

EVALUATION OF MICROBIAL RISK IN SOIL AMENDED WITH ORGANIC FERTILIZERS FROM STABILIZED SWINE MANURE WASTE

Il Han^{1,3}, Young Shin Lee², and Joonhong Park^{3,†}

¹Department of Civil Engineering and Environmental Sciences, Korea Military Academy, Nowon-Gu, Gongneung-Dong PO Box 77, Seoul, 139-799 Korea

²Department of Environmental Engineering, Hanseu University, Daegok Ri 360, Haemi-Myun, Seosan-Si, Chungcheongnam-Do, 356-706 Korea

³Department of Civil and Environmental Engineering, Yonsei University, Shinchon-Dong 134, Seodaemun-Gu, Seoul, 120-749 Korea

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Abstract : This study evaluated microbial risk that could develop within soil microbial communities after amended with organic fertilizers from stabilized swine manure waste. For this purpose, we assessed the occurrences and competitiveness of antibiotic resistance and pathogenicity in soil microbial communities that were amended with swine manure wastes stabilized by a traditional lagoon fermentation process and an autothermal thermophilic aerobic digestion process, respectively. According to laboratory cultivation detection analysis, soil applications of the stabilized organic fertilizers resulted in increases in absolute abundances of antibiotic resistant bacteria and of two tested pathogenic bacteria indicators. The increase in occurrences might be due to the overall growth of microbial communities by the supplement of nutrients from the fertilizers. Meanwhile, the soil applications were found to reduce competitiveness for various types of antibiotic resistant bacteria in the soil microbial communities, as indicated by the decrease in relative abundances (of total viable heterotrophic bacteria). However, competitiveness of pathogens in response to the fertilization was pathogens-specific, since the relative abundance of *Staphylococcus* was decreased by the soil applications, while the relative abundance of *Salmonella* was increased. Further testes revealed that no MAR (multiple antibiotic resistance) occurrence was detected among cultivated pathogen colonies. These findings suggest that microbial risk in the soil amended with the fertilizers may not be critical to public health. However, because of the increased occurrences of antibiotic resistance and pathogenicity resulted from the overall microbial growth by the nutrient supply from the fertilizers, potential microbial risk could not be completely ruled out in the organic-fertilized soil samples.

Key Words : Antibiotic resistant bacteria, Autothermal thermophilic aerobic digestion, Lagoon fermentation, Multiple antibiotic resistance (MAR), Pathogen

INTRODUCTION

The 'antibiotic era' was started in 1928 form the discovery of the first antimicrobial agent, penicillin. Since then antibiotic have saved mil-

lions of lives by making once fatal bacterial infection treatable.¹⁾ But due to their prolong misuses, antibiotic resistant (AR) bacteria started to emerge. For example, AR *Staphylococcus* species were observed in 1946, and 4 years later AR *Streptococcus* species were reported.²⁾ In 1992, MAR (multiple antibiotic resistant)-bacterial infections caused 19,000 deaths in the United

[†] Corresponding author

E-mail: parkj@yonsei.ac.kr

Tel: +82-02-2123-5798, Fax: +82-02-312-5798

States.³⁾ The rising number of AR bacteria species and their quick adaptation to newly-formulated antibiotics have been serious concerns to public health, environmental regulation and management.⁴⁾ In agricultural industry, vast amount of antibiotics are being used in livestock feeds (~0.2% w/w) for growth stimulation and infection control while little degradation through animal digestive system occurs. Because of this, the concentrations of antimicrobial substances in animal manure wastes are generally high.^{1,5)} The high antibiotic concentration can result in the selection and occurrence of AR bacteria in livestock manure waste, and its accidental release into environment can have devastating effects on bacterial infections to human.¹⁾

