Insights into the Microbiological Safety of Vermicompost and Vermicompost Tea Produced by South African Smallholder Farmers

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Abstract Feedstock used in the production of vermicompost (VC) and vermicompost tea (VCT) may harbour various pathogenic bacteria responsible for a number of animal and human diseases worldwide. The identification and characterisation of such pathogenic organisms is necessary for assessing the safety of these products. In the present study, our goal was to determine the presence of possible pathogens in VC and VCT and, if present, to determine the antimicrobial susceptibility profiles of the organisms. VC and VCT samples were collected from five

different farms in the Winterveldt, South Africa. Only one out of 60 VC and VCT samples was found to contain a potentially pathogenic organism. The use of phenotypic procedures aided the final identification of the isolate, which was confirmed to be *Salmonella enterica* serovar Typhimurium. This isolate tested positive for species specific *invA* genes. Antibiotic testing using the agar diffusion technique showed that the *Salmonella* isolate was resistant to only kanamycin. The *Salmonella* counts that were observed in this study were lower than the generally accepted infective doses of these bacteria. In the light of these findings, it was concluded that VC and VCT produced by the farmers involved presented a low risk in terms of the safety of the products.

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Introduction

Vermicompost (VC) is a composted material providing adequate drainage, good aeration, porosity, water-holding capability, microbial activity and a variety of nutrient substances when worked into the soil [1]. The watery extract obtained from VC is known as vermicompost tea (VCT). Vermicompost tea is an aqueous extract of VC, which is used on soil as a soil drench and for its favourable effect on plants [2]. The nutrients and the large populations of beneficial microbes contained in the VC and VCT can be useful as a supplement for chemical fertilisers [1, 2]. The materials used in the production of VC and VCT are generally waste products from a wide range of industrial, municipal and agricultural sources amongst others [1, 3]. As a result of the variety of feedstock materials utilised in

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the process of vermicomposting, various bacteria are present in abundance [3]. These bacteria are mostly beneficial. For instance, some bacterial that possess special attributes that aid in the mobilisation of important nutrients in the soil [4] could be found in both VC and VCT [1, 3]. In spite of the presence of large numbers of beneficial microbes, pathogenic microorganisms could also be present [3]. It is therefore important to detect the presence of foodborne pathogens in VC and VCT in order to determine the extent of risk associated with the usage of these materials [5]. In addition, a secondary concern about the presence of pathogenic microorganisms in VC and VCT products is the antibiotic resistance of pathogens that might occur. Hence, it is important not only to isolate and identify potential pathogens associated with these products but also to investigate the antibiotic resistance of such pathogens. To ascertain the microbiological safety of VC and VCT, a robust approach is needed which should include isolation, characterisation and antimicrobial resistance of potential pathogens.

Different techniques have been developed over the years for the identification and characterisation of foodborne pathogens [6], which are usually viruses, fungi and/or bacteria. Bacteria are the major foodborne pathogenic organisms that are commonly encountered [6]. General examples of such bacterial pathogens include *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium perfringens*, *Clostridium botulinum*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes* and *Yersinia enterocolitica* [7]. Standard microbiological methods are used to identify and detect these bacteria. Such methods are broadly classified into culture-based and culture-independent techniques [8]. These two approaches have been widely utilised in the detection of such microorganisms in substrates such as vermicompost and vermicompost tea.

Resistance to antimicrobial drugs by bacterial pathogens is a global phenomenon and at the same time a growing concern in the effective management of bacterial infections [9]. The antibiotic resistance (AMR) phenomenon in pathogenic bacteria is increasing, partly due to the administration of large quantities of antibiotics to food-producing animals with a resultant increase in cases of serious bacterial infections, high mortality rates, morbidity and treatment failure [10]. Common examples of foodborne pathogens exhibiting increasing resistance to antimicrobials are *Salmonella* spp. and *C. jejuni* [11]. These two bacterial types constitute a great public health risk and are responsible for salmonellosis and campylobacteriosis respectively [11, 12]. Resistance or susceptibility to a particular antibiotic by pathogenic bacteria can be determined using various techniques such as dilution methods, the disk diffusion method, automated

antimicrobial susceptibility testing systems, mechanism-specific tests and genotypic methods. In this study, the Kirby–Bauer disc diffusion method was used to determine antimicrobial resistance [13].

