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The effects of garlic-supplemented diets on antibacterial activities against *Photobacterium damselae* subsp. *piscicida* and *Streptococcus iniae* and on growth in Cobia, *Rachycentron canadum*

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ABSTRACT

Photobacteriosis and streptococcosis are the most threatening diseases in cage-cultured cobia, *Rachycentron canadum*, due to high mortality of 50–80% and annual outbreaks in Taiwan. Garlic, *Allium sativum*, has long been known to have broad antibacterial properties. This study aimed to examine the in vitro antibacterial activities of garlic and the effects of dietary garlic on disease resistance against *Photobacterium damselae* subsp. *piscicida* and *Streptococcus iniae* and on growth in cobia. The results revealed the marked inhibitory effect of garlic against both *P. damselae* subsp. *piscicida* and *S. iniae*, and feeding garlic diet significantly conferred resistance to challenge with *P. damselae* subsp. *piscicida* or/and *S. iniae*. Cobia fed garlic powder at doses of 0.5 and 1.5 g/kg b.w. for 28 days produced significantly ($p < 0.05$) lower mortality after a challenge with *P. damselae* subsp. *piscicida* and higher percent weight gain. Cobia fed garlic powder at a dose of 1.2 g/kg b.w. for 21 days and at doses of 0.4 and 1.2 g/kg b.w. for 28 days provided significant ($p < 0.01$) resistance against *S. iniae* infection. A diet containing garlic powder at a dose of 1.2 g/kg b.w. for 28 days provided significant ($p < 0.05$) protection against *P. damselae* subsp. *piscicida* plus *S. iniae* combined infection in cobia.

1. Introduction

Cobia, *Rachycentron canadum*, is a commercially important species for cage culture and takes a leading distribution in both annual total production (60.0%) and total value production (65.8%) among cage-cultured fishes in Taiwan (Fisheries Agency, 2013). Its culture technology has rapidly developed in the past several years because it is comparatively easy to spawn, rear, and feed (Arnold et al., 2002; Benetti et al., 2008; Holt et al., 2007; Liao et al., 2004). Cobia is well suited for intensive grow-out culture in offshore cages and super-intensive nursery production in tanks, ponds and nearshore cages.

Concomitant with the growth of the industry, however, has been the emergence of *Photobacterium damselae* subsp. *piscicida* and *Streptococcus iniae* which are two major pathogenic bacteria of cobia (Liao et al., 2004; Liu et al., 2003). *Photobacterium damselae* subsp. *piscicida* is the etiologic agent of fish photobacteriosis, also referred to as pasteurellosis and pseudotuberculosis, that presents with whitish granulomatous deposits on the kidney, liver and spleen of diseased fish. Streptococcosis is caused by *S. iniae*. Infected fish show clinical features of dark body coloration, erratic swimming, haemorrhage around the eye and anus, exophthalmia and corneal opacity. Some surviving fish have characteristics of blindness. The feeding activity of blind cobia is highly affected resulting in slower growth and lower market value. Mass mortality is usually experienced when outbreaks of pasteurellosis and streptococcosis occur.

Garlic, *Allium sativum*, has a history of dietary and medicinal applications for curing various diseases. The medical effects of garlic are based on organosulfur compounds, particularly allicin (Rose et al., 2005) which has potential antibacterial, antiprotozoal, antifungal and antiviral properties (Afzal et al., 2000; Buchmann et al., 2003; Madsen et al., 2000; Nya et al., 2010; Peyghan et al., 2008; Soko and Barker, 2005). The antibacterial activities of garlic have been widely studied. Indeed, allicin and garlic preparations (fresh, oven- and freeze-dried garlic, etc.) have been shown to have a wide spectrum of antibacterial activity, including effects on *Aeromonas*, *Bacillus*, *Clostridium*, *Cryptocaryon*, *Escherichia*, *Helicobacter*, *Klebsiella*, *Mycobacterium*, *Photobacterium*, *Proteus*, *Pseudomonas*, *Salmonella*, *Staphylococcus*, *Streptococcus* and *Vibrio* species, as well as the common plant pathogenic bacteria, i.e., *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae*, *Xanthomonas campestris* (Ankri and Mirelman, 1999; Bakri and Douglas, 2005; Curtis et al., 2004; Guo et al., 2012; Vaseeharan et al., 2011). Furthermore, the use of garlic in aquaculture can promote growth, stimulate appetite, provide a tonic to improve the immune system and antistress protection, and can be a proven prophylactic and therapeutic agent as has been proven (Aly et al., 2010; Dias et al., 2002; Metwally, 2009; Ndong and Fall, 2011; Nya and Austin, 2009; Sahu et al., 2007; Talpur and Ikhwanuddin, 2012). The purpose of this study was to examine the in vitro antibacterial activities of garlic

