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Sulfonamide resistance genes in soils treated with waste from animal production in an organic production system

Genes de resistência a sulfonamida em solos tratados com resíduos da produção animal em sistema orgânico de produção

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Highlights

Animal waste is a source of sulfonamide resistance genes *sul*1 and *sul*2.
Composting process may not be enough to eliminate antimicrobial resistance genes.
Animal waste can increase genes for resistance to antimicrobials in agricultural soils.

Abstract

Animal waste is widely used in organic production systems. However, these residues can increase antimicrobial determinants in the soil. In this perspective, this study was developed to evaluate the presence of sulfonamide resistance genes in soils from an organic production system that received animal waste as organic fertilizer. Soil samples were collected from four properties with different management practices to increase soil fertility. Three properties use the animal waste from the conventional system and the other use plant residues as soil cover and a legal reserve. The extraction of total DNA from soil was carried out followed by the amplification of genes encoding sulfonamide resistance (*sul*1 and *sul*2) by the PCR (polymerase chain reaction) technique. The *sul*1 and *sul*2 genes were detected only in soils treated with animal waste. The

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genes were not detected in soils from the legal reserve and the property that used plant residues as soil cover. These results indicate that the use of animal waste as agricultural fertilizer can increase genes for resistance to antimicrobials in the soil and the composting process may not be enough to eliminate them. This information reiterates the need to implement standards that establish quality parameters for animal waste, considering resistance to antimicrobials, as well as the development of management strategies that reduce the risk of spreading resistance to antimicrobials when these residues are applied to soils.

Key words: Animal waste. Antimicrobial resistance. Bovine manure. Poultry litter.

Resumo

Resíduos animais são amplamente utilizados em sistemas de produção orgânicos. No entanto, esses resíduos podem incrementar determinantes antimicrobianos no solo. Nesta perspectiva, este estudo foi desenvolvido para avaliar a presença de genes de resistência à sulfonamida em solos do sistema de produção orgânico que receberam resíduos de animais como fertilizante orgânico. Amostras de solo foram coletadas de quatro propriedades com diferentes formas de manejo para aumentar a fertilidade do solo. Três utilizaram resíduos animais do sistema convencional; uma utilizou resíduos vegetais como cobertura do solo, além de uma reserva legal. Foi realizada a extração do DNA total do solo, seguida da amplificação dos genes que codificam resistência sulfonamida (*sul*1 e *sul*2) pela técnica de PCR (*Polymerase Chain Reaction*). Os genes *sul*1 e *sul*2 foram detectados apenas nos solos tratados com dejetos de animais. Não foram detectados nos solos da reserva legal e das propriedades que utilizavam resíduos vegetais como cobertura do solo. Esses resultados indicam que o uso de resíduos animais como fertilizante agrícola pode incrementar genes de resistência aos antimicrobianos no solo e que o processo de compostagem pode não ser suficiente para eliminá-los. Essas informações reiteram a necessidade de implementar padrões que estabeleçam parâmetros de qualidade para os resíduos animais, levando em conta a resistência aos antimicrobianos, bem como o desenvolvimento de estratégias de manejo que reduzam o risco de disseminação da resistência aos antimicrobianos quando esses resíduos são aplicados no solo.

Palavras-chave: Resíduos animais. Resistência a antimicrobianos. Esterco bovino. Esterco de galinha.

Animal wastes are widely used in organic production systems to provide nutrients to crops and promote soil attributes due to organic matter addition (Blum et al., 2006). This practice is in line with Federal Law No. 10,831/2003, which recommends the recycling of organic waste to minimize the use of non-renewable resources in organic production systems (Lei 10.831, 2003).

According to Normative 64/2008, which decides on the organic systems of animal and plant production, the residue that contains animal excrements must be composted and bio-stabilized to be applied as fertilizer. Also, it is recommended that the residues come from animals managed in organic systems. The use of animal waste produced conventionally is allowed when organic animal waste is not available in the region, provided that, in addition to being composted, it does not exceed the maximum limit allowed for contaminants, such as heavy metals and some pathogens (Instrução Normativa nº 64, 2008).

