Global Journal of Medical Research: K
Interdisciplinary
Volume 22 Issue 3 Version 1.0 Year 2022
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

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GJMR-K Classification: DDC Code: 398.2109481 LCC Code: PZ8.A89

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Dispersion of Hazardous Bacteria

Katsuji Watanabe

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Core ideas

- Higher ratio of composts for organic farmers included multidrug resistant bacteria (MRB).
- Higher ratio of composts for organic farmers included fecal bacteria and pathogenic bacteria.
- Numbers of MRB and thermotolerant bacteria in composts varied in reverse trend.
- MRB in composts might be eliminated by controlling an abiotic factor.
- The used method was effective to evaluate MRB and fecal bacteria in compost.

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I. Introduction

At present, almost all farmers have been encouraged to spread organic fertilizer into field soil under a government policy to improve soil fertility and maintain field conditions (FAO 2018; Rayne and Aula 2020). The spreading of organic fertilizer into field soils has also been encouraged to reduce and to recycle organic wastes under social demand to construct sustainable societies in all over the world (Chatterjee et al., 2017, Sharma et al.,2019). As organic fertilizer originates from raw livestock feces which includes pathogenic bacteria (Gerba & Smith 2005) or multidrug resistant bacteria (MRB) (Agga et al., 2015; Loofta et al., 2012), organic fertilizer application has a possibility to enhance contamination of such the hazardous bacteria to various environments (Smith et al.,2019; Watanabe 2008, 2009;Watanabe et al.,2008; Watanabe and Koga 2009; Watanabe et al., 2015a) and foods (Hölzel et al., 2018; Marti et al., 2013; Zekar et al., 2017; Zhang et al., 2019). Although fecal bacteria might be reduced during composting process, huge amount of organic fertilizer, which is made from livestock feces by various ways and includes various amount of such the hazardous bacteria, has annually been dispersed onto field soils without checking. In order to reduce their contamination to various environments and food, an appropriate method to check such hazardous bacteria in organic fertilizer is required before their application on field soil (Watanabe 2008, 2009; Watanabe et al., 2008; Watanabe and Koga 2009).

Whereas with respect to MBR, conventional surveillance method targeting specific nosocomial bacteria was not suitable, because the susceptibility tests and taxonomy determinations of isolates should be expanded broadly over various kinds of bacterial groups (Burgos et al., 2005; DebMandal et al., 2011; Ghosh. & LaPara 2007; Kilonzo-Nthenge et al., 2013; Oliver et al., 2020; Sawant et al., 2007, Yang et al., 2016; Young quist et al., 2016). Furthermore, un-culture-based molecular methods such as quantitative polymerase chain reaction (qPCR) and next generation sequencing (NGS) was also not suitable. Because the antibiotic resistant genes (ARGs) targeted by these methods were not intrinsic

virulence genes, such as the Vero toxin gene (Kudo et al., 2007), or the Shiga toxin gene (Parsons et al., 2016), which could differentiate pathogenic bacteria from the other harmless bacteria, but harmless genes, which were widely distributed into indigenous bacteria in natural environments (D'Costaet al.,2011; Nesme et al., 2014), and into natural mammalian intestines (Stanton et al.2011; Zhang et al., 2011) before the modern selective pressure of clinical antibiotic use (D'Costa et al.,2011), and the hazard level of the samples could not be evaluated by the detection of ARGs.

MRB groups in the sample were found to be rapidly identified and quantified by analyzing the bacteria that proliferated under antibiotics (Watanabe et al., 2016). In this manuscript, each MRB group included in compost had been identified by multiple enzyme restriction fragment length polymorphism (MERFLP) (Watanabe et al., 2008; Watanabe & Koga 2009) and quantified by the most probable number method by using an originally developed method (Watanabe et al., 2015a, 2015b, 2016).

The author had explored MRB and fecal bacteria in nine composts that originated from diverse livestock feces and had annually been applied on soils of organic farms in various regions of Japan. The purposes of this experiment were 1) to speculate how widely MRBs and the other fecal bacteria had spread into the field soil of Japanese organic farms through compost application, 2) to know what kinds of MRB and fecal bacteria had introduced into field soil though compost application, and 3) to find out a way to reduce MRB and fecal bacteria during composting process. In order to speculate composting conditions of the tested composts, which might had affected the residual MRB and fecal bacteria, the composition and numbers of numerically dominant bacteria were also searched.

