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Establishment of evaluation method to determine effects of veterinary medicinal products on manure fermentation using small-scale composting apparatus

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To evaluate on a laboratory scale the influence of veterinary medicinal products (VMPs) excreted into feces on manure fermentation, we have developed an evaluation method that uses a small-scale composting apparatus. Each run is of approximately 3 kg scale and the operation can be conducted in an environmentally controlled laboratory. The main evaluation parameter is calorific value generated by aerobic fermentation. At the sulfadimethoxine (SDM) trial, the volume of CO_2 generated during fermentation and the disappearance of the inhibitory effect of immature manure on sprouting (using Komatsuna (*Brassica rapa* var. *perviridis*)) were measured. In addition, DNA of 16S rRNA was extracted from a manure sample and subjected to denaturing gradient gel electrophoresis (DGGE). The results suggest that the presence of such VMPs in feces affected the microbial community in manure fermentation, and indicate that the evaluation method may be used as a standard method to evaluate the effect of VMPs on the microbial community. Using the method, we obtained data of the influence of five VMPs approved for stockbreeding in Japan on swine manure fermentation. Erythromycin (EM) affected the calorific value even at a relatively low concentration (105 mg/3 kg manure). In contrast, oxytetracycline hydrochloride (OTC), norfloxacin (NFLX), and tylosin tartrate (TS) had no effect at that concentration. These VMPs also affected the increase of fermentation temperature when added at high concentrations.

[**Key words:** Ecotoxicity; Pharmaceutics; Manure; Fermentation; Stockbreeding]

The use of appropriate doses of veterinary medicinal products (VMPs) is indispensable in modern stockbreeding. However, some kind of VMPs are excreted into feces or urine without being metabolized. Because of this, it is suspected that the excreted VMPs may affect the ecosystem.

This problem has been causing much anxiety since the 1970s. Coats et al. evaluated the ingredients of VMPs by using a model ecosystem (1). Wall and Strong reported that an anthelmintic excreted into bovine feces affected the degradation of the feces (2). It has been pointed out since the late 1980s that certain drugs excreted by farm animals may adversely affect the ecosystem.

Boxall et al. published a review of the detection of drugs (not only from animal but also from human) in the environment (3). The fact that drugs are detected in the environment has led us to suspect that such drugs may have some impact on the ecosystem.

To estimate the environmental risk posed by a particular drug, information of the load applied to the ecosystem and the toxicity is needed. According to the guidelines issued by the International Cooperation on Harmonization of Technical Requirement for Registration of Veterinary Medicinal Products (VICH), environmental risk is estimated from the load applied to the ecosystem (which can be calculated from the dosage, the dosage form, and the feces disposal method) and the toxicity data (which are essentially based on experimental data along with OECD guidelines for testing chemicals) (4,5). One example of such work is that of Halling-Sørensen (6). Furthermore, the use of specialized testing methods for drugs is suggested because drugs have specific physiological function and use. Ando et al. suggested a new assay for the toxicity of prokaryotes by using cyanobacterium (7). In this regard, cyanobacteria have been added to the OECD test guidelines 201 (algal growth inhibition test) as a recommended strain (8).

It is assumed that excreted VMPs are found in feedlots, grazing land, or manure retention tanks. The excreted VMPs may affect numerous microorganisms in the field. In addition, as biological treatment is widely performed on livestock wastewater, the excreted VMPs may affect microorganisms used in the biological treatment. Schlüsener et al. investigated the fate of antibiotics in liquid manure tanks and estimated their half-lives (9). De Liguoro et al. administered sulfadimethoxine (SDM) to bovine and monitored its fate from the feedlot to the field (10).

In Japan, waste from livestock is first divided into liquid and solid. The liquid portion is treated with activated sludge and the solid portion is fermented. The fermentation aims to control

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carbon/nitrogen (C/N) ratio, degrade substances that inhibit germination, and remove harmful microorganisms. Feces is mixed with rice straw, sawdust, bran or other plant biomass and fermented for 1–3 months. As fermentation is promoted by microbial activity, substances that are harmful to microorganisms may affect fermentation. Nevertheless, there is no report of the effect of VMP-derived contaminated substances on fermentation. However, there are reports of the detection of VMPs in manure or their fate in various circumstances, but no reports of their effect on fermentation. We have noted such effects and the possibility of using fermentation as an environmental toxicology test for VMPs.