Ensuring the microbiological safety of VC and VCT from processing to application is essential because there is an increasing demand on the utilisation of such products and the well-being of the users is essential. Only limited information concerning the microbiological safety of VC and VCT produced in the Winterveldt region of South Africa is available. In addition, information relating to the antimicrobial resistance of potentially pathogenic foodborne isolates obtained from VC and VCT in Winterveldt is virtually non-existent. The present study was aimed at providing insights into microbiological safety of typical low-cost VC and VCT being produced by smallholder farmers with a focus on the Winterveldt region of South Africa. The purpose of this study was, consequently, to determine the presence of possible pathogens in VC and VCT and, if present, to determine the antimicrobial susceptibility profiles of the organisms.

Materials and Methods

Study Area and Sample Collection

Vermicompost producing farms located in the Winterveldt region, north-west of Pretoria, South Africa were used for this study. Winterveldt falls under the Tshwane Metropolitan Municipality. Its geographical coordinates are 25°25'4"S, 28°1'50"E and it is surrounded by many townships (South African term used to describe underdeveloped and segregated urban settlements) such as Mabopane and Soshanguve among others [14]. In this region, the bin method of vermicomposting is employed in small-scale productions. The bin method involves the use of rectangular bins that are constructed from aluminium and have a lid to shield the worms from the sunlight. The heights of these bins is 40 cm and the production is outdoors. The bottom of the bin is perforated for the drainage of the vermicompost tea and other holes are introduced on the side of the bin for aeration. Different kinds of waste are utilised for the production of VC and VCT by farmers in Winterveldt, including municipal waste, animal waste and plant waste (Mlokotwa-Dube, Diphokos, Mahlangu, Tlhapane and Moela, 2014—Personal communication). Thirty VC and VCT samples were collected in duplicate in both summer and winter seasons from five vermicomposting farms. These included 2, 6, 2, 2 and 4 samples collected in summer from Farms 1, 2, 3, 4 and 5 respectively (16 samples for VC and 16 for VCT in duplicates). In addition, for winter, 2, 6, 2, 2 and 2 samples were collected from

Farms 1, 2, 3, 4 and 5 respectively (14 samples for VC and 14 for VCT in duplicates). Vermicompost samples weighing approximately 500 g from each farm were placed in sterile Ziploc plastic bags, while approximately 500 mL of VCT were collected in sterile containers. All samples were transported on ice to the Feed and Food Analysis Laboratory, Bacteriology section of the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) within 24 h of collection and stored at 4 °C until processing. Altogether 60 samples were collected for the study, comprising 30 VC and 30 VCT samples in both summer and winter seasons.

Isolation of Potentially Pathogenic Bacteria Using Classical Microbiological Techniques

The media and reagents used for classical microbiological techniques are shown in Table 1. Detection of *Salmonella* spp. was done in three different steps according to ISO 6579 standard guidelines with slight modifications. In the first step, pre-enrichment entailed transferring 1 g of VC and 1 mL of VCT into 9 mL of buffered peptone water and incubating at 37 °C for 18 h. In the second step, 1 mL of pre-enriched culture was transferred into 10 mL of Rappaport–Vassiliadis Soy (RVS) broth and incubated at 42 °C for 24 h. In addition, 1 mL of pre-enriched culture was transferred to 10 mL of Muller-Kauffmann Tetrathionate-Novobiocin (MKTn) broth and incubated at 37 °C for

24 h. In the third step, the latter two selective enrichment broths were streaked onto Xylose Lysine Desoxycholate (XLD) and Brilliant Green Agars (BGA), followed by incubation at 37 °C for 24 h. Positive and negative control organisms were always included with each batch of samples (Table 2). After incubation, the agar plates were evaluated for the presence of typical *Salmonella* colonies. The typical *Salmonella* colonies on XLD agar appeared as pinkish-red colonies with a black centre. Typical presumptive *Salmonella* appeared as reddish/pink colonies on BGA agar.

In attempting to isolate *Escherichia coli* O157:H7, the immunomagnetic concentration technique was employed to increase the probability of isolating these bacteria. For the selective enrichment of *E. coli* O157:H7, 1 g of VC and 1 mL of VCT were transferred separately into different bottles containing 10 mL of Modified Tryptone-Soya broth supplemented with 20 mg/L Novobiocin and incubated at 42 °C for 24 h. Dynabeads pre-coated with anti-O157 antibodies were used in an attempt to concentrate any cells of *E. coli* O157:H7 that may have been present. Selective isolation entailed the subculturing of the enriched cultures onto Tellurite Cefixime-Sorbitol MacConkey agar (CT-SMAC) and incubating at 37 °C for 24 h. Positive control organism was included with each batch of samples. After incubation, the agar plates were scrutinised for the presence of typical colourless or smoky brown colonies of *E. coli* O157:H7.

