Exploring the Animal Waste Resistome: The Spread of Antimicrobial Resistance Genes Through the Use of Livestock Manure

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Antibiotic resistance is a public health problem of growing concern. Animal manure application to soil is considered to be a main cause of the propagation and dissemination of antibiotic residues, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) in the soil-water system. In recent decades, studies on the impact of antibiotic-contaminated manure on soil microbiomes have increased exponentially, in particular for taxonomical diversity and ARGs' diffusion. Antibiotic resistance genes are often located on mobile genetic elements (MGEs). Horizontal transfer of MGEs toward a broad range of bacteria (pathogens and human commensals included) has been identified as the main cause for their persistence and dissemination. Chemical and bio-sanitizing treatments reduce the antibiotic load and ARB. Nevertheless, effects of these treatments on the persistence of resistance genes must be carefully considered. This review analyzed the most recent research on antibiotic and ARG environmental dissemination conveyed by livestock waste. Strategies to control ARG dissemination and antibiotic persistence were reviewed with the aim to identify methods for monitoring DNA transferability and environmental conditions promoting such diffusion.

Keywords: veterinary antibiotics, animal manure, antibiotic resistance genes, crop soils, antimicrobial resistance

INTRODUCTION

In recent decades, the overuse and misuse of antibiotics in human and veterinary medicine has become a serious public health issue (World Health Organization, 2014; Aidara-Kane et al., 2018). The increased number of resistant pathogens and commensal bacteria has been associated with the environmental spread of antibiotics and the propagation of antimicrobial resistant genes (ARGs; Levy, 1998; Witte, 1998; He et al., 2020). Furthermore, the environmental diffusion of antibiotics may lead to the change (Han et al., 2018) and loss (Chen et al., 2019) of microbial community diversity in soil (Kemper, 2008).

Antibiotics are used worldwide in livestock production, thus increasing the risk of antimicrobial resistance (AMR) spread. When administered for prophylactic treatments, antibiotics can directly increase selective pressure, thus favoring the generation of antibiotic-resistant bacteria (ARB;
ARGs in the environment

The majority of antibiotics are naturally produced by microbes as a self-protection mechanism against other microorganisms. ARGs have been always present in the environment. ARGs encoding resistance for a large set of antibiotics have been found in 30,000-year-old Beringian permafrost and in bacteria isolated from prehistoric caves (D’Costa et al., 2011; Berglund, 2015). When present in the environment at a sub-inhibitory concentration, antibiotics frequently play a role in transcription regulation and in the exchange of signals among cells (i.e., quorum sensing mechanism and conjugation) (Reygaert, 2018).

Antibiotic resistance consists of a large variety of mechanisms, such as inactivation by specific cleaving enzymes, exclusion from cells via efflux pumps, interference with protein synthesis, limitation of drug uptake, and modification of antibiotic target.
Resistance acquired through MGEs and plasmids is responsible for the last two mechanisms in which the resistance extent depends on bacterial species and acquired ARGs (Reygaert, 2018; Kraemer et al., 2019). The antibiotic selective pressure driving the acquired resistance determines accurate ARGs’ specialization, thus making the environment a potential reservoir.

Anthropogenic activities affect antibiotic and ARGs’ spread with somewhat predictable effects (Vikesland et al., 2017). In livestock farming, the use of antibiotics varies depending on the farming type and location, having a considerable effect on ARGs’ concentration. Among the ARGs most frequently detected in livestock production, those related to sulfonamide resistance (sul) (Table 1) are particularly diffused in aquatic systems (Chen et al., 2015; Makowska et al., 2016). In surface and fresh waters, sul genes were found in IncQ plasmid group (Sköld, 2001; Berglund, 2015). Similarly, diaminopyrimidine genes (dfr), which confer resistance to antimicrobial trimethoprim, have been identified in both class 1 and class 2 integrons (Deng et al., 2015). Similarly, quinolone resistance qnr genes have been frequently associated with different plasmid groups. Both dfr and qnr genes easily disseminate in the environment, being found in surface waters (Berglund, 2015), wastewaters, and related irrigated soils (Dalkmann et al., 2012). Tetracycline resistance genes (tet) are widely diffused in different pathogenic and environmental bacteria (Roberts, 2005) and are often detected in sewage treatment plants, soil, and surface and ground water (Chee-Sanford et al., 2001; Berglund, 2015). In the same environments, erm genes, which are the most widespread macrolides resistance gene, were isolated.

