Colegio de Postgraduados

Sanitary analysis of compost derived from canine origin: pilot study

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ABSTRACT

Objective: To evaluate the sanitary quality of compost derived from canine origin through parasitological, bacteriological, biochemical, and molecular analyses, focusing on the detection of enteropathogens relevant to public health.

Design/methodology/approach: Samples of compost derived from canine origin underwent parasitological, bacteriological, biochemical, and molecular analyses to detect enteropathogens such as *Escherichia coli* (ETEC) and *Salmonella* spp. Additionally, 16S rRNA gene sequencing was performed for species-level identification.

Results: No parasite structures were found, and molecular tests for *E. coli* and *Salmonella* spp. were negative. However, colonies suggestive of Salmonella arizonae were isolated by the research team. Subsequent 16S rRNA sequencing analysis revealed homology with *Citrobacter arsenatis*, a recently described bacterium.

Limitations on study/implications: The study was limited to a single compost source and focused on a few bacterial pathogens reported in canines (*Escherichia coli* y *Salmonella* spp.); broader microbiome analysis and pathogenicity assessments are recommended. The findings underline the need for rigorous characterization before the agricultural application of animal-origin compost.

Findings/conclusions: Compost derived from canine origin does not appear to pose a significant public health risk due to the presence of enteropathogens such as *Escherichia coli* (ETEC) and *Salmonella*, according to our analyses, although the pathogenicity of *Citrobacter arsenatis* is unknown. However, as it is compost derived from dog carcasses, it may contain opportunistic or pathogenic microorganisms of sanitary importance. Thus, its use is recommended for ornamental plants, lawn, trees.

Keywords: Carcasses, Composting, Public Health, Zoonosis.

INTRODUCTION

Composting is a biological process that takes place under aerobic conditions, in which microorganisms carry out the biotransformation of organic matter through the biogeochemical cycles of carbon and nitrogen. The process includes three stages of temperature that result from microbial metabolism: a) Mesophilic I, b) Thermophilic (up to 60 °C), and c) Cooling or Mesophilic II, in addition to a stage of maturation of variable duration (Román, 2013). As a result, a biological product whose microbiological

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complexity is related to biofertilizing activities is obtained. Composting constitutes one of the best alternatives for treatment and valuation of organic urban solid residues (Pérez, 2019).

Animal residues in Mexico have scarce specific regulations in relation to compost elaboration (Rodríguez and Córdova, 2006). In livestock production, mortality composting is an alternative for the management of animal residues, following a locally adapted methodology, with an emphasis on cost savings, reduction of environmental risks, and generation of potential biofertilizers (Bass and Lupis, 2010). Regarding the remains of companion animals, there has been an increase in private incineration services or those offered by public institutions (PROFECO, 2022), although there are scenarios such as shelters or animal control centers where there is a lack of equipment or elevated costs, generating a problem for the safe elimination of biological residues, for which composting could be an alternative.

Although aerobic composts harbor high microbiological diversity, including microorganisms with effects that promote plant growth and biological control of plant diseases (Martínez-Yáñez *et al.*, 2023), in the case of compost of animal origin, the presence of microorganisms with potential risks for public health is likely. Therefore, it is necessary to determine the sanitary quality of animal-based composts (Bass and Lupis, 2010). In this context, thermo-tolerant coliforms are a group of Gram-negative bacteria that belong to enterobacteria with pathogenic potential. According to the United States Environmental Protection Agency, a high level of thermotolerant coliforms is an indicator of the likely presence of pathogenic bacteria such as enterotoxigenic *Salmonella*, *Shigella*, *Leptospira*, *Listeria* and *E. coli*, which cause public health problems (Román, 2013).

Escherichia coli, Gram-negative facultative anaerobic bacillus, is a microorganism that is part of the normal microbiota of canines, and can become an important nosocomial pathogen (Alberca, 2017).

Salmonella, Gram-negative facultative anaerobic bacillus, non-sporulated, generally mobile by peritrichous flagella, is a microorganism found in asymptomatic carrier dogs of both antimicrobial-resistant and potentially pathogenic *Salmonella* strains. Dogs can harbor a large bacterial load in their intestines and mesenteric lymphatic ganglia, which can be eliminated through feces, with possibility of transmission to humans (Usmael *et al.*, 2022).

Another opportunistic nosocomial pathogen is *Citrobacter* spp., Gram-negative facultative anaerobic bacteria, which does not form spores and is rod-forming, reported in intestinal tracts of animals and humans; it is commonly associated with infections of various organs in adults and neonates (Jabeen *et al.*, 2023).