For *Y. enterocolitica* enrichment, 1 g of VC and 1 mL of VCT were transferred separately into different bottles containing 9 mL of buffered peptone water and incubated at 37 °C for 24 h. For selective isolation, the enriched cultures were streaked onto Cefsulodin–Irgasan–Novobiocin (CIN) agar supplemented with 20 mg/L of *Yersinia* supplement and incubated at 37 °C for 24 h. Positive and negative control organisms were included with each batch of samples. After incubation, the agar plates were evaluated for the presence of typical *Y. enterocolitica* colonies. On CIN agar, these appear as ‘bull’s eye’ colonies having a deep-red centre with a transparent surrounding zone.

For the selective enrichment of *Shigella* spp., 1 g of VC and 1 mL of VCT were transferred separately into different bottles containing 10 mL of Selenite F broth and incubated at 37 °C for 24 h. For selective isolation, the enriched cultures were streaked onto MacConkey (MAC) agar and incubated at 37 °C for 24 h. A negative control organism was included with each batch of samples. After incubation, the agar plates were scrutinised for the presence of typical (colourless or clear) *Shigella* colonies.

For *C. perfringens* selective enrichment, 1 g of VC and 1 mL of VCT were transferred separately into different bottles containing 10 mL of Robertson’s cooked meat broth and incubated anaerobically at 37 °C for 48 h. For

Table 1 Bacteriological media and reagents utilised in the study

Medium used	Source
STEC enrichment broth	Quantum Biotechnologies
Sorbitol-MacConkey agar supplemented with Cefixime and tellurite (CT-SMAC)	
Buffered peptone water	Quantum Biotechnologies/Feed and Food Laboratory (ARC-OVI)
Rappaport–Vassiliadis broth	
Muller-Kauffmann Tetrathionate-Novobiocin (MKTn)	
Xylose Lysine Deoxycholate agar	
Brilliant green agar	
Selenite F broth	Quantum Biotechnologies
MacConkey (MAC) agar	
Buffered peptone water	Quantum Biotechnologies
Cefsulodin–Irgasan–Novobiocin (CIN) agar	
Robertson’s cooked meat broth	Feed and Food Laboratory (ARC-OVI)
Mannitol Egg Yolk Polymyxin (MYP) agar	
TSC <i>Perfringens</i> agar base	
Buffered peptone water	Quantum Biotechnologies
MacConkey (MAC) agar	

Table 2 List of control organisms used in the study

Organism	Positive control	Negative control
<i>Salmonella</i> spp.	<i>Salmonella typhimurium</i> 14028	<i>Escherichia coli</i> O157:H7 ATCC 43888
<i>Escherichia coli</i> O157:H7	<i>Escherichia coli</i> O157:H7 ATCC 43888	<i>Staphylococcus aureus</i> ATCC 25923
<i>Yersinia enterocolitica</i>	<i>Yersinia enterocolitica</i> ATCC 27729	<i>Salmonella typhimurium</i> 14028
<i>Shigella</i> spp.	–	<i>Staphylococcus aureus</i> ATCC 25923
<i>Clostridium perfringens</i>	<i>Clostridium perfringens</i> ATCC 13124	<i>Escherichia coli</i> O157:H7 ATCC 43888
<i>Klebsiella</i> spp.	<i>Klebsiella pneumonia</i> in-house reference	–

selective isolation, the enriched cultures were streaked onto TSC Perfringens Selective agar with a Perfringens TSC Selective supplement (D-cycloserine) and incubated anaerobically at 37 °C for 24–48 h. Positive and negative control organisms were always included with each batch of samples. After incubation, the agar plates were scrutinised for the presence of typical (black coloured) *C. perfringens* colonies.

For *Klebsiella* spp. enrichment, 1 g of VC and 1 mL of VCT were transferred separately into different bottles containing 10 mL of Buffered peptone water and incubated at 37 °C for 24 h. For selective isolation, the enriched cultures were streaked onto Oxoid MacConkey (MAC) agar and incubated at 37 °C for 24 h. Positive control organism was included with each batch of samples. After incubation, the agar plates were evaluated for the presence of typical large, moist, shiny, mucoid and pink coloured *Klebsiella* colonies.

Phenotypic Characterisation of the Potentially Pathogenic Isolates

Preliminary tests for the identification of potential members of the *Enterobacteriaceae* family included the Gram stain as well as oxidase and catalase tests. More specific tests for the identification of the *Salmonella* isolate included lactose fermentation, H₂S production and mode of fermentation in triple sugar iron agar slants. Additional tests included urease and lysine decarboxylase production, motility, and fermentation of dulcitol.