Animal treatment with antimicrobials is prohibited in the organic animal production system. It is only allowed in cases of illnesses or injuries in which the use of the permitted medicines is not effective and the animal presents suffering or risk of death. If applied, the waste from these animals during the treatment and grace period cannot be sold or used as organic (Instrução Normativa nº 64, 2008). However, antimicrobials are commonly used in conventional production systems for therapeutic and prophylactic purposes or as zootechnical additives added to the diets at therapeutic and subtherapeutic doses for almost the entire life of the animal to accelerate weight gain and increase feed efficiency (Thomas et al., 2015). A large proportion of the administered antimicrobials is usually excreted as an unchanged substance or as metabolites that may still be active, which favors the selection of the antimicrobial resistance of bacteria present in the feces of these animals (Zhu et al., 2013). Thus, despite the benefits obtained with the use of animal waste in agricultural environments, these compounds can be a source of antimicrobials, bacteria resistant to antimicrobials, and antimicrobial resistance genes (Reginato & Leal, 2010).

Soil represents a natural reservoir of bacteria resistant to antimicrobials, carrying a diverse set of known and unknown determinants of resistance. However, activities that release resistant antimicrobials and/or antimicrobial-resistant bacteria, and/or antimicrobial resistance genes, such as animal manure applications as organic fertilizer, can increase the abundance and diversity of resistance genes intrinsic to the soil, affect its resistome, and favor the dissemination of determinants of antimicrobial resistance to commensal bacteria and pathogens of humans and animals (Xie, Shen, & Zhao, 2018). Therefore, this study aimed to evaluate the presence of genes of resistance to sulfonamide *sul*1 and *sul*2 in soils from properties with different management practices to increase soil fertility.

The study was conducted on properties that adopt the organic production system, located in the municipality of Seropédica, Rio de Janeiro, Brazil. The properties belong to producers associated with the Participatory Guarantee System-ABIO (SPG-ABIO), Seropédica Nucleus - RJ. According to Law Nº 12,651/2012, all rural properties must maintain an area covered with native vegetation, named as a legal reserve.

The main soil classes identified in the municipality of Seropédica are Red-Yellow Argisols, Haplic Planosols, and Haplic Gleysols (Ramos, Castro, & Camargo, 1973). The first two soil classes occur in a larger extension in the region, being those of higher agricultural use.

Soil samples were collected from four properties with different management practices to increase soil fertility (Table 1). Cattle and chicken manure were acquired in properties that adopt the conventional production system, being previously composted before the incorporation into the soil. Two samples were collected from each property. A soil sample from the legal reserve was also collected in the fourth property for comparative purposes, totaling nine soil samples for analysis. Each sample consisted of a composite sample prepared from three simple samples, collected at a depth of 0-5 cm. The soils were characterized according to their physical and chemical characteristics, following the methodology described by Donagema, Campos, Calderano, Teixeira and Viana (2011).

Table 1
Types of management used to increase soil fertility and identification of the collected samples

Property	Type of management	Identification
1	Plant residues covering the soil	P1A P1B
2	Limestone, natural phosphate, "Yorin" phosphate fertilizer, and cattle manure	P2A P2B
3	Limestone, natural phosphate, and cattle and chicken manure	P3A P3B
4	Natural phosphate and chicken and cattle manure	P4A P4B
4	Legal reserve	LR

The soil samples were processed (air-dried and sieved in a 2.0-mm diameter mesh) to determine the contents of organic matter (OM), pH (H_2O), P, K, Ca, Mg, exchangeable Al, H+Al, sum of bases, sand, silt, and clay, according to Embrapa methodology (Donagema et al., 2011). The physicochemical characterization isshown in Table 2.