For many years, in agricultural industry, stabilized livestock manures have been used as alternatives to chemical fertilizers.⁶⁾ The conventional and most frequently used manure stabilization method for "organic" fertilizer production is a lagoon fermentation process.⁷⁾ This process is a very primitive stabilization technique in which livestock manure waste is stabilized through microbial fermentation processes by simply storing the wastes in a deep lagoon tank for several months.⁷⁻⁹⁾ In Korea a rising demand for "organically-cultured" crop products stimulated the production and utilization of organic fertilizers.¹⁰⁾ A major disadvantage in use of lagoon fermentation is its slow process. Because this, relatively quick and efficient aerobic processes have been considered as an alternative stabilization approach.¹¹⁾ Autothermal thermophilic aerobic digestion (ATAD) has been regarded as a preferred aerobic process in stabilizing livestock manure waste not only because its stabilization generally takes relatively short time (i.e., couple of days), but also because the heat developed during the aerobic digestion has a potential for effective microbial risk reduction.¹²⁻¹⁴⁾ For example, we previously reported detection of relatively high microbial risk in manure of chlortetracyclin-fed pig and in its 6-month lagoon-fermented products, while ATAD could significantly reduce the occurrences and competitiveness of AR bacteria and

pathogenic bacteria indicators from microbial communities.¹²⁾ There are several studies that have evaluated microbial risk in stabilized livestock manure products and dissipation of antimicrobial substance in soil.¹³⁾ But little is known about the occurrence and competitiveness of AR bacteria and pathogens in soil microbial communities in response to soil application with organic fertilizers.

The aim of this study was to evaluate microbial risk in soil microbial communities in response to amendment of organic fertilizers. For this, we assessed the occurrences and competitiveness of AR bacteria and pathogenic indicator bacteria in soil microbial communities, when amended with the swine manure fertilizers stabilized by lagoon fermentation and ATAD treatments, respectively. To examine whether microbial risk in the organic-fertilized soils was critical to public health, a potential existence of MAR-exhibiting pathogenic bacteria was also assayed.

MATERIALS AND METHODS

Stabilized Swine Manure Products

Swine manure was stabilized by a field-scale lagoon fermentation process and a pilot-scale ATAD process in Daun swine farm (Seosan, Chungcheongnamdo). Pigs were fed with feeds that contained 0.2% (w/w) chlortetracycline. Fermented manure products were collected from a bottom of the field lagoon tank after 6 months of fermentation stabilization. ATAD treated products were collected after 3 days of the ATAD operation in which the operational temperature rose up to 65-70°C after 1.5 days of operation period, and the developed thermophilic condition was sustained until the end of the operation. More detail information on the stabilization operations and the sampling of stabilized products is described elsewhere.¹²⁾

Soil Amendments with Stabilized Swine Manure Products

To examine the effects of organic fertilization in soil microbial communities, previously unfertilized soil from a forest area was collected

(Ahn Mountain, 20 cm below top soil). After homogenization of the soil using sterilized garden shovels, it was divided into six cylindrical pots (height \times diameter, 200 mm \times 250 mm) which were further divided into three sets of duplicated pots. The first set was left unfertilized as a control (Untreated), the second set was fertilized with 150 mL of fermented swine manure (Fermented), and the final set with 150 mL of ATAD treated swine manure (ATAD). The prepared pots were incubated under outdoor conditions for five weeks (May 16~Jun 20, 2005), triplicate soil samples (0.5-1.0 g) were taken and prepared for the following microbial assays (viable counts for heterotrophic bacteria, antibiotic resistant bacteria, and pathogenic bacteria indicators).

Viable Counting for Heterotrophic Bacteria

To quantify overall heterotrophic bacteria in soil samples, a standard spreading plate assay¹⁵⁾ was conducted under aerobic conditions using R2A solid agar medium (Difco). For the experiment, cells were extracted from soil samples (approximately 0.5 g of wet soil weight) by vortexing for 15 min. with 1 mL of a sterilized potassium phosphate buffer (0.1 M, pH 6.8). The soil micro-organism suspensions were diluted in series with the same potassium phosphate buffer and 0.1 mL of the each diluted cell suspension was spread onto separate R2A plate. The spread plates were incubated at 24°C for 3 days, and the number of colonies formed on each plate were counted. For absolute abundance report, colony counts were normalized by the weight of dried soil, i.e., CFUs (colony formation units) per gram of dried soil. For weighing of dried soil, the taken soil samples were oven-dried overnight at 105°C.

