INVESTIGATION OF ANTIMICROBIAL RESISTANCE GENES IN ORGANIC FERTILIZERS FROM ANIMAL RESIDUES

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ABSTRACT – Animal wastes have been commonly reused in agriculture due to improve organic matter and nutritional input. However, organic animal fertilizers can be sources of antimicrobials, bacteria resistant to antimicrobials, and antimicrobial resistance genes. In this perspective, this work aimed to investigate the presence of antimicrobial resistance genes in organic animal wastes, such as fresh bovine manure from organic and conventional production systems, fresh and tanned organic poultry litter, conventional poultry litter tanned and bone meal. Sulfonamide resistance genes were detected in all residues. On the other hand, colistin and carbapenem resistance genes were not detected in any samples. A tetracycline resistance gene was detected in poultry litter. Quinolone resistance genes were detected in all samples, except bone meal. Despite the advantage of applying animal wastes in agricultural production, the existence of resistance genes in all residues, regardless of whether they come from organic or conventional systems, fresh or tanned, represents a public health risk since we can increase antimicrobial resistance genes in the soil which can favor the transfer to pathogenic bacteria of humans and animals.

Keywords: organic agriculture, tanned manures, agricultural fertilizers, one health, bacterial resistance.

INVESTIGAÇÃO DE GENES DE RESISTÊNCIA A ANTIMICROBIANOS EM ADUBOS ORGÂNICOS PROVENIENTES DE RESÍDUOS ANIMAIS

RESUMO – Reutilizar resíduos de origem animal é comum na agricultura devido ao incremento de matéria orgânica e aporte nutricional. Contudo, esses resíduos podem ser fonte de antimicrobianos, bactérias resistentes a antimicrobianos e genes de resistência a antimicrobianos. Nesse panorama, esse estudo objetivou investigar a presença de genes de resistência a antimicrobianos em adubos orgânicos de origem animal como esterco bovino fresco, proveniente de sistema de produção orgânico e convencional, cama de aviário orgânica fresca e curtida, cama de aviário convencional curtida e farinha de osso. Genes de resistência a sulfonamida foram detectados em todos os resíduos. Entretanto, genes de resistência a colistina e carbapenêmicos não foram detectados em nenhuma amostra. Um gene de resistência a tetracilina foi detectado somente na cama de aviário. Genes de resistência a quinolona foram detectados em todas as amostras, exceto farinha de osso. Apesar das vantagens desses resíduos nas produções agrícolas, a presença de genes de resistência em todos os resíduos, independente se foram provenientes de sistema orgânico e convencional, ou se eram frescos ou curtidos, representam um risco a saúde pública, visto que podem incrementar genes de resistência a antimicrobianos nos solos, e consequentemente favorecer sua transferência para bactérias patogênicas de humanos e animais.

Palavras-chave: agricultura orgânica, adubos curtidos, fertilizantes agrícolas, one health, resistência bacteriana.

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INTRODUCTION

The expansion of agricultural production has resulted in a progressive increase of waste-overs produced all over the years (Trani et al., 2013; Wichmann et al., 2014; Dalolio et al., 2017). One of the most promising ways to dispose of these wastes is to use them as organic fertilizers in agricultural production. This practice increases organic matter in the soil, keeping it fertile, reducing the dependence on non-renewable energy and aims at environmental protection (Dalolio et al., 2017). This activity complies with Federal Law 10.831/2003, which regulates the use of animal waste in organic agriculture to improve the soil's physical and chemical properties and, consequently, increase agricultural productivity.

Despite the various advantages of its reuse, residues from animal production can carry resistant bacteria, antimicrobial resistance genes or even antimicrobial residues (Ji et al., 2012; Cui et al., 2016). Antimicrobials are used in agriculture for various purposes. In conventional production, these drugs are common for therapeutic and prophylactic use and a zootechnical additive, known as growth promoters (Zwald et al., 2004). In organic production, the use of antimicrobials is prohibited. However, an exception is made only when there are no alternatives for treating animals that are still suffering or at risk of death, even after carrying out the recommended treatments (Brasil, 2021).