The extraction of total DNA from the soil was performed using the Power Soil DNA Isolation kit (MOBIO Laboratories, Inc, USA), according to the protocol provided by the manufacturer. The quantity and quality of the DNA obtained were evaluated using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). The DNA integrity was assessed by electrophoresis on 0.8% agarose gel plus SYBR green (Invitrogen, USA). The gel was visualized under 254 nm UV light and the images were captured using the L-PIX EX photo-documentation system (Loccus Biotechnology, Brazil).

The PCR (polymerase chain reaction) was performed in a 20-µL volume containing 1X of reaction buffer, 2.5 mM $MgCl_2$ (Invitrogen, USA), 0.2 mM of each dNTP (Invitrogen, USA), 0.4 µM of each primer (Invitrogen, USA), 1 U of Taq DNA polymerase (Invitrogen, USA), and 10-30 ng of DNA. Primers 27-F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Suzuki & Giovannoni, 1996) and 1512-R (5′-ACGGCTACCTTGTTACGACT-3′) (Kane et al., 1993) were used in the amplification of the *rrs* gene encoding the 16S rDNA. Primers sul1-F (5′-CGCACCGGAAACATCGCTGCAC-3′) and sul1-R (5′-TGAAGTTCCGCCGCAAGGCTCG-3′) and sul2-F (5′-TCCGGTGGAGGCCGGTATCTGG-3′), and sul2-R (5′-GGGAATGCCATCTGCCTTGAG-3′) (Pei, Kim, Carlson, & Pruden, 2006) were used in the amplification of genes related to sulfonamide resistance. Amplifications of the *rrs* gene were carried out according to the following parameters: 5 min of initial denaturation at 94 °C, 30 cycles of 94 °C for 60 s, 58 °C for 60 s, 72 °C for 60 s, followed by a final elongation at 72 °C for 10 min. The amplification conditions of the sul1 and *sul*2 genes were optimized: 5 min of initial denaturation at 95 °C, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, followed by a final elongation at 72 °C for 7 min. The

Table 2

Physicochemical characterization and detection of genes encoding 16S rDNA (*rrs*) and resistance to sulfonamide (*sul*1 and *sul*2) in soils from an organic production system

Sample	pH	Ca	Mg	Al	H+Al	SB	P	K	OM	Clay	Sand	Silt	*rrs*	*sul1*	*sul2*
	$CaCl_2$			$cmol_c\,dm^{-3}$			$mg\,kg^{-1}$		%	(%)	(%)	%			
P1A	5.10	1.40	0.80	0.30	1.80	2.33	11.00	41.00	1.75	24.28	56.24	19.49	+	–	–
P1B	7.00	3.90	1.20	0.00	0.90	5.42	87.00	76.00	1.81	6.75	82.90	10.34	+	–	–
P2A	5.80	2.60	1.30	0.00	1.80	4.03	17.00	36.00	4.52	16.77	78.36	4.87	+	–	+
P2B	5.60	3.00	1.50	0.00	1.80	4.65	42.00	38.00	5.47	25.86	11.95	62.19	+	–	+
P3A	4.80	1.00	0.60	0.60	3.30	2.05	7.00	162.00	1.58	18.68	75.37	5.95	+	–	–
P3B	5.60	1.40	0.70	0.00	3.00	2.39	6.00	105.00	0.51	5.49	86.95	7.56	+	–	+
P4A	4.80	0.80	0.50	0.70	4.00	1.39	3.00	29.00	4.35	18.53	74.73	6.73	+	–	+
P4B	4.71	0.00	0.66	1.00	3.03	0.76	1.00	33.00	2.16	25.17	66.65	8.18	+	+	+
LR	4.79	0.00	2.31	0.32	4.59	2.35	2.00	12.00	4.75	14.33	81.83	3.85	+	–	–

SB = sum of bases; OM = organic matter. Presence (+) and absence (–) of the gene.

products of PCR reactions were visualized by electrophoresis on a 1.5% agarose gel under UV light in the photo documentation system L-PIX EX (LoccusBiotechnology, Brazil). All reactions were performed in triplicates to confirm the results. The positive control of PCR reactions was obtained by extracting DNA from a sulfonamide-resistant strain of *Escherichia coli* isolated from chickens from poultry farms located in the state of Rio de Janeiro (Pimenta, 2018).