Abundance and Competitiveness of Antibiotic Resistant Bacteria

AR bacteria in tested soils were quantified by counting the number of viable colonies formed on antibiotic applied culture plates after 5 days of incubation at 25°C. For this, modified standard spread plate method was adapted, in which each R2A solid medium were mixed with each

antibiotic agent (100 μ g/mL).¹⁵⁾ Viable AR bacteria counts per gram of dried soil was reported as absolute abundance. For competitiveness measurement, relative abundance was calculated by dividing viable AR bacteria counts with viable heterotrophic bacteria counts (on no-antibiotic-amended R2A plate). Antibacterial agents used in this study were tetracycline (Tet), kanamycin (Ka), ampicillin (Amp), and rifampicin (Rif). These agents were purchased from Sigma-Aldrich. These antibiotics were selected because they are representative compounds for different antimicrobial mechanisms, i.e., tetracycline and kanamycin inhibit bacterial protein synthesis including those of ribosomal proteins; ampicillin disturbs cell walls and membranes; and rifampicin inhibits DNA/RNA synthesis.^{1,16)}

Abundance and Competitiveness of Pathogenic Bacteria Indicators

Salmonella (Sal) and *Staphylococcus* (Sta) were pathogenic bacterial indicators assessed in this study. Desoxycholate Citrate agar¹⁷⁾ (BBL) and Mannitol Salt agar¹⁸⁾ (BBL) were used for the selective growth and isolation of *Salmonella* and *Staphylococcus*, respectively. For the selective growth and isolation of pathogenic indicator bacteria method specified on manufacturers protocols were used. After 5 days of incubation at 37°C, colonies on each plate were counted. Viable pathogen indicator count per gram of dried soil was reported for absolute abundance of a pathogenic bacteria indicator in a soil sample. For competitiveness measurement, relative abundance was calculated by dividing viable pathogen indicator count with viable heterotrophic bacteria count (on no-antibiotic-amended R2A plate).

Multiple Antibiotic Resistance (MAR) Assays

To examine whether pathogenic bacteria exhibit MAR phenotype, eighty of either *Staphylococcus* or *Salmonella* colonies formed on the corresponding pathogen indicator plates were transferred onto multiple antibiotic amended plates (MAAP), in which a combination of three antibiotics was added in R2A media. The MAAP contained

either i) a combination of tetracycline, kanamycin and rifampicin (Tet + Kan + Rif, 100 µg/mL each) or ii) a combination of tetracycline, kanamycin and ampicillin each (Tet + Kan + Amp, 100 µg/mL). MAAPs were then incubated for 3 days at 25°C and 37°C respectively, the number of colonies formed on each plate was counted.

RESULTS & DISCUSSION

Abundance of Heterotrophic Bacteria in response to Organic Fertilizers

After 5 weeks of outdoor incubation, the amendments with organic fertilizers resulted in increased heterotrophic bacteria viable counts in the tested soil samples (Table 1). The absolute abundance of heterotrophic bacteria for the lagoon-fermented fertilizer applied soil (Fermented) was approximately 67-time greater than that for no-fertilization control (Untreated), while the soil application with ATAD-stabilized fertilizer (ATAD) resulted in approximately 552-time increase in the absolute abundance of heterotrophic bacteria. Between the two differently fertilized soils (Fermented and ATAD), the amendment with ATAD-stabilized fertilizer showed approximately 7.2-time higher population growth. This difference might be partially attributed to the greater organic content in the ATAD fertilizer. The concentration of volatile suspended solid (VSS) in the ATAD fertilized (36.7 ± 0.5 g/L) was approximately 3-time greater than the VSS concentration in the Fermented fertilizer (12.4 ± 1.1 g/L). However, only 3 times greater VSS concentration cannot explain the more preferential growth in the ATAD fertilized case. Although the mechanism for this observation was unclear, these findings strongly suggested that the ATAD-stabilized fertilizer provided a better nutrient environment for the

indigenous soil microbial communities to grow.