The practice of tanning or composting is recommended to ensure animal waste safety as an organic fertilizer to regard pathogenic microorganisms (Brasil, 2021). The tanning process is different from the composting process since there is no temperature control, turnover, and moisture of the material. That makes it a more practical process. Nevertheless, there is no consensus on the effectiveness of these processes to reduce or eliminate antimicrobial resistance determinants (Le Devendec et al., 2016; Lin et al., 2017; Gou et al., 2018; Xu et al., 2018).

The use of animal waste as organic fertilizer can increase and introduce resistance genes in soils. It may change the environmental resistome, which may advantage of the transfer of these genes to animal and human pathogens, becoming a risk to public health. Therefore, this work's objective was to investigate antimicrobial resistance genes in organic fertilizers from different animal origins, production systems, and treatment types.

Materials and Methods

Characterization of fertilizers and bacteria used as a positive control in PCR reactions

Six animal fertilizers were selected. In an organic production system, fresh bovine manure (FOBM), fresh poultry litter (FOPL) and tanned poultry litter (TOPL) were purchased. In conventional production systems were acquired fresh bovine manure (FCBM), tanned poultry litter (TCPL) and bone meal (BM).

For each residue, five simple samples were collected. These samples were mixed, forming composite samples. Then they were packed in plastic bags and transported to the Laboratory of Molecular Genetics of Microorganisms of the Veterinary Institute of the Federal Rural University of Rio de Janeiro, where they were stored at -20ºC. The positive control of PCR reactions was obtained by DNA extraction from strains of *Escherichia coli* resistant to sulfonamide, colistin, tetracycline and quinolone, isolated from poultry farms in the state of Rio de Janeiro, by the doctoral student Ramon Loureiro Pimenta, oriented by the professor Miliane Moreira Soares de Souza.

Total DNA extraction

The extraction of total DNA from *Escherichia coli* strains was performed according to Buyukcangaz et al. (2013). The extraction of total DNA from the fertilizers was carried out according to the adapted protocol described by Tito et al. (2015). The quantity and quality of the DNA were evaluated by spectrophotometer (NanoDrop ND-1000 Spectrophotometer- Thermo Fisher Scientific). DNA integrity was assessed by electrophoresis on 0.8% agarose gel plus SYBR green (Invitrogen). The gel was visualized under 254 nm UV light, and the images were captured using the photo-documentation system L-PIX EX (Loccus Biotecnologia).

Amplification of genes related to antimicrobial resistance

For the amplification of antimicrobial resistance genes, the *primers* used are described in Table 1, as well as their sequences and fragment sizes. The amplification conditions used are following the recommendations of the references listed in this same table. For the PCR reactions, 1X buffer, 2.5 mM $MgCl^2$, 0.2 mM dNTP, 0.4 µM of each *primer*, 1 U Taq DNA Polymerase, about 20 ng of total DNA, and ultrapure water for complete the total volume of 25 µL. The PCR products were separated by electrophoresis on 3% agarose gel containing SYBR Green (Life Technologies). The images were captured using the photo documentation system L-PIX EX (Loccus Biotecnologia).

Table 1 - Sequence of primers and size of fragments generated in the amplification of the *rrs* gene and resistance genes *sul*1, *sul*2, *tet*A, *tet*B, *qnr*A, *qnr*B, *qnr*S, *mcr*-1 e *mcr*-3.

