Journal of Bioscience and Bioengineering
VOL. 121 No. 5, 530–535, 2016

www.elsevier.com/locate/jbiosc

Thermophile-fermented compost as a fish feed additive modulates lipid peroxidation and free amino acid contents in the muscle of the carp, *Cyprinus carpio*

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Received 24 July 2015; accepted 6 October 2015
Available online 17 December 2015

Recently, a compost fermented with marine animals with thermophilic *Bacillaceae* in a clean and exclusive process at high temperature was reported as a possible feed additive to improve the healthy balance in sea fish and mammals (i.e., pigs and rodents). Here, the effects of the oral administration of the compost on the muscle and internal organs of carp (*Cyprinus carpio*) as a freshwater fish model were investigated. The fatty acid composition was different in the muscle of the carp fed with or without the compost extract, but there was little difference in the hepatopancreas. The accumulation of triacylglycerols, cholesterol, lipid peroxide and hydroxyl lipids decreased in the muscle after the oral administration of the compost extract in the carps over 12 weeks, but the accumulation did not always decrease in the hepatopancreas. In contrast, free-radical-scavenging activities and the concentrations of free amino acids in the muscle did not always increase and was dependent on the dose of the compost at 12 weeks. The scavenging activities and part of free amino acid levels in the muscle of the carp were improved at 24 weeks after a high dose of compost exposure, and then the survival rates of the carp were maintained. Thus, the oral administration of thermophile-fermented compost can prevent peroxidation and increase the content of free amino acids in the muscle of the freshwater fish, depending on the dose and term of the administration, and may be associated with the viability of the fish.

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[**Key words:** Compost; Feed additive; Thermophile; Lipid peroxidation; Amino acid; Carp]

Lipid peroxidation is associated with various pathogenic conditions in many mammals and fish, and it also affects their physiological conditions (1–4). For example, the levels of lipid peroxide in fish are associated with fish diseases (2,4) and oxidative stress (1,3). Based on these observations, lipid peroxidation in fish affects the efficiency of fish production in the nursery. The control of lipid peroxidation is important for sustainable food production. In addition, lipid peroxidation in fish also affects the quality of food. Therefore, it is necessary to develop a feed to prevent lipid peroxidation.

Recently, we investigated the microbial diversity of a compost produced from marine animal resources by a unique fed-batch fermentation system with three bioreactors (5). Marine animal resources (i.e., small fishes, shrimp, and crabs that were not available for human consumption) were used as a compost material. The composting process was carried out at high temperatures (around 75°C) by fermentation-associated self-heating. The final product contained thermophiles, and the majority of thermophiles in the compost were *Bacillaceae* (5). This composting process likely limited the proliferation of general pathogens; therefore, the cost for sterilization was low. The compost also contained a bacterial strain expressing a cyclic lipopeptide with antifungal activities, which was heat stable and stable over a broad pH range.

The compost was used as a feed additive for farmed fish, such as flatfish (*Paralichthys olivaceus*), because the numbers of dead and diseased fish were reduced in the nursery given the compost. We studied the effects of the consumption of the compost on the physiological responses of flatfish, and the results showed that total levels of free amino acids in the muscle, which is an index of food quality and healthy balance, increased in the compost-treated groups (10).

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http://dx.doi.org/10.1016/j.jbiosc.2015.10.006

The effects of the oral administration of this compost on the physiological responses in several animal species were also investigated. When the compost extract was orally administered to pigs, the rate of stillbirths in the sows decreased and the growth of their piglets was promoted (6). Oral administration of the compost extract to rats activated the gut mucosal immune system (7). In addition, the number of pigs with visceral lesions at a swine farm had a tendency to decrease after the extract of the thermophile-fermented compost was orally administered to the pigs for 6 months or more. These observations suggested that the oral administration of this compost or its extract, which can be used as an organic fertilizer in agriculture (8), could regulate the farrow, immune functions and other internal organs in the animals. In addition, we examined the mechanism by which the compost extract could influence the physiological functions of animals using rats, and showed that the oral administration of the compost extract to rats reduced lipid peroxidation in the liver, where antioxidant activity increased, although the antioxidant activity of the extract itself was low (9). The extract of thermophile-fermented compost might be a novel type of functional feed that could regulate certain physiological functions of animals.

In the present study, using the experiments with a setting of an appropriate control group, we examined the effects of the compost on the physiological functions of fish, such as the reduction of lipid peroxidation in the liver and the increase in free amino acid levels in muscle, similar to that found in rats (9) and flatfish (10). Carp (*Cyprinus carpio*) was used for the experiments, due to their availability and equipment for breeding.