Abundance and Competitiveness of AR Bacteria in response to Organic Fertilizers

The unfertilized soil samples showed the presence of bacteria that exhibited resistance against the tested antibiotic agents (Fig. 1). A possible explanation for this observation is that indigenous bacteria, which probably had defense mechanisms against naturally-occurring antimicrobial agents, have fortuitously exhibited resistance against tested antibiotics. The amendments with the Fermented and ATAD fertilizers resulted in increases in absolute abundances of kanamycin-resistant and ampicillin-resistant bacteria (p-values < 0.005), and constant tetracycline resistance occurrences. These suggested that, although the fertilizer production methods may be biologically different, the amendments of the two fertilizers have similar effects on the abundances of particular bacteria

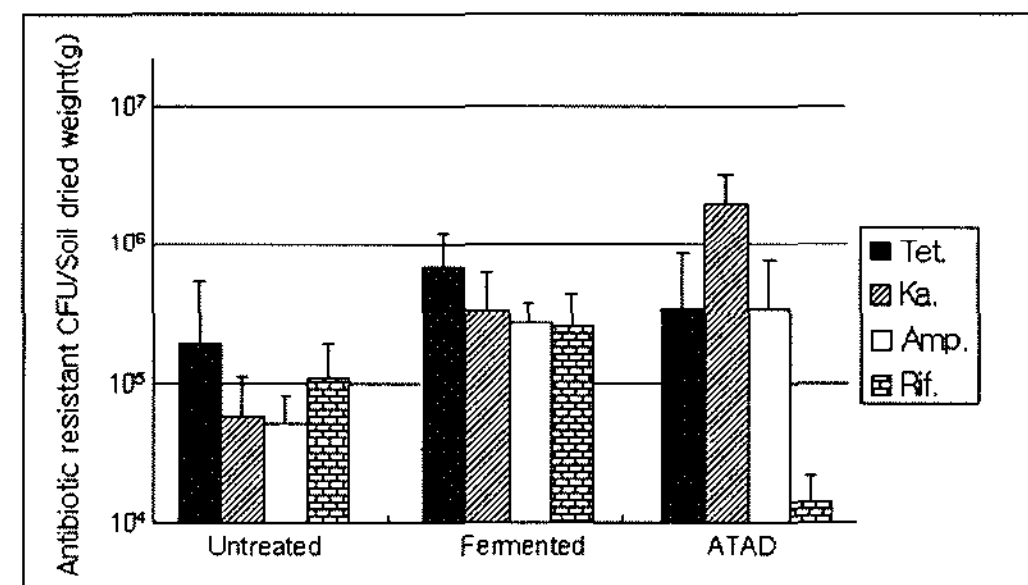


Figure 1. Absolute abundance (viable counts cfu [colony formation unit] per gram of dried soil) for antibiotic resistant bacteria in response to soil application with swine manure fertilizers, i.e., no-soil treatment (Untreated), lagoon-fermented treated (Fermented), and ATAD-stabilized treated (ATAD). Four different types of antibiotic resistance were assayed. "Tet" indicates tetracycline; "Ka" kanamycin; "Amp" ampicillin; "Rif" rifampicin.

Table 1. Viable counts for heterotrophic bacteria

Soil Sample	Untreated*	Fermented	ATAD
Heterotrophic counts per Soil dried weight (10 ⁶ CFU/g)	193.0 (±12.0) [#]	12,950 (±11,290)	106,600 (±39,200)

* "Untreated" indicates no-fertilization control; "Fermented" indicates soil samples amended with lagoon fermented swine manure fertilizer; "ATAD" indicate soil samples amended with ATAD-stabilized swine manure fertilizer).

