Journal of Bioscience and Bioengineering
VOL. 116 No. 2, 203–208, 2013

www.elsevier.com/locate/jbiosc

Thermophile-fermented compost as a possible scavenging feed additive to prevent peroxidation

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Received 9 November 2012; accepted 31 January 2013
Available online 19 March 2013

The extract of compost from fermented marine animals and thermophiles, including *Bacillaceae*, confers health benefits as a feed additive for fish and pigs. However, little research has explored how such compost extracts affect the physiological functions of the animals. In this study, the physiological effects of oral administration of the compost extract on the liver and muscle of rats are evaluated. After long-term administration of the compost extract in rats fed with either a normal diet or a high-fat diet over 3 months, accumulation of lipid peroxide and malondialdehyde, a marker of peroxidation, in the livers was reduced. Under such conditions, the unsaturated fatty acid composition in the liver was not significantly different in the rats fed either with or without the compost extract. In contrast, analyzes of 2,2-diphenyl-1-picrylhydrazyl (DPPH) revealed that free-radical-scavenging activity was increased in the livers of rats fed with the compost extract, although the extract itself had little of this activity. Glutathione, an antioxidant, was slightly increased following compost exposure. In addition, the levels of glutamate and glutamine, sources of glutathione, were slightly raised. Such a tendency was also observed in the muscle. Thus, thermophile-fermented compost can be a fermented feed additive to prevent peroxidation in the liver and muscle, and the effects of this additive may, in part, be associated with the retention of antioxidants and free amino acids within the organs.

[**Key words:** Compost; Feed additive; Thermophilic bacteria; Lipid peroxidation; Antioxidant; Free amino acid]

Several fermented foods, such as cheese and yogurt, confer health benefits (1–3). These foods are produced through fermentation with specific mesophilic probiotic microbes, such as *Lactobacillus* sp., *Bifidobacterium* sp., and *Saccharomyces cerevisiae*. The materials used for the fermentation of foods are important for maintaining freshness and should be cleaned immediately before the fermentation process. The fermentation process must avoid contamination and the proliferation of harmful microbes. Recycled feed for livestock is another type of fermented food produced from food waste through fermentation with probiotic bacteria. Such recycled feed can be regarded as a compost, which is defined as a fermented organic matter (4). Especially it can be a clean type of compost in case that it is fermented with fresh food waste and specific nonpathogenic microbes. In contrast, typical compost is produced by the fermentation of unclean food waste, animal feces or sewage sludge with unspecified microbes (5–7). Typical compost is utilized as an organic fertilizer or a soil conditioner (4–7). The microbial structure of such compost has been difficult to control, unlike fermented food. Therefore, such compost does not always have a stable function.

Recently we clarified the microbial diversity of a compost made from fermented fresh marine animals, i.e., small fishes, shrimp, and crabs that were not available for human consumption. This compost was produced by a fed-batch fermenter system with a clean and exclusive process (8). The fermentation temperature reached approximately 75°C within 6 h. The microbial community in the compost is stably dominated by specific *Bacillaceae* bacteria, especially bacteria with sequence similar to the 16S rDNA of *Bacillus*

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http://dx.doi.org/10.1016/j.jbiosc.2013.01.024

thermoamylovorans and *Bacillus thermocloaceae* (8). The compost and its extract have been used as an organic fertilizer to decrease pathogenic effects on plants in Japan. The compost contains a novel *Bacillus* strain expressing a cyclic lipopeptide with anti-fungal activity (8), indicating that it plays a role in plant growth-promoting rhizobacteria (PGPR). In addition, the compost denitrify in the soil (9). The denitrification reduces nitrate accumulation in the plants. Based on these observations, this compost is a novel type of functional fertilizer that stably modifies the growth of plants and soil environments.