II. Materials and Methods

a) Samples

The nine tested composts had been used on organic farms in various regions of Japan. Compost A, which was a marketable good originating from chicken droppings, has been applied on organic farm A in Nagano Prefecture in the Chubu region, where organic rice has been cultivated. Compost B, which was handmade from chicken droppings, has been applied on organic farm B in Niigata Prefecture in the Hokuriku region, where organic rice has been cultivated. Compost BB was a so-called "Bokashi-compost", which was a handmade from several kinds of organic waste through fermentation, and has also been applied on farm B, where organic rice has been cultivated. Compost C, which was a marketable good originating from pig feces, has been applied on organic farm C in Chiba Prefecture in the Kanto region, where organic vegetables have been cultivated. Compost D, which was a marketable good originating from pig feces, has been applied on organic farm D in Ibaraki Prefecture in the Kanto region, where organic vegetable has been cultivated. Compost E, which was a marketable good made from cattle feces, has been applied on organic farm E in Fukushima Prefecture in the Tohoku region, where organic vegetables have been cultivated. Compost F1, F2, and F3, which were made from cattle feces by the National Agricultural Research Center for the Kyushu-Okinawa region in Kumamoto Prefecture in the Kyushu region, have been applied on experimental fields in the research center, where vegetables and rice have been cultivated. Although there was not such a large difference in the composting process among these three composts (Watanabe et al., 2015b), recycled paper was added to adjust the moisture content (60%) of the starting material in the composting process of compost F1, rice straw was added for composting to compost F2, and wood chips were added to compost F3.

b) MPN and used antibiotics

For analysis of general bacteria (B), serial 10-fold dilutions (10-8 to 10-12) prepared from samples (1g fresh wt.) were inoculated to centrifuge tubes (5 replicates) including an LB medium. After 5 days of incubation at 30℃, the bacterial DNA in each tube was extracted as described previously and purified by conventional methods (Watanabe et al., 2015a, 2015b). For analysis of MRB (M), the following antibiotics were simultaneously added to the LB medium: streptomycin (25 mgl-1), chloramphenicol (25 mgl-1), and ampicillin (25 mgl-1). Serial 10-fold dilutions (10-4 to 10-7) prepared from samples (1g fresh wt.) were inoculated to centrifuge tubes (5 replicates) including an LB medium and the antibiotics. As the MRB detected by the method was bacteria that proliferated under a mixture of 25 ppm each of three antibiotics, they had higher resistance to those detected by conventional susceptibility tests such as the disk diffusion test, where the resistance of each antibiotic was separately tested. Until now, the MRB had been exceptionally detected in limited samples only, such as livestock feces, composts (Watanabe et al., 2016), feces applied to field soils, activated sludges, a few fresh meats, river water, and fresh vegetables (Watanabe unpublished results).

c) MERFLP of the amplified 16S rDNA

Using the V2 forward primer (41f), and the V6 reverse primer (1066r)(Weidner et al., 1996), 16S rDNA was amplified, as described previously (Watanabe et al., 2008). Their restriction fragment lengths were measured by microchip electrophoresis systems (MCE-202 MultiNA; Shimadzu Co., Ltd. Kyoto Japan) after digestion of the PCR product (10μl) using each restriction enzyme, HaeIII or Hhal or Rsa I (10 units, Takara Bio Co. Ltd. Shiga Japan) in a buffer solution (10xLow salt buffer, Takara Bio Co. Ltd.) and 5 folds

dilution by de-ionized water, as described previously (Watanabe et al., 2015b, 2016).

d) Reference database used for the phylogenetic estimation

The reference database used for this research included 30,844 post-amplification sequence files of 16S rDNA amplified by 41f/1066r primers (Watanabe et al., 2016), which were mainly re-edited from small subunit rRNA files in the Ribosomal Database Project (RDP) II release 9_61 (Cole et al., 2007) under 5-bases mismatches in both primer annealing sites, and consisted of 1,379 bacterial genera, including uncultured and unidentified bacteria (Watanabe et al., 2016). From post-amplification sequence files, fragment size for each restriction enzyme was calculated and save in the restriction fragment database and used for similarity search as described previously (Watanabe et al., 2008; Watanabe and Koga 2009).

e) Data processing to select homogenous 16S rDNA and phylogenetic estimation

For precise phylogenetic estimation by MERFL, the measured MERFL originating from homogeneous 16S rDNAs had to be selected among the mixed MERFLs by data processing (Watanabe et al., 2015a, 2015b). Because all the reference MERFLs were calculated from the homogeneous 16S rDNA sequence in the RDP II database, while the measured MERFL was obtained by restriction digestions of a mixture of 16S rDNAs, which were amplified using DNAs from different bacteria in each MPN tube as described previously (Watanabe et al., 2015a, 2015b). The selected restriction fragments (RFs) with the highest relative mole concentrations (ratio of fluorescent intensity to fragment size) was summed up until to leach the 16S rDNA size before restriction digestion, which was treated as the major RFLP (represented as H in Table S1 and S2) originated from a the major homogenous 16S rDNA in a MPN vial. The 2nd major RFs (represented as M in Table S1 and S2), and the 3rd major RFs (represented as L in Table S1 and S2) were similarly selected as described in the former manuscript (Watanabe et al., 2015a, 2015b).