Essentially, ARGs’ diffusion is associated with a stress response activated by exposure to antibiotics as well as with the mobilization of several integrative and conjugative elements. ARGs’ maintenance depends on their considerably low fitness cost. In fact, once a specific ARG has been acquired by a bacterial cell, it must evolve to produce more benefits than costs in order for multiple copies of the same gene to be kept and to maintain the expression control of genes in MGEs (Bengtsson-Palme et al., 2017). Furthermore, as already mentioned, nutrient rich environments can positively influence the ARGs’ spread and facilitate cell–cell interactions (Manaia et al., 2018) (Figure 1).

**THE USE OF VETERINARY ANTIBIOTICS**

In veterinary medicine, antimicrobials can be used as therapeutics and/or growth promoters. Antibiotic growth promoters (AGPs) are antimicrobial substances administered at a sub-therapeutic dose for a prolonged time with the main purpose being to improve the feed conversion rate, especially in young animals, raising the economical profit of farmers. Since 2006, both the European Union and Australia have forbidden the use of AGPs. Nevertheless, in most other countries the use of AGPs is still permitted (Guardabassi et al., 2009).

Among breeding farms, poultry and pig livestock have received the majority of antibiotics for therapeutic or prophylactic use (Ungemach, 2000; Kim et al., 2011), resulting in an abundance of ARGs greater than three orders of magnitude compared to other farming systems, such as fish and cattle farming. Several studies confirmed swine farms as a hot-spot for ARB and ARGs (Rosen, 1995; Cromwell, 2002; de Greeff et al., 2019; Petrin et al., 2019). Recently, the scientific community investigated prevalence, abundance, and possible mobilization of ARGs in pig farms and surrounding environments (Hölzel et al., 2010; Marti Serrano, 2014; Petrin et al., 2019; Van den Meersche et al., 2019; Wu et al., 2019).
| Antibiotic family | Most used | Animal Farming | Use | Contrasted bacteria and recognized main targets | Resistance mechanism | Main ARGs |
|-------------------|-----------|----------------|-----|-------------------------------------------------|----------------------|-----------|
| Macrolides        | Tylosin   | Cattle         | Gastrointestinal and respiratory infections | Gram-positive bacteria. | Interference with protein synthesis (sequestration of mRNA ribosome-binding site) | erm, msr, mef genes |
|                   | Erythromycin | Pig            | Main target: *Lawsonia intracellularis* | Staphylococcus aureus | | |
|                   | Clarithromycin | Pig/ Poultry | Urinary tract infections | Gram-positive and Gram-negative bacteria. Main target: *Enterobacteriaceae, Pasteurellaceae* | Interference with folic acid synthesis competing for the enzyme DHPS | sll, sull genes |
| Sulfonamides      | Sulfamethazine | Cattle/ Pig | Gastrointestinal and respiratory infections | Interference with efflux pump systems | tet genes |
|                   |            | Pig | Respiratory infections | | |
| Tetracyclines     | Chlortetracycline | Cattle | Systemic and local infections | Gram-positive and Gram-negative bacteria | Interference with efflux pump systems | tet genes |
|                   |            | Pig | Gastrointestinal and respiratory infections | | |
|                   | Oxytetracyclines | Pig | | | |
|                   | Doxycycline | Pig | | | |
| Quinolones        | Fluoroquinolones (Enrofloxacin, Danofloxacin, Marbofloxacine) | Pig | Intestinal infections | Gram-positive and Gram-negative bacteria, including mycobacteria, and anaerobes | Mutations in the genes encoding quinolone target DNA gyrase and topoisomerase IV. Interference with efflux pump systems | qnr genes |
