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Continuing Environmental Effects of Antibiotics Used in Livestock Farms

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ABSTRACT

The overuse of antibiotics to counteract animal diseases and in arable farming, such as the utilization of manure and bio solids as fertilizers and the use of reclaimed water, have been significantly contributing to the environmental contamination from antibiotics and to the selection of antibiotic resistance genes. The selection and transmission of ARGs, which give resistance bacteria (ARB) the ability to overcome the effects of antibiotics, is a phenomenon well known in hospitals, where antibiotic resistant pathogenic bacteria can persist and infect patients in the nosocomial environment in different ways.

KEYWORDS

Anaerobic digestion; ARGs; Cattle manure; Ciprofloxacin; Enrofloxacin; Microbial Community; Sulfamethoxazole.

1. Introduction

Italy is the second country in Europe for the number of anaerobic digestion plants (Statistical report 2018 of European Biogas Association). Although they were initially set up with the main purpose of solving the issue of farm waste disposal, the production of electricity (by on-site combustion of biogas) now constitutes another source of income for agricultural firms, like milk and meat production. It is well known that it is common in cattle farms to use antibiotics for treating animal diseases [1]. However, residues of antibiotics and antibiotic resistance genes (ARGs) are nowadays considered environmentally emerging contaminants [2-4] posing a risk of spreading of antibiotic resistance among natural, human and animal microbial populations. Moreover, antibiotics themselves can also affect some sensitive natural microbial populations with possible consequences for some key ecosystem functions [5].

Consequently, an increasing number of infections are becoming harder to treat owing to antibiotic ineffectiveness [13-16]. The origin of antibiotic resistance genes in microorganisms is older than the antibiotic era [17, 18], because ARGs are commonly found in natural bacteria [9]. However, the scientific community is now aware that the spreading of ARB and ARGs is a complex phenomenon, involving both the indoor environments where antibiotics are used and soil and water ecosystems, where they are present as micro-contaminants [19,20], in line with the One Health approach, which recognizes that human health is connected to that of animals and the environment. The holistic One Health concept is ancient and up-to-date

at the same time and is based on the recognition that human, animal and ecosystem health are inextricably linked. This model has been recognized by several Ministries of Health and Environment worldwide, the European Commission and the World Health Organization [21- 3].

In antibiotic treated animals, gut bacteria can act as ARG reservoirs [24], potentially transferring genetic material to soil and water environmental microorganisms and from the latter directly or indirectly to humans, for example through the consumption of fresh vegetables (Fig. 1) [25-28]. The European Commission has recently promoted the European Green Deal [29], a series of actions aimed at boosting the efficient use of resources, with a view to achieving a clean, circular economy and restoring biodiversity and reducing pollution. Among the various actions, the so called « Farm to Fork » one [30] recognizes the link between humans and ecosystems, where the One Health concept is reiterated. Antimicrobial resistance due to the massive use of antibiotics worldwide has led to thousands of human deaths and considerable healthcare costs. The Commission aims to reduce the overall use of antibiotics for farmed animals by 50% by 2030.

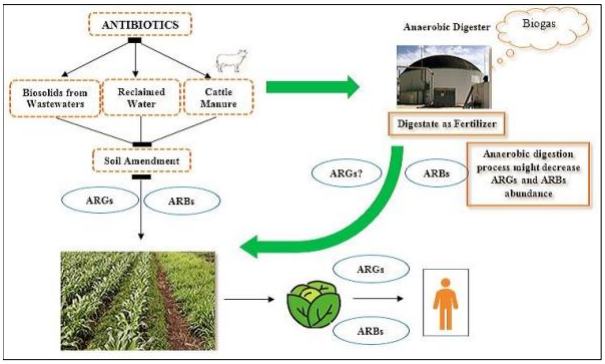

Figure 1. Farming practices and the potential path for the spreading of ARB and ARGs. Can Anaerobic Digestion influence (decrease/increase) ARB and ARGs in the digestate used as a good quality and environmentally friendly fertilizer?

Moreover, the spread of ARGs through microbial communities can also be favored by other chemicals [31]. The coexistence of antibiotic-producing and non-producing bacterial strains has led to the co-evolution of resistance mechanisms in environments where antibiotics are not present, due to the co-selection of ARGs with other genes conferring, for example, resistance to heavy metals or other pollutants such as polycyclic aromatic hydrocarbons [32, 33]. This is due to the fact that ARGs are commonly located on genetic cassettes associated with several genes and linked to integrases, placed on plasmids. ARGs can be transferred between different bacterial species due to their association with integrons and mobile genetic elements (MGEs), such as transposons and plasmids [34, 35].

