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Independent and combined effects of oxytetracycline and antibiotic-resistant *Escherichia coli* O157:H7 on soil microbial activity and partial nitrification processes

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ABSTRACT

The common practice of field-spreading animal manure on agricultural land contributes to the dissemination of antibiotics and antibiotic-resistant pathogens, which may disrupt important soil microbial functions. In this study, the potential independent and combined effects of oxytetracycline (OTC) and antibiotic-resistant *Escherichia coli* O157:H7 (*E. coli* O157:H7) on soil microbial activity and partial nitrification processes were investigated by testing the abundance of 16S rRNA and 18S rRNA, ammonia-oxidizing bacteria (AOB) and archaea (AOA). Treatments included manure-amended soil inoculated vs. not inoculated with *E. coli* O157:H7, treated or not treated with OTC at environmentally releant concentration. Results indicated that OTC did not affect soil bacterial diversity or abundance but increased the abundance of 18S rRNA, the AOB *amoA* gene, and the activity of urease. The invasion of *E. coli* O157:H7 significantly decreased the abundance of 16S rRNA, the AOA *amoA* gene, and soil microbial diversity from 1 to 14 days, while there was no significant impact of *E. coli* O157:H7 on soil microbial activity and function from 14 to 28 days. The dual treatment with OTC and *E. coli* O157:H7 significantly increased the abundance of AOB at day 14 and 28, which resulted in higher concentrations of NO_3^-–N in the soil. The interaction between OTC and *E. coli* O157:H7 on decreasing the abundance of 16S rRNA and microbial diversity was statistically significant after 1 day of incubation. Additionally, OTC and *E. coli* O157:H7 had significant interactive effects on urease activity, which may be also attributed to the impact on the partial nitrification process.

1. Introduction

Globally, thousands of tons of veterinary pharmaceuticals are used annually for disease control or growth promotion in intensive animal husbandry (Thiele-Bruhn, 2003; Sarmah et al., 2006; Liu et al., 2009). A large proportion (30–90%) of the antibiotics may be excreted in manure due to incomplete metabolism. Meanwhile, farm animals often harbor a variety of zoonotic pathogens; for example, dairy cattle are the reservoir of antibiotic-resistant bacteria *Escherichia Coli* O157:H7 (Fang et al., 2014). The common practice of field-spreading animal manure on agricultural land is a major pathway for the dissemination of antibiotic residues and antibiotic-resistant pathogens into the agoecosystem and beyond. Indeed, antibiotics and antibiotic-resistant bacteria related to food animal production have been detected in agricultural soils receiving manures (Qiao et al., 2012; Fang et al., 2014), raising concerns over potential soil health as well as food security and ecological sustainability issues (Jechalke et al., 2014).

Oxytetracycline (OTC) is one of the tetracycline class of antibiotics widely used on animal farms with broad-spectrum antimicrobial activities against a variety of bacterial infections (Gao et al., 2013). Residues of OTC have been detected in farm animal manure, water and soils (Qiao et al., 2012; Hou et al., 2009). Several studies have reported adverse effects of OTC on soil enzymes (Yang et al., 2009; Gao et al., 2013; Thiele-Bruhn and Beck, 2005), soil microbial community (Liu et al., 2009), microbial function (Liu et al., 2012; Kong et al., 2006) and root and shoot elongation of wheat (Jin et al., 2009). OTC has also been shown to decrease the rate of

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nitrification through inhibiting the activity of soil nitrifying bacteria (Piotrowska-Seget et al., 2008). However, it is not clear how OTC, when added with manure at environmentally relevant concentration, may affect soil microbial activities or key soil processes such as the N cycling.

E. coli is a Gram-negative, rod-shaped bacterium commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some serotypes are pathogenic and can cause serious disease. Among the pathogenic *E. coli* strains, *E. coli* O157:H7 has been shown to pose a significant threat to environmental safety and public health because of its low infective dose (as few as 10 cells), high pathogenicity and ability to survive under harsh environmental conditions (Griffin and Tauxe, 1991; Haznedaroglu et al., 2009). Survival of *E. coli* O157:H7 in different substrates, such as soil, manure and soil-manure mixtures, can range from several weeks to more than a year (Kudva et al., 1998; Jiang et al., 2002; Nicholson et al., 2005). Once *E. coli* O157:H7 is introduced into soil through animal manure, they start to interact with the autochthonous microbial community and influence soil microbial activity and diversity. The invasion of *E. coli* O157:H7 changed the soil microbial community to some extent, making the environment stressful and unstable (Yao et al., 2014). However, with regard to pathogenicity, most of the research has focused on the survival and transport of *E. coli* O157:H7 in soil (Van Elsas et al., 2007; Liang et al., 2011; Franz et al., 2008; Semenov et al., 2008). Little information is available on the impact of *E. coli* O157:H7 on soil functional microorganisms, such as N cycle-related microorganisms.

The widespread use of veterinary pharmaceuticals (e.g. OTC) and the high prevalence of *E. coli* O157:H7 in livestock animals (particularly cattle) inevitably lead to manure containing both the chemical and the bacterial agents and their subsequent spreading to agricultural field. Once applied, OTC and resistant *E. coli* O157:H7 may affect the abundance of bacteria and fungi, microbial diversity and activity, and soil microbial function. Therefore, it is important to examine the potential impacts of OTC and resistant *E. coli* O157:H7, when simultaneously released into the soil environment, on disruption of soil microbial functions. To our knowledge, there has not been research addressing this issue.

