RESEARCH REPORT
PORK SAFETY

Title: Macrolide, lincosamide, and streptogramin B (MLS_B) resistance in soils amended with swine manure – **NPB #05-068**

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Abstract:

The land application of treated swine manure has been proposed as a possible factor in the environmental development and dissemination of antimicrobial resistance, but this possibility has not been clearly demonstrated. This work included specific experiments addressing (i) levels of MLS_B antimicrobials and resistance in fields regularly amended with manure from conventional and organic swine farms as compared to fields not amended with manure, (ii) the level of resistance in specific populations of microorganisms, and (iii) the contributions of the antimicrobials lincomycin and tetracycline versus MLS_B resistant microorganisms to MLS_B resistance in soil batch tests. MLS_B antimicrobials were quantified in manure and soil samples by solid phase extraction and liquid chromatography tandem mass spectrometry (LC-MS). MLS_B resistance was quantified in the same samples using fluorescence *in situ* hybridization (FISH) to detect the ribosomal modification responsible for MLS_B resistance. The combination of this method with phylogenetic identification protocols allows the quantification of resistance in particular organisms or groups of organisms. MLS_B antimicrobials were generally not detected in the soil samples, while tetracyclines were more persistent. No significant difference in MLS_B resistance was observed between amended and unamended soils, either in the soil samples or in laboratory batch tests. The results suggest that land application is not contributing to the development and spread of MLS_B resistance. However, given the persistence of tetracycline antimicrobials observed in this and previous work, it would be prudent to also examine the levels of tetracycline resistance in these samples.

Introduction:

This study focuses on evaluating the effects of land application of treated swine manure on environmental levels of antimicrobial resistance and responds to the research priority of investigating the relationship between the use of antimicrobials in swine production and the dissemination and persistence of antimicrobial resistant bacteria and/or resistance genes in the environment, including water. Treated swine manure has been shown to contain substantial levels of antimicrobial resistant microorganisms and antimicrobials [1-4], and land application therefore has the *potential* to have a significant impact on levels of antimicrobial resistance in the soil and subsequently in water. The word potential must be emphasized because available data on the actual impact are limited [4-7]. Furthermore, there is also a scarcity of data on *naturally occurring* levels of antimicrobials and antimicrobial resistance, which are needed to provide the appropriate comparison and to accurately assess the impact of land application of swine manure. This work is therefore designed to assess the impact of land application of swine manure with and without antimicrobials on the levels of both antimicrobial resistance and antimicrobials. In addition, it will differentiate between the effects of antimicrobial resistance and antimicrobials, thereby identifying the relevant target for revising practices, should that be necessary.

In this work, we used a novel method of quantifying antimicrobial resistance that is uniquely suited for environmental work. There are two existing types of methods for measuring antimicrobial resistance. Culture-based methods rely on our ability to grow the microorganisms in the laboratory. These methods are simple, inexpensive, and compatible with the characterization of resistant bacterial strains. However, because microorganisms vary greatly in their growth requirements, culture-based methods typically only take into account a small fraction of the microbial community and may provide a biased view of resistance. To avoid such biases, environmental microbiologists are commonly using nucleic acid based molecular methods, which do not require the growth of microorganisms. Molecular methods are suitable for the assessment of the whole community of microorganisms, and they can provide a high level of resolution, but one major disadvantage of existing molecular methods is that they do not allow the identification of the resistant microorganisms. To address these concerns, we recently developed a molecular microscopy method permitting the direct quantification and identification of microorganisms resistant to macrolide, lincosamide, and streptogramin B (MLS_B) antimicrobials, without the biases of existing culture-based and molecular methods [8]. This method detects cells containing the specific methylation that leads to MLS_B resistance using a fluorescent label. Among MLS_B antimicrobials, tylosin, lincomycin, and virginiamycin are commonly used in swine production. In this work, we have applied this method to determine the levels and identities of resistant microorganisms in manure-amended and unamended soils.

Objectives:

Objective #1: Quantify MLS_B antimicrobials and MLS_B resistance in soils amended with swine manure. A comparison of concentrations of antimicrobials and levels of antimicrobial resistance in soils that have received no, organic, and conventional swine

manure will determine whether the practice of land application affects levels of MLS_B antimicrobials and/or MLS_B resistance.

Objective #2: Identify proportion of MLS_B resistance that results from common manure and soil microorganisms. To accurately assess potential risks associated with high levels of antimicrobial resistance, it is important to know which types of organisms are resistant. Experiments are designed to determine resistance in specific classes of bacteria, which are abundant in manure storage pits and lagoons, and soil, namely *Clostridium* spp. in manure and low G+C Gram positives and gamma-Proteobacteria in soil.

Objective #3: Compare the contributions of antimicrobials and MLS_B resistant microorganisms to MLS_B resistance in soil batch tests. This set of experiments is needed to determine the specific contributions of the addition of antimicrobials (lincomycin, tetracycline, and both) and MLS_B resistant microorganisms to MLS_B resistance levels.