The organic management of soils in the evaluated properties is inefficient to maintain soil fertility at adequate levels, especially regarding the neutralization of Al^{3+}, which is toxic to plants, and the maintenance of the pH around 6.5 (Table 2). The liming process is fundamental for any agricultural production system. It is the most efficient way to increase the soil pH and, consequently, decrease the Al^{3+}, in addition to increasing soil Ca and Mg contents (Barbosa & Silva, 1994; Caires, Kusman, Barth, Garbuio, & Padilha, 2004). Under Brazilian Legislation (Lei 10.831, 2003), organic production systems can use commercial fertilizers if they are poorly soluble and hence nutrients are made available to the soil gradually and slowly. However, we can observe that the properties that use natural phosphate with phosphorus source (P3 and P4) have this nutrient well below that of the others. Moreover, the organic matter content was higher than 1.5% in all areas and, for some samples, the levels were higher than the control (4.75%). It indicates an improvement in soil quality and process restoration of agroecosystems (Faucon, Houben, & Lambers, 2017), mainly due to plant biodiversity (recommended in organic systems) and fertilization with organic residues (manure).

The *rrs* gene encoding 16S rDNA was detected in all soil samples (Table 2), confirming there is DNA in sufficient quantity and quality to be used as a template for PCR amplification of genes encoding antimicrobial resistance. The *sul*1 gene, encoding sulfonamide resistance, was detected in only one soil sample from property 4, in which bovine manure was used, representing 11% (1/9) of the samples. The *sul*2 gene was detected in 55% (5/9): in both samples from property 2 and 4, which used bovine manure as fertilizer, and also in a sample from property 3, where bovine and chicken manure were used as fertilizer. Neither gene was detected in soils from property 1, which use plant residues covering the soil, and in the legal reserve area (Table 2).

Independent cultivation techniques based on nucleic acids have been increasingly used in environmental microbiology studies, as they allow reaching a larger portion of the bacterial community in their natural habitat since cultivable microorganisms represent less than 1% of the microbial population in the soil (Amann, Ludwig, & Schleifer, 1995). We can extrapolate this advantage to studies of antimicrobial resistance, in which we have access to antimicrobial resistance genes directly from DNA of a wider range of soil bacteria and not only those grown in the culture medium.

Soils from four properties under the organic system and a legal reserve in the municipality of Seropédica-RJ were analyzed to evaluate the presence of sulfonamide resistance genes (*sul*1 and *sul*2). Sulfonamides were the first antimicrobials used systematically to prevent and treat diarrhea and other infectious diseases (Heuer & Smalla, 2007). The action of this drug is related to the inhibition of the formation of dihydrofolic acid by binding to the enzyme dihydropteroate synthase (DHPS) in the folic acid pathway. Bacterial resistance to sulfonamides mainly occurs because of mutations in the DHPS gene (*fol*P) or due to the acquisition of alternative DHPS genes *sul*1, *sul*2, and *sul*3 (Sköld, 2000). Property 1, where only plant residues are used in the soil cover, as well as the legal reserve area, did not present any of the two evaluated resistance genes, which suggests that the resistance genes detected in properties 2, 3, and 4 come from animal residues applied as organic fertilizers in the soils. The difference in animal waste used as organic fertilizers in the properties analyzed in this study (2 and 3 - cattle manure; 4 - cattle and chicken manure) can reflect the increase of different genes in soils, as the *sul*1 gene was detected only on property 4 and *sul*2 was detected on properties 2, 3, and 4.

Soils have a high abundance of microorganisms and represent a large natural reservoir of antimicrobial resistance genes. Although the *sul*1 and *sul*2 genes have not been detected in the legal reserve area, there is evidence that genes from antimicrobial resistance are originated in the environment even before the use of antimicrobial by humanity (Wright, 2007; Xie et al., 2018).

