be correlated with interference in the contact between the surfaces of host and bacteria.

Anti-adherence effect of antibacterial peptides and oligosaccharides

Although there is a controversy on the anti-adherence of ABPs, increased evidences show the anti-adhesive property of ABPs. Islam et al. (2001) reported that the down-regulated expression of the ABPs LL-37 and human β-defensin-1 might promote bacterial adherence into host epithelium during early in *Shigella* spp. infections. Lactoferrin with a sub-MIC of 10 mg/ml (0.125 mM) can inhibit the adhesion of enteropathogenic *Escherichia coli* to HEp2 cells (Ochoa et al., 2003). A 23-mer derived from fibronectin at concentrations of 4 to 5 μM inhibited the adherence of *Candida albicans* to 7-mer (KLRIPSV) peptide-coated PEG beads (Klotz, 2004). Recently, Guerra et al. (2005) observed that nisin, an ABP produced by lactobacillus, reduced the attachment of *Listeria monocytogenes* strains.

There are many reports on anti-adherence of glucan (Mata et al., 1997), mannan (Ofek et al., 2003), fructo-oligosaccharides (Bornet et al., 2002), galactan (Sharon, 2006), and chitosan (Sano et al., 2001). However, it has been rarely reported that KGLM and CBGAM inhibited adhesion of bacteria, especially on relationship of anti-adhesion and growth promotion.

Results of adhesion assays *in vivo* and *in vitro* in our study indicated that the tested ABPs and oligosaccharides

had anti-adhesive effects. Antibiotics at concentrations lower than MICs can inhibit adherence of bacteria in alimentary canal, which is one of the mechanisms of antibiotic promoting animal growth and preventing bacterial infection (Shibl, 1985; Schifferli and Beachey, 1988). Therefore, anti-adhesion of the tested agents may be one of the mechanisms of growth promotion and disease resistance for tilapia.

Mechanism of anti-adhesion of antibacterial peptides and botanic oligosaccharides

The bacterial adhesion is a complex biological process involving many physicochemical forces, which primarily include hydrophobic interactions, cation bridging and receptor-ligand binding. The surface components responsible for the hydrophobic interactions include hydrocarbons, aromatic amino acids, fatty acids and mycolic acids. The adhesion and hydrophobicity of interface has the positive correlation when other physicochemical forces are same (Mack and Sherman, 1999; Doyle, 2000). The mutual repulsion between the surfaces of bacteria and host cells with net negative charge can be counteracted by divalent metal ions, which thereby act as a cation bridge between the two surfaces. The receptor on the surface of host cell can 'recognize' the ligand on the bacteria surface with a complementary structure and form a strong (but non-covalent) bond. The change of three physicochemical forces mentioned above, will influence the adherence between bacteria and host cells (Wilson et al., 2002).

Intestinal ABPs are amphiphilic peptides with a net positive charge and a substantial portion of hydrophobic residues (Selsted et al., 1992; Hancock and Lehrer, 1998). Therefore, the ABPs can influence the net negative charge of surface of bacteria and host cell. Three tested intestinal ABPs decreased the adhesion of the pro-incubated BJCP-5, but they enhanced the hydrophobicity of bacteria. Moreover, intestinal ABPs interfered with the adherence between BJCP-5 and epithelia through cation bridging and/or receptor-ligand binding, because the adhesion enhanced with the increased concentration of intestinal ABPs in experiment II. It suggests that the adhesive inhibition of the ABPs correlate with cation bridging and/or receptor-ligand binding, but not with hydrophobicity. However, the mechanisms of reciprocal dose-depended inhibition need further investigation.

That KGLM and CBGAM influenced the adhesive characteristic of BJCP-5 through altering the hydrophobicity of cell surface, and impacted on the adhesive process of BJCP-5 with tilapia intestinal epithelia through interfering receptor-ligand binding has been previously reported in other oligosaccharides (Ofek et al., 2003; Sharon, 2006).

ACKNOWLEDGEMENTS

This research was supported by a grant from the China National Science Foundation (No. 30471301). We would like to thank Madam Yuejuan Gong, Dr Jiufeng Wang and Professor Bin Wang for the English revision.

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