Materials & Methods:

Farm selection and sampling. The three conventional (LF, MF, HF) and two natural farms (OF and NF) were selected based on antimicrobial usage and manure handling and treatment processes. At the natural farms no antimicrobials were used in production. For all of the farms except NF, data had already been collected on antimicrobial usage, manure handling, treatment, and application practices, the performance of the manure treatment processes, MLS_B resistance throughout the treatment process, and, for LF, concentrations of MLS_B antimicrobials throughout the treatment process and in amended soils [3, 9]. From each farm, fields were identified that routinely receive treated manure. To ensure that the soil samples were representative, five locations were sampled from each field (from the four corners and the center of the field), and for each of these locations, five soil cores were taken (at the chosen site and from locations about 1 m away in each direction). The 25 soil cores collected from each field were pooled. For the soil batch tests, manure was collected from the building floor at farm NF and from the settling basin at conventional farm LF in December 2005. The soil used in the batch tests was from a field at the UIUC South Farms that had not received manure. The manure and soil were stored at 4°C for 1 and 177 days before the start of the first and second batch tests, respectively.

For the molecular analysis, samples were fixed using ethanol as described previously [10]. Briefly, samples were incubated in 100% ethanol (AAPer Alcohol & Chemical, Shelbyville, KY) for 2 h on ice. Following fixation, the samples were resuspended in 1:1 (vol/vol) PBS:ethanol and stored at -20°C. Subsequently, the fixed samples were diluted (1:100) in 0.1% sodium pyrophosphate (NaPPi) buffer [11]. Then, 10 µl diluted sample was sonicated in 2 ml 0.1% NaPPi buffer with a sonic dismembrator (5 s pulse on/off, output: 250 W, 60s, FISHER Scientific, Model 500, Pittsburgh, PA) and filtered through a black 0.22-µm pore size polycarbonate membrane (diameter: 25 mm, Osmonics, Minnetonka, MN). Cells were transferred from the filters to gelatin coated slides by manually pressing the filters onto the slides for at least 10 s. The gelatin coated slides were prepared following the protocol of Amann et al. [12].

Quantification of MLS_B antimicrobials. The manure and soil samples for MLS_B antimicrobial analysis were shipped on ice to the Institute of Agriculture and Natural Resources at the University of Nebraska (Lincoln, Nebraska) for analysis. The manure

samples were diluted in 0.5-M potassium phosphate/citric acid buffer pH 2.5 (tetracyclines) or a neutral phosphate solution (macrolides) and extracted using Oasis HLB cartridges (Waters Corporation, Milford, Massachusetts). The soil samples were extracted twice with 1-M citric acid/sodium citrate pH 4 and twice with a mixture of acetone and formic acid pH 4 [3]. Following solid phase extraction, the concentrations of tetracycline, macrolide, and lincosamide antimicrobials were quantified using triple quadrupole liquid chromatography tandem mass spectrometry (LC-MS) as previously described [13, 14].

Molecular microscopy methods. MLS_B resistance was quantified using a fluorescence *in situ* hybridization (FISH) method developed in our research group [8, 15]. The FISH technique detects the common mechanism of MLS_B resistance, which involves methylation of a specific adenine residue (A2058) in the 23S rRNA, using a probe (MLS_B in Table 1) that binds only to the unmethylated (MLS_B sensitive) 23S rRNA. FISH was performed as previously described [10] except that the hybridization temperature was lowered to 37°C for the MLS_B probe. Pure cultures of MLS_B-sensitive (*Enterococcus faecalis JH2-2*) and MLS_B-resistant (*Enterococcus faecalis JH2-2* pAMβ1) strains were used to determine that 12.5% formamide was the optimal concentration for distinguishing sensitive and resistant strains [8]. 4', 6-diamidino-2-phenylindole (DAPI) staining was used for total cell counts. The level of MLS_B resistance was calculated by subtracting the number of sensitive cells from the total number of bacterial cells ($\%R = 1 - MLS_B$ / Bact0338).

To assess the resistance in specific groups of microorganisms, we combined this method with differentially labeled phylogenetic probes (Table 1). The dual labeling FISH experiments followed the same protocol, except that the samples were first hybridized using the probe with a more stringent (higher) formamide concentration. After the washing step samples were air dried and the protocol was repeated with the second probe under its specified hybridization conditions.

FISH slides were observed under 630x magnification using a Zeiss Axioskop 40 microscope equipped for both light and fluorescence microscopy (Carl Zeiss, Oberkochen, Germany) using a green filter set (excitation 480 nm/ emission 535 nm, Chroma Technology Corp, Model 41001, Rockingham, VT) for cells hybridized with FAM/Alexa488 labeled probes, a red filter set (excitation 535 nm/ emission 610 nm, Chroma Technology Corp, Model 41002, Rockingham, VT) for cells hybridized with Cy3/Alexa555 labeled probes, and a near UV filter set (excitation 350 nm/ emission 460 nm, Chroma Technology Corp, Model 31000, Rockingham, VT) for DAPI stained cells. Images were acquired from 10 random locations using a monochrome camera (AxioCam MRm, Carl Zeiss MicroImaging, Inc. Thornwood, NY) and an exposure time of 5, 2, 2, and 0.5 s for FAM, Alexa555, Alexa488, and DAPI images, respectively. All experiments were carried out in triplicate. The resulting images were analyzed in an automated fashion using image analysis software Visilog (version 6, Noésis, Les Ulis, France) for expedience and consistency. This method was developed in our laboratory and was recently validated with swine manure samples through comparison with existing methods [8].