Studies have shown that sulfonamide resistance genes are often detected in manure and soil fertilized with manure, and the frequency in which they are found is closely related to the frequency in which they are used (Lin et al., 2016). There is a lower occurrence of resistance genes in bovine manure compared to chicken manure (Wichmann, Udikovic-Kolic, Andrew, & Handelsman, 2014). This variation occurs due to differences in management practices, in which antimicrobials are used less frequently in cattle production for the prevention and treatment of diseases. At the same time, it is added to the feed as a zootechnical additive in poultry farming, besides its prophylactic and therapeutic use, resulting in approximately four times more antimicrobials for chicken production compared to cattle (Kim et al., 2011).

The current legislation allows the use of animal waste from conventional production in organic production systems, when organic animal waste is no available in the region, as is the case of the studied properties (Instrução Normativa nº 64, 2008). The use of antimicrobials in the organic animal production system is prohibited and they can only be used when the treatment with permitted medicines is not effective, the animal is suffering, or is at risk of death. In this case, a grace period is imposed during and after treatment so that products, by-products, and manure from these animals are not sold or used as organic (Instrução Normativa nº 64, 2008).

Studies demonstrating the occurrence of resistance genes on agricultural soils are scarce. In Brazil, most studies are related to aquatic environments (Serafim & Ruiz, 2018). However, Cadena et al. (2018) conducted a study with soil from organic production systems treated with organic animal residues in Nebraska to evaluate the presence of genes for resistance to tetracycline and sulfonamide in these environments and found that about 93% of the analyzed soil samples (182/196) of 12 organic properties showed resistance genes to these antimicrobials. Antimicrobial resistance genes are also present in bacteria in the intestinal tract of animals not treated with antimicrobials. They confer an intrinsic resistance, that is, the organism is not susceptible to the compound due to an inherent structural or functional characteristic (Heuer & Smalla, 2007). Thus, residues from organic or conventional animal production can carry resistance genes and management techniques that aim to eliminate these genes must be used to avoid contamination of the environment.

Composting is alternative management that can result in the reduction or elimination of undesirable pathogens of animal residues. Composting consists of an aerobic process by which several groups of microorganisms break down organic materials and produce stable organic and inorganic products. During this process, the intense proliferation of microorganisms causes an increase in temperature capable of destroying pathogens and guaranteeing the final product (Orrico, Orrico, & Lucas, 2009). However, there is still no consensus on its effect on the elimination of antimicrobial residues and particularly antimicrobial resistance genes, which can vary depending on the type of material of animal origin and the conditions of the composting procedure (Maccari, Segat, Testa, Maluche-Baretta, & Baretta, 2020).

Lin et al. (2017) investigated the effect of different temperatures in the composting process of swine and chicken manure on the degradation of sulfonamide, resistant bacteria, and resistance genes for this antimicrobial and observed an efficient degradation in chicken manure for the antimicrobial at a temperature of 30 °C, different from the result of swine manure, which had a higher degradation efficiency at thermophilic temperatures (above 60 °C). However, no incubation effectively decreased the relative abundance of *sul*1 and *sul*2 resistance genes and resistant bacteria in the animal waste. It highlights the varied effects of composting on the different contaminants present in the substrate, being dependent on the conditions and materials used in the process. According to Qian et al. (2016), the composting process was efficient in decreasing the abundance of genes resistant to tetracyclines, that is, *tet*Q, *tet*M, and *tet*W, in bovine manure. However, it increased the abundance of *tet*C, *tet*X, *sul*1, and *sul*2.

The animal waste used in organic production properties in this study came from a conventional system, being previously composted before the incorporation into the soils. Thus, the results shown in this study corroborate the hypothesis that using animal manure as a fertilizer in agricultural soils can be a source of resistance genes. It also reinforces the need for management strategies that effectively reduce the risk to spread antimicrobial resistance in agricultural environments and the implementation of standards that establish animal waste quality parameters to be applied safely in organic production.

This study showed that the use of animal residues from conventional production, though composting, may favor the occurrence of sulfonamide resistance genes in soils from an organic production system.

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