[#] The number in each parenthesis indicates one-standard-deviation among replicate experiments.

that were resistant against protein synthesis (kanamycin and tetracycline) and/or cell wall/membranes (ampicillin) inhibitors. In contrast, the response of soil microbial communities to antibiotic agents disturbing the nucleic acids synthesis (rifampicin) seemed to depend on fertilizer stabilization methods, i.e., rifampicin-resistant bacteria were decreased when soil was amended with the ATAD fertilizer (p-value < 0.005) while rifampicin-resistant bacteria were increased when same were done with the fermented fertilizer (p-value < 0.01).

According to our previous study, no AR bacterial colonies were detected in the ATAD-stabilized fertilizer while a significant number of AR bacterial colonies were observed in the Fermented fertilizer.¹²⁾ Thus, the increase in AR bacteria for the ATAD-fertilizer application was not attributed to the transfer of AR bacteria from ATAD fertilizer to soil or their selective stimulation. Instead, it might be simply due to the overall microbial growth, which was stimulated by the nutritious fertilizer. This is also supported by the fact that the fertilizer applications resulted in decreases in their relative abundances (Fig. 2). This suggests that the fertilization selectively reduced the competitiveness of AR bacteria in the soil communities, which is beneficial in controlling microbial risk.

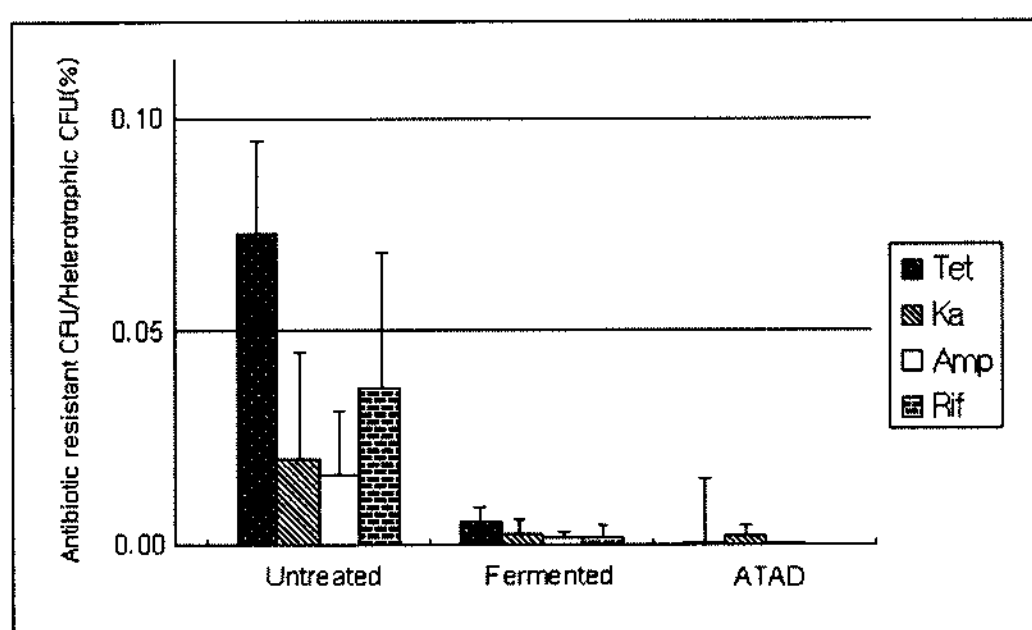


Figure 2. Relative abundances (total heterotrophic viable counts) for antibiotic resistant bacteria in soil microbial communities in response to soil applications with stabilized swine manure fertilizers i.e., no-soil treatment (Untreated), lagoon-fermented treated (Fermented), and ATAD-stabilized treated (ATAD). “Tet” indicates tetracycline; “Ka” kanamycin; “Amp” ampicillin; “Rif” rifampicin.