If the completely identical theoretical MERFL was not found out by using all the measured MERFL data, combinations of restriction enzymes used for the analysis was changed (Table 1, and Table 2) (Watanabe et al., 2015a, 2015b). Because measured RFs with near DNA length could not always be separated by electrophoresis, which resulted in lower similarity in similarity search for RFLP (Watanabe et al., 2015a, 2015b). As to the measured MERFL which had not completely identical theoretical MERFL, the theoretical MERFL having the highest similarity to the measured MERFL was indicated in Table 1 and Table 2.

f) Enumeration of antibiotic resistant bacterial groups by MPN

Based on the results of phylogenetic estimation, each 16S rDNA was differentiated into the following 12 groups: Actinobacteria (A), Bacillus group (bF), Staphylococcus sp. (sF), other Firmicutes (F), Sphingomonadaceae (sP), other α-Proteobacteria (aP), β-Proteobacteria (bP), γ-Proteobacteria (rP), δ-Proteobacteria (dP), ε-Proteobacteria (eP), Cytophaga (C), other bacteria (O), and unidentified or uncultured bacterial (U), as shown in Table S1 and Table S2. By using MPN score for each groups (Table 1 and Table 2) and a table for a five-tube and three-decimal-dilution experiment (Blodgett 2010), the MPN of each bacterial group and MRB group were estimated (Table 1, 2). Using the FDA's Bacterial Analytical Manual (Blodgett 2010), confidence limits were obtained and shown in Table 1 and Table 2.

III. Results and Discussion

a) Phylogenetic estimation and enumeration of general bacteria

There was a large difference in the total bacteria numbers included in the tested composts (from 7.08x109 MNP g-1 dry matter to 316.2x109 MNP g-1) (Table 1), which were higher than those of the reported numbers by plate count (Rebollido et al., 2008; Vishan et al., 2014). Although there was no report of a bacterial number by the culture-independent method (Schloss et al., 2005), the higher bacterial numbers by the method might be caused by included unculturable bacteria (Watanabe et al., 2015b).

In composts originating from chicken droppings and pig feces (compost A, B, BB, C, and D), the major bacteria were gram-positive bacterial groups, such as Actinobacteria and Firmicutes, which occupied 79.7% to 98.3% of the total bacterial number when unidentified bacterial numbers were subtracted (Table 1, and Figure 1). Extremely high numbers of total bacteria in compost D (316.2x109 MNP g-1) are attributed to the higher number of Staphylococcus sp. (297x109 MNP/g), where Staphylococcus aureus occupied most of them (95.7%) (Table 1). Staphylococcus sp. was also the numerically dominant bacteria in compost BB (55.2x109 MNP g-1), which occupied 67.9% of the total bacterial number (86.03 x109 MNP g-1) (Table 1, Figure 1). The higher number of total bacteria in compost C (146.4x x109 MNP g-1) is attributed to the number of spore-forming bacteria group, such as Bacillus sp.(21.0 x109 MNP), Paenibacillus sp., and Clostiridium sp. (43.6 x109 MNP g-1; 65.6%) (Table 1 and S1). The number of spore-forming bacteria was also higher in compost B (25.7 x109 MNP g-1), which occupied 65.8% of the total bacterial number (41.2 x109 MNP g-1) (Table 1, Figure 1). As our former results about bacterial compositional changes during each composting process indicated

that the ratio of Bacillus groups increased to 54.4% and the bacterial number decreased after the thermophilic phase (Watanabe et al., 2015b), which were similar to those of the other reports (Cahyani et al., 2003; Partanen et al., 2010; Rebollido et al., 2008; Sasaki et al., 2009; Schloss et al., 2005; Yamamoto et al., 2009), the higher ratio of gram-positive bacterial groups seemed be caused by a higher survival ratio of relatively thermotolerant gram-positive bacterial groups during the thermophilic phase (Roman et al.,2015).

