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Effects of Swine Manure on Macrolide, Lincosamide, and Streptogramin B Antimicrobial Resistance in Soils[▽]

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Current agricultural practices involve inclusion of antimicrobials in animal feed and result in manure containing antimicrobials and antimicrobial-resistant microorganisms. This work evaluated the effects of land application of swine manure on the levels of tetracycline, macrolide, and lincosamide antimicrobials and on macrolide, lincosamide, and streptogramin B (MLS_B) resistance in field soil samples and laboratory soil batch tests. MLS_B and tetracycline antimicrobials were quantified after solid-phase extraction using liquid chromatography-tandem mass spectrometry. The prevalence of the ribosomal modification responsible for MLS_B resistance in the same samples was quantified using fluorescence *in situ* hybridization. Macrolide antimicrobials were not detected in soil samples, while tetracyclines were detected, suggesting that the latter compounds persist in soil. No significant differences in ribosomal methylation or presumed MLS_B resistance were observed when amended and unamended field soils were compared, although a transient (<20-day) increase was observed in most batch tests. *Clostridium* cluster XIVa accounted for the largest fraction of resistant bacteria identified in amended soils. Overall, this study did not detect a persistent increase in the prevalence of MLS_B resistance due to land application of treated swine manure.

Treated swine manure contains substantial levels of both antimicrobial-resistant microorganisms (10, 26) and antimicrobials (7, 18, 33). Land application of manure could therefore contribute to public health risks associated with the increasing prevalence of antimicrobial resistance in pathogens both directly, through the dissemination of antimicrobial-resistant pathogens, and indirectly, through the introduction of and selection for antimicrobial resistance genes. Because limited data are available, this connection is largely a theoretical connection, particularly for the indirect effects. However, a recent retrospective study of antimicrobial resistance in soil did support the hypothesis that there is an environmental connection by documenting that there was an increase in the abundance of antibiotic resistance genes in samples collected from 1940 to 2008, during which time antimicrobial production increased dramatically (12).

The fate of antimicrobials in amended soils is a function of their sorptive properties, the soil characteristics, and the potential for abiotic and biotic degradation of the antimicrobials. Tetracyclines tend to adsorb to soil (21, 23), which leads to persistence in amended soils (3, 7, 11), although they are also susceptible to degradation (3, 4). The macrolide tylosin frequently is not detected (3, 4, 7, 11, 33) and is likely rapidly degraded in manure and soils (8, 16, 24). However, persistence of tylosin for several months in amended soil has also been reported (6). The differences in degradation rates may be caused by differences in soil characteristics, manure-to-soil ratios, and/or microbial communities (15, 16, 21).

Addition of both antimicrobials and antimicrobial-resistant microorganisms might be expected to result in an increase in the levels of resistance. However, most studies have not shown that there is a long-term increase in antimicrobial resistance due to land application of manure at agronomically prescribed rates (5, 9, 26). Transient (i.e., <45-day) increases have been reported (9, 26), as have elevated levels of resistance at sites near manure piles (5). In contrast, another report showed that there were significantly higher levels of tylosin resistance in soils that received animal manure from operations that used subtherapeutic levels of antimicrobials than in soils at sites where there was no use of subtherapeutic levels of antimicrobials (19). One limitation of these studies was their use of culture-based methods to quantify resistance; the results may not be representative of the entire microbial community. The molecular methods that have been used to quantify resistance also have limitations, and the most serious limitation is the inability of these methods to examine the full diversity of known and unknown resistance genes. The previous molecular studies of the impact of land application on resistance were largely restricted to qualitative analyses (10, 25), although quantitative PCR methods for analysis of tetracycline resistance genes have recently been used for cattle and swine lagoons (14, 20). In a retrospective soil study, Knapp et al. (12), who also used quantitative PCR, found multiple site differences, which made it difficult to evaluate the impact of manure application. However, the site with the highest manure application rate did not show the highest levels of antimicrobial resistance, suggesting that there are other factors that have a greater influence on the prevalence of resistance.