Furthermore, this compost can be utilized as a feed additive for fish and livestock animals. The oral administration of the compost or its extract to the flatfish *Paralichthys olivaceus* was shown to increase amino-acid levels in the muscle and decrease the number of dead flatfish in a nursery (10). When the compost extract was orally administered to pigs, the rate of stillbirth in the sows was decreased, and the growth of their piglets was promoted (11). Oral administration of the compost extract to rats activated the gut mucosal immune system (12). In addition, the number of pigs with visceral lesions at a swine farm had a tendency to be decreased after the extract of the thermophile-fermented compost was orally administered to the pigs for 6 months or more. These observations suggest that the oral administration of this compost or its extract, which can be used as an organic fertilizer in agriculture, could regulate the farrow and the conditions of immune and other internal organs in the animals. This study was conducted to examine the mechanism by which the compost extract could influence the physiological functions of animals.

Here, the effects of the compost as a feed additive on the organs of rats, which are used as a mammalian model, are investigated. Interestingly, our observations revealed that oral administration of the compost extract to rats reduced lipid peroxidation in the liver as well as muscle, where antioxidant activity was increased, although the antioxidant activity of the extract itself was low. Thus, the extract of thermophile-fermented compost may be a novel type of functional feed that can regulate certain physiological functions of animals.

MATERIALS AND METHODS

Animal and feeding conditions Male Wistar rats aged 3 weeks were purchased from Kyudo Co. Ltd. (Saga, Japan) and fed a pelleted commercial diet (MF; Oriental Yeast Co. Ltd, Tokyo, Japan) for 5 d. All rats were housed in a room on a 12 h:12 h light cycle at 24 ± 2°C and a relative humidity of 50 ± 10%. All of the animal treatments followed the guidelines for the care and use of laboratory animals at the National Fisheries University. The conventional rats were individually fed in each cage. After acclimatization, the animals received potable water *ad libitum*. The potable water was supplemented with 0.5% or 1.0% (v/v) compost extract, 0.5% of the filterated compost extract, or water only. The animals received water and the following diet *ad libitum* over 13 weeks. In addition, a pellet type of normal diet or a powdered high-fat diet, which was made by Oriental Yeast, was given to the rats. The normal and high-fat diet was designed as follows, respectively. The normal diet was composed of the following: 7.70% moisture, 23.60% protein, 5.30% lipid (0.00% lard), 6.10% ash, 2.90% fiber, and 54.40% carbohydrate; and 360.00 calorie per 100 g. The high-fat diet was composed of the following: 6.20% moisture, 18.90% protein, 24.20% lipid (20% lard), 4.90% ash, 2.30% fiber, 43.50% carbohydrate; and 476.00 calorie per 100 g. After the feeding periods were finished, the livers and thigh muscles of the rats were collected.

Preparation of the compost extract As previously described, the compost was produced using an aerobic repeated fed-batch fermentation system (8). This compost has been marketed as an organic fertilizer or fermented feed for fish, pigs and chickens (Miroku Co. Ltd., Oita, Japan; Keiyo Plant Engineering Co. Ltd., Chiba, Japan). The compost was diluted 1/100 with potable water (as v/v) and incubated under aerobic conditions at 60°C for at least 10 h as previously described (11,12); the diluted compost solution, 10^6 colony-forming units (CFU) per 1 mL, was used in this experiment. The compost extract was filtered through a Millipore filter (0.2-μm pore size). The compost extract and the filtered extract were frozen at −20°C until use.

Analysis of serum components Blood was collected from individual rats at the end of the feeding test, and the serum was divided and stored at −80°C. Aspartate aminotransferase (AST or GOT), alanine aminotransferase (ALT or GPT), alkaline phosphatase (ALP), cholinesterase (ChE), albumin (Alb), HDL-cholesterol, LDL-cholesterol, and triglyceride in the serum were analyzed with BM2250 (Nihon Denshi Ltd., Tokyo, Japan) according to the manufacturer's instructions.