In particular, ARGs can be transferred by one microorganism to another by vertical gene transfer (VGT) inside the same bacterial species, and by horizontal gene transfer (HGT) between different species through MGEs [36]. Once in the soil, intestinal bacteria mix with soil ones carrying genetic material useful for evolutionary adaptation [37]. Several studies have investigated if residual antibiotics and ARGs from cattle manure can alter the natural environmental resistome, showing a general increase in ARG abundances [38]. For example, different classes of antimicrobials have been detected in cattle manure, in particular fluoroquinolones, sulfonamides and tetracyclines in US beef cattle manure [39]. In a comprehensive study, Zhao et al. found seven fluoroquinolones, eight sulfonamides, and four tetracyclines in manure from large- scale animal feedlots in China [40]. Overall, fluoroquinolones and tetracyclines were detected more frequently and at higher concentrations than sulfonamides (oxytetracycline, 1.24 mg/kg; enrofloxacin, 6.79 mg/kg; ciprofloxacin, 3.44 mg/kg; sulfamethoxazole, not detected). In a similar way, the number of genes associated with resistance to these antibiotics and found in manure and in soil amended with it was considerable [28].

2. Anaerobic Digestion

An alternative to the direct application of manure on agricultural land is its use as feed for anaerobic digesters, in order to obtain biogas to produce electricity, heat or fuel [41, 42]. Anaerobic digestion (AD) is a spontaneous process, widespread in environments rich in organic matter but depleted of oxygen and other electron acceptors such as nitrate, sulfate, iron or oxidized manganese [43]. These ecosystems include shallow freshwaters such as swamps, rice fields and submerged soils, but also human and animal intestinal tracts (large ruminant and non-ruminant herbivores, as well as termites and woodworms). Anthropogenic and engineered environments, such as landfills and anaerobic digesters, are also included [44]. The AD technology is spreading rapidly [45] due to the numerous advantages it offers. In addition to the well-known possibility of connecting waste disposal with sustainable energy production and using the digestate as a fertilizer [46], other benefits for society and the environment are the increase in nutrient recovery and the reduction of greenhouse emissions [47]. Moreover, from a technological point of view, AD has been developed as a low- cost organic waste treatment technology with a simple setup and relatively limited investment and operating costs [48]. All these reasons currently place the anaerobic digestion process and biogas in a hub position in the development of the circular economy, especially in the biomethane production perspective.

The AD process consists of a sequence of anaerobic biochemical reactions mediated by populations of microorganisms that, cooperating sequentially [49], convert complex organic molecules, such as polysaccharides, lipids, and proteins, into simple substances, mainly CH_4 (50-75%) and CO_2 (25-45%). During AD small quantities of other gases are also produced, such as H_2O, H_2, CO, N_2, NH_3, O_2, and H_2S, whose overall fraction is approximately 5% of the biogas produced [50,51].

The AD process includes four main metabolic steps: (i) hydrolysis, (ii) acidogenesis and (iii) acetogenesis, which are performed by Bacteria and (iv) methanogenesis, the last step, carried out by the methanogenic Archaea.

Since an unbalanced composition of the microbial community affects biogas production efficiency, microbial ecology studies are fundamental for successful AD. In the last decade, many works have been focusing on understanding the structure and dynamics of the archaeal and bacterial communities during the AD process. [6,46,48]. For example, some authors showed that due to their bacteriostatic and/or biocide effects, antibiotics can affect AD performance and reduce biogas production, primarily by selectively influencing microbial components and thus changing the general structure of the microbial community [52]. Key populations of acetogenic bacteria, capable of converting volatile acids such as propionate and butyrate into acetate (which in turn can be directly used by acetoclastic archaea to produce methane) are reported to be sensitive to the detrimental actions of antibiotics [53]. Other authors reported that antibiotics such as sulfamethoxazole can trigger significant changes in methanogen composition, by driving it towards a predominance of the acetotrophic or hydrogenotrophic metabolic pathway in the production of methane [54, 55]. The consequences in terms of overall AD efficiency are still unclear.

To date, few studies have been conducted on the interaction between antibiotics and sulfate-reducing bacteria (SRB), developing H_2S during AD. A microbial compositional analysis revealed that anaerobic reactors receiving antibiotic-bearing (mainly streptomycin) wastewater were dominated by Deltaproteobacteria (51%) affiliated mainly with sulfatereducing bacteria (SRB) [56]. The latter can have a detrimental effect on biogas production because they can compete with
Archaea [57] and because H_2S can be toxic for other anaerobes.

Although these studies showed that the presence of antibiotics can affect AD performance, the possible removal of antibiotic residues during the AD process has not been sufficiently investigated so far, especially in cattle manure fed anaerobic digesters [58, 59]. Some studies reported that degradation of various antibiotics

during AD can occur; in fact, a complete removal of ampicillin, florfenicol, sulfadimethoxine, sulfamerazine, sulfamethoxazole, sulfamethoxydiazine, tetracycline, trimethoprim, and tylosin was observed [60-62].