In contrast, there was no numerically dominant bacterial group in composts originating from cattle feces (E, F1, F2, and F3), and the ratios of gram-positive bacterial groups became lower (22.5% to 57.4%) than those of the former (A, B, BB, C, and D) (Table 1, Figure 1). This difference might be caused from the lower maximum temperature attained during the thermophilic phase, which was not enough to eliminate fecal bacteria and increase thermotolerant bacterial groups (Roman et al.,2015). As compost F1, F2, and F3 was made from the same cattle feces with the same composting process, differences in bacterial composition were caused from the difference in thermophilic condition, which was resulted from difference in starting condition as described in Material and Method (Table 1, Figure 1). As typical fecal bacteria and pathogenic bacteria was detected in the tested composts, such as Clostridium perfringens (C.perfri50, C.perfring, CP000246 ,M59103), Fusobacterium nucleatum (Fus.nuclea, AE009951, AJ133496) or F.sunuae (Fus.simiae) in compost B, Clostridium botulinum (L37585, L37587, C.botulin6), Mycoplasma salivarium (M.salivari), and Prevotellaoris (L16474) or Bacteroides eggerthii (L16485) in compost BB, Bacteroides sp. (AY008308), Clostridium butyricum (AY442812, C.butyric2, C.butyric3, C.butyric4), and Fusobacterium sp. (AF287805, AF385575, AF432130) in compost C, Ehrlichiasp. (Ehr.ris081, Ehr.risKEN, Her.ristic, M73225) or E.sennetsu (M73225), and Leptonema illini (Lpn.illini,Z21632) in compost D, Bordetella sp. (DQ132877) and Fusobacterium nucleatum (Fus.nuclea) in compost E, and Fusobacterium nucleatum (Fus.nuclea) and Parachlamydia sp. (AF366365, AJ715410) or Spirillum winogradskii (AY845251) in compost F3 (Table S1), these composts were indicated to include bacteria of fecal origin. As typical fecal bacteria such as Fusobacterium sp., Borrelia anserine, and Leptospira fainei, were also detected in the former studies (Watanabe et al.2015b), the fecal bacteria and pathogenic bacteria were not always completely eliminated during the composting process, as reported in other results (Brinton et al., 2009; Reynnells et al., 2014).

As typical fecal bacteria such as Mycoplasma sualvi (M. sualvi), Prevotellanuminicola (AB003401), P. oralis (L16480), and Spiroplasma sp. (M24662, Spp.cit2HP, Spp.poulsn) had been detected in paddy field soil annually applied with compost (Watanabe et al.,2015a), compost application was suggested to disperse fecal bacteria originating from livestock to field soil.

b) Phylogenetic estimation and enumeration of MRB

In the composts originating from chicken droppings and pig feces (compost A, B, BB, C, and D), compost C included a considerable number of MRB (1.0 x104MPN g-1), and compost D included a higher number of MRB (84.9 x104MPN g-1) (Table 2). The composts originating from cattle feces (compost E, F1, F2, and F3) included MRB from 43.2 x104MPN to 84.9 x104MPN g-1 (Table 2). There was a large difference between the composition of general bacteria and that of MRB, where the gram-positive bacterial group was not numerically dominant (Figure 1, and 2). Uncultured Sphigomonadaceae (AF408325) was the numerically dominant MRB in compost E (67.1x x104MPN) and various Sphnigomonas sp. were the numerically dominant MRB in compost F1 (Table 2, and Figure 2). As composts F1, F2, and F3 were made from the same cattle feces under the same composting process, the compositional difference of MRB among the composts was suggested to be caused from a slight difference in starting conditions (Table 2, Figure 2).

As typical fecal bacteria, such as Bacteroides coprocola (AB200223, AB200225, AB200225) and Borrelia recurrent is(AF107356, U42300), were detected as MRB in compost E, and Bacteroides bacterium (AY162121) was detected in compost F2 (Table 2S), these composts were indicated to include MRB of fecal origin.

The present data indicated that most composts used by organic farmers in various region of Japan not only included MRB but also pathogenic bacteria of livestock origin. The present results were enough to promote awareness that these hazardous bacteria might contaminate fresh vegetables from field soils, as suggested by the other reports (Watanabe 2008, 2009; Watanabe et al.,2015a; Yong et al., 2016).As elimination of contaminated hazardous bacteria from field soils was difficult (Watanabe 2008, 2009; Watanabe et al.,2015a), their existence in compost had to be checked before spreading into field soil. For this purpose, the method used in this manuscript was found to be suitable.

IV. Conclusions

Composting is a biological aerobic decomposition process of biomaterials consisting of two different phases: first, the thermophilic phase and next, the maturing phase (Misra et al., 2003; Roman et al., 2015). First, in the thermophilic phase, microbiological degradation of easily degradable biomaterial in feces elevated the temperatures and diminished the moisture content, while fecal bacteria in livestock feces were eliminated and only thermotolerant bacterial groups

survived (Cahyani et al., 2003; Partanen et al., 2010; Rebollido et al., 2008; Roman et al., 2015; Sasaki et al., 2009; Schloss et al., 2005; Yamamoto et al., 2009). However, the attained maximum temperature and reduction of moisture content were varied depend on starting conditions and air supply during this phase (Roman et al., 2015), which would affect numbers of residual fecal bacteria.

Significant positive correlation of the total bacterial number (2) with those of the gram-positive bacterial group (3) ($R=0.976$, $P<0.001$ $n=9$, Table 3) suggested that variation in number of thermotolerant gram-positive bacteria caused the major bacterial difference among the tested 9 composts, which might be mainly affected by a conditional difference in thermophilic phase and could be used as an index to speculate the condition of the thermophilic phase for each compost.