Occurrence and Competitiveness of Pathogenicity in response to Organic Fertilizers

In the no-fertilization control soil samples, *Staphylococcus* was detected, but *Salmonella* was not (Fig. 3). But in comparison, both the ATAD and Fermented fertilizer applications led to increases in absolute abundances of *Staphylococcus* and of *Salmonella*, respectively. The increases in *Staphylococcus* colonies may be due to the nonselective overall microbial growth by the addition of nutritious fertilizers. *Salmonella* colonies, which were not detected in Untreated, were significantly increased in the organo-fertilized soils. According to our previous study no *Salmonella* and *Staphylococcus* colonies were not detected in the ATAD-stabilized fertilizer.¹²⁾ These suggests that the significant increases of *Salmonella*-like microorganisms by the fertilization is attributed to nonselective growth of microorganisms which already existed at a minuscule number in either soil or ATAD-fertilizer. Nevertheless, according to the relative abundance data (Fig. 4), the organic fertilizer applications significantly reduced competitiveness of tested pathogen indicator bacteria.

Examination of MAR-exhibiting Pathogenic Bacteria

The existence of pathogens with MAR pheno-

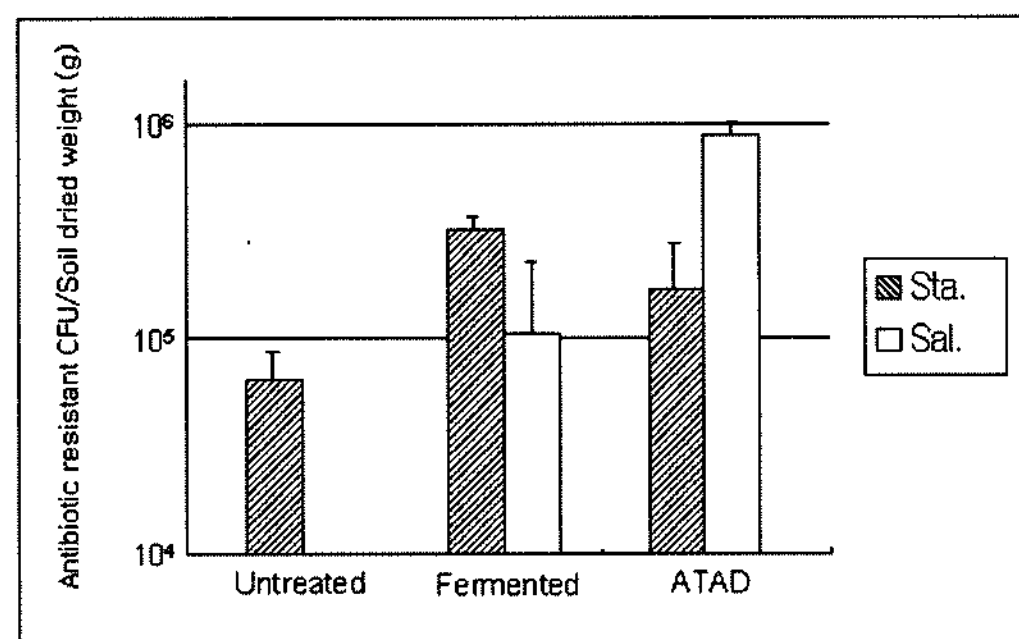


Figure 3. Absolute abundance (viable counts CFU [colony formation unit] per gram of dried soil) for pathogenic bacteria indicators (*Staphylococcus* [Sta] and *Salmonella* [Sal]), in response to soil application with swine manure fertilizers i.e., no-soil treatment (Untreated), lagoon-fermented treated (Fermented), and ATAD-stabilized treated (ATAD).