In this study, the hypothesis is set out that compost elaborated from canine carcasses will contain enteropathogenic microorganisms with zoonotic potential. With this purpose, the objectives were to identify parasites and enterobacteria of sanitary importance through isolation methods, biochemical characterization, and molecular confirmation. This analysis is relevant given the absence of specific regulations for the handling of animal carcasses such as dogs and cats, and the need for environmentally safe alternatives and socially viable for their disposal.

MATERIALS AND METHODS

Compost

Humus from compost elaborated with the technique based on the model by Rodríguez and Córdova (2006) was received, where a 3-month-old canine carcass, sawdust (product from sterilized wood, 11 kg), plant carbon ash (5 kg), zeolite of volcanic origin (1 kg), plant residues (2.5 kg), and water (8 L), were used in a 56 cm by 36 cm drum-type plastic recipient, with aeration holes and temperature monitoring. No clinical analysis or previous treatments were carried out with the live animal. The compost was monitored for 35 days, performing the first turning at 15 days and then once per week. During the thermophilic stage, 60 °C and 60% relative humidity were recorded. Personal protection gear was used both for the elaboration and while turning the compost.

Molecular analysis: DNA extraction from composts and molecular detection of thermotolerant enterobacteria

For the DNA extraction of the compost sample by triplicate, the DNeasy Powersoil Kit (QIAGEN Cat. No. 47014) was used, following the instructions from the manufacturer. For the molecular detection of thermotolerant enterobacteria, *Escherichia coli* pathotype ETEC (López-Saucedo, 2003) and *Salmonella* sp. (Liu, 2002), the pairs of oligonucleotides and PCR conditions described in Table 1 were used.

The cycling program for PCR was 5 min at 94 °C initial denaturalization, followed by 35 cycles for 45 s at 94 °C denaturalization, 45 s at the alignment temperature for each fragment and 90 s at 72 °C for polymerization.

The amplification of PCR fragments was verified through electrophoresis in agarose gel at 1% with TAE 1X buffer (Tris Base 4.84 g/L; Acetic acid 11.4 ml/L; EDTA 7.44 g/L). The gels were stained with ethidium bromide (1 μg/1ml) and visualized with UV light with a photo-documenter device (Labnet).

Table 1. Oligonucleotides used for PCR for the molecular detection of *E. coli* pathotype ETEC and *Salmonella* sp.

Enterobacteria	Gene	Oligonucleotide sequence	Tm	Expected product (bp)	Reference
E. coli ETEC H10407	lt	F: GGCGACAGATTATACCGTGC R: CGGTCTCTATATTCCCTGTT	55 °C	450	López-Saucedo, 2003
	st	F: ATTTTTCTTTCTGTATTGTCTT R: CACCCGGTACAAGCAGGATT	48 °C	190	
Salmonella spp.	invA	invA1: CTGTTGAACAACCCATTTGT invA2: CGGATCTCATTAATCAACAAT	57.4 °C		Liu, 2002
Universal	16S rRNA	F27: AGAGTTTGATCMTGGCTCAG R1492: TACGGYTACCTTGTTACGACTT	55 °C	1500	Heuer *et al.*, 1997

Bacterial culture

Isolation and identification of Salmonella

For the isolation and identification of *Salmonella*, the protocols established by norm NOM-210-SSA1-2014 "Microbiological testing methods: determination of indicator microorganisms and determination of pathogenic microorganisms" were used. A brief dilution of 1:10 of the compost sample in peptone water was made, and then it was passed to Rappaport Vassiliadis Soya (RVS) broth and incubated at 37 °C. Then, it was plated in Salmonella-Shigella and brilliant green agars and incubated at 37 °C. Bacterial colonies with characteristic growth suggestive of *Salmonella* spp. were subjected to short biochemistry analysis, using the TSI tests, urea, citrate and SIM tests.

Molecular identification of colonies with characteristics suggestive of thermotolerant enterobacteria

DNA extraction from isolated colonies suggestive of *Salmonella* spp. was carried out with a lysis buffer based on 50 mM Tris base, 50 mM EDTA and 3% sodium dodecyl sulfate (SDS). It was done by preparing a bacterial culture in 3 ml of King's B liquid medium incubated 24 hours at 30 °C; the cells were harvested by centrifugation for 3 minutes at 10,000 rpm and the supernatant was discarded. It was suspended in 500 μl of lysis buffer (Tris base 50 mM, EDTA 50 mM, 3% SDS) and incubated in a water bath for 40 minutes at 65 °C. Then, 500 μl of phenol chloroform isoamyl alcohol was added and mixed by inversion. It was centrifuged at 10,000 rpm for 10 minutes, the aqueous (superior) phase was separated, and it was transferred to another tube. Then, 100 μl of sodium acetate 3M was added and mixed by inversion; 1 ml of absolute ethanol was added and incubated in ice for 25 minutes. It was centrifuged for 20 minutes at 10,000 rpm, the ethanol was eliminated, and the pellet was washed with 400 μl ethanol at 70%. It was centrifuged for 5 minutes at 10,000, the ethanol was eliminated, and it was left to dry at 37 °C. It was suspended in 40 μl of TE water and stored at 20 °C. To amplify the fragment of 1500 pb of the 16S rRNA of the isolated colonies, the oligonucleotides F27 and R1492 were used with the PCR software mentioned before (Table 1). The PCR products of the expected size were purified with the commercial kit DNA Clean & Concentrator (Zymo Research), according to the manufacturer's indications and were sent for sequencing at the National Laboratory of Agricultural, Medical and Environmental Biotechnology (Laboratorio Nacional de Biotecnología Agrícola Medica y Ambiental, LANBAMA, IPICyT). The sequences obtained were analyzed taxonomically through BLASTn (Basic Local Aligment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Parasitological analysis