Soil batch tests. Land application rates were used to estimate the appropriate manure-to-soil ratio for the soil batch tests; based on this a ratio of 8 g manure to 200 g soil was selected for the first batch test. For increased resolution, conditions with higher concentrations of amendments were also included in the second test. The second test included a range of concentrations for each of the following five amendments: natural farm

manure alone, natural farm manure with resistant *Clostridium* spp., lincomycin, or chlortetracycline, and conventional farm manure, as well as an unamended control. For all soil batch tests, the materials were completely mixed in 250 ml glass bottles and incubated at 25°C with soil wetness maintained at 25% of capacity. Full spectrum fluorescence light was supplied for 12 of every 24 hours. At specified intervals the contents of each batch test were completely mixed prior to taking a five gram sample and fixing it for molecular analysis. Samples for antimicrobial analysis were stored at -80 °C until shipment.

Table 1. Probes used for quantification of MLS_B resistance and microbial community analysis.

Short Name	Systematic Name	Label (5')	Target Organisms	Formamide[a]	Specificity[b]	Coverage[c]	Probe Sequence (5'-3')	Ref.
MLS_B	L-*-MSS-2053-a-S-13	Alexa 555	MLS_B sensitive bacteria	12.5%	99.0%	NA[d]	GGG TCT TTC CGT C	[8]
HGC69A	L-P-Grps-1901-a-A-18	Alexa 488	*Actinobacteria* (High G+C gram-positive bacteria)	25%	NA	82.5%	TAT AGT TAC CAC CGC CGT	[16]
LGC354	S-P-Firm-0354-b-A-18	Alexa 488	*Firmicutes* (Low G+C gram-positive bacteria)	35%	99.6%	32.7%	YSG AAG ATT CCC TAC TGC	[17]
ClostXIVa	S-S-Clos-0129-a-A-15	FAM	*Clostridium* cluster XIVa	30%	99.3%	8.8%	CTG TAT GAG GCA GGT	[18]
aP19	S-Sc-aProt-0019-a-A-17	Cy3	*alpha-Proteobacteria*	20%	66.1%	16.5%	CGT TCG YTC TGA GCC AG	[19]
bP1027	L-C-bProt-1027-a-A-17	FAM	*beta-Proteobacteria*	35%	92.6%	NA	GCC TTC CCA CTT CGT TT	[19]
gP1027	L-C-gProt-1027-a-A-17	FAM	*gamma-Proteobacteria*	35%	90.8%	NA	GCC TTC CCA CAT CGT TT	[19]
StrepB	S-G-Strp-0139-a-A-20	Alexa 488	*Streptomyces*	25%	95.0%	86.5%	ACC CCG TTT CCA GGG CTT GT	[20]
Bact338	S-D-Bact-0338-a-A-18	Cy3	*Bacteria*	20%	70.2%	90%	GCT GCC TCC CGT AGG AGT	[21]

[a] Formamide: The percent of formamide in the hybridization buffer for optimal hybridization conditions in FISH experiments.

[b] Specificity: The percentage of sequences matching the probe from within the target group over the total number of sequences matching the probe.

[c] Coverage: The percentage of sequences matching the probe from within the target group over the total number of sequences in the target group.

Results:

Objective #1: Quantify MLS_B antimicrobials and MLS_B resistance in soils amended with swine manure.

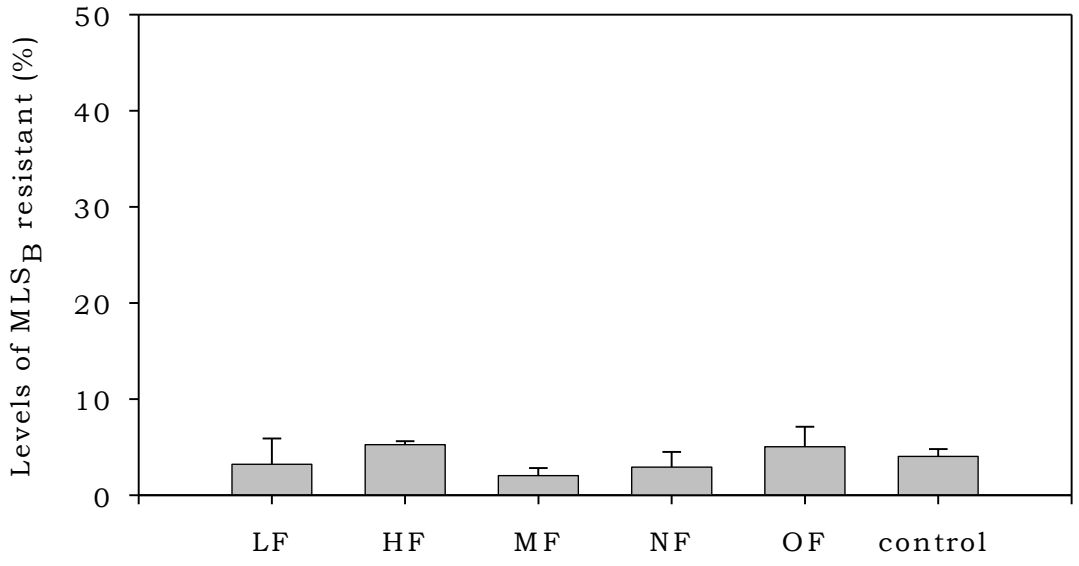
The concentrations of MLS_B and tetracycline antimicrobials in soils that had received no (control), natural (OF, NF), and conventional (LF, MF, HF) swine manure were determined with LC-MS analysis. Erythromycin A, spiramycin, oleandomycin, tilmicosin, tylosin, tiamulin, and minocycline were below the detection limit in all samples; detection limits were ≤5 ng/g except for minocycline (10ng/g). Lincomycin and several tetracyclines were detected as shown in Table 2. Concentrations of tetracyclines were higher in soils receiving conventional swine manure (LF, MF, and HF).