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In the present study, a variation of the fluorescence *in situ* hybridization (FISH) technique was used to assess the impact of land application of swine manure on the levels of macrolide-lincosamide-streptogramin B (MLS_B) resistance. Although the MLS_B antimicrobials are chemically distinct, methylation or mutation of a single base of the 23S rRNA prevents binding and results in cross-resistance to all three classes (29). The prevalence of MLS_B antimicrobial resistance in the microbial community can therefore be quantified indirectly by hybridization of an oligonucleotide probe to unmethylated, MLS_B-sensitive ribosomes, using either membrane hybridization (1, 10) or FISH (31). These methods do not require culturing or a comprehensive knowledge of the diversity of resistance gene sequences, but they do not detect resistance to specific antimicrobials that results from other mechanisms, such as macrolide efflux.

This study focused on evaluating the impact of land application of swine manure on the levels of antimicrobials and the prevalence of antimicrobial resistance in the soil environment. The concentrations of tetracycline, macrolide, and lincosamide antimicrobials and the prevalence of MLS_B resistance were compared for field soils that received no manure, swine manure from farms that did not use antimicrobials (referred to below as organic farms), and swine manure from conventional farms to determine whether land application affects the levels of antimicrobials and MLS_B resistance. The effects of addition of manure, antimicrobials (lincomycin and chlortetracycline), and MLS_B-resistant microorganisms on the prevalence of MLS_B resistance were also compared using soil batch tests.

MATERIALS AND METHODS

Farms and sampling. Three Illinois swine farms that routinely used antimicrobials (farms LF, MF, and HF), designated conventional farms, and two Illinois swine farms that used no antimicrobials (farms OF and OF2), designated organic farms, were included in this study. Details concerning antimicrobial use and farm management have been reported previously (10, 31, 33). Manure was applied using surface broadcasting or injection into fields with a corn-soybean crop rotation. The field soils were predominately silt loams (farms MF, LF, OF, and OF2), while the field sampled at farm HF contained silty clay loam and in some areas silt loam (28). The moisture content was between 0.28 and 0.30 g water/g sample. For each farm, five soil cores from each of five systematic locations were pooled to form a single composite sample that was used for antimicrobial quantification and molecular analysis.

For the soil batch tests, manure was collected from the building floor at farm OF2 and from the settling basin at farm LF in December 2005. The unamended soil used in the batch tests was a silty clay loam collected from a single location in an agricultural field at the University of Illinois at Urbana-Champaign South Farm (farm SF) that had not received manure in at least 40 years.

For molecular analysis, samples were fixed using ethanol as described previously (30) and stored at −20°C. The manure and soil samples used for liquid chromatography-mass spectrometry (LC-MS) were kept at temperatures below −20°C until they were shipped on ice. The manure and soil samples used in the batch tests were stored at 4°C.

Quantification of antimicrobials. Antimicrobials were quantified by triple quadrupole liquid chromatography-tandem mass spectrometry at the Institute of Agriculture and Natural Resources at the University of Nebraska (Lincoln, NE) (27, 32). Prior to analysis, the manure samples were diluted in 0.5 M potassium phosphate-citric acid buffer (pH 2.5) (tetracycline analysis) or a neutral phosphate solution (macrolide and lincosamide analysis) and extracted using Oasis HLB cartridges (Waters Corporation, Milford, MA). 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) (27).

FISH methods. Fixed soil samples were diluted (1:100) in 0.1% sodium pyrophosphate (NaPPi) buffer (13). Then 10 μl of a diluted sample was sonicated in 2 ml 0.1% NaPPi buffer with a sonic dismembrator (5-s on-off pulses; output, 250 W; 60 s; model 500; Fisher Scientific, Pittsburgh, PA). Slides were prepared and FISH was performed as previously described (30). Details concerning oligonucleotide probes Bact338, Arc0915, Alf1b, Bet42a, Gam42a, HGC69A, and LGC354 are available at probeBase (17). The MLS_B and ClosXIVa probes and the FISH protocols used have been described previously (31). The *Streptomyces* probe (5′-ACC CCG TTT CCA GGG CTT GT) was originally developed for PCR (22); for use in FISH, a hybridization condition of 25% formamide was selected based on the fluorescence intensities of pure-culture positive and negative controls (negative control, *Kitasatospora phosalacinea*; positive controls, *Streptomyces albus* and *Streptomyces chatrensis*). In dual labeling experiments, the samples were first hybridized with the probe requiring more stringent conditions. Total cell counts were obtained after staining with 4′,6-diamidino-2-phenylindole (DAPI), and the cell counts obtained from the 10 random images for each replicate ranged from 325 to 8,277. The images were analyzed in an automated fashion using the Visilog image analysis software (version 6; Noésis, Les Ulis, France) as previously described (30). The prevalence of ribosomal methylation and presumed MLS_B resistance was calculated as follows: 1 − number of MLS_B-sensitive cells/total number of cells or Bact338-hybridized cells. Detection limits could not be quantified because for all of the soils sampled there were detectable levels of MLS_B resistance, as expected for naturally occurring antimicrobials.