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and the effects of dietary garlic on disease resistance against *P. damselae* subsp. *piscicida* and *S. iniae* and on growth in cobia.

2. Materials and methods

2.1. Garlic and sample preparation

Fresh garlic was purchased from the local market in Pingtung (Taiwan, ROC). Peeled fresh garlic was stored in a −80 °C freezer and dried in a partial vacuum using the freeze-dry system (Labconco, USA) and then was ground to a fine texture in a mechanical grinder and sieved to obtain garlic powder. Garlic powder was stored in an airtight container in a dry cabinet until use. Before the start of each experiment, the garlic powder was soaked thoroughly in sterile distilled water and vortexed using a vortex mixer at room temperature. An aliquot (aqueous extract of garlic powder, AEGP) was centrifuged and filtered through a 0.2-μm pore size filter for the in vitro antibacterial experiments. Garlic powder and the mixture of garlic powder suspended in water, respectively, were used for the in vivo experiments on disease control against *P. damselae* subsp. *piscicida* and *S. iniae* in cobia.

2.2. Allicin level

Routine analysis of the concentration of allicin in the garlic preparations was performed with a Waters (Massachusetts, USA) 616 pump and a Waters 996 photodiode-array detector (Massachusetts, USA) described by Guo et al. (2012). The allicin level of freeze-dried garlic powder in water was 13.55 ± 0.37 mg/g.

2.3. Pathogenic bacteria

Bacteria of *P. damselae* subsp. *piscicida* maintained on brain heart infusion agar supplemented with 2% NaCl (BHIA, Difco) and *S. iniae* maintained on tryptic soy agar (TSA, Difco) blood agar (5% sheep blood) supplemented with 1.5% NaCl at 28 °C, respectively, were isolated during natural photobacteriosis and streptococcosis outbreak in cage-culture cobia and identified by 16S rRNA sequence analysis. The subcultures were grown under the same conditions for 24 h for all experiments. Growth and desired density of bacteria were measured with optical density at 540 nm and then with plate counting.

2.4. In vitro antibacterial experiments

The inhibitory zone (IZ), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of AEGP, an aqueous extract of 1 g garlic powder in 10 ml water, was evaluated with the agar well diffusion method and microdilution assay, respectively, described by Guo et al. (2012). Briefly, 100 μl of Muller Hinton broth (Difco, supplemented with 1.5% NaCl; MHB) containing *P. damselae* subsp. *piscicida* at 1.4×10^9 cells/ml and *S. iniae* at 3.5×10^9 cells/ml, respectively, was seeded over the Muller Hinton agar (Difco, supplemented with 1.5% NaCl; MHA) plates. Wells were punched in the agar and each well filled with 100 μl of AEGP. One solvent blank (sterile water) was used per plate, and each test was run in triplicate. The plates were incubated at 28 °C for 24 h. The diameter of the clear IZ (mm) formed around the 8-mm diameter well was determined.