For possible *E. coli* O157:H7, the differentiating tests included the determination of cellular motility, indole production from tryptone broth, citrate utilisation and also the methyl red and Voges Proskauer tests.

For *Y. enterocolitica*, differentiating tests included the lactose fermentation, and the production of H₂S, lysine decarboxylase and urease.

For *Shigella* spp., differentiating tests included the production of H₂S, urease and lysine decarboxylase l.

Differentiating tests for *Klebsiella* spp. included lactose and dulcitol fermentation, H₂S, indole and urease

production, citrate and malonate utilisation, and determination of the Voges Proskauer and Methyl Red reactions.

Confirmation tests for potential isolates of *C. perfringens* included the Gram reaction, oxidase, and catalase production, as well as lactose, lecithinase and indole production and the fermentation of sucrose.

Determination of the *Salmonella* Serotype

The isolated *Salmonella* strain was referred to the Bacteriology Laboratory of Agricultural Research Council–Onderstepoort Veterinary Institute for serotyping. This was performed using the slide agglutination technique with O, H and Vi antigen antisera (BD Diagnostics Systems, Woodmead, Gauteng, South Africa). The results were interpreted according to the Le Minor Kauffmann–White scheme [14].

Determination of the *Salmonella* Viable Counts

For microbial counts of *Salmonella*, 10 g of VC were aseptically added to 90 mL of Ringer's solution (Quantum Biotechnologies, Johannesburg, South Africa) followed by thorough mixing. Serial dilutions (10⁻¹–10⁻⁵) were made in Ringer's solutions. Of the diluted samples, 100 µL volumes were streaked onto XLD, followed by incubation at 37 °C for 24–48 h. The presumptive *Salmonella* count was done according to ISO methods (ISO 4833-1:2013). The *Salmonella* colonies were confirmed as described in the section on biochemical identification. The counts were reported as colony forming units per gram (cfu/g).

Molecular Identification of Pure Cultures of the presumptive *Salmonella* Isolate

DNA Extraction of the presumptive *Salmonella* Isolate

Prior to the DNA extraction, a pure culture of the isolate was sub-cultured on Nutrient agar. Genomic DNA was extracted from the sub-cultured isolate using Zymo extraction kit (Quick gDNATM Miniprep) (Inqaba Biotechnology, Pretoria, South Africa). DNA purity and

quantity of the extract were determined using a spectrophotometer (ND-1000; Nanodrop technologies, Inc, USA). The DNA extracts were analysed twice to minimise quantitative error. Tubes containing the extracted DNA templates were stored at -20°C prior to downstream processing.

Detection of the invA Gene in the Salmonella Isolate by Conventional Polymerase Chain Reaction (PCR)

The total genomic DNA was used as template in PCR amplification, targeting the *invA* gene, which is known to be specific for *Salmonella* (Table 3). The final volume of the PCR reaction mixture was 25 μL and consisted of the following: 12.5 μL of 2 $\times$ PCR master mix (0.2 mMol/L dNTP, 2.5 mMol/L MgCl_2 , 1 μL of Taq DNA polymerase, 1 μL of PCR buffer) (Inqaba Biotechnology, Pretoria, South Africa), 1.0 μL forward and reverse primers each (Inqaba Biotechnology, Pretoria, South Africa) (Table 3), 2 μL of genomic DNA as template and 8.5 μL molecular grade water. A negative control containing nuclease free water (Fermentas Life Sciences, USA) and a positive control containing a DNA template from *Salmonella* Typhimurium ATCC 14028 were included in the amplification process. PCR was performed with a thermal cycler (Bio-Rad, USA) under the conditions that are shown in Table 4. The PCR products were analysed on 1% agarose gel (Seakem® L E Agarose) stained with ethidium bromide in 1 $\times$ TAE electrophoresis buffer at 60 V for 60 min. A 100 bp DNA ladder (Fermentas Life Sciences, USA; Inqaba Biotechnology, Pretoria, South Africa) was included as molecular size marker when running the gel. After electrophoresis, the PCR fragments were visualised using a Gel Doc™ UV transilluminator (Bio-Rad, USA; Inqaba Biotechnology, Pretoria, South Africa).