Table 2. Concentration of antimicrobials in soils amended with swine manure. Values are given in ng antimicrobial/g wet soil.

	LF	**HF**	**MF**	**NF**	**OF**	**control**
Lincomycin	9.2	<2	<2	<2	<2	<2
Tetracycline	9.0	7.5	13.8	<1	<1	<1
Chlortetracycline	245.1	40.8	150	<1	<1	<1
Oxytetracycline	4.1	<1	2.6	<1	<1	8.9
Anhydrotetracycline	<2	3	6.1	<1	<2	<2
Anhydrochlortetracycline	<2	<2	<2	<2	<2	<2
Beta-Apo-oxytetracycline	<2	<2	2.4	<2	2	8.6

The levels of MLS_B resistance in the soil samples were measured by FISH in triplicate experiments (Fig. 1). In all soil samples, the levels of MLS_B antimicrobials resistance were low, less than 6% of cells hybridizing with the bacterial domain probe, and no significant difference was observed among the three types of soil samples (one-way ANOVA test with the statistical software R).

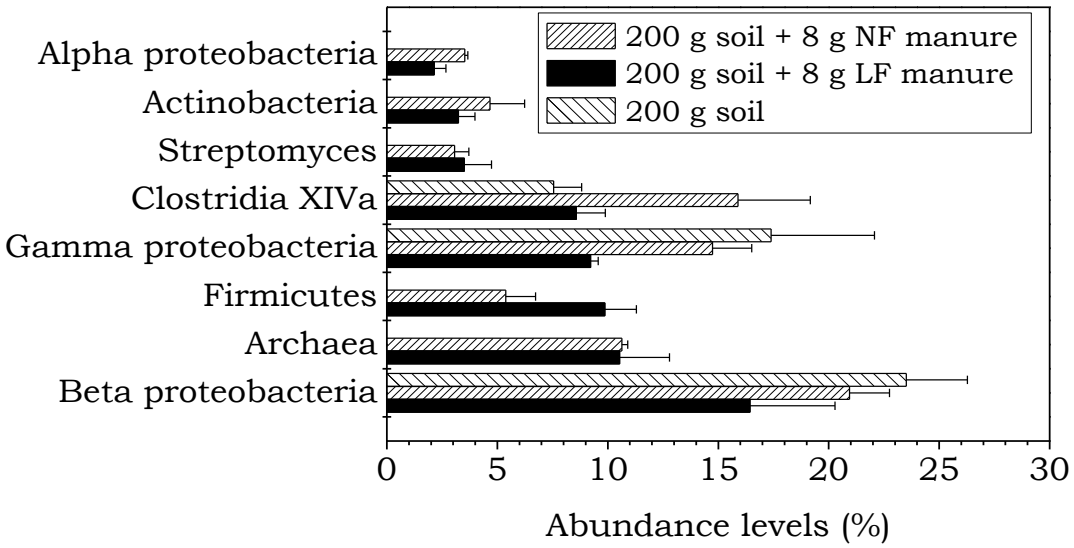
Figure 1. Level of MLS_B resistance in soils amended with swine manure. Error bars represent standard errors.



Objective #2: Identify proportion of MLS_B resistance that results from common manure and soil microorganisms.

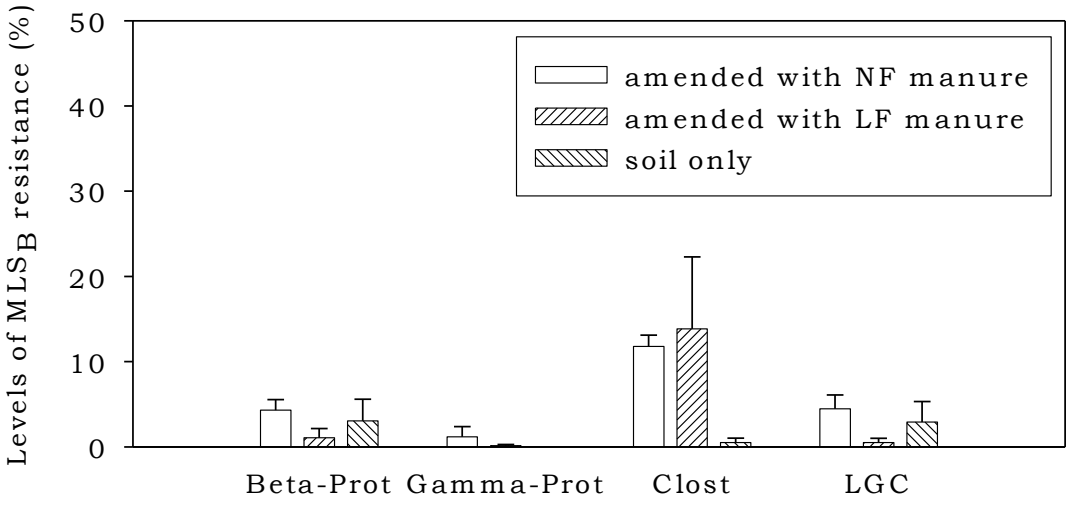
Even the low levels of resistance observed in Objective #1 can be a health risk if they are concentrated in pathogenic organisms. We therefore characterized the microbial community in manure-amended soils and assessed the level of MLS_B resistance in the most abundant groups. The low level of MLS_B resistance in the soil samples analyzed in Objective #1 presented technical difficulties for this analysis, so it was performed on the batch test samples, immediately after the soil had been amended with natural or conventional swine manure. This initial analysis used a wide variety of phylogenetic probes (Table 1) and accounted for 71% of the total microorganisms (Fig. 2).