Soil batch tests. The manure-to-soil ratio was estimated using recommended land application rates of manure based on the Illinois Best Management Plan (BMP) (https://webs.extension.uiuc.edu/immp/), measurements of soil density, and an assumed direct impact on the first 20 cm of soil (based on injection depth). Concentrations of resistant microorganisms and antimicrobials were based on previous farm measurements (33). The standard amendments (1×) were 8 g manure, 1.0×10^9 cells of *Clostridium* spp., 160 μg lincomycin, and 80 μg chlortetracycline in 200 g soil. For increased resolution, rather than identical replicates, a concentration series was used for each amendment. Unamended soil was used as a control. The MLS_B-resistant *Clostridium* spp. were isolated from farm OF. For all soil batch tests, the materials were completely mixed in 250-ml glass bottles and incubated at 25°C and the soil wetness was maintained at the initial value (25% of the capacity). The bottles were sealed with Parafilm, and full-spectrum fluorescence light was supplied for 12 of every 24 h. To obtain a bulk measurement of resistance, for each batch test the contents were mixed before a 5-g sample was removed and fixed for molecular analysis.

Statistics. Student *t* tests were performed to compare the levels of antimicrobial resistance of manure-amended soils and unamended soils. Where specified below, one-way analysis of variance (ANOVA) was used for pairwise comparisons. Differences were considered significant at a P value of <0.05. Statistical analyses were carried out with the software R (http://www.r-project.org/).

RESULTS

Levels of antimicrobials and MLS_B resistance in field soils. The concentrations of MLS_B and tetracycline antimicrobials in soils that received no swine manure (unamended) and in soils that received swine manure from organic farms (farms OF and OF2) or swine manure from conventional farms (farms LF, MF, and HF), along with a summary of the antimicrobials used at the different farms, are shown in Table 1. Of the MLS_B antimicrobials, tiamulin, tilmicosin, and tylosin were not detected despite the fact that they were used at one or more farms. Of the tetracycline antimicrobials, chlortetracycline was the compound that was most commonly used and detected (Table 1). The concentrations of tetracyclines were significantly higher ($P = 0.020$) in soils that received swine manure from conventional farms (farms LF, MF, and HF) than in soils that received swine manure from organic farms (farms OF and OF2) or no manure (unamended).

For the six composite soil samples, each from a different farm, the prevalence of ribosomal methylation—and presumed resultant MLS_B antimicrobial resistance—was between 2.0 and 5.2% of the cells that hybridized with the bacterial domain probe. No significant differences were observed among the soil samples from unamended fields and the soil samples that received either organic or conventional farm manure ($P = 0.690$, ANOVA).

TABLE 1. Use of antimicrobials on swine farms and concentrations of antimicrobials in soils that received manure from these farms[a]

Antimicrobial	Farm LF[b]		Farm MF		Farm HF		Concn in soil (ng/g [dry wt])			Detection limit (ng/g [dry wt])
	Use[c]	Concn in soil (ng/g [wet wt])	Use[c]	Concn in soil (ng/g [dry wt])	Use[c]	Concn in soil (ng/g [dry wt])	Farm OF[d]	Farm OF2[d]	Unamended	
MLS_B antimicrobials										
Erythromycin	−	BD[e]	−	BD	−	BD	BD	BD	BD	5
Spiramycin	−	BD	−	BD	−	BD	BD	BD	BD	5
Oleandomycin	−	BD	−	BD	−	BD	BD	BD	BD	2
Tiamulin	−	BD	−	BD	+	BD	BD	BD	BD	5
Tilmicosin	+	BD	−	BD	−	BD	BD	BD	BD	5
Tylosin A	+	BD	+	BD	−	BD	BD	BD	BD	2
Lincomycin	+	9.2	−	BD	−	BD	BD	BD	BD	2
Tetracycline antimicrobials										
Tetracycline	−	9.0	−	16.1 (1.5)	−	35.4 (25.8)	BD	BD	BD	1
Chlortetracycline	+	245.1	+	211.8 (19.8)	+	209.8 (157.6)	BD	BD	BD	1
Oxytetracycline	+	4.1	−	21.9 (18.6)	−	≤4.0	≤3.2	≤3.1	7.8 (3.7)	1
Anhydrotetracycline	−	BD	−	≤4.9	−	3.6 (0.3)	≤3.2	≤4.0	≤3.6	2
Anhydrochlortetracycline	−	BD	−	BD	−	BD	BD	BD	≤4.5	2
β-Apo-oxytetracycline	−	NA[f]	−	9.3 (6.3)	−	≤2.6	4.0 (1.4)	≤3.3	≤6.5	2