| β-lactams         | Penicillins (Amoxicilline, Ampicillines) Cephalosporins, Carbapenems | Pig | | | |
|                   |            | Cattle | Respiratory diseases | Gram-positive and Gram-negative bacteria | Interference with cell wall synthesis and permeability, inactivation through β-Lactamase enzyme | bla, amp, pen genes, |
|                   |            | Cattle/ Poultry/ Dog/ Cat | Necrotic enteritis | | |
| Aminoglycosides   | Streptomycin, Spectinomycin, Neomycin, Aspramyycin, Gentamycin, Lincomycin | Pig | Intestinal infections | Gram-positive, and Gram-negative bacteria, if aerobic | Inhibition of protein synthesis (ribosome interference) | aac, aad, aad aph genes |
| Phenics           | Chloramphenicol | Pig | Respiratory disease, foot rot | Broad spectrum. Main target: *Photobacterium, Salmonella, E. coli* | Enzymatic modification of antibiotic molecules | cat, pp-flo, flo genes |
|                   | Thiamphenicols (thiamphenicol, florfenicol) | Pig | | | |
| Diaminopyrimidines | Trimethoprim | Horse | Post-weaning scours | Gram-positive and many Gram-negative bacteria. Main target: *Enterobacteriaceae* | Interference with folic acid synthesis by binding the enzyme DHFR | dfr genes |
|                   |            | Pig | | | (Continued)
Table 1 summarizes the main antibiotic families and the most used antimicrobics in livestock animals for therapeutic use. Nowadays, more than 150 antimicrobial compounds in livestock production are used. The residues inevitably end up in the environment because of manure application on agricultural lands (Baguer et al., 2000). In 2010, more than 63,000 tons of antimicrobials were consumed by livestock across the globe. The predicted growth of the world’s population allows for an estimated increase in antibiotic consumption of up to 105,000 tons by 2030 (Tasho and Cho, 2016). For this reason, specific action plans have been defined to reduce the use of antibiotics as therapeutics for livestock in several countries (i.e., the European One Health Action Plan against Antimicrobial Resistance, 2017; the National Strategy to Combat Antibiotic-Resistant Bacteria, proposed by the White House, 2014; the National Action Plan to Contain Antimicrobial Resistance issued by the Chinese National Health and Family Planning Commission, 2016–2020).

**MANURE TREATMENTS**

Besides direct collection into aerobic or anaerobic lagoons, animal manure can undergo drying and liquid-solid phase separation. Manure solid phase, as well as whole manure if shovelable, is traditionally composted to produce biofertilizer. Currently, anaerobic digestion and biological treatments of animal manure are often adopted on intensive animal farms (Van Epps and Blaney, 2016).

Composting can substantially reduce the antibiotic load, especially during the thermophilic phase (Zhang et al., 2019), but recalcitrant antibiotics accumulate in compost products and in amended soil (Bohrer et al., 2019; Zang et al., 2019). A general ARG abatement (0.7–2.0 log decrease) is obtained through thermophilic composting of swine, cattle, and poultry manure, depending on manure type and operational conditions (He et al., 2020).

Biological treatments of animal manure and wastewater, which are adopted to reduce the environmental input of nitrates, slightly decreases the levels of antibiotic residues and pathogenic bacteria (Van den Meersche et al., 2019). Antimicrobial resistant gene reduction of 0.1–3.3 log is observed in swine manure after treatment (He et al., 2020).