Figure 2. Microbial community analysis for manure-soil mixtures. Abundance data were normalized with total cell counts by DAPI staining. The abundance of beta-Proteobacteria, gamma-Proteobacteria, and *Clostridium* spp. was determined for soil samples. Error bars represent half ranges.



For the most abundant groups, the level of MLS_B resistance within that group was quantified using dual labeling FISH (Fig. 3). Archaea were not included in this analysis because they are not affected by MLS_B antimicrobials. Although the abundance of *Clostridium* spp. was not specific to amended samples (Figure 2), the resistance levels within the *Clostridium* spp. were different between amended and unamended samples. In amended samples, a higher fraction of the *Clostridium* spp. was resistant, and they accounted for the largest identified fraction of MLS_B resistance in both amended samples.

Figure 3. Quantification of MLS_B resistance in specific populations. Unamended soil samples were compared to samples amended with 8 g NF or LF manure. Error bars represent standard errors.



Objective #3: Compare the contributions of antimicrobials and MLS_B resistant microorganisms to MLS_B resistance in soil batch test.

The batch tests were designed to determine the specific contributions of the addition of antimicrobials (lincomycin or tetracycline) and MLS_B resistant microorganisms to MLS_B resistance levels. Although the focus of this work was on MLS_B resistance rather than the fate of antimicrobials in the soil, the two are interrelated. Measurements of antimicrobials were made on the individual components of the batch tests and on selected batch tests after 90 days (Table 3). Using these data and the known proportions in the batch tests, antimicrobial removal efficiencies of 69%, and 78% were calculated for lincomycin and tetracyclines, respectively.

Table 3. Concentrations of antimicrobials in soil batch tests.

	Antimicrobials[a]						
	Lin	**Tet**	**Chl**	**Oxy**	**Atet**	**Achl**	**BAoxy**
individual component							
LF manure	5.8	18.4	3.8	1794	6.9	<2	1215
NF manure	<2	66	623	139	35.8	<2	48.5
soil	<2	<1	<1	8.9	<2	<2	8.6
amendment (in 200g soil)							
8g NF manure		<1	5.7	<1	<2	<2	<2
32g NF manure		<1	17.0	1.1	<2	<2	3.1
8g NF manure + 160 μg lincomycin	212						
8g NF manure + 640 μg lincomycin	1069						
8g NF manure + 80 μg chlortetracycline		5.2	28.4	8.6	14.2	7.5	12.4
8g NF manure + 320 μg chlortetracycline		4.1	107.0	1.0	6.6	<2	3.3
8g LF manure		<1	4.8	14.4	4.0	<2	7.8
32g LF manure		<1	<1	101.0	<2	<2	41.1

[a] Abbreviations of antimicrobials: Lin: Lincomycin; Tet: Tetracycline; Chl: Chlortetracycline; Oxy: Oxytetracycline; Atet: Anhydrotetracycline; Achl: Anhydrochlortetracycline; BAoxy: Beta-Apo-oxytetracycline

The MLS_B resistance levels in the soil batch tests receiving the highest concentrations of amendments (32g NF manure, $4*10^9$ resistant *Clostridium* spp., 640 μg lincomycin, 320 μg chlortetracycline, or 32g LF manure) are presented in Figure 4. After 20 days incubation, no significant difference was observed in MLS_B resistance levels between amended and unamended samples (one-way ANOVA test with the statistical software R). This pattern was not strongly influenced by varying concentrations of either resistant *Clostridium* spp. or lincomycin (Figs 5 & 6), respectively.

Figure 4. MLS_B resistance in batch tests with highest concentrations of amendments. Resistant organisms and antimicrobials were added to 8 g NF manure and 200 g soil, while samples containing only manure and soil had 32 g manure and 200 g soil. Error bars represent standard errors.