As the ratio of the gram-positive bacterial number to the total bacterial number (4) had significant negative correlation to those of gram-negative MRB (6) ($r=-0.723, <0.01$, $n=9$, Table 3), those of the MRB of Sphingomonadacea (7) ($r=-0.687, <0.05$, $n=9$), and those of the MRB of the other α-Proteobacteria (8)($r=-0.901, <0.001$, $n=9$), numbers of most ofthe MRB in the composts varied inreversal trend against that of the gram-positive bacterial group (Oliver et al.,2020; Sharma et al., 2009; Wang et al.,2015; Youngquist et al., 2016). As the ratio of gram-positive bacteria would become higher by an effective thermophilic phase, where a higher maximum temperature and lower moisture content was attained (Misra et al., 2003; Roman et al.,2015), MRB might be reduced by the same abiotic factors during thermophilic phase.

Moisture content (1), which had decreased by an effective thermophilic phase (Misra et al., 2003; Roman et al., 2015), had positive correlations with the ratios of MRBs ((5)-(8) from $r=0.384$ to $r=0.791$; Table 3). Significant positive correlation between moisture content (1) and the ratio of gram-negative MRB (7) ($R=0.791$, $P<0.05$, Table 3) suggested that moisture content might be a critical factor to eliminate gram-negative MRB during a thermophilic phase. The elimination of MRB by controlling the composting process will be presented in the next manuscript.

Acknowledgments

Part of this research was achieved in the Research Team for Biomass Recycling System, in the National Agriculture and Food Research Organization of Japan, supported by a grant under the theme of "Analyses of heavy metal and antibiotic resistant bacteria in composts," funded by Japanese Ministry of Agriculture, Forestry and Fisheries from April, 2008 to March, 2010. The author thanks the organic farmers in various regions of Japan for sending the composts used in this experiment. Thank is also given to Mrs. K. Matsuoka for supporting the experiments. The other part of this research was achieved in Department of Life, Environment and Applied Chemistry, Fukuoka Institute of Technology (FIT) supported by a grant (2010) under the theme of "Development of rapid analysis method for microorganisms in aim to contribute environmental protection and recycling biological resources," funded by FIT, a grant (2014) under the theme of "Development of new method for identification and quantification of bacteria using microchip electrophoresis" funded by the Japan Science and Technology Agency, and a grant (2018) under the theme of "Practical realization of the method for fact-finding survey of antibiotic resistant bacteria in environment and foods" funded by the economic council for the Kyushu region. The author thank Mr.PatrickSulsar in the Office of International Education in FIT for corrections of grammatical errors in this manuscript.

Supplemental Material

Supplemental Table 1 shows the phylogenetic estimations of general bacteria in each dilution vial, whose DNA was extracted after the incubation of diluted samples in an LB medium.

Abbreviations: ARG antibiotic resistant gene; MERFLP multiple enzyme restriction fragment length polymorphism; MPN most provable number; MRB multidrug resistant bacteria; NGS next generation sequencing; qPCR quantitative polymerase chain reaction; RDP the Ribosomal Database Project.

References Références Referencias

1. Agga, G.E., T.M. Arthur, L.M. Durso, D.M. Harhay, & J.W. Schmidt. 2015. Antimicrobial-Resistant Bacterial Populations and Antimicrobial Resistance Genes Obtained from Environments Impacted by Livestock and Municipal Waste. PLoS ONE. 10: e0132586. doi:10.1371/journal.pone.0132586.
2. Blodgett, R. 2010. FDA, Bacterial Analytical Manual, Appendix 2: Most Probable Number from Serial Dilutions. http://www.fda.gov/Food/FoodScience Research/LaboratoryMethods/ucm109656.htm/
3. Brinton Jr, W.F., P. Storms, & T.C. Blewett. 2009. Occurrence and Levels of Fecal Indicators and Pathogenic Bacteria in Market-Ready Recycled Organic Matter Composts. Journal of Food Protection. 72: 332–339.doi: 10.4315/0362-028x-72.2.332.
4. Burgos, J.M., B.A. Ellington, & M.F. Varela. 2005. Presence of Multidrug Resistant Enteric Bacteria in Dairy Farm Topsoil. Journal of Dairy Science. 88: 1391-1398. http://dx.doi.org/10.3168/jds.S0022-0302(05)72806-X.
5. Cahyani, V.R., K. Matsuya, S. Asakawa, & M. Kimura. 2003. Succession and Phylogenetic Composition of Bacterial Communities Responsible