Anerobic digestion (AD) is adopted to stabilize manure with a final production of methane (Fubin et al., 2016, 2017). A 0.3–52 log decrease of ARGs was observed in digestate from swine wastewater (He et al., 2020). Interestingly, the higher the content of volatile solids in manure and the mixing rate, the higher the ARGs number in the digestate (Turker et al., 2018). The combined pasteurization and AD of swine manure reduced sole archaeal communities, whereas simple AD affected bacteria and archaea (Fubin et al., 2020). Manure pretreatment with bacterial strains is effective in degrading antibiotics (Liu et al., 2019) and enhancing biogas production, but the overall effect on ARB and ARGs was not addressed.

 Constructed wetlands are vegetated aquatic systems that can be adopted for the treatment of wastewater and agricultural drainage water (Lavrnic et al., 2018). Their ability to reduce

### Table 1

| Antibiotic family | Main ARGs | Contrasted bacteria and recognized main targets | Resistance mechanism | Animal farming | Use | Most used | References |
|------------------|-----------|-----------------------------------------------|----------------------|---------------|-----|-----------|------------|
| Polypeptides     | Bacitracin, Colistin | Gram-positive (Bacitracin) or Gram-negative (Colistin) bacteria. Main Gram negative target: E. coli, Salmonella spp., Pseudomonas aeruginosa, Klebsiella pneumoniae, or Acinetobacter. | LPS modification, efflux pump systems regulation | Pig | Intestinal diseases | Gram-positive (Bacitracin) or Gram-negative (Colistin) bacteria. Main Gram negative target: E. coli, Salmonella spp., Pseudomonas aeruginosa, Klebsiella pneumoniae, or Acinetobacter. | petinaki et al., 2008; Guardabassi et al., 2009; Abbas et al., 2011; Van Hoek et al., 2011; Li et al., 2013; Shang et al., 2013; Tasho and Cho, 2016; Deng et al., 2017; Aghapour et al., 2019; https://www.msdvetmanual.com/pharmacology/antibacterial-agents) |
| Lincosamides     | Lincomycin | Pig | Respiratory and Intestinal infections | Campylobacter | Gram positive target: Campylobacter | Alteration of the antibiotic target site | lnu, lin, erm genes |
| Pleuromutilins   | Tiamulin | Pig | Respiratory and Intestinal infections | Pasteurellaceae, Brachyspira, Mycoplasma | Pasteurella | Alteration/protection of the antibiotic target site | vga, sal, lsa genes |
| Valnemulin       | None | Poultry | Respiratory and Intestinal infections | Mycoplasma | Mycoplasma | Additional mechanisms may be involved in the resistance mechanism | |

**References:** Schwarz et al., 2001; Petinaki et al., 2008; Guardabassi et al., 2009; Abbas et al., 2011; Van Hoek et al., 2011; Li et al., 2013; Shang et al., 2013; Tasho and Cho, 2016; Deng et al., 2017; Aghapour et al., 2019; https://www.msdvetmanual.com/pharmacology/antibacterial-agents.
ARGs in swine wastewater resulted in a 0.18–3 log decrease (He et al., 2020).

Oxidizing post-treatments, as ozonation or Fenton conditions, can be used on animal or treated wastewaters to degrade antibiotics and bacteria thanks to the activity of reactive oxygen species (Balcıoğlu and Ötker, 2003; Ikehata et al., 2006; Uslu and Balcıoğlu, 2009). Among advanced oxidation processes, highly costly ionizing radiations are known for their ability to destroy microbial DNA. Therefore, affordable combinations of ionizing radiation and oxidation allows for the degradation of antibiotics and ARGs in organic matrices, although with a high biological and environmental risk (Chu et al., 2019, 2020).

DIFFERENT APPROACHES TO RESISTOME PROFILING STUDY

Even though AMRs introduced in the environment with animal manure have been largely explored (Dolliver et al., 2008; Selvam et al., 2012b), contradictory information exists regarding the fate of ARGs (Selvam et al., 2012a; Wang et al., 2013; Xie et al., 2016). The growing need for the control of ARGs’ spread prompted the scientific community to set up and to validate refined molecular methods for the study of ARGs’ dissemination dynamics among environmental microbial communities.

