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Cow excrements enhance the occurrence of tetracycline resistance genes in soil regardless of their oxytetracycline content

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HIGHLIGHTS

- Oxytetracycline-rich and -free manure equally enriched soil tetracycline resistome.
- Tetracycline resistance genes differed in their persistence in manure-amended soils.
- *tet*(M), *tet*(Z) and *tet*(W) persisted in manure-amended soils for at least 3 weeks.
- *tet*(Q), though highly abundant in manure, did not persist in manure-amended soils.
- *tet*(L) and *tet*(V) were found in soils with no recent history of manuring.

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ABSTRACT

Fertilizing soils with animal excrements from farms with common antibiotic use represents a risk of disseminating antibiotic resistance genes into the environment. In the case of tetracycline antibiotics, it is not clear, however, whether the presence of antibiotic residues further enhances the gene occurrence in manured soils. We established a microcosm experiment in which 3 farm soils that had no recent history of fertilization with animal excrements were amended on a weekly basis (9 times) with excrements from either an oxytetracycline-treated or an untreated cow. Throughout the study, the concentration of oxytetracycline in excrements from the treated cow was above 500 $\mu g\ g^{-1}$ dw, whereas no oxytetracycline was detected in excrements from the healthy cow. Both excrements contained tetracycline resistance (TC-r) genes *tet*(L), *tet*(M), *tet*(V), *tet*(Z), *tet*(Q) and *tet*(W). The excrements from the treated cow also contained the *tet*(B) gene, and a higher abundance of *tet*(Z), *tet*(Q) and *tet*(W). Three weeks after the last excrement addition, the individual TC-r genes differed in their persistence in soil: *tet*(Q) and *tet*(B) were not detectable while *tet*(L), *tet*(M), *tet*(Z) and *tet*(W) were found in all 3 soils. There were, however, no significant differences in the total number, nor in the abundance, of TC-r genes between soil samples amended with each excrement type. The oxytetracycline-rich and the oxytetracycline-free excrement therefore contributed equally to the increase of tetracycline resistome in soil. Our results indicate that other mechanisms than OTC-selection pressure may be involved in the maintenance of TC-r genes in manured soils.

1. Introduction

Antibiotics are routinely administered to farm animals in order to treat and prevent diseases or, in sub-therapeutic doses, to promote growth. Among these, tetracyclines (TCs) are a group of broad spectrum antibiotics that are widely used to treat infections of the digestive and urinary tracts (Chee-Sanford et al., 2009). It has been shown, however, that TCs may select for resistant (TC-r) bacteria in the animals' intestinal tracts (Hart et al., 2006). Furthermore, it is estimated that as much as 75–80% of the TCs administered to farm animals are poorly metabolized and remain in their active form during their passage through the animal's gut (Hamscher et al., 2003). Thus, TC-r bacteria along with non-metabolized TCs are recycled to the soil via animal excrements, which may increase TC resistance in the environment (Chee-Sanford et al., 2009).

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Indeed, it was shown that the abundance of TC-r genes in soils rose considerably during the antibiotic era (Knapp et al., 2010), and this may also have consequences for human health, as antibiotic resistomes may be shared among soil, animal and human microflora (Pasquali et al., 2004; Kobashi et al., 2007; Forsberg et al., 2012).

The presence of antibiotic residues in animal excrements may increase the persistence of antibiotic resistance genes in soils either by selecting for resistant populations, or by enhancing horizontal gene transfer (Chee-Sanford et al., 2009). For example, Heuer and Smalla (2007) treated soil samples with a combination of sulfonamide and hog manure and observed an increase in cultivable sulfonamide-resistant bacteria and *sul1* gene abundance, as well as in transfer frequencies of resistance plasmids. These findings were further corroborated in mesocosm and field experiments, where manure from sulfadiazine-treated animals increased the abundance of *sul* genes to a higher extent than manure from untreated animals (Jechalke et al., 2013; Kopmann et al., 2013). It is not clear, however, whether soils amended with TC-rich manure likewise would increase TC resistance. In comparison to sulfonamides, TCs bind more strongly to the soil matrix (Chee-Sanford et al., 2009) such that their effect on soil microbial community structure and function may be more limited (Nelson et al., 2011; Liu et al., 2012). A previous microcosm study (Schmitt et al., 2006) showed that the persistence of certain TC-r genes in soil amended with pig manure was enhanced only when rather high oxytetracycline doses (above 15 mg kg^{-1}) were spiked into soil. The immediate effect of an antibiotic added into soil in a form of solution is, however, likely different from that of an antibiotic present in manure, and the fate of TC-r genes in soils amended with manure from TC-treated animals remains unknown. In this study, we focused on the fate of TC-r genes in soil after amendments with excrements from either an oxytetracycline (OTC)-treated or an untreated dairy cow. We hypothesized that (i) soil resistome would be enriched with TC-r genes originating from cow excrements at several weeks after excrement addition, and (ii) the TC-r gene abundance and diversity would be higher in soils amended with excrement from an OTC-treated cow, compared to the excrement from a healthy cow.

We monitored, therefore, presence or abundance of 8 TC-r genes in soil samples collected from 3 non-manured agricultural fields, after repeated additions of excrements from either an OTC-treated or an untreated cow. We chose the genes *otr*(B), *tet*(B), *tet*(L), *tet*(V) and *tet*(Z) coding for TC-efflux pumps, and the genes *tet*(M), *tet*(Q) and *tet*(W) coding for ribosomal protection proteins. The genes *tet*(Q) and *tet*(W) were monitored with quantitative PCR (qPCR) based on previously published protocols (Smith et al., 2004), and a new qPCR protocol was developed for *tet*(Z). The other genes were assessed with conventional PCR with published primers (Aminov et al., 2001, 2002; Kyselková et al., 2012).

2. Materials and methods

2.1. Soil and excrement sampling

Soils used in our incubation experiment were collected from 3 private farms near the city of Sherbrooke, Québec, Canada. Each field had not been fertilized with manure for at least 20 years. The cropping history and soil physicochemical characteristics are given in Table 1.

Excrement samples were taken from 1 healthy and 1 Oxymycine-treated dairy cow (Holstein breed), both quartered at the Dairy and Swine Research and Development Centre, Lennoxville, QC, Canada. Both cows were fed micro-nutrient supplements in a standard way, which included typically (approx. mg per cow and day) Fe (840), Zn (1040), Cu (170), Mn (700), Co (20) and Se (7).

Table 1
Soil characteristics.

Texture	pH ($CaCl_2$)	Dry matter (%)	C organic (%)	N total (%)	P (mg kg^{-1})	K (mg kg^{-1})	Mg (mg kg^{-1})	Ca (mg kg^{-1})	Cropping history
Farm I									
Sand	5.95	82.1	3.71	0.24	42	122	261	1811	Tree-based intercropping since 2004, combining rows of hardwood species with cereals as the intercrop; periodic applications of lime and mineral fertilizers
Farm II									
Loam	7.02	76.6	2.43	0.19	62	177	103	2781	Certified organic farm since early 1980's; annual rotation of various vegetable crops; fertilized with vegetable compost
Farm III									
Sandy loam	5.39	79.0	2.03	0.14	56	108	78	748	Continuous corn production for over 20 years; periodic applications of lime and mineral fertilizers