Gene	Primer	Sequence	Fragment	Reference
rrs	27F	AGAGTTTGATCCTGGCTCAG	1500 bp	Lane (1991)
	1512R	ACGGCTACCTTGTTACGACT		
*sul*1	Sul 1 F	CGCACCGGAAACATCGCTGCA	162 bp	Pei et al. (2006)
	Sul 1 R	TGAAGTTCCGCCGCAAGGCTC		
*sul*2	Sul 2 F	TCCGGTGGAGGCCGGTATCTGG	190 bp	Pei et al. (2006)
	Sul 2 R	CGGGAATGCCATCTGCCTTGAG		
tetA	tetA F	GCGCGATCTGGTTCACTCG	164 bp	Aminov et al. (2004)
	tetA R	AGTCGACAGYRGCGCCGGC		
tetB	tetB F	TACGTGAATTTATTGCTTCGG	206 bp	Aminov et al. (2004)
	tetB R	ATACAGCATCCAAAGCGCAC		
*qnr*A	qnr F	TCAGCAAGAGGATTTCTCA	627 bp	Wang et al. (2004)
	qnr R	GGCAGCACTATTACTCCCA		
*qnr*B	qnrB F	GATCGTGAAAGCCAGAAAGG	469 bp	Robicsek et al., (2006)
	qnrB R	ACGATGCCTGGTAGTTGTCC		
*qnr*S	qnrS F	ACGACATTCGTCAACTGCAA	417 bp	Robicsek et al. (2006)
	qnrS R	TAAATTGGCACCCTGTAGGC		
*mcr*1	mcr1-q F	CGGTCAGTCCGTTTGTTC	309 bp	Liu et al. (2015)
	mcr1-q R	CTTGGTCGGTCTGTAGGG		
mcr-3	mcr-3q F	ACCTCCAGCGTGAGATTGTTCCA	169 bp	Li et al. (2017)
	mcr-3q R	GCGGTTTCACCAACGACCAGAA		
oxa-23	oxa-23 F	ACTTGCTATGTGGTTGCTTCTTCTT	797 bp	Chen et al. (2018)
	oxa-23 R	TTCAGCTGTTTTAATGATTTCATCA		
oxa-24	oxa-24 F	CAGTGCATGTTCATCTATT	702 bp	Chen et al. (2018)
	oxa-24 R	TCTAAGTTGAGCGAAAAG		
oxa-51	oxa-51 F	TCCAAATCACAGCGCTTCAAAA	639 bp	Chen et al. (2018)
	oxa-51 R	TGAGGCTGAACAACCCATCCA		
oxa-58	oxa-58 F	AAGTATTGGGGCTTGTGCTG	599 bp	Chen et al. (2018)
	oxa-58 R	CCCCTCTGCGCTCTACATAC		

RESULTS AND DISCUSSION

The *rrs* gene encoding 16S rRNA was detected in all samples (Table 2). It shows that there was DNA in sufficient quantity and quality to be used as a template for PCR amplification of genes encoding antimicrobial resistance. In addition to the *rrs* gene, sulfonamides resistance genes (*sul*1 and *sul*2) were also detected in all evaluated residues.

Table 2 - Detection of *rrs* genes and antimicrobial resistance genes in organic fertilizers.

Sample	*rrs*	*sul*1	*sul*2	*tet*A	*tet*B	*qnr*A	*qnr*B	*qnr*S	*mcr*-1	*mcr*-3	*oxa*-23	*oxa*-24	*oxa*-51	*oxa-58*
FOBM	+	+	+	-	-	+	-	+	-	-	-	-	-	-
FCBM	+	+	+	-	-	-	-	+	-	-	-	-	-	-
FOPL	+	+	+	-	+	+	+	+	-	-	-	-	-	-
TOPL	+	+	+	-	-	-	-	+	-	-	-	-	-	-
TCPL	+	+	+	-	+	-	-	+	-	-	-	-	-	-
BM	+	+	+	-	-	-	-	-	-	-	-	-	-	-

Legend: fresh organic bovine manure (FOBM); fresh conventional bovine manure (FCBM); fresh organic poultry litter (FOPL); tanned organic poultry litter (TOPL); tanned conventional poultry litter (TCPL); bone meal (BM); Where results that are positive (+) and negative (-).

Sulfonamide is a high priority antimicrobial in veterinary medicine since its constant use to treat diarrhea and other infections in cattle, pigs and birds (Heuer & Smalla, 2007; Lin et al., 2017). For this reason, the genes that confer resistance to this antimicrobial are often found in animal manures and fertilized soils (Heuer & Smalla, 2007; Ji et al., 2012).