[a] Concentrations in dry soil are shown, except for farm LF, for which the concentrations in wet soil are shown because the moisture content was not available for the samples. The values in parentheses are the half-ranges for duplicate measurements. In several cases, values above and below the detection limit were recorded for the same samples. In these cases, the values below the detection limit were included in calculations as the detection limit value, and the affected values are indicated by the ≤ symbol.
[b] The antimicrobial values for the farm LF soil sample have been reported previously (33) and are included for comparison.
[c] +, antimicrobial was used; −, antimicrobial was not used. Details concerning antimicrobial doses and timing have been described previously (10).
[d] No antimicrobials were used on farms OF and OF2.
[e] BD, below the detection limit.
[f] NA, not analyzed.

Effects of antimicrobials and antimicrobial-resistant microorganisms on MLS_B resistance in soil batch tests. The impact of land application of conventional and organic farm manure was also evaluated under controlled conditions using soil batch tests. To distinguish the effects of antimicrobials and MLS_B-resistant microorganisms, the test conditions included amendment with organic farm manure spiked with lincomycin, chlortetracycline, or MLS_B-resistant *Clostridium* spp. The conventional farm manure contained primarily oxytetracycline and its degradation product, β-apo-oxytetracycline (Table 2). Although, as expected, the organic farm manure contained lower concentrations of antimicrobials, surprising amounts of chlortetracycline and oxytetracycline were present (Table 2). Sorption and/or degradation of the antimicrobials occurred during the batch tests but was not complete, as the concentrations at day 90 were 7 to 35% of the estimated initial values (Table 2).

The prevalence of ribosomal methylation—and presumed resultant MLS_B antimicrobial resistance—in the soil batch tests for soils that received the highest concentrations of amendments (4×) is shown in Fig. 1A. As expected, the 4× amendments increased the prevalence of ribosomal methylation in the initial samples ($P = 0.010$). The increase was still evident in the day 10 samples ($P = 0.008$), but no significant difference from the unamended control was observed by day 20 ($P = 0.084$). Similar results were obtained for samples amended with 1× farm OF2 manure and various concentrations of resistant *Clostridium* spp. (Fig. 1B). Specifically, addition of resistant *Clostridium* spp. increased the prevalence of ribosomal methylation in the initial samples ($P = 0.039$), and the difference was still evident at day 10 ($P = 0.019$), but after 20 days of incubation there was no significant difference ($P = 0.240$). Throughout the experiment, there was no significant difference between the prevalence of MLS_B resistance in the unamended soil and the prevalence of MLS_B resistance in the soil amended with 1× farm OF2 manure. The prevalence of ribosomal methylation in soil samples amended with 1× farm OF2 manure and some concentrations of lincomycin appeared to increase over the first 10 days (Fig. 1C). However, like the results of the other batch tests, by day 20 the results were not significantly different from those for unamended samples (for comparisons of unamended and lincomycin-amended samples, at day zero $P = 0.067$, at day 10 $P = 0.015$, and at day 20 $P = 0.076$). Small differences in the prevalence of resistance may have been obscured by the variability.