To conduct the parasitological study, a modified sedimentation/floating technique was used with sodium nitrate solution ($NaNO_3$) with 1.25 spg. (Raissi *et al.*, 2020). The samples were analyzed by duplicate, with 10 grams of sieved humus (fine fraction) (Raissi *et al.*, 2020). As positive control, substrate and concentrated canine feces positive to parasites such as *Toxocara* sp., *Dipylidium* sp., *Ancylostoma* sp. and *Trichuris* sp., were used. After performing the technique, the samples were observed with the microscope to carry out the analysis.

RESULTS AND DISCUSSION

Molecular analysis

Detection of *E. coli* ETEC and *Salmonella* sp., through amplification by PCR of gene fragments it and invA, respectively, had negative results. In this regard, it is likely that populations of the microorganisms mentioned are reduced and, therefore, detection by conventional PCR in a complex matrix such as composts cannot be achieved (Chen *et al.*, 2023; Prosser *et al.*, 2007).

Bacteriological analysis

Given the likely reduced population density of *E. coli* ETEC and *Salmonella* sp., the isolation *Salmonella* spp. was carried out, following specific protocols. This was done because composts have biofertilizer applications in agricultural crops and vegetables that could be susceptible to contamination by these pathogens and transmitted to humans through consumption, thus risking the safety of those foods (Sharma *et al.*, 2016).

After incubation of the enriched and diluted compost sample, it was observed that in the Salmonella-Shigella agar, 2 colonies grew with morphology suggestive of *Salmonella*.

Short biochemistry was performed on those colonies, which showed the following results: TSI reaction 4, negative citrate, negative urea, negative SIM to indole and positive to motility and production of H_2S, which, when compared with identification tables, matched with *Salmonella arizonae* although the citrate was negative (Table 2).

However, bacteria from genus *Citrobacter* present high genotypical similarities with *Salmonella* which is why it is frequent for them to be identified incorrectly when differential biochemical tests are used (Pławińska-Czarnak *et al.*, 2021).

Bioinformatic analysis of 16S rRNA sequences from isolated strains

Knowing that short biochemistry presents limitations for identification (Pławińska-Czarnak *et al.*, 2021), the 1500 pb fragment of 16S RNAr gene from the colonies identified as *S. arizonae* was amplified by PCR. Sequencing of both 16S RNAr fragments revealed an identity of 74.73 and 93.3% with *Citrobacter arsenatis* (Table 3).

Taxonomically, the *Citrobacter* genus is closely related to *Salmonella* spp. and *Escherichia coli* (Katzenellenbogen *et al.*, 2017; Schoch *et al.*, 2020). Based on antigen O of the liposaccharide (LPS), a total of 43 serogroups O of *Citrobacter* are recognized, and a total of 20 chemical groups have also been classified according to the sugar composition of their lipopolysaccharides (LPS) and 19 genomospecies of *Citrobacter* based on the DNA relationship (Jabeen *et al.*, 2023). This is probably why similarity was found at the

Table 2. Biochemical tests used.

Bacteria	TSI	Gas in glucose	H_2S	Indole/ motility	Citrate	Urea
Salmonella arizonae	A/A	d	+	−/+	+	−
Citrobacter spp.	A/A	d	+	−/+	+	+
Isolated bacteria	A/A	+	+	−/+	−	−

TSI: triple sugar iron, A/A: acid/acid, d: different reactions (Barrow, 1993).

Table 3. Sequencing results of the 16S rRNA region.

Sample	BLASTn	Evalue	Cover	Identity	Accession
Grey strain grown in McConkey cultivation medium	*Citrobacter arsenatis* strain LY-1 16S ribosomal RNA, partial sequence.	5e-85	81%	74.73%	NR_180353.1
Black strain growin in Salmonella-Shigella cultivation medium	*Citrobacter arsenatis strain* LY-1 16S ribosomal RNA, partial sequence.	1e-157	93%	93.33%	NR_180353.1

level of culture and short biochemistry with Salmonella, although with negative results in the PCR.