In this study, we developed an evaluation method that uses a small-scale composting apparatus set in the laboratory. Further investigation was carried out on SDM by denaturing gradient gel electrophoresis (DGGE). The variations of C/N ratio of manure by fermentation and the inhibition of Komatsuna seed sprouting were observed. Such observation parameters are useful to qualitatively evaluate the effects of the tested VMPs. And we carried out the test also for oxytetracycline (OTC), norfloxacin (NFLX) erythromycin (EM), and tylosin tartrate (TS) to investigate the generality of the method.

MATERIALS AND METHODS

Small-scale composting apparatus FN-1500 "Kaguyahime" (Fujihira Industry Co., Ltd., Tokyo, Japan) was used as the composting apparatus. Its structure has been previously reported (11). It can execute 3–5 kg laboratory-scale aerobic fermentation. The composting apparatus is kept warm by a wooden jacket without any heat source and fresh air is injected from the bottom at a constant flow rate. Water traps are set at the top and bottom portions of the composting apparatus, which can collect water generated by fermentation. Exhaust gas is led to a trap set containing sulfuric acid that traps basic gas. Gas can be sampled before flowing into the trap set.

Material for fermentation Swine feces was provided by the livestock center of the Ome branch of Tokyo Development Foundation for Agriculture, Forestry, and Fisheries. Feces from swine administered VMPs was excluded to prevent possible contamination of the feces with VMPs. Rice straw cut to 5 cm length was mixed with the feces as additional material for adjusting water content. Three kg of feces and 300 g of rice straw were used for each run.

Chemicals Reagent-grade OTC, NFLX, SDM, EM, and TS were purchased from Sigma–Aldrich Co. (St. Louis, MO) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The concentrations of the tested VMPs were determined as follows. We assumed that VMP administered at the maximum dose approved in Japan was not metabolized at all and excreted into feces, and defined the concentration of VMP in the feces as the "estimated maximum concentration in feces on administration of the approved dose in Japan" (MCF). The concentrations of the tested VMPs are 1/10 of MCF, MCF, 10 times (except SDM, 5 times) of MCF, and control (Table 1).

Fermentation Twelve kg of swine feces collected in the day and 1200 g of cut rice straw were mixed uniformly with a hayfork. A 3.3 kg amount of the feces-rice straw mixture was weighed out and mixed with the tested VMP, and the whole was set into the composting apparatus carefully in order to not vary the condition between composting apparatuses. Air was injected from the bottom of the composting apparatus at a constant flow rate (0.8 L/min). The flow rate was determined by a preliminary test (data not shown) that revealed that the temperature increase was not suppressed by setting the flow rate at 0.8 L/min. On the other hand, a lower flow rate suppressed the temperature increase.

The temperatures of the manure and the laboratory were recorded at 10 min intervals with a thermo recorder. Room temperature was controlled to around 23°C with an air conditioner. When manure temperature was around 30°C, the composting apparatus was opened and the manure was mixed. Afterward, the composting apparatus was set and fermentation was started again. The lack of any temperature increase after the second mixing signaled the end of the test. The tests were conducted in duplicate for each tested VMP (trial 1 and 2).

In addition, a control test was carried out in which no test VMPs were added. This was used to determine whether the obtained data have a significant difference or not (see "Statistical analysis").

TABLE 1. Carbon/nitrogen ratio of fermentation products after SDM addition (MCF = 5 g/kg).

	C/N ratio
Control	9.48
MCF × 1/10 SDM	10.8
MCF × 1 SDM	10.5
MCF × 5 SDM	8.46
Before fermentation	14.3

Evaluation of calorific value We defined manure having a temperature that is 10°C higher than room temperature as "fermenting" manure. Then, we tallied temperature data recorded at 10 min intervals.

Total Calorie (TC) is expressed as $\sum(t_i - tr_i)$. (We adopt only data that are $(t_i - tr_i) > 10$. t_i is the temperature of manure at measuring point "i" and tr_i is room temperature at measuring point "i".)

Calorific period (CP) is the number of measuring points where $(t_i - tr_i) > 10$.

As the definition of TC and CP, TC/CP indicates the average of calorific values by fermentation.