Initial microbial communities in soil batch tests. Laboratory incubation of soil changes the microbial community even when no amendments are added, and addition of the organic material and microorganisms present in manure was expected to affect the microbial community structure. Coarse characterization of the initial microbial community structure in soils that received the standard amendment of organic or conventional farm manure was performed to select groups to monitor during the batch tests (Fig. 2). This analysis characterized 71% of the total microorganisms (average value for cells hybridized with the Bact0338 and Arc0915 probes normalized with the total number of DAPI-stained cells) and 95% of the total bacteria (average value for cells hybridized with all phylogenetic probes except Arc0915 normalized with the number of cells hybridized with Bact0338) and identified *Betaproteobacteria*, *Gammaproteobacteria*, *Clostridium* cluster XIVa, and *Firmicutes* as the most abundant bacterial groups. The dynamics of these groups were then monitored during the batch tests (Fig. 3A). At the coarse resolution used, the levels of specific groups of bacteria were relatively stable during the soil batch tests.

MLS_B resistance in specific microbial populations. While the results described above indicated that land application of manure did not result in increased levels of MLS_B resistance in the soil microbial community as a whole, measuring MLS_B resistance as a bulk parameter could have concealed important effects of land application, such as changes in the types of microorganisms exhibiting resistance. Therefore, we combined the phylogenetic FISH analysis with the MLS_B probe to characterize the prevalence of ribosomal methylation and the prevalence of presumed MLS_B antimicrobial resistance in the most abundant groups of bacteria throughout the batch tests (Fig. 3B). By combining data for the abundance and prevalence of resistance for specific groups, the relative contribution of each group to the total resistance could be calculated (Fig. 3C). The initial prevalence of resistance in *Clostridium* cluster XIVa was significantly higher for amended samples ($P = 0.006$) than for unamended samples. A decrease in the prevalence of resistance in this cluster corresponded with the overall decrease in resistance observed during the batch tests. *Clostridium* cluster XIVa accounted for the largest fraction of the identified resistant bacteria, comprising an average of 47.0% of the MLS_B-resistant microorganisms throughout the batch tests (Fig. 3C).

DISCUSSION

This work was designed to investigate the impact of land application of manure on the levels of MLS_B resistance in soils. Specific antimicrobials were detected in the soil samples, but the results for field soil samples and the results of laboratory batch tests indicate that land application leads to only a transient increase in MLS_B resistance in the microbial community as a whole. Although our study considered only one resistance mechanism, ribosomal modification, our results are consistent with the results of previous studies in which a subset of the microbial community was monitored using culture-based measurements of resistance to the macrolides tylosin (6) and erythromycin (9, 26). Over months to years, land application of manure does not appear to result in increased levels of MLS_B resistance in soil. It is important to note that this study did not investigate changes in the diversity or the distribution of antimicrobial resistance genes that may occur as a result of manure application. The results may also be different for different antimicrobials.

Specific antimicrobials persisted in the amended soils, and the connection between the use of antimicrobials in swine production and the presence of antimicrobials in the soil was supported by the agreement between the usage data and the antimicrobial measurements (Table 1). The absence of macrolides and the persistence of tetracyclines and, to a lesser degree, lincomycin were consistent with most available data on degradation of these antimicrobials (3, 4). In a recent study that focused on the fate of tylosin and chlortetracycline, analysis of tylosin degradation products resulted in an increase in the half-life of tylosin (6). No tylosin degradation products were monitored in the present study, nor was the potency of the antimicrobials in the soil matrix assessed. Based on the antimicrobial data, a greater increase in tetracycline resistance than in MLS_B resistance might be predicted, as observed by Knapp et al. (12).