Parasitical identification

No structures suggesting canine parasites were found in the compost sample, although mites, mite eggs, and free-living nematode eggs were found. The free-living mites, although not taxonomically identified, were very similar to *Klinckowstroemia bifurcata*, which are commonly found associated to environments in or under decomposing logs and soil (Villegas-Guzmán *et al.*, 2012), considering that materials such as sawdust and plant residues were used which could transport them.

There are reports that indicate that to inactivate helminth eggs, it is recommended to raise the temperature to >40 °C, decrease moisture <5% or keep both for a period of time (Escobar *et al.*, 2014); and from temperatures of 60 °C for 30 to 60 minutes, eggs of parasites such as *Trichuris* sp., *Strongyloides* spp., *Trichostrongylus* sp. and *Haemonchus* sp. are inactivated, among others (Mahmud *et al.*, 2016). This suggests that, through the thermal and biological composting process, potentially zoonotic pathogens in different animal species are eliminated (canines, Nemiroff and Patterson, 2007; bovine and human, Mahmud *et al.*, 2016; human and birds, Manga *et al.*, 2016).

However, no parasitological analyses were conducted of the individual used in the composting process, so the presence or absence of common parasites in puppies cannot be assured.

The composting process applied to dead animals is an alternative whose popularity is increasing and it is a viable alternative; it has been shown that there is low risk of environmental contamination when using pig carcasses. In addition, the composting technique reduces the risk of transmission of external animal diseases to production (Pepin *et al.*, 2021). Because of this, the compost elaborated based on dog carcasses could be an alternative to use to eliminate these organic residues; however, in our country there is a lack of guidelines and regulations concerning the risks and use that must be considered and avoided in composting of carcasses. Likewise, the carcasses used for compost can imply a risk of contamination of soil and water, because of their organic composition and the potentially pathogenic agents that present what would represent an impact on the environment (Vithanage, 2021).

In this study, bacterial colonies that showed high similarity at the level of 16S RNAr sequence with *Citrobacter arsenate* were isolated from the sample of compost from dog carcasses. This bacterium is a microorganism of recent isolation in freshwater sediments in China, which presents the ability to reduce arsenate to arsenite (Wang *et al.*, 2021). Likewise, the *Citrobacter* genus has been described as a bacteria susceptible of acquiring resistance genes through horizontal transference, in equines in Europe and birds in Asia and Nigeria (Anyanwu *et al.*, 2022; Anyanwu *et al.*, 2023). Because it is a species of recent identification, there are no reports of association with pathologies in humans. However, the *Citrobacter* genus is associated to pathologies in humans as opportunist and is frequently found in various environments including the soil (Wang *et al.*, 2021), and because of this we cannot dismiss the likely pathogenicity of this new species.

Compost elaborated from carcasses of different animals has been used as a safe environmental method to eliminate the carcasses (Pepin *et al.*, 2021; Guan *et al.*, 2010), and it has even been observed that there is a low risk of contamination of air or underground water by important viruses in the pork industry (Pepin *et al.*, 2021). Although no important parasites or bacteria for public health were identified in this study in the compost from dog carcass, it should be highlighted that future metagenomic and metabolome studies are required, to assess the behavior of the microbial communities in the different states of the compost. Something to underline is that various metagenomic studies in composts made from pig, ruminant and bird manure have suggested the presence of bacterial genes involved in resistance to antibiotics, and therefore, inherent risks of transmission and proliferation of those phenotypes in the ecosystem (Zhang *et al.*, 2023; Fan *et al.*, 2023; Zhao *et al.*, 2024). In this regard, the thermophilic step during the composting process significantly reduces the risks of antibiotic resistance transfer, as a result of the low population density of the communities that harbor them and the dynamic interactions with other microbial communities (Werner, 2022; Eckstrom and Barlow, 2019).

In addition to researching the taxonomic and metabolomic profiles of the microbial communities that develop after the application of compost to the soil (Meneghine, 2017) to assess whether it can be used to grow vegetables, the proposed approaches aim to ensure the lowest possible risk for public, livestock and environmental health.

CONCLUSIONS

Bacterial and molecular analyses indicate that compost derived from canine origin presents low sanitary risk regarding enteropathogens such as *E. coli* ETEC and *Salmonella*. However, the detection of *Citrobacter arsenati*, whose pathogenic relevance is still not well-established, points to the need for additional studies that include more specific methods and a greater number of samples.

Evidence suggests that this compost could be viable for agricultural uses not destined for human consumption, although it is necessary to evaluate its safety in various contexts of application. Finally, these results highlight the convenience of advancing towards specific normative criteria for composts derived from companion animals, to ensure their safe handling and use.

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