Multiplex Polymerase Chain Reaction Screening Analysis for Detection of C. botulinum Toxino-Types A, B, E, and F

For the detection of the presence of possible *C. botulinum* toxinotypes, DNA was extracted directly from VC and VCT samples using Zymo extraction kit (Quick gDNA™ Miniprep) (Inqaba Biotechnology, Pretoria, South Africa). DNA purity and quantity of each extract was determined

using gel electrophoresis and a spectrophotometer (ND-1000; Nanodrop technologies, Inc, USA). Tubes containing the extracted DNA templates were stored at -20°C prior to downstream processing. The extracted DNA was used in multiplex PCR for the determination of A, B, E and F toxinotypes of *C. botulinum*, using four pairs of primers (Inqaba Biotechnology, Pretoria, South Africa). The primer set used was based on its suitability for the detection of a gene regulating the production of botulinum neurotoxin toxinotypes (BoNT) [15, 16] as shown in Table 5.

The final volume of the PCR reaction mixture was 25 μL and consisted of the following; 12.5 μL of 2 $\times$ PCR master mix (0.2 mMol/L dNTP, 2.5 mMol/L MgCl_2 , 1 μL of Taq DNA polymerase, 1 μL of PCR buffer) (Thermo Fisher Scientific, USA; Inqaba Biotechnology, Pretoria, South Africa). Four sets of primers were used, 0.5 μL forward and reverse primers each (Inqaba Biotechnology, Pretoria, South Africa) (Table 5), 2 μL of genomics DNA as template and 6.5 μL molecular grade water. A negative control consisting of nuclease free water (Fermentas Life Sciences, USA; Inqaba Biotechnology, Pretoria, South Africa) and a positive control (field strain) respectively were included in the amplification.

PCR was performed with a thermal cycler (Bio-Rad, USA) under the following conditions, an initial denaturation step at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 53°C for 30 s, and at 72°C for 1 min. The final extension was carried out at 72°C for 7 min. The PCR products were analysed on 1% agarose gel (Seakem® L E Agarose) stained with ethidium bromide in 1 $\times$ TAE electrophoresis buffer at 100 V for 100 min. A 100 bp DNA ladder (Fermentas Life Sciences; Inqaba Biotechnology, Pretoria, South Africa) was included as molecular size marker. After electrophoresis, the PCR fragments were visualised using a Gel Doc™ UV transilluminator (Bio-Rad, USA).

Duplex Polymerase Chain Reaction Screening Analysis for Detection of C. botulinum Toxinotypes C and D

For determination of the possible presence of C and D toxinotypes of *C. botulinum*, two sets of primers were used in duplex PCR as shown in Table 6. The primer sets were utilised because they facilitate the identification of the gene

Table 3 Specific primers used for detection of the *invA* gene in the *Salmonella* isolate from vermicompost and vermicompost tea

Potential pathogen	Primer	Primer sequence	Product size (bp)	References
<i>Salmonella</i> isolate	<i>InvA</i>	F: 5'-GTGAAATTATCGCCACGTTCTGGGCAA-3' R: 5'-TCATCGCACCGTCAAAGGAACC-3'	284	[23, 24]

Table 4 PCR thermal cycling conditions used in the study

Potential pathogen	PCR thermal cycling conditions	No. of cycles	References
<i>Salmonella</i> isolate	Initial denaturation: 95 °C at 3 min	30	[23, 24]
	Denaturation: 94 °C at 30 s		
	Annealing: 56 °C at 30 s		
	Extension: 72 °C at 30 s		
	Final extension: 72 °C at 5 min		

Table 5 Primers utilised for the Multiplex PCR for the determination of A, B, E and F *Clostridium botulinum* toxinotypes from vermicompost and vermicompost tea samples

Toxinotype	Primer	Sequence	Amplicon size (bp)	References
A	IA_03_F	5'-GGGCCTAGAGGTAGCGTARTG-3'	101	[26, 27]
	IA_03_R	5'-TCTTYATTTCCAGAAGCATATTTT-3'		
B	CBMLB1	5'-CAGGAGAAGTGGAGCGAAAA-3'	205	[25, 27]
	CBMLB2	5'-CTTGCGCCTTTGTTTCTTG-3'		
E	CBMLE1	5'-CCAAGATTTTCATCCGCCTA-3'	389	[25, 27]
	CBMLE2	5'-GCTATTGATCCAAAACGGTGA-3'		
F	CBMLF1	5'-CGGCTTCATTAGAGAACGGA-3'	543	[25, 27]
	CBMLF2	5'-TAACTCCCCTAGCCCCGTAT-3'		

region, determining the creation of the light chain in *botulinum* neurotoxin (BoNT) [15, 16].