Table 2. Result of MAR(multiple antibiotic resistant)-pathogenicity analysis

	Pathogen Indicator	Antibiotics	Untreated	Fermented	ATAD
Soil % sample	<i>Staphylococcus</i>	Tet+Ka+Amp	ND [#]	ND	ND
		Tet+Ka+Rif	ND	ND	ND
	<i>Salmonella</i>	Tet+Ka+Amp	ND	ND	ND
		Tet+Ka+Rif	ND	ND	ND

[#] ND indicates "non-detected"

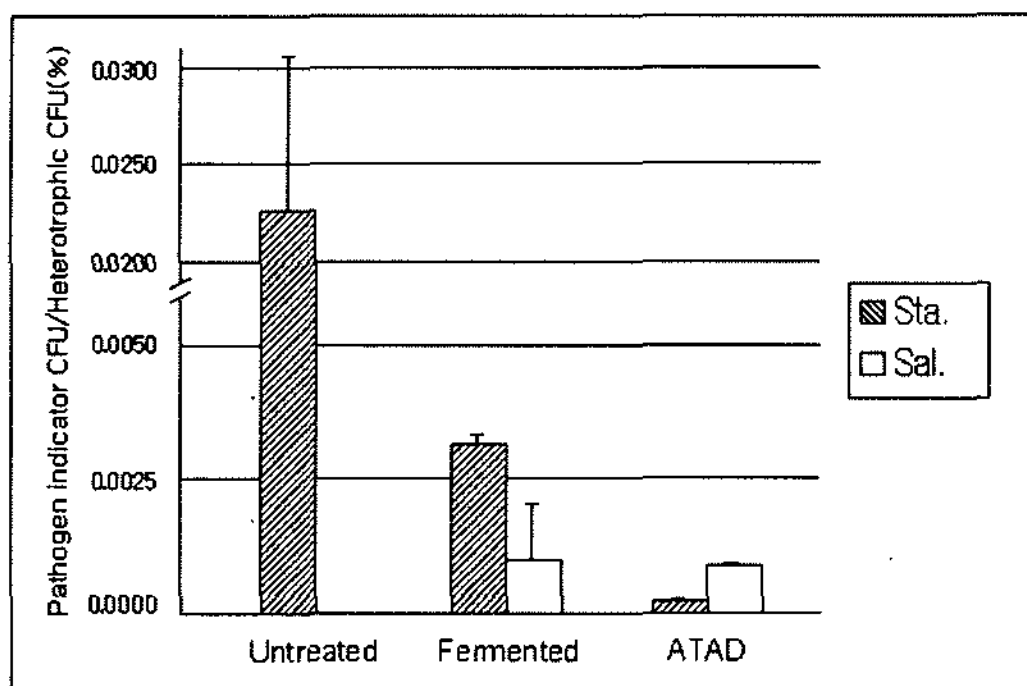


Figure 4. Relative abundances (total heterotrophic viable counts) for pathogenic bacteria indicators (*Staphylococcus* [Sta] and *Salmonella* [Sal]) in soil microbial communities in response to soil applications with stabilized swine manure fertilizers, i.e., no-soil treatment (Untreated), lagoon-fermented treated (Fermented), and ATAD-stabilized treated (ATAD).

types was also examined in microbial communities of the organo-fertilized soils (Table 2). Fortunately no MAR-exhibiting pathogenic colonies were detected. This suggests that the degree of microbial risk associated in the organo-fertilized soils are not critical to public health.

CONCLUSION

In this study microbiological evaluations were carried out to assess AR and pathogenicity of soils amended with biologically-stabilized swine manure fertilizers. In all the tested soil samples, no MAR-exhibiting pathogenic colonies were detected. In addition, the organic fertilizer applications resulted in reduced competitiveness of AR bacteria and pathogenic indicator bacteria. It is possible to conclude that the use of biologically-stabilized fertilizers did not cause critical risk to

public health. However, the nonselective growth of microbial communities in response to the addition of nutritious fertilizers could have increased the occurrences of AR bacteria and pathogenic indicator bacteria in soil. Because of this possibility, potential microbial risk could not be completely ruled out.

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