MATERIALS AND METHODS

Compost, compost extract and test diets preparation The compost was produced using an aerobic repeated fed-batch fermentation system (5). This compost was marketed as an organic fertilizer or fermented feed for flatfish, pigs and chickens (Miroku Co. Ltd. and Keiyo Plant Engineering Co. Ltd., Japan) as previously described (5–7,9,10). The powdered compost contained <25% water, <25% protein, <1% lipid, <25% fiber, <10% ash. In addition, the compost contained <1.5% Urea, <500 ppm 5-aminolevulinate, <500 ppm serine, <500 ppm glycine, <500 ppm alanine, <500 ppm glutamic acid, <500 ppm glutamine, <500 ppm aspartic acid, <500 ppm isoleucine, <500 ppm valine, <500 ppm leucine, <500 ppm phenylalanine, <500 ppm ornithine, <500 ppm histidine, <250 ppm arginine, <250 ppm lysine, <100 ppm proline, <100 ppm malate, <100 ppm citrate, <0.5% lactate, <0.5% butyrate. The powdered compost (compost powder) was diluted 1/100 with potable water (as vol/vol) (this was used as the compost extract) and incubated under aerobic conditions at 60°C for at least 10 h (6,7). The composition of bacterial species in the compost is stable (5). The number of culturable thermophilic bacteria in the compost extract was less than 5.0×10^6 CFU/mL as previously described (6,7,9,10). The compost grain or extract was diluted to administer to the carp based on the experimental conditions. A commercial diet for carp P-3 (Hayashikane Sangyo Co., Ltd.; Shimonoseki, Yamaguchi, Japan) was used for the basic control group. The powdered compost or the compost extract was added to the powdered basic control diet in the ratio of 0.5–5% (compost powder) or 1–20% (compost extract) for the diets of the experimental groups. After the addition of distilled water to the mixed powdered diet, pellets of the diet were produced using a garlic press. The dried experimental diets were stored at 4°C until use for feeding. The control diet was composed of the following: 11% moisture, 39% protein, 5% lipid, 20% ash, 8% fiber and 16% carbohydrate.

Fish and feeding conditions Carp were hatched and cultured in the ponds and aquariums of the National Fisheries University. The juvenile fish (body weight 20–30 g) were divided into three 60 L tanks with 13–15 fish per tank and acclimated to the commercial diet for two weeks. The water temperature was held at 23 ± 1°C, and water quality was maintained via circulation through polyester wool filters (8.0 L/min), supplemental aeration, periodic aquaria cleaning, and water changes with preconditioned water twice a week. Each tank of carp was fed the experimental diet (1% of initial body weight) twice a day for 3 months or 6 months. After feeding for 3 months or 6 months, carp were sacrificed, and the removed hepatopancreas and muscle were stored at −30°C until the determination of lipid peroxides or hydroxyl lipid levels and other analyses.

Lipid extraction Lipids were extracted by homogenizing the sample (1.0–3.0 g for hepatopancreas, 5.0 g for muscle) from the muscle or the whole hepatopancreas with chloroform/methanol (2/1, vol/vol) containing 0.005% butylated hydroxyl-toluene under ice-cold conditions and recovered in a manner similar to that described by Folch et al. (11). The volume of the solution containing the extracted lipids was adjusted to 10 mL by the addition of chloroform/methanol. The lipids solution was stored at −80°C until the analysis. The lipid contents were determined by weighing the aliquot of the lipids solution after the removal of solvent.

Analysis of lipid classes The lipid classes were analyzed by thin-layer chromatography with flame-ionization detection (TLC-FID) using an Iatroscan-MK5 (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) (12). A microdispenser (The Drummond Scientific Co., Broomall, PA, USA) was used to spot 5 μL of lipid solution (7.5 mg/mL) onto S-III chromarods (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). Before spotting, the chromarods were scanned twice in the Iatroscan-MK5. In total, three sets of 10 chromarods were simultaneously used in the experiments. After spotting, the set of S-III chromarods was placed in the developing tank for 20 min. The developing solvents contained n-hexane/diethyl ether/formic acid (42/28/0.3, vol/vol). The rods were then dried in an oven at 120°C for 2 min using a Rod Dryer TK-8 (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) and left in a vacuum desiccator for 5 min to reach room temperature. Next, the chromarods were transferred to the Iatroscan-MK5 and scanned. FID scans and data acquisitions were performed using an Iatroscan-MK5 analyzer connected to a Chromatocorder 21 (System Instruments, Tokyo, Japan). The hydrogen and air flow rates of the FID were 160 mL/min and 2000 mL/min, respectively. The scanning speed was set at 30 cm/min.