Even the relatively low prevalence of resistance observed in

TABLE 2. Concentrations of antimicrobials in soil batch tests

Component(s)[a]	Concn (ng/ml or ng/g [dry wt]) of [b]:						
	Lincomycin	Tetracycline	Chlortetracycline	Oxytetracycline	Anhydrotetracycline	Anhydrochlortetracycline	β-Apo-oxytetracycline
Individual components[c]							
Farm LF manure[d]	4.4 (1.4)	19.8 (1.4)	5.3 (1.5)	2,630.0 (836.0)	≤6.0	<5	1,230.5 (15.5)
Farm OF2 manure[d]	<2	60.1 (6.0)	723.9 (100.9)	126.5 (12.5)	20.9 (14.9)	≤4	45.8 (2.8)
Soil	<2	<1	<1	8.1 (3.4)	≤3.8	≤4.9	≤6.5
Batch tests							
1× farm LF manure	NA/NA[e]	1.1/<1	0.3/7.1	159.9/21.3	4.9/5.9	5.8/<2	78.2/11.5
4× farm LF manure	NA/NA	4.7/<1	1.3/<1	631.4/171.7	6.1/<2	5.9/<2	298.9/69.9
1× farm OF2 manure	NA/NA	3.4/<1	40.7/8.2	15.8/<1	5.6/<2	5.9/<2	9.9/<2
4× farm OF2 manure	NA/NA	13.2/<1	159.1/26.7	36.3/1.7	8.9/<2	6.8/<2	17.2/4.9
1× farm OF2 manure + 1× chlortetracycline	NA/NA	3.4/7.6	602.8/41.5	16.0/12.6	5.7/20.7	6.0/11	10.0/18.1
1× farm OF2 manure + 4× chlortetracycline	NA/NA	3.7/6.4	2,444.1/166.9	17.1/1.6	6.1/10.3	6.4/<2	10.7/5.1
1× farm OF2 manure + 1× lincomycin	1,130.8/311.6	NA/NA	NA/NA	NA/NA	NA/NA	NA/NA	NA/NA
1× farm OF2 manure + 4× lincomycin	4,492.3/1,560.7	NA/NA	NA/NA	NA/NA	NA/NA	NA/NA	NA/NA

[a] For 1× amendments 8 g manure was added to 200 g soil.

[b] The concentrations in manure are expressed in ng/ml, and the concentrations in soil and amended soil are expressed in ng/g (dry weight). Erythromycin, spiramycin, oleandomycin, tiamulin, tilmicosin, and tylosin A concentrations were analyzed for the same samples as lincomycin concentrations and were always below the detection limit. The values in parentheses for individual components are the half-ranges for duplicate measurements. Measurements below the detection limit for individual components were handled as described in Table 1, footnote *a*. The values for the batch tests are initial concentrations (estimated using data for individual components)/final concentrations (measured after 90 days of incubation). For estimation of the initial concentrations values below the detection limit were assumed to be zero.

[c] Individual components were sampled for analysis on day 0.

[d] Antimicrobial concentrations in farm LF and OF2 manure were reported previously (31) and are included for comparison.

[e] NA, not analyzed or calculated.