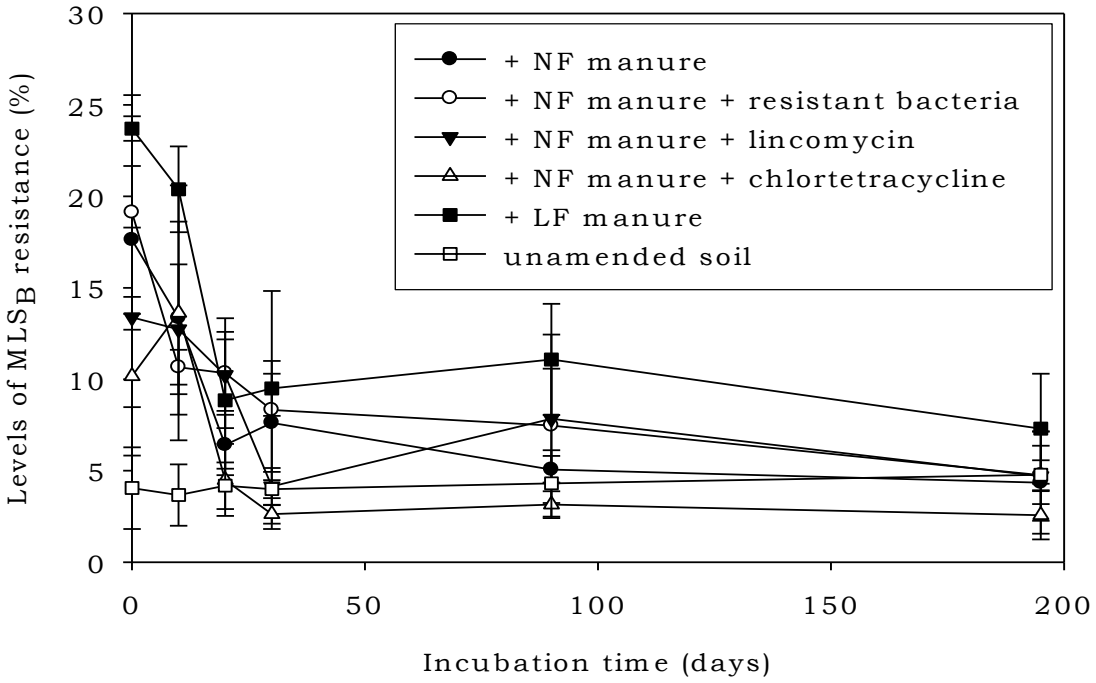


Figure 5. Influence of resistant *Clostridium* spp. on levels of MLS_B resistance The specified amounts of resistant *Clostridium* spp. were added to 200 g soil and 8 g NF manure. Error bars represent standard errors.

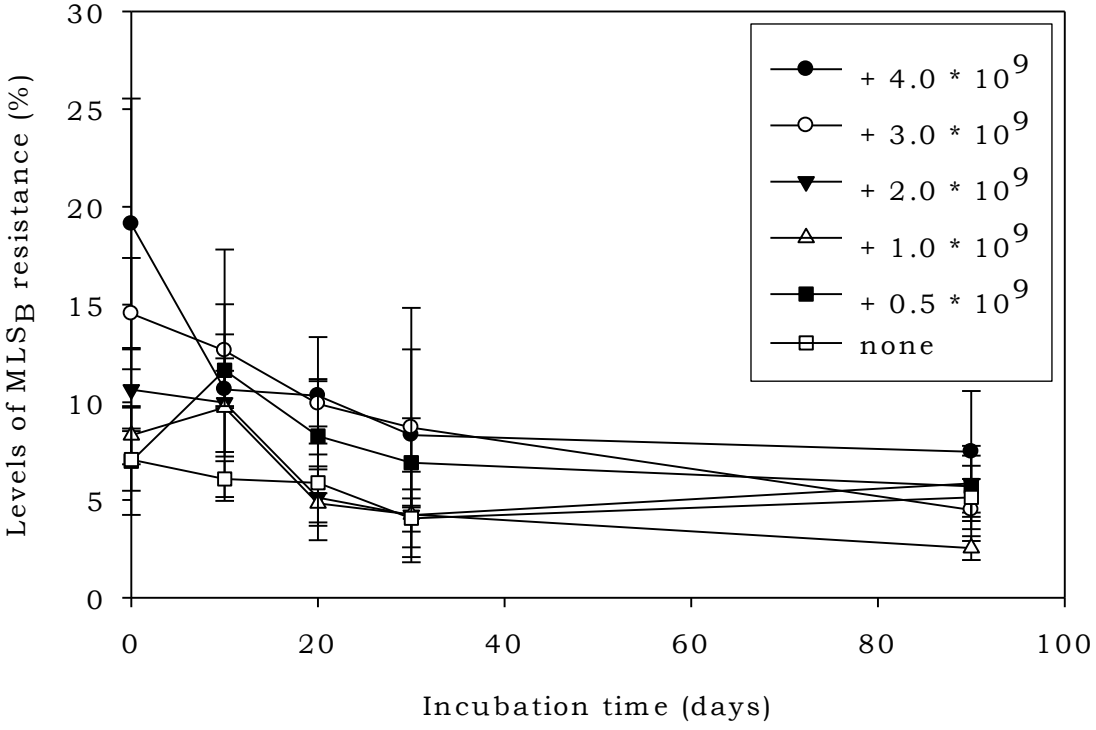
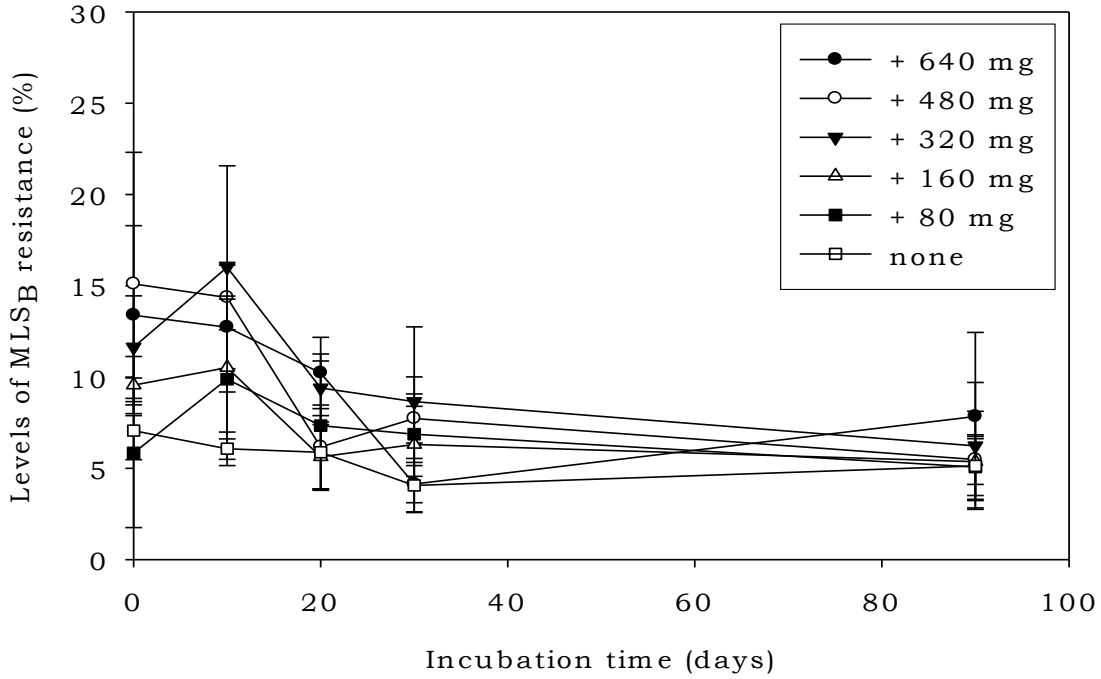