Both 16S rRNA amplicon and untargeted sequencing can be considered exhaustive methods for the exploration of microbial community structure in manure-fertilized soil and farm waste. Several studies on resistome diffusion in wastewater treatment plants (Yadav and Kapley, 2019), sewage sludge composting units (Su et al., 2015), and urban sewage support the metagenomic approach (Hendriksen et al., 2019) in monitoring ARGs’ level during treatments and seasonal changes. A recent work (Han et al., 2018) showed that the shift in soil bacterial communities caused by manure application leads to changes in the soil bacteria resistome.

Recently, studies on the detection of genetic markers associated with AMR (transposases and class 1 integron-integrase genes) and ARGs have been markedly increasing. The quantification of ARGs in soils amended with livestock and swine manure (Brooks et al., 2014; Tao et al., 2014) was performed with high-throughput qPCR assay (Rocha et al., 2018; Blau et al., 2019). In a recent study, both intracellular and extracellular DNA containing ARGs were quantified in sludge at about $10^{10}$ and $10^{12}$ copies per gram, respectively (Dong et al., 2019). Here, the intracellular ARGs were assessed through conjugation with cell–cell contact, whereas the extracellular ARGs were assessed through natural transformation. Several works on different manure types focused on the quantification of targeted genes intI1 and intI2 for class 1 and 2 integron-integrase genes and korB gene, specific for IncP-1 plasmids, together with ARGs (Hu et al., 2016; Blau et al., 2018, 2019).

As already reported, plasmid-mediated ARGs’ diffusion is frequently used, especially for the role of plasmids in the rapid bacterial adaptation and fitness improvement (Smalla et al., 2000). Exogenous plasmid isolation techniques (Bale et al., 1988) clarified how plasmids diffuse in different environments. Recently, plasmids from municipal sewage sludge and recipient bacteria were analyzed for their transferability by exogenous isolation (Blau et al., 2018; Wolters et al., 2018). Referring to pig manure samples, four IncQ-like plasmids were isolated in recipient strains: Pseudomonas putida UWC1, Acinetobacter sp., Ralstonia eutropha, Agrobacterium tumefaciens, and E. coli. The plasmid transferability in E. coli strains was not efficient, underlying a broad but highly specific host range (Smalla et al., 2000).

Recently, simplified mathematical models have been applied to predict and quantify ARGs’ spread in livestock animal gut microbiomes (Andersen et al., 2020) and in agricultural waste (Baker et al., 2016). In such environments, the variables involved in the ARGs’ spread are countless and depend on a wide range of intrinsic and extrinsic factors, such as genetic mechanisms of ARB replication, HGT dynamics, environmental and stressor conditions, and microbiota composition. Therefore, future research should focus on the improvement of predictive models of ARGs’ dissemination mechanism, exploitable for targeted operations in livestock waste management.

CONCLUSION

Although a decrease in the use of antibiotics in livestock production is highly recommended, antibiotics’ overuse remains an important issue to solve. The uncontrolled spread of ARB and ARGs in the environment due to soil manuring is of serious concern. Many studies highlight ARGs’ presence in microbial communities of livestock manure and manured agricultural fields, despite the improved livestock and waste management strategies to contain in-farm ARGs’ spread. In the last thirty years, knowledge on pathways of ARGs’ diffusion from animal waste to the environment was enriched by multidisciplinary research approaches.

In light of the current knowledge, the study of the dynamics of AMR and ARGs’ spread in manure and environments surrounding livestock farms should combine molecular and functional genetics strategies with prediction models of the diffusion of MGEs (integrons and plasmids) and metagenomic data.

AUTHOR CONTRIBUTIONS

AC: original draft preparation, figure and table conceptualization, review, and editing. PT, MM, and SB: review. DL: original draft preparation and table preparation. IB and PM: original draft preparation and table preparation. IB and PM: original draft preparation and table preparation. DL: original draft preparation and review. All authors contributed to critically revising the manuscript and gave final approval for publication.

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