Soil N cycle is one of the fundamental processes critical to soil health, productivity, and food security, driven by soil microorganisms. Of the N transformation processes, ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) are the drivers for the first and rate-limiting step in the nitrification processes (Zhang et al., 2015). Monitoring AOB and AOA activities has been used as a way for assessing microbial response to soil changes as a result of agricultural management practices, such as oxygen limitation and pH changes (Francis et al., 2007; Schleper and Nicol, 2010; Prosser and Nicol, 2012). It was also suggested that studying AOB and AOA dynamics may help us better understand the impact of climate change on key soil processes, linking microbial community composition with functions (Prosser and Nicol, 2008).

In the present study, we elucidate the potential impact of manure-borne antibiotics OTC (at environmentally relevant rate) and antibiotic-resistant pathogen *E. coli* O157:H7 on soil N processes using AOB and AOA as sentinels, in addition to other soil microbial activity indicators. Our objective was to assess the individual and combined effects of OTC and *E. coli* O157:H7 on the abundance of soil bacteria and fungi, microbial diversity and activity, and the partial nitrification process (by measuring the abundance of AOA and AOB). Based on the rationale above, we hypothesized that OTC and *E. coli* O157:H7 could affect soil N cycling by regulating soil microbial activity and suppressing specific groups of ammonia-oxidizer (Fig. 1). Soil microbial diversity and communities were tested by the terminal restriction fragment length polymorphism (T-RFLP) technique. The abundance of AOB and AOA were assessed using quantitative polymerase chain reaction (qPCR).

2. Materials and methods

2.1. Soil, antibiotic and E. coli O157:H7

The soil, mapped as a silt loam, was obtained from the 0- to 20-cm layer of long-term non-farmed land in the Shangzhuang experimental station, Beijing (116.25°E, 40.13°N), and had received no fertilizer or manure containing veterinary antibiotics for at least 10 years. The bulk soil sample was air-dried and passed through a 2-mm sieve, and stored in a plastic container at ambient temperature. The soil had a total nitrogen concentration of 0.10%, an organic matter concentration of 1.25%, a pH of 7.40 (H_2O, soil 2.5:1, v/v), a cation exchange capacity of 10.43 cmolc kg^{-1}, an Olsen-P of 13.65 mg kg^{-1} and an NH_4Cl-exchangeable K of 143.0 mg kg^{-1}.

The antibiotic used in the study was OTC (Terramycin®, 200 mg mL^{-1} base as OTC dihydrate). The international reference strain of enterohemorrhagic *E. coli* O157:H7, EDL933, was purchased from the Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine, and antibiotic-resistant strains were screened out using 1 mg L^{-1} OTC. The *E. coli* O157:H7 was cultured on eosin methylene blue medium (EMB) containing 1 mg kg^{-1} OTC, and incubated aerobically at 37 °C for 24–48 h, then the growing colonies were transferred into Luria–Bertani (LB) medium. After 24 h of growth, the *E. coli* O157:H7 was inoculated onto the surface of EMB agar plates. Subsequently, filter paper discs (6 mm in diameter) saturated with 1 mg kg^{-1} OTC were placed on the surface of each inoculated plate. The plates were incubated at 37 °C for 24 h, and there was no inhibition zone observed. In all the *E. coli* O157:H7 treatments, cells were pre-grown overnight in LB medium, harvested by centrifugation (12,000 *g*) for 10 min, and washed with phosphate buffer (10 mM, pH 7.2) three times. The cell pellets were resuspended in sterile deionized water and used as inoculum. The cells were added to the soil to a final concentration of approximately 10^7 CFU gdw^{-1} according to a method adapted from Franz et al. (2008).

2.2. Experimental design and incubation

Treatments included manure-amended soil inoculated vs. not inoculated with antibiotic-resistant *E. coli* O157:H7 (10^7 CFU gdw^{-1}), treated or not treated with OTC [1 mg kg^{-1} soil DM, which was selected to encompass environmentally relevant concentrations (Toth et al., 2011)]. Each treatment had three replications. Both the antibiotic and *E. coli* O157:H7 were spiked to cattle manure. The cattle manure, testing negative for presence of *E. coli* O157, was obtained from the floor of a free-stall dairy immediately following excretion from cows that did not receive any antibiotics. After thoroughly mixing with a sterile wooden spatula, the spiked manures were mixed with the prepared soil. The final soil-manure mixture consisted of 35 g cattle manure per kilogram of DM soil, and the manure/soil ratios were calculated to approximate an agronomic nitrogen-based manure application of 180 kg N ha^{-1}. The mixtures were then brought to 80% of field capacity (soil field capacity was 31.3% gravimetric water content), having accounted for the water contained in manure, PSS and antibiotic liquid. After thorough mixing, portions of 500 g were weighed into sterilized 800 mL polypropylene beakers for incubation and the beakers were covered in parafilm to retard moisture loss. During the course of the incubation, beakers were arranged on a laboratory bench in a completely random design, and the ambient temperature was approximately 25 °C. Throughout the incubation,