Figure 6. Influence of lincomycin on MLS_B resistance in soil samples. The specified amounts of lincomycin were added to 200 g soil and 8 g NF manure. Error bars represent standard errors.



Although the levels of MLS_B resistance did not differ among the amended and unamended batch tests, we expected to see changes in the microbial community composition and potentially in the types of microorganisms exhibiting resistance. For this analysis, we selected the groups that were most common in the initial community analysis (Fig. 2). The results of this analysis are shown in Figure 7 for the three basic conditions of soil amended with organic or conventional manure and unamended soil. *Clostridium* spp. represented the largest identified fraction of the resistant microorganisms. While the level of resistance among *Clostridium* spp. tended to decrease in amended batch tests, in the unamended control test resistance increased among *Clostridium* spp.

Figure 7. Contributions of resistance in specific populations to total MLS_B resistance.

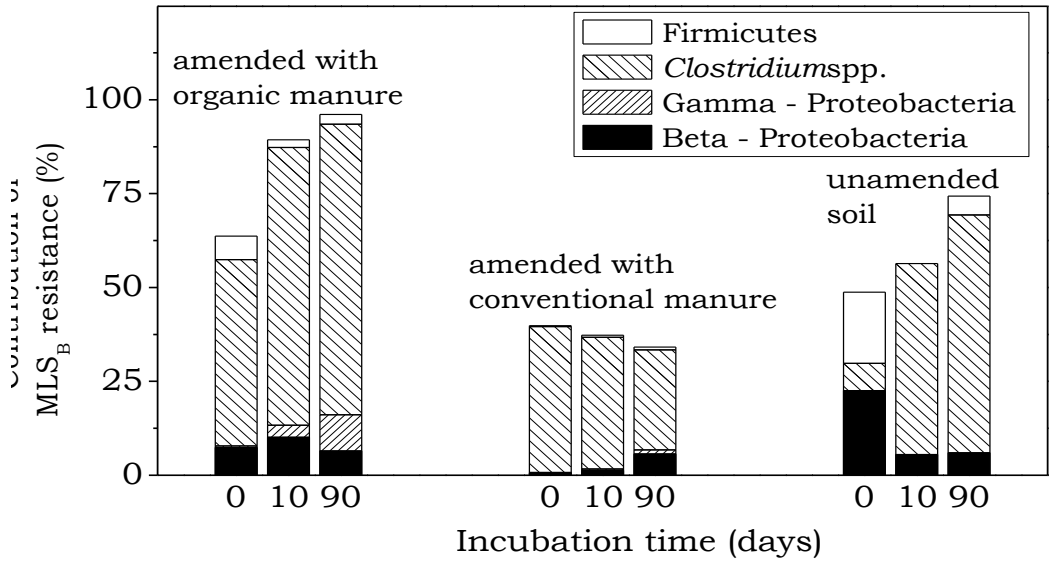
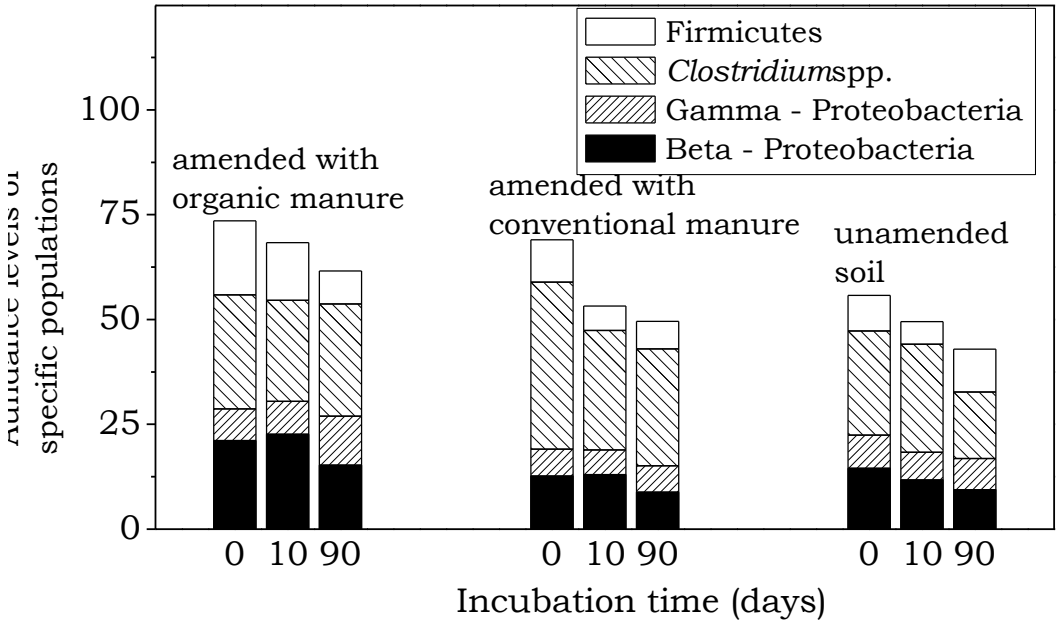