The MIC was determined as the lowest concentration that significantly inhibited bacterial growth, and the MBC was calculated as the lowest concentration that abrogated bacterial growth. Briefly, 20 μl of the AEGP was added to column 1 of each 96-well microplate and then serially diluted (dilution factor 1:1) in MHB. Each well in the first three rows of the plate was then inoculated with 90 μl of bacterial suspension (*P. damselae* subsp. *piscicida* 1.4 ± 10^8 cells/ml and *S. iniae* 3.5×10^8 cells/ml, respectively). Three rows of each plate were inoculated with bacterial suspension alone without any AEGP (positive control), and one row was incubated with MHB medium alone (negative control). The microplates were incubated at 28 °C for 24 and 48 h. After incubation, bacterial growth was measured at 600 nm using a multi-mode microplate reader (BioTek, Vermont, USA). Abrogation of growth was confirmed by plating 10 μl from each well onto MHA plates and incubating for 24 h.

2.5. In vivo experiments on disease resistance

2.5.1. Fish

Two batches of cobia were involved in two in vivo experiments. Fish were obtained from the Tungkang Biotechnology Research Center of the Fisheries Research Institute (Pingtung, Taiwan, ROC) and acclimated for 2 weeks in 1.8-ton tanks with sand-filtered seawater. During the acclimation period, fish were fed with control diet twice a day at 10:00 and 16:00 h. The water was maintained at a temperature of 24 ± 2 °C, a pH of 8.0, and a salinity of 30 psu. Before the start of the experiment, the cobia were starved for 24 h and weighed.

2.5.2. P. damselae subsp. piscicida challenge and growth trial

Three diets were prepared with 0% garlic powder as a control and 1.0 and 3.0% garlic powder as treatments in commercial powder feed of eel (Dongli, Taiwan, ROC) (wt%). Water of 35.0% (v/w) was added to above each powder diet in the agitator tank and mixed thoroughly using the mixer for 15 min. The resulting dough was extruded through a die into small strands of pellets. The diets were dried at room temperature for 48 h.

The fish (29.5 ± 3.8 g) were divided into six groups of 25 each in 18 individual 0.5-ton capacity flow-through tanks in triplicate. The fish were weighed individually before (initial body weight) and after (final body weight) the 14- and 28-day feeding experiments at a daily rate of 5% of their body weight. For each treatment, all the fish were used to quantify the percent weight gain (%WG). This parameter was calculated as follows: %WG = (final body weight − initial body weight) × 100 / initial body weight. Meanwhile, the fish were challenged intraperitoneally with 0.05 ml of phosphate buffer saline (PBS, Sigma-Aldrich) suspensions containing *P. damselae* subsp. *piscicida* at approximately 2.9×10^6 cells/ml, maintained in 0.5-ton tanks as mentioned to ensure water quality, and observed for a period of 14 days for mortality.

2.5.3. S. iniae challenge trial

Three diets were prepared respectively containing 0% garlic powder as a control, 0.7% and 2.0% garlic powder as treatments in commercial pellet feed (Dongli, Taiwan, ROC) (wt%). The garlic powders were soaked thoroughly in water (the ratio of water to feed was 15%), vortexed, and then sprayed on the commercial pellet feed slowly and mixed evenly. The control diet contained the same volume of water without the garlic.

The fish (10.5 ± 1.2 g) were divided into three groups of 40 each in 9 individual 0.5-ton capacity flow-through tanks in triplicate. After the 21- and 28-day feeding experiments at a daily rate of 6% of their body weight, the fish were challenged intraperitoneally with 0.05 ml of PBS suspensions containing *S. iniae* at approximately 1.8×10^6 and 7×10^5 cells/ml, respectively, maintained in 0.5-ton tanks as mentioned to ensure water quality, and observed for a period of 14 days for mortality and lesion score.

2.5.4. P. damselae subsp. piscicida plus S. iniae challenge trial

The fish (10.5 ± 1.2 g) were divided into three groups of 40 each in 9 individual 0.5-ton capacity flow-through tanks in triplicate and were fed with the same 3 diets as above *S. iniae* challenge trial. After the 21- and 28-day feeding experiments, the fish were challenged intraperitoneally with 0.05 ml of PBS suspensions containing *P. damselae* subsp. *piscicida* (1.4×10^2 cells/ml) and *S. iniae* (1.8×10^6 cells/ml), maintained in 0.5-ton tanks as mentioned to ensure water quality, and observed for a period of 14 days for mortality and lesion score.