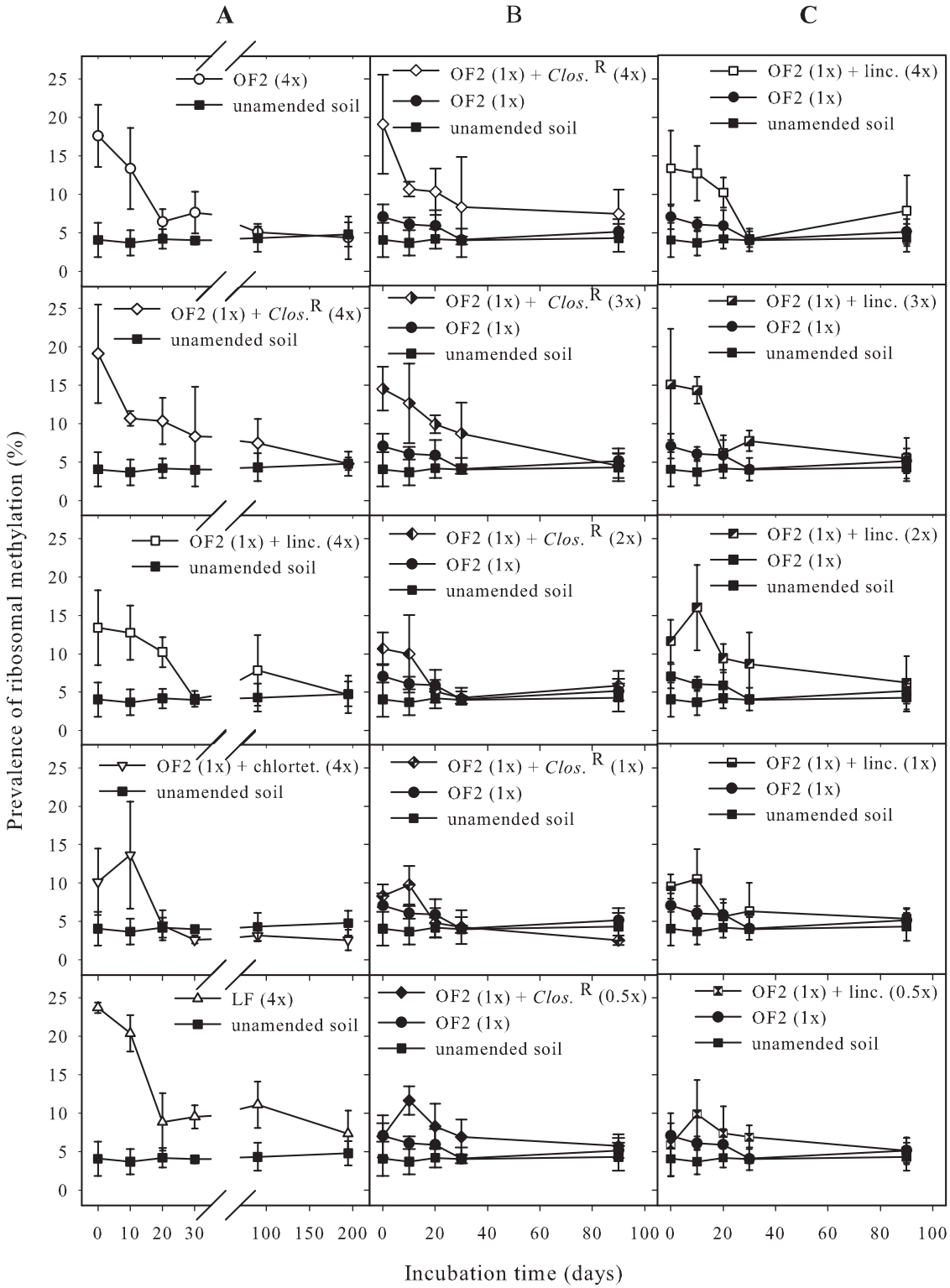

FIG. 1. Influence of manure application on MLS_B resistance in batch tests. The prevalence of ribosomal methylation and the prevalence of presumed MLS_B resistance are shown for the batch tests in which soil was amended with the maximum concentrations (4×) of manure from farms OF2 and LF, with MLS_B-resistant *Clostridium* spp. (*Clos.*^{R}), with lincomycin (linc.), and with chlortetracycline (chlortet.) (A). The responses to a range of concentrations of MLS_B-resistant *Clostridium* spp. and lincomycin were also determined (B and C, respectively). The error bars indicate standard errors for triplicate hybridizations.

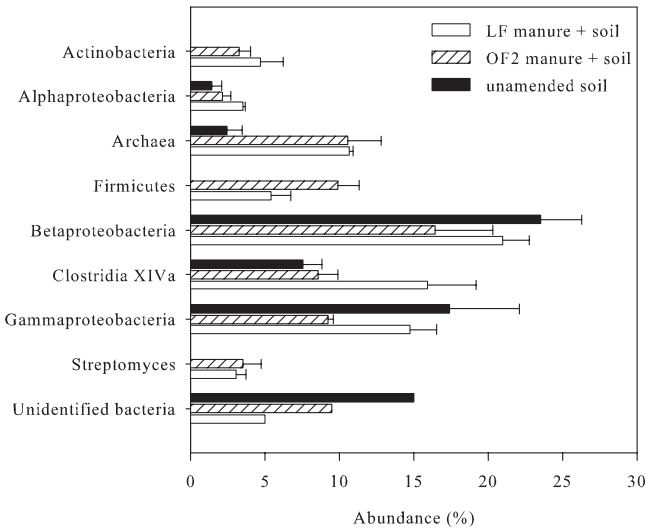

FIG. 2. Microbial community analysis for manure-soil mixtures. Abundance was normalized with total cell counts by DAPI staining. *Firmicutes* and *Streptomyces* in the unamended soil were not quantified. The error bars indicate half-ranges for duplicate hybridizations.

this study could represent a threat to public health if the resistance genes are concentrated in pathogenic organisms or are readily transferred to such organisms. Using dual labeling experiments, *Clostridium* cluster XIVa has been identified as a key group. Not only were members of this cluster abundant, in agreement with a previous analysis of swine manure collection pits and lagoons (2), but they also accounted for a large fraction (up to 77%) of the MLS_B resistance in this study. In the batch tests in which soil was amended with conventional farm manure, the relative abundance of *Clostridium* cluster XIVa remained fairly constant, while the fraction of resistant members in the population decreased, perhaps because the introduced microorganisms did not proliferate in the soil. Nevertheless, these bacteria continued to account for a large fraction of the observed resistance. These results suggest that additional analysis of this group of organisms, including analysis of the location and mobility of their resistance genes, is necessary to improve our understanding of the potential consequences of land application of manure.