for the Composting Process of Rice Straw Estimated by PCR-DGGE Analysis. Soil Science and Plant Nutrition. 49: 619-630. http://dx.doi.org/10.1080/00380768.2003.10410052.
6. Chatterjee, R, S. Gajjela, & R.K. Thirumdasu. 2017. Recycling of Organic Wastes for Sustainable Soil Health and Crop Growth. International Journal Waste. Resources. 7:3 doi: 10.4172/2252-5211.1000296.
7. Cole, J.R., B. Chai, R.J. Farris, Q.Wang, A.S. Kulam-Syed-Mohideen, D.M. McGarrell, A.M. Bandela, E. Cardenas, G.M. Garrity, &J.M.Tiedje. 2007. The Ribosomal Database Project (RDP-II): Introducing myRDP Space and Quality Controlled Public Data. Nucleic Acids Research. 35: D169-D172. http://dx.doi.org/10.1093/nar/gkl889.
8. D'Costa, V.M., C.E. King, L. Kalan, M. Morar, W.W.L. Sung, C. Schwarz, D. Froese, G. Zazula, F. Calmels, R. Debruyne, G.B. Golding, H.N. Poinar, & W.D. Gerard. 2 0 1 1. Antibiotic resistance is ancient. Nature. 457: 457-461. https://www.nature.com/articles/nature10388.
9. DebMandal, M., S. Mandal, & N.K.Pal. 2011. Antibiotic Resistance Prevalence and Pattern in Environmental Bacterial Isolates. The Open Antimicrobial Agents Journal. 3:45-52. http://dx.doi.org/10.2174/1876518101103010045.
10. Food and Agriculture Organization of the United Nations (FAO). 2018. Nitrogen Inputs to Agricultural Soils from Livestock Manure: New Statistics. Available online: http://www.fao.org/3/I8153EN/i8153en.pdf.
11. Gerba, C.P., &J.E.Smith. 2005. Sources of pathogenic microorganisms and their fate during land application of wastes. Journal of Environmental Quality. 34:42–48. https://doi.org/10.2134/jeq2005.0042a.
12. Ghosh, S., & T.M. LaPara. 2007. The Effects of Subtherapeutic Antibiotic Use in Farm Animals on the Proliferation and Persistence of Antibiotic Resistance among Soil Bacteria. Multidisciplinary Journal of Microbial Ecology. 1:191-203. http://dx.doi.org/10.1038/ismej.2007.31.
13. Hölzel, C.S., J. L.Tetens, & K. Schwaiger. 2018. Unraveling the Role of Vegetables in Spreading Antimicrobial-Resistant Bacteria: A Need for Quantitative Risk Assessment. Foodborne Pathogens and Disease. 15:671-687. doi: 10.1089/fpd.2018.2501.
14. Kilonzo-Nthenge, A., E.Rotich, & S.N. Nahashon. 2013. Evaluation of Drug-Resistant Enterobacteriaceae in Retail Poultry and Beef. Poultry Science. 92: 1098-1107. http://dx.doi.org/10.3382/ps.2012-02581.
15. Kudo, H.Y., J. Nemoto, K. Ohtsuka, Y. Segawa, K. Takatori, T. Kojima, & M. Ikedo. 2007. Sensitive and rapid detection of Vero toxin-producing Escherichia coli using loop-mediated isothermal amplification. Journal of Medical Microbiology. 56:398–406. doi: 10.1099/jmm.0.46819-0.
16. Loofta, T, T.A. Johnson, H.K. Allena, D.O. Baylesa, D.P. Alta, R.D. Stedtfeld, W.J. Sulb, T.M. Stedtfeld, B. Chaib, J.R. Cole, S.A. Hashsham, J.M.Tiedje, & T.B. Stantona. 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proceedings of the National Academy of Sciences of the United States of America. 109:1691–1696. https://doi.org/10.1073/pnas.1120238109.
17. Marti R, Scott A, Tien YC, Murray R, Sabourin L, Zhang Y, &Toppa E. 2013. Impact of Manure Fertilization on the Abundance of Antibiotic-Resistant Bacteria and Frequency of Detection of Antibiotic Resistance Genes in Soil and on Vegetables at Harvest. Applied and Environmental Microbiology. 79: 5701–5709.doi: 10.1128/AEM.01682-13.
18. Misra R.V., R.N.Rat, &H.Hraoka. 2003. On-farm composting methods. Land and Water Discussion paper 2. FAO. pp.1-36. www.fao.org/3/y5104e/y5104e.pdf.
19. Nei, M., & W.H. Li. 1979. Mathematical Model for Studying Genetic Variation in Terms of Restriction Endonucleases. Proceedings of the National Academy of Sciences of the United States of America. 76: 5269-5273. http://dx.doi.org/10.1073/pnas.76.10.5269.
20. Nesme, J., S. Cécillon, T.O. Delmont, J.M. Monier, T.M. Vogel, & P. Simonet. 2014. Large-scale metagenomic-based study of antibiotic resistance in the environment. Current Biology. 24: 1096–1100.doi:10.1016/j.cub.2014.03.036.
21. Oliver,J.P., C.A. Gooch, S. Lansing, J. Schueler, J.J. Hurst, L. Sassoubre, E.M. Crossette, & D.S. Aga. 2020. Invited review: Fate of antibiotic residues, antibiotic-resistant bacteria, and antibiotic resistance genes in US dairy manure management systems. Journal of Dairy Science. 103:1051–1071. https://doi.org/10.3168/jds.2019-16778.
22. Parsons, B.D., N. Zelyas, B.M. Berenger, & L. Chui. 2016. Detection, Characterization, and Typing of Shiga Toxin-Producing Escherichia coli. Frontiers in Microbiology. 7: Article 478. doi: 10.3389/fmicb.2016.00478.
23. Partanen P., J. Hultman, L.Paulin, P. Auvinen, & M. Romantschuk. 2010. Bacterial diversity at different stages of the composting process. BMC Microbiology. 10:94 http://www.biomedcentral.com/1471-2180/10/94.
24. Rayne, N., & L. Aula (2020) Livestock Manure and the Impacts on Soil Health: A Review. Soil Systems. 4: 64; https://doi.org/10.3390/soilsystems4040064.
25. Rebollido, R., J. Martizez, Y. Aguilera, K. Melichor, I. Koerner, & R. Stegmann. 2008. Microbail populations during composting process of organic