Analysis of fatty acids To analyze the fatty acid composition, the extracted lipids were transmethylated by saponification followed by a modification of the previously described protocol (13). A lipid sample (approximately 10 mg) and 1 mg of heptadecanoic acid as an internal standard in a screw-capped glass tube were hydrolyzed with 0.75 mL of 0.5 mol/L KOH in methanol at 100°C for 9 min. The reaction mixture was added to 1 mL of 14% BF_3 in methanol at 100°C for 7 min. Next, 1 mL of water and 2.5 mL of saturated NaCl solution were added to the solution. The mixture was vortexed and centrifuged at 2000 g for 10 min. The upper layer, containing fatty acid methyl esters (FAMEs), was transferred to Sep-Pak Silica-630 mg (Waters, Milford, MA, USA) and pre-washed with hexane. Then, the FAMEs were eluted with 10 mL hexane/diethyl ether (96/4). The eluted solution was evaporated to dryness using a centrifuged concentrator (Taitex, Koshigaya, Japan), and then the FAMEs were dissolved in 200 mL acetone for gas liquid chromatography analysis. The gas liquid chromatography system was a gas chromatograph (G-6000; Hitachi, Japan) equipped with a flame-ionization detector and a capillary column (TC-FFAP, 30 m × 0.25 mm i.d.; GL Science, Japan). The column temperature was programmed for a linear increase of 2°C/min from 180°C to 230°C. The injection and detector port temperatures were both 250°C. The FAMEs on the chromatogram were identified and calculated with conventional methods using the retention time of standards.

Analysis of lipid peroxide levels The lipid peroxide levels were determined using a modification of the previously described protocol (14). An aliquot (10 mg) of the lipids was placed in a screw-capped tube, 100 μL of cyclohexane and 100 μL of triphenylphosphine reagent (11 mg triphenylphosphine in 10 mL cyclohexane) were added, and the mixture was shaken gently at 30°C in the dark for 30 min. Stoichiometrically generated triphenylphosphine oxide was determined by high-performance liquid chromatograph (HPLC). The system included a Nova-pack Silica column (3.9 mm × 150 mm, Waters) and an ultraviolet detector 875-UV (Jasco, Tokyo, Japan) at 230 nm. The flow rate of the mobile phase, n-hexane/2-propanol (95/5, vol/vol), was 1.0 mL/min and delivered with an 880-PU pump (Jasco, Tokyo, Japan). The system and column were maintained at room temperature.

Analysis of hydroxyl lipids levels Hydroxyl lipids levels were determined by measuring anthroyl esters (1). A 100 μL aliquot of 1-hexadecanol (internal standard) in benzene, 100 μL of 1-anthroyl cyanide reagent (8 mg/mL in acetonitrile), and 50 μL of quinuclidine reagent (2.4 mg/mL in acetonitrile) were added to a screw-capped test tube containing the extracted lipid (approximately 1 mg). The mixture was stirred gently for 40 min at 60°C. After cooling, the reaction was terminated by adding 1 mL methanol. A 10 μL aliquot of the resulting mixture was injected into the HPLC system. The anthroyl derivatives of hydroxyl lipids were analyzed using an HPLC system that included a PU-1580 pump, a DG-1580-53 degasser, a CO-1565 column oven (40°C), an FP-1520S fluorescence detector (excitation, 370 nm; emission, 470 nm) (Jasco), a 7125i Rheodyne sampling valve connected to a 10 μL sample loop (Rheodyne), and a 150 mm × 4.6 mm CAPCELL PAK C-18 column with a diameter of 5 μM (Shiseido) connected to a Guard-Pak containing NovaPak-C18 (Waters). Mobile phase solutions composed of acetonitrile/water (80/20, vol/vol) (A) and acetone (B) were used. The gradient was run from 0% B to 70% B from 0 min to 30 min and maintained isocratic at 70% B from 30 min to 50 min. The flow rate was 1.0 mL/min. New samples were injected at intervals of 55 min. HPLC was performed at room temperature and controlled by Jasco-Borwin chromatography software (Jasco). Hydroxyl lipids that eluted between 1-hexadecanol (the internal standard) and cholesterol, corresponding to a retention time of approximately 35–45 min, were tentatively identified as hydroxyl triacylglycerols, depending on the agreement between its retention time and that of authentic hydroxyl triacylglycerols (castor oil and reduced–autoxidized tuna triacylglycerols).

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