Histochemical detection Frozen 6-μm sections of the liver were prepared by Cryostat (Leica Ltd., Tokyo, Japan). To detect lipid droplets in the liver, staining with Sudan III (Wako Pure Chemical Industries, Osaka, Japan) and Nile blue (Wako Pure Chemical Industries) were performed, respectively, as previously described (13,14).

Lipid extraction Lipids were extracted by homogenizing the sample (*ca* 5 g) from the whole liver with $CHCl_3$/methanol (2/1) under ice-cold conditions and recovered in a manner similar to that described by Folch et al. (15). The extracted lipids were added to 10 mL of $CHCl_3$/methanol (2/1) and stored at −30°C.

Analysis of lipid classes The concentrations of phospholipid, free fatty acid, cholesterol, and triglyceride, as lipid classes in the liver, were analyzed by using thin-layer chromatography with flame-ionization detection (TLC–FID) using an Iatroscan-MK5 (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) (16). A microdispenser (Drummond Scientific Co., Broomall, PA, USA) was used to spot 5 μL of lipid solution (7.5 mg mL^{-1}) onto S-III chromarods (Mitsubishi Chemical Medience Corporation). 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/diethylether/formic acid (42/28/0.3). The rods were then dried in an oven at 120°C for 2 min using a Rod Dryer TK-8 (Mitsubishi Chemical Medience Corporation) and then 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 and 2000 mL min^{-1}, respectively. The scanning speed was set at 30 cm min^{-1}.

Analysis of lipid peroxide levels The lipid peroxide (LPO) levels were determined using a modification of the protocol described previously (17). An aliquot (10 mg) of the lipids was placed in a screw-capped tube, 100 μL of cyclohexane and 100 μL of triphenylphosphine (TP) reagent (11 mg TP in 10 mL cyclohexane) were added, and the mixture was shaken gently at 30°C in the dark for 30 min. Stoichiometrically generated TP oxide was determined by HPLC. The system included a Nova-pack Silica column (3.9 × 150 mm) (Waters, Milford, MA, USA) 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 v/v) was 1.0 mL min^{-1}, delivered with an 880-PU pump (JASCO). The system and column were maintained at room temperature.

Analysis of malondialdehyde concentration The concentrations of malondialdehyde (MDA) in the liver samples were determined using an MDA assay kit, Bioxytech MDA-586 (Percipio Bioscience, CA, USA) according to the manufacturer's protocol.

Analysis of fatty acids To analyze fatty acid composition, the extracted lipids were transmethylated by saponification followed by a modification of the protocol described previously (18). 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^{-1} 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) 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, Saitama, Japan), and then the FAMEs were dissolved in 200 mL acetone for gas liquid chromatography (GLC) analysis. The GLC system was a gas chromatograph (G-6000; Hitachi, Tokyo, Japan) equipped with a flame-ionization detector and a capillary column (TC-FFAP, 30 m × 0.25 mm i.d.; GL Science, Tokyo, Japan). The column temperature was programmed for a linear increase of 2°C min^{-1} from 180° 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.

DPPH radical-scavenging assay The DPPH radical-scavenging activity assay (19) was performed. An aliquot (0.2–1.0 g) of rat liver was homogenized with 30 mL of ethanol under ice-cold conditions, and the mixture was centrifuged at 2000 × *g* for 10 min. The upper ethanol layer was evaporated, and the volume of extract solution was adjusted to 10 mL. This sample solution (0.3 mL) in the assay tube was added to 0.7 mL of ethanol, 1.0 mL of distilled water, 1.0 mL of 50 mM Tris buffer solution (pH 7.4) and 1.0 mL of 0.1 mM DPPH solution in ethanol. The mixture was incubated at 37°C for 20 min, and the absorbance at 517 nm was measured with a spectrophotometer (UVmini 1240; Shimadzu, Kyoto, Japan). The antioxidant activity was calculated from a calibration curve that had been prepared using a set of standard colors obtained by mixing 0–50 μM α-tocopherol in ethanol solution. Each value was expressed as the α-tocopherol equivalent per gram of tissue (nmol/g liver).