However, these works focused more on the effects of antibiotics on the AD process than on the removal of antibiotics by the microbial community. It has been hypothesized that antibiotics can act as a selective pressure on some microbial components, which can develop resistance. In some cases, microorganisms can resist the toxic effects of antibiotics; in others they can show the capability to degrade and remove them as a homeostatic response to stress. [5]. This hypothesis is supported by the fact that there is also substantial evidence that antibiotic compounds (at some concentrations) do not completely affect AD process stability as measured by biogas production and composition, pH, volatile fatty acids (VFAs) concentrations, soluble organic content in the AD process, volatile solids removal or nitrogen content [28,63,64]. In any case, the effects of antibiotics on the AD microbial community depend on several parameters, and, primarily, their concentration [65].

Another aspect to be still clarified is if the AD process can influence the fate of ARGs; some studies report that anaerobic digestion can reduce tet genes (tetracycline resistance genes), [63] and others that AD can promote an increase in sul genes at the end of the process. [28, 64]. Further studies are therefore necessary to investigate more thoroughly the possible interactions between antibiotic residues, the AD process and ARGs.

3. Sulfonamides and Fluoroquinolones

Among the different classes of antibiotics used for cattle, there are sulfonamides and fluoroquinolones. Sulfonamides are one of the oldest classes of drugs used systemically. Gerhard Domagk, a Nobel Prize winner in 1939, first discovered their anti-bacterial properties; he observed that prontosil, a sulfonamide dye, was able to restrain selectively infectious bacteria cells. Prontosil is a pro-drug, which is transformed by the human body to sulfanilamide, the anti-bacterial active agent. This finding led to the discovery of other anti-bacterial compounds derived from this chemical group, with the same main core, but different bioactivities [66]. Currently, sulfamethoxazole (SMX) is one of the antibiotics most commonly prescribed and consumed in both human and veterinary medicine. In particular, SMX can be used in combination with the antibiotic Trimethoprim to treat and prevent respiratory infections and mastitis in cattle. SMX is a sulfonamide compound with aniline and an isoxazole group. Its action is bacteriostatic; it is able to inhibit the synthesis of folic acid, necessary for the synthesis of nucleotides, in bacterial cells (Fig. 2a). Many bacteria are able to convert paraaminobenzoic acid to folic acid. Since sulfonamides are very similar in structure to para-amino benzoic acid, they can act as competitive inhibitors of it, by interrupting its role in the synthesis of folic acid and ultimately, of purine and DNA [67]. Once administered, SMX is not completely metabolized: approximately 43% is transformed to N4-acetylsulfamethoxazole, 9-15% to sulfamethoxazole N1- glucuronide and 15-25% is excreted unchanged [68, 69]. Some authors found SMX degradation from 50% to 80% in biologically active soils in about 20 days, under respectively aerobic and anaerobic conditions [70, 71]. Although this antibiotic can be biodegraded [7], a reduction in soil bacterial diversity and increase in the number of ARB is expected in agricultural soils. Finally, another issue to be investigated is the possible accumulation of SMX into fresh edible vegetables [72, 73] which can be a source of ARB and ARG for humans.

Resistance to sulfonamides, clinically present in gramnegative enteric bacteria, is transmitted by plasmids and influenced by genes that encode alternative variants of drugresistant DHPS enzymes. Dihydropteroate synthase (DHPS) catalyzes the reaction of 6-hydroxymethyl-dihydropterin 1'diphosphate with 4-aminobenzoate producing dihydropteroate and inorganic pyrophosphate [74]. Sulfonamides act as competitive inhibitors of DHPS. Enzymes encoded by resistance-plasmids correspond to two genes, sul1 and sul2. The sul1 gene is usually found linked to other resistance genes in the Tn21 type integron, while sul2 is usually found on small plasmids of the IncQ family (RSF1010) and of the pBP1 one. DHPS products of both sul1 and sul2 show low Km values (0.6 μM) for para-aminobenzoic acid (PABA), resulting in resistance to high concentrations of sulfonamide (Fig. 3a). In particular, sul2's DHPS appears to show a very acute specificity in

distinguishing between its normal PABA substrate and sulfonamide. Moreover, a third resistance gene, sul3, has been characterized by Perreten and Boerlin [75], coding for a 263amino-acid protein similar to a dihydropteroate synthase encoded by the 54-kb conjugative plasmid pVP440 from Escherichia coli.

Quinolones are one of the most frequently prescribed types of antimicrobials in the world and are used to treat various human bacterial infections [76]. Due to their widespread use and overuse, the number of quinolone-resistant bacterial strains has steadily increased since the 1990s [77]. As well as other antimicrobials, the increase in quinolone resistance threatens the clinical applicability of this class of drugs. Quinolones are able to convert their targets, gyrase and topoisomerase IV, into toxic enzymes that fragment bacterial chromosomes [78] (Fig. 2b). Norfloxacin, the first broad-spectrum quinolone, was restricted to use in the treatment of urinary tract infections and sexually transmitted diseases. Currently, fluoroquinolones (FQs), fully synthetic and broad-spectrum antibiotics, are the most frequently used in animal husbandry [79]. They derive from quinolones by modifying their structure with a fluorine