fraction of municipal solid waste. Applied Ecology and Environmental Research.6: 61-67. www.aloki.hu/pdf/0603_061067.pdf.
26. Reynnells, R., D.T. Ingram, C. Roberts, R. Stonebraker, E.T. Handy, G. Felton, B.T. Vinyard, P.D. Millner, & M. Sharma. 2014. Comparison of U.S. Environmental Protection Agency and U.S. Composting Council Microbial Detection Methods in Finished Compost and Regrowth Potential of Salmonella spp. and Escherichia coli O157:H7 in Finished Compost. Foodborne Pathogens and Disease. 11: doi: 10.1089/fpd.2013.1698.
27. Roman, R., M.M.Marinez, &A.Pantoja. 2015. Famer's Compost Handbook; Experiences in Latin America. FAO. pp. 1-87. www. fao.org/3/i3388e/i3388e.pdf.
28. Sasaki, H., J. Nonaka, K. Otawa, O. Kitazume, R. Asano, T. Sasaki, & Y. Nakai. 2009. Analysis of the Structure of the Bacterial Community in the Livestock Manure-Based Composting Process. Asian-Australasian. Journal of Animal Science. 22:113-118.
29. Sawant, A.A., N.V. Hegde, B.A. Straley, S.C. Donaldson, B.C. Love, S.J. Knabel, & B.M. Jayarao. 2007. Antimicrobial-Resistant Enteric Bacteria from Dairy Cattle. Applied and Environmental Microbiology. 73: 156-163. http://dx.doi.org/10.1128/AEM.01551-06
30. Schloss, P.D., A.G. Hay, D.B. Wilson, J.M. Gossett, & L.P. Walker. 2005. Quantifying bacterial population dynamics in compost using 16S rRNA gene probes. Applied Microbiology and Biotechnology. 66: 457–463. doi: 10.1007/s00253-004-1727-y.
31. Sharma, R., F.J. Larney, J. Chen, L.J. Yanke, M. Morrison, E. Topp, T.A. McAllister, & Z. Yu. 2009. Selected antimicrobial resistance during composting of manure from cattle administered sub-therapeutic antimicrobials. Journal of Environmental Quality. 38: 567–575. doi: 10.2134/jeq2007.0638.
32. Sharma, B., B. Vaish, M. Mahajan, U.K.Singh, P. Singh, & R.P. Singh. 2019. Recycling of Organic Wastes in Agriculture: An Environmental Perspective. International Journal of Environmental Research.13: https://doi.org/10.1007/s41742-019-00175-y.
33. Stanton, T.B., S.B.Humphrey, & W.C. Stoffregen. 2011. Chlortetracycline-resistant intestinal bacteria in organically raised and feral swine. Applied and Environmental Microbiology. 77: 7167–7170. doi: 10.1128/AEM.00688-11.
34. Smith, S., P. Colgan, F. Yang, E.L. Rieke, M. Soupir, T.B. Moorman, H.K. Allen, & A. Howe. 2019. Investigating the dispersal of antibiotic resistance associated genes from manure application to soil and drainage waters in simulated agricultural farmland systems. PloS ONE.14: e0222470. doi: 10.1371/journal.pone.0222470.
35. Vishan, I., H. Kanekar, & A. Kalamdhad. 2014. Microbial population, stability and maturity analysis of rotary drum composting of water hyacinth. Biologia. 69: 1303—1313. doi: https://doi.org/10.2478/s11756-014-0450-0.
36. Wang, L., A. Gutek, S. Grewal, F.C. Michel, Jr., & Z. Yu. 2015. Changes in diversity of cultured bacteria resistant to erythromycin and tetracycline in swine manure during simulated composting and lagoon storage. Letters in Applied Microbiology. 61: 245–251. doi:10.1111/lam.12450.
37. Watanabe, K. 2008.Application of multiple enzyme restriction fragment length polymorphism analysis and microchip electrophoresis for estimation of antibiotic-tolerant bacterial group. Journal of Pesticide Science. 33: 249–260. https://doi.org/10.1584/jpestics.G08-04
38. Watanabe, K., M. Okuda, & N. Koga. 2008. Newly developed system based on multiple enzyme restriction fragment length polymorphism-an application to proteolytic bacterial flora analysis. Soil Science and Plant Nutrition. 54: 204-215. https://doi.org/10.1111/j.1747-0765.2007.00230.x.
39. Watanabe, K. 2009. Detection of protease gene in the field soil applied with liquid livestock feces and speculation of their function and origin. Soil Science and Plant Nutrition. 55: 42-52. doi: https://doi.org/10.1111/j.1747-0765.2008.00323.x
40. Watanabe, K., & N. Koga. 2009. Use of a Microchip Electrophoresis System for Estimation of Bacterial Phylogeny and Analysis of NO3 Reducing Bacterial Flora in Field Soils. Bioscience, Biotechnology, and Biochemistry.73: 479-488. doi: https://doi.org/10.1271/bbb.70712.
41. Watanabe, K., N. Horinishi, & K. Matumoto, 2015a. Antibiotic-Resistant Bacterial Group in Field Soil Evaluated by a Newly Developed Method Based on Restriction Fragment Length Polymorphism Analysis. Advances in Microbiology. 5:807-816.doi: 10.4236/aim.2015.512085
42. Watanabe, K., N. Horinishi, K. Matumoto, A. Tanaka, & K. Yakushido. 2015b. Bacterial Groups Concerned with Maturing Process in Manure Production Analyzed by a Method Based on Restriction Fragment Length Polymorphism Analysis. Advances in Microbiology. 5:832-841. doi: 10.4236/aim.2015.513088.
43. Watanabe, K., N. Horinishi, K. Matsumoto, A. Tanaka, & K. Yakushido. 2016. A new evaluation method for antibiotic-resistant bacterial groups in environment. Advances in Microbiology. 6:133-151. doi: 10.4236/aim.2016.63014
44. Weidner, S., W. Arnold, & A. Puhler. 1996. Diversity of Uncultured Microorganisms Associated with the Seagrass Halophila stipulacea Estimated by