Figure 8. Abundance of specific populations during batch tests. Abundance data were normalized with total cell counts by probe hybridization with universal bacterial probe (Bact0338).



Discussion:

The land application of swine manure could impact the level of antimicrobial resistance in the environment through the addition of antimicrobials, antimicrobial resistance genes, or both. This work was designed to evaluate the combined impact of these additions, to differentiate the contributions of each, and to investigate whether any observed increases in resistance were due to proliferation of microorganisms originating in the manure or the soil. Overall, although particular antimicrobials do persist in the soil, the results indicate that land application does not lead to an increase in MLS_B resistance.

1) Land application of manure results in accumulation of specific antimicrobials.

Tiamulin, tylosin, and tilmicosin were not detected in the soil samples, despite their use at farms H, M and L, and L, respectively. Lincomycin was only in use at farm L and was detected in the soil sample from that farm, although the concentration was low and near the detection limit. All three of the conventional farms used chlortetracycline, and chlortetracycline were present in the corresponding soil samples. These results confirm that the potential for dissemination of antimicrobials into the environment must be considered for a particular compound rather than for antimicrobials as a whole.

The relationship between the use of antimicrobials in swine production and antimicrobials in the soil was confirmed both by the correlation between usage data and antimicrobial measurements described above and by the results of control samples. Negligible concentrations of antimicrobials were found in the soils amended with manure from natural farms and unamended soils. Endogenous synthesis antimicrobials cannot be ruled out, but if it is occurring, it is not resulting in accumulation of antimicrobials substantially above the detection limits.

The potential impact of these relatively low concentrations of antimicrobials is difficult to assess. The observed concentrations of lincomycin are well below the minimum inhibitory concentration (16 μg/ml) [22], and insufficient (and sometimes contradictory) data is available on the effects of subinhibitory concentrations on the development of resistance [23-26]. For chlortetracycline, the measured concentrations are above the minimum inhibitory concentration (16 μg/ml) and should inhibit sensitive microorganisms, thereby promoting the growth of tetracycline resistant microorganisms. In some cases, tetracycline resistance genes and MLS_B resistance genes are linked [27, 28], so this could also lead to a selective pressure for the maintenance of MLS_B resistance.

2) MLS_B resistance was not increased by land application of swine manure.

Levels of MLS_B resistance in the soil samples were all below 5.1%, and no significant difference was observed among soils that received no manure, natural farm manure, and conventional farm manure (ANOVA test with the statistical software R). After twenty days, the levels of MLS_B resistance in the soil batch tests were also independent of amendment, even at the highest

concentrations of swine manure, resistant *Clostridium* spp., lincomycin, or chlortetracycline. Although the focus of these tests was on long-term impacts, a transient increase in resistance was observed in the first ten days of some tests. This is consistent with previous results [5, 29]. The results from both the operational farms and the soil batch tests indicate that the land application of manure did not increase the levels of MLS_B resistance. One important caveat is that the levels of tetracycline resistance were not monitored in the current work. Although linkage has been reported between MLS_B and tetracycline resistance genes, these genes may also occur independently, and given the persistence of chlortetracycline observed above, it would be prudent to examine the levels of tetracycline resistance in these samples. As no increase in resistance was observed, the question of whether it was related to antimicrobials or resistant microorganisms was moot.

3) *Clostridium* spp. in amended soils were more likely to be resistant than those in unamended soils, and in all sample types they accounted for the largest identified fraction of resistant organisms.

Clostridium spp. were clearly an important population, accounting for the largest identified fraction of resistant organisms in most samples. In the day zero samples, the fraction of MLS_B resistant *Clostridium* spp. was higher in amended conditions. The level of resistance in *Clostridium* spp. dropped during the amended batch tests, suggesting that the introduced microorganisms were not persisting. Interestingly, the level of resistance in *Clostridium* spp. increased during the batch tests of unamended soils.

Lay Interpretation: As stated in the contract, we require a lay interpretation of the project, suitable for public release by the Board. Please include contact information, should the public want to contact you directly.

The land application of treated swine manure has been proposed to contribute to the spread of antibiotic resistance, but this possibility has not been confirmed. In this work we measured antibiotics and antibiotic resistance in manure amended fields and in laboratory tests. Some of the antibiotics were found in the soil. However, no long-term increase in the level of antibiotic resistance was seen. These results suggest land application is not contributing to the spread of antibiotic resistance. However, this study only measured one type of antibiotic resistance. It is possible that resistance to a different antibiotic does increase with land application.

References

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