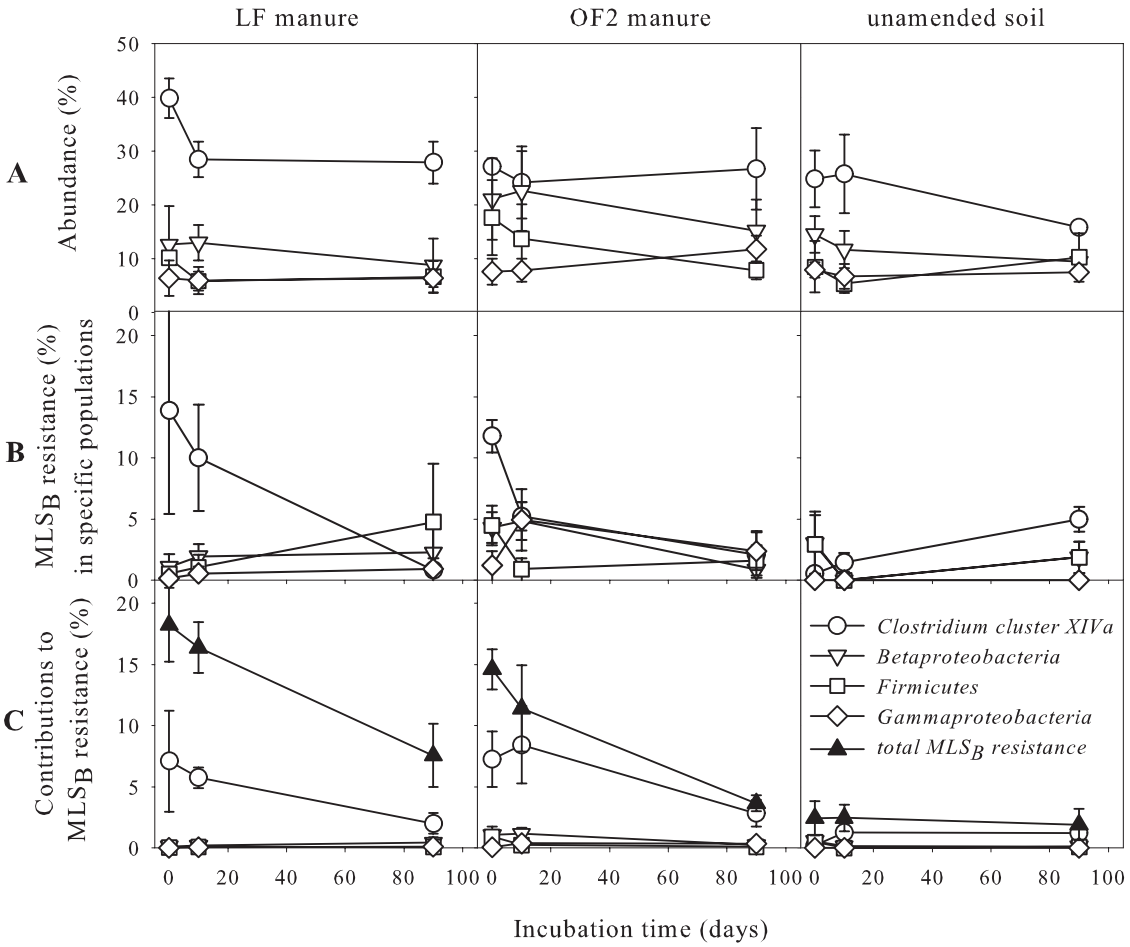

FIG. 3. Abundance and prevalence of the MLS_B resistance of specific populations during batch tests. Unamended soil samples were compared to samples amended with 4× farm LF manure or 4× farm OF2 manure. (A) Abundance data, normalized to total cell counts by DAPI staining. (B) Prevalence of MLS_B resistance in specific populations. (C) Contributions of specific populations to overall resistance levels. The error bars indicate standard errors for triplicate hybridizations.

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