Restriction Fragment Length Polymorphism Analysis of PCR-Amplified 16S rRNA Genes. Applied and Environmental Microbiology. 62:766-771. doi: 10.1128/AEM.62.3.766-771.1996.

45. Yamamoto, N., K. Otawa, & Y. Nakai. 2009. Bacterial Communities Developing during Composting Processes in Animal Manure Treatment Facilities. Asian-Australasian. Journal of Animal Science. 22: 900-905. https://www.animbiosci.org/upload/pdf/22-120.pdf.
46. Yang, Q., H.Zhang, Y. Guo, & T. Tian. 2016. Influence of Chicken Manure Fertilization on Antibiotic-Resistant Bacteria in Soil and the Endophytic Bacteria of Pakchoi. International Journal of Environmental Research and Public Health. 13:662. doi:10.3390/ijerph13070662.
47. Youngquist, C.P., S.M. Mitchell, & C.G. Cogger. 2016. Fate of Antibiotics and Antibiotic Resistance during Digestion and Composting: A Review. Journal of Environmental Quality. 45:537-545.doi: 10.2134/jeq2015.05.0256.
48. Zekar, F.M.,S.A. Granier, M. Marault, L.Yaici, B. Gassilloud,C. Manceau, A. Touati&Y. Millemann. 2017. From Farms to Markets: Gram-Negative Bacteria Resistant to Third-Generation Cephalosporins in Fruits and Vegetables in a Region of North Africa. Frontiers in Microbiolgy. 24: Article 1569. doi: 10.3389/fmicb.2017.01569.
49. Zhang, L., D. Kinkelaar, Y. Huang, Y. Li, X. Li, &H.H.Wang 2011. Acquired Antibiotic Resistance: Are We Born with It? Applied and Environmental Microbiology. 77: 7134–7141. doi: 10.1128/AEM.05087-11.
50. Zhang, Y.J., H.W. Hua, Q.L. Chena, B.K. Singhb, H. Yand, D. Chena, & J.Z. Hea. 2019. Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. Environmental International. 130:104912. https://doi.org/10.1016/j.envint.2019.104912.All in-text reference citations must be formatted using the author-year system and must be listed in alphabetical order. Please do not use numbering for your references.