DGGE analysis DNA extraction from manure was performed as follows. Total DNA was extracted from compost sample using a MoBio PowerSoil™ DNA isolation kit (Carlsbad, CA, USA) with the following modifications: 0.2 g manure was used and samples were homogenized with a Micro Smash (TOMY, Tokyo, Japan). The concentrations of the DNA extracts were determined with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

PCR amplification was performed as follows. Fragments of the 16S rRNA gene were enzymatically amplified using an I-cycler (Japan Bio-Rad Laboratories, Tokyo, Japan) with primers 341f and 907r from total DNA samples (12,13). A GC clamp was attached to the forward primer for DGGE-PCR as described by Muyzer et al. (12). The amplification mixture consisted of 1× Ex Taq buffer (Takara, Japan), 0.2 mM of each dNTP, 0.25 μM of each primer, 1 μL of template DNA, and 1.25 units of Ex Taq polymerase (Takara), in a final volume of 50 μL. The amplification conditions were as follows: 94°C for 2 min, 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min.

Five microliters of each PCR product was analyzed by electrophoresis on 1.2% agarose gel and visualized by ethidium bromide staining.

Analysis of PCR products by DGGE was performed as follows. DGGE of the PCR products was performed using a D-code universal mutation detection system (Bio-Rad Laboratories, Richmond, CA, USA). Polyacrylamide gel [8% (wt/vol) of a 37.5:1 acrylamide–bisacrylamide mixture in 1× TAE buffer (0.04 M Tris base, 0.02 M acetate, and 1.0 mM EDTA)] was used. Denaturant gradients from 30% to 70% [100% denaturant was 7 M urea plus 40% (wt/vol) formamide] in the direction of the electrophoresis were used. Gels were exposed to a constant voltage of 100 V for 8 h at 60°C. After the electrophoresis, the gels were stained for 10 min with GelStar® stain (Cambrex, Baltimore, MD, USA) and photographed with a UV transilluminator (Printgraph, ATTO, Tokyo, Japan). Digital images of the gels were acquired with a CCD video camera module (ATTO).

Small pieces of selected DGGE bands were excised from the gel using sterilized cutter blades, transferred into 50 μL of sterile water, and incubated overnight at 4°C. One microliter of the eluted DNA was used for re-amplification without the GC-clamped primer and the PCR products were checked by electrophoresis for their specificity. Amplified products were treated with ExoSAP-IT (Amersham Biosciences, Tokyo, Japan) to remove excess primers and dNTPs and sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing kit v1.1 (Applied Biosystems) according to the manufacturer's instructions on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Searches in GenBank with the BLAST program were performed to search the most similar sequence type. The nucleotide sequence data reported in 16S rRNA gene analysis were deposited in DDBJ/EMBL/GenBank under Accession numbers AB699226–AB699239.

Analysis of CO_2 concentration in exhaust gas of SDM trial CO_2 concentrations of control and 5 times of MCF were measured with PNA-100 (Teijin Engineering, Tokyo, Japan) and LI-800 (LI-COR Biosciences, Lincoln, NE) non-diffused infrared CO_2 analyzer. Data were logged every 10 min on a connected personal computer.

Manure C/N ratio Manure sample was dried at 40°C for 48 h in an MIR-253 refrigerated incubator (Sanyo Electric Co., Ltd., Osaka, Japan), smashed with a ZM100 centrifugal mill (Retsch GmbH, Haan, Germany), and analyzed with an NC-220F automatic C/N analyzer (Sumika Chemical Analysis Service, Osaka, Japan). Humidity was measured as follows. The manure sample was dried at 105°C in an electric oven (Hirasawa Works, Tokyo, Japan) and weighed every hour. The weight was confirmed to have reached dry weight when its difference from the previously determined weight is 0.1%. Humidity was calculated as the difference between wet and dry weights.

Komatsuna sprouting test Fifty Komatsuna seeds were distributed on 12.5 cm size filter paper set in a glass dish. One hundred mL of boiled water was added to 10 g of manure sample and the mixture was stirred. After cooling to room temperature, the mixture was passed through 2-ply gauze and 10 mL of the filtrate was poured into the glass dish. The dish was covered with a glass lid and set in a light incubator (FLI-301NH, Tokyo Rika, Tokyo, Japan) at 23°C, 4000 lx, for 96 h. After that, root length was measured with a caliper. A control sample that contained no manure sample was also subjected to the same treatment conditions at the same time. The results were expressed as the ratio of root length to control. All tests were performed in duplicate and the results are the average of two tests. The Komatsuna sprouting test is widely used to judge manure maturity in Japan. Chanyasak et al. (14) also performed the test.