The final volume of the PCR reaction mixture was 25 µL and consisted of the following: 12.5 µL of 2× PCR master mix (0.2 mMol/L dNTP, 2.5 mMol/L MgCl₂, 1 µL of Taq DNA polymerase, 1 µL of PCR buffer) (Thermo Fisher Scientific, USA; Inqaba Biotechnology, Pretoria, South Africa). Two sets of duplex PCR primers were utilised, 0.5 µL forward and reverse primers each (Inqaba Biotechnology, Pretoria, South Africa) (Table 6), 2 µL of genomics DNA as template and 8.5 µL molecular grade water. A negative control consisting of nuclease free water (Fermentas Life Sciences, USA; Inqaba Biotechnology, Pretoria, South Africa) and a positive control (field strain) respectively were included in the amplification. PCR was performed with a thermal cycler (Bio-Rad, USA) under the following conditions: an initial denaturation step at 95 °C for 5 min, with the reaction subsequently being subjected to 35 cycles at 95 °C for 30 s, at 53 °C for 30 s, and at 72 °C for 1 min. The final extension was carried out at 72 °C for 7 min. The PCR products were analysed on 1% agarose gel (Seakem® L E Agarose) stained with ethidium bromide in 1xTAE electrophoresis buffer at 100 V for 100 min. A 100 bp DNA ladder (Fermentas Life Sciences;

Inqaba Biotechnology, Pretoria, South Africa) was included as molecular size marker. After electrophoresis, the PCR fragments were visualised using a Gel Doc™ UV transilluminator (Bio-Rad, USA; Inqaba Biotechnology, Pretoria, South Africa).

Antimicrobial Resistance and Susceptibility Profiles

Preparation of Mueller–Hinton Agar

According to the manufacturer's instruction, 38 g of Mueller–Hinton agar was added to 1000 mL of distilled water and autoclaved at 121 °C for 15 min. After cooling, the agar (20 mL) was poured into sterile 90 mm petri dishes and allowed to set at ambient temperature prior to use.

Inoculum Preparation

An overnight culture of the *Salmonella* isolate was inoculated into Nutrient broth and vortexed. The turbidity was standardised to that of a 0.5 McFarland standard (this was verified using a spectrophotometer). The analysis was done according to CLSI (2015) guidelines.

Table 6 Primers utilised for the Duplex PCR for the determination of C and D *Clostridium botulinum* toxinotypes from vermicompost and vermicompost tea samples

Toxinotype	Primer	Sequence	Amplicon size (bp)	References
C	CS-11	5'-ATACACTAGCTAATGAGCCTG-3'	290	[28, 27]
	CS-22	5'-TGGAGTATTGTTATTTCCAGG-3'		
D	DS-11	5'-GTGATCCTGTGAATGACAATG-3'	497	[28, 27]
	DS-22	5'-TCCTTGCAATGTAAGGGATGC-3'		

Choice of Antimicrobials

Filter paper discs with several types of antimicrobials (Oxoid, England) were utilised to evaluate the antibiotic susceptibility profiles of *Salmonella* isolate. The antibiotics selected were the types most often used for effective treatment of Gram-negative *Enterobacteriaceae* infections in humans and animals [17]. The antimicrobial susceptibility profiles were determined according to the CLS of January 2015, formerly known as the National Committee for Clinical Laboratory Standards (NCCLS).

Antimicrobial Susceptibility Profiles

Cotton swabs were immersed in the standardised bacterial suspension (inoculum) and then rotated against the inner surface of the test tube to express the excess fluid. The entire surface of the Mueller–Hinton agar plate was streaked three times with the moistened cotton swab. The Mueller–Hinton agar plates were rotated between each streaking, in order to obtain even distribution of the bacterial suspension (inoculum). The streaked agar plates were allowed to dry for 5 min for the absorption of moisture. The antibiotic impregnated disks, (using an Oxoid Disc dispenser) were applied to the surface of streaked agar plates. The disks contained the following antibiotics: Ampicillin (10 µg), Cefotaxime (30 µg), Enrofloxacin (5 µg), Florfenicol (30 µg), Kanamycin (30 µg), Oxytetracycline (30 µg) and Trimethoprim (5 µg). Subsequently, the streaked agar plates were inverted and incubated at 37 °C for 24 h. After 24 h of incubation, the agar plates were examined for a zone of inhibition around the antibiotic disks. The width of the zone of inhibition was measured using Vernier callipers. The results were interpreted using the criteria recommended in the CLS (2015) guidelines.

Results

Classical Microbiological Analysis

The targeted organisms included *Salmonella* spp., *Shigella* spp., *E. coli* O157:H7, *Y. enterocolitica*, *Klebsiella* spp., *C. botulinum* and *C. perfringens*. Only one of the sixty samples (30 VC and 30 VCT) tested yielded a potentially pathogenic isolate. The isolate was identified as a *Salmonella* strain. No other potentially pathogenic isolates were detected in any other VC sample or in any of the VCT samples.