Bone meal is a residue from animal production widely used in agriculture because it is an essential nutrient source, especially phosphorus (Trani et al., 2013). However, its potential as a disseminator of antimicrobial resistance has been poorly elucidated nowadays. In the present study, the *sul*1 and *sul*2 genes were detected in bone meal.

The *qnr*S gene was detected in all samples that contained animal feces, except for bone meal. In a study by Mu et al. (2015), this same gene and the *sul*1 and *sul*2 genes were detected in all feces samples from chicken, swine and beef cattle in feedlots and adjacent soils in northern China.

In the fresh organic poultry litter, many antimicrobial resistance genes were detected compared to fresh organic bovine manure. For example, *tet*B was detected only in poultry litter samples. This fact can be explained from the comparative perspective of the number of antimicrobials used in each animal's management. In cattle, the use of antimicrobials is used for prophylactic and therapeutic purposes of diseases, while for poultry, they are also used as growth promoters, which results in the use of four times more antimicrobials for chicken production than to cattle production (Wichmann et al., 2014).

About production systems, when comparing a tanned conventional poultry litter with a tanned organic poultry litter, a more significant number of resistance genes can be observed in the conventional system. Studies indicate that it is common to have a higher prevalence of quantity and diversity of bacteria resistant to antimicrobials in this production system due to the continuous use of antimicrobials that increases the selection of these resistant bacteria (Luangtongkum et al., 2006; Mota et al., 2018). However, when comparing fresh organic bovine manure with fresh conventional bovine manure, the opposite behaviour is observed. In this case, it is essential to mention that bacterial resistance also occurs intrinsically. That is a natural part of a bacterial evolution process, which may cause resistance to antimicrobials, even in systems where the use of antimicrobials is prohibited, such as organic (Heuer & Smalla, 2007; Brasil, 2021). Therefore, it is common to find resistance determinants in animals that have not been treated with antimicrobials due to the aforementioned natural phenomenon (Sancheza et al., 2016; Bush, 2018).

The tanning process is related to the stack and storage of the material. It allows the temperature to rise, making it possible to eliminate most pathogens (Knapik & Angelo, 2007). Regarding the treatment of waste, when comparing fresh organic poultry litter with tanned organic poultry litter, it is observed that the fresh residue has a higher number of antimicrobial resistance genes. However, some of them still have been detected in the tanned residues. This fact proves that the tanning process decreased the abundance of genes, but it was not enough to eliminate them. In this context, despite the efficiency and low cost of the tanning and composting process, it is necessary a further improvement of this process since studies prove that the elimination of resistance genes is not sufficient even after both processes (Lin et al., 2017; Manyi-Loh et al., 2018).

In that regard, it is important to clarify that residues from animal production represents a huge part of agriculture, considering that they are used as organic fertilizers. However, as observed in this work, these residues can carry determinants of resistance. When these residues are used in agriculture, they can modify the environmental resistome. In addition to this, these determinants of resistances found in those organic fertilizers can contaminate vegetables. When the vegetables are eaten *in natura*, they can contaminate humans and animals whom subsequently go through an infectious process with a higher chance of therapeutic failure, so that can cause an increase in morbidity and mortality and, consequently, resulting in health risk.

The World Health Organization classifies bacterial resistance as one of the biggest public health impasses (Who, 2017). However, few studies address this issue in a "One Health" approach, which aims to connect humans, animals and the environment. The results in this work confirm that, regardless of the origin, production system or type of treatment, animal residues in agricultural production represent a risk to public health since it can increase antimicrobial resistance genes in soils and consequently favor their transfer to human and animal pathogenic bacteria.

CONCLUSIONS

1. Sulfonamide resistance genes are prevalent in organic animal fertilizers.

2. Resistance genes are present in residues from conventional and organic systems of animal production.

3. A more significant number of resistance genes is detected in poultry residues and fresh residues.

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