Phenotypic Characterisations, Serotyping and Viable Counts of the *Salmonella* Strain

The phenotypic characteristics of the *Salmonella* strain are shown in Table 7 as compared to a *Salmonella* type strain

Salmonella serovar Typhimurium ATCC 14028. Serotyping assigned the *Salmonella* isolate to *Salmonella enterica* serovar Typhimurium.

Evaluations of the counts of potential pathogens are significant in the assessment of the safety and suitability of VC samples. The counts of the *S. enterica* serovar Typhimurium in the sample from Farm 4 were < 10 cfu/mL.

DNA Extraction and Detection of *invA* Species-Specific Genes of the *Salmonella*

The *Salmonella* isolate (strain VC-28) from Farm 4 was subsequently subjected to molecular analysis to determine the presence of species-specific *invA* genes in the isolate. In this analysis the quantified DNA was pure and of good quality. For the *Salmonella* spp. isolate, the PCR results showed an expected amplicon size of 284 bp.

Screening Analysis for Toxino-Types in *C. botulinum*

The *Clostridium botulinum* screening analysis performed using Multiplex and Duplex PCR techniques for the identification of the toxin genes did not show the presence of any of the toxinotypes tested for in the VC or VCT samples.

Antibiotic Susceptibility

The positive identification of *Salmonella* spp. by biochemical and molecular techniques proved that potential pathogens can occur in VC, albeit in one sample of VC and in very low numbers. The *Salmonella* strain was subjected to antibiotic susceptibility testing. The resistance profile of the *Salmonella* isolate (strain VC-28) against a wide range of antimicrobial agents is shown in Table 8.

Discussion

In this study, vermicompost and vermicompost tea samples were screened for a range of potentially pathogenic bacteria. The targeted organisms included *Salmonella* spp., *Shigella* spp., *Escherichia coli* O157:H7, *Y. enterocolitica*, *Klebsiella* spp., *C. botulinum* and *C. perfringens*. These bacteria were selected because they have been reported to be involved in various foodborne diseases worldwide. In addition, the selected bacteria may occur in the feedstock materials utilised in vermicomposting. The present results are in agreement with a previous finding [18], which reported that VC and its derivatives may contain pathogenic *Enterobacteriaceae* genera, including *Salmonella* spp. Other potential pathogens that have been encountered include *Staphylococcus aureus* and a variety of other

Table 7 Morphological, physiological and biochemical characteristics of a single *Salmonella* strain isolated from a vermicompost sample

Identification	Tests											
	Morphological	Physiological		Biochemical								
		Ox ^b	Cat ^c	Ind ^d	Urea ^e	Mal ^f	Dulc ^g	H ₂ S ^h	Sor ⁱ	Lac ^j	Dcc ^k	Ldc ^l
<i>Salmonella</i> spp. isolate												
VC-28	–	–	+	–	–	–	+	+	+	–	–	+
ATCC 14028	–	–	+	–	–	–	–	+	+	–	–	+

+, positive; –, negative

^aGram staining^bOxidase^cCatalase^dIndole production^eUrease^fMaltose^gDulcitol^hH₂S (TSI)ⁱSorbitol^jLactose^kDecarboxylase control^lLysine Decarboxylase**Table 8** Antimicrobial susceptibility of *Salmonella* isolate VC-28 to several antibiotics and minimum inhibitory concentration (MIC) breakpoints

Antibiotic	MIC (µg/ml) breakpoints			<i>Salmonella</i> isolate VC-28		
	Resistant (R)	Susceptible (S)	Intermediate (I)	R	S	I
Ampicillin (AMP)	≤ 13	≥ 17	14–16	–	–	+
Cefotaxime (CTX)	≤ 14	≥ 23	15–22	–	+	–
Enrofloxacin (ENR)	≤ 16	≥ 23	17–22	–	+	–
Florfenicol (FFC)	≤ 14	≥ 19	15–18	–	+	–
Kanamycin (K)	≤ 13	≥ 18	14–17	+	–	–
Oxytetracycline (OT)	≤ 14	≥ 19	15–18	–	+	–
Trimethoprim (W)	≤ 10	≥ 16	11–15	–	+	–

R resistant, S susceptible, I intermediate, + positive, – negative

possible pathogens. The fact that only one potentially pathogenic bacterial strain was found in 60 samples (30 VC and 30 VCT) analysed in the present study may be ascribed to the effective removal of such organisms by the earthworms involved during vermicomposting [19]. One of the reasons may be the elimination of these organisms by the earthworms through secretion of antibiotic-like substances, which destroy any pathogenic bacteria or make them non-viable and unable to multiply any further [20]. Sinha et al. [21] reported that earthworms normally consume and digest microorganisms such as fungi, bacteria and protozoa present in feedstock waste in their habitat. In addition, it is possible that the original feedstock materials were generally not contaminated with the pathogenic bacteria tested for. Alternatively, the population of potentially pathogenic bacteria in VC could have been reduced by the

vermicomposting process [1, 6]. The isolation of the single *Salmonella* isolate could be due to contamination of the specific wastes used in vermicomposting in farm 4. The waste materials including plant/vegetable waste, cattle manure and municipal wastes such as paper sludge were used at this farm and could have been a source of this microorganism.

The result for *Salmonella* spp. counts in this study suggests that fewer cells than the estimated infectious dose of *Salmonella* were present. Taking the relatively high infectious dose into consideration, the low counts (< 10 organisms) of *S. enterica* serovar Typhimurium obtained in the VC sample in this study would not be expected to constitute a significant health risk. A report by Kothary and Babu [22] stated that the infectious dose for the various serovars of *Salmonella* was relatively large (10⁵–10¹⁰

organisms). It has been demonstrated that the diseases caused by *Salmonella* are most frequently related to the intake of commonly consumed foods in which bacteria have undergone considerable multiplication before ingestion [12]. The fact that this organism was not consistently found in the VC of the farm in question in subsequent samplings showed that the contamination from whatever source was not repeated.

The determination of the presence of toxinotypes in any *Clostridium* spp. is an important technique in the characterisation of the organism [23]. All the Multiplex and Duplex PCR results were negative for the toxinotypes A, B, E and F, as well as for toxinotypes C and D of *C. botulinum*. The absence of these toxinotypes in *C. botulinum* is a good indicator of the quality of the VC and VCT samples studied based on the fact that the toxinotypes are responsible for botulism [17].

Monitoring antimicrobial resistance tendencies among foodborne bacteria isolated from VC is important, in order to gain knowledge of the possible resistance of such a pathogen to antimicrobial agents. If multiple drug resistance is found to occur in such isolates from VC, it could imply that they could be transmitted to humans through soil amended with VC. The antibiotics selected were the types mostly used for effective treatment of Gram-negative *Enterobacteriaceae* infections in humans and animals [24]. A study by Zhang et al. [25] stated that high prevalence of antibiotic resistance is common among *Enterobacteriaceae* isolated from sludge waste used in vermicomposting. The tested bacterial isolate was, however, susceptible to most antibiotics tested, namely CFX, ENR, FFC and OT (see Table 8). It was intermediately resistant to AMP and resistant only to K. The occurrence of intrinsic resistance to kanamycin is generally extremely high in members of the *Enterobacteriaceae* family [26]. The effects of intermediate resistance to a specific antibiotic by the tested bacterial strain should not be overlooked. An organism is intermediately resistant to a particular antibiotic, when the tested organism's MICs exceeds or falls within the dose level of the antibiotics that is normally prescribed [27]. The implication of an intermediate resistance is that it can lead to ineffective application of the drug or to the administering of a higher dosage.

Conclusion

In conclusion, the results presented in this study suggest that the vermicompost and vermicompost tea produced by farmers in the Winterveldt region, constituted a low risk in terms of the safety of the products. This is based on the finding that only one out of 60 VC and VCT samples were found to contain a potentially pathogenic organism, namely

S. enterica serovar Typhimurium. The counts of the *Salmonella* in the vermicompost from the specific farm were less than 10 cfu/g, which was much lower than the reported infective doses (10^5 – 10^{10} organisms) for these bacteria. Antibiotic testing using the agar diffusion technique, showed that the *Salmonella* isolate was resistant to only kanamycin. It may be concluded from these results that the VC and VCT products produced by the farmers in the Winterveldt region could be regarded as safe for their use as fertilisers. However, farmers should be vigilant because there is always the possibility of zoonotic foodborne pathogens occurring in some batches. The vermicomposting process, however, seems to play a large role in the relative safety of these products.

It would nevertheless be interesting to do further research on the prevalence of potential pathogens in the waste materials used by the farmers in the vermicomposting process and to compare these findings with those from vermicomposting products such as VC and VCT. This could assist in developing a protocol to eliminate unsuitable waste materials. It could also highlight the important role played by the vermicomposting process in reducing pathogens and acting as a source of beneficial bacteria. These bacteria could enrich the plant rhizosphere and enhance the growth and development of vegetables and other food plants above and beyond the physical and chemical contribution of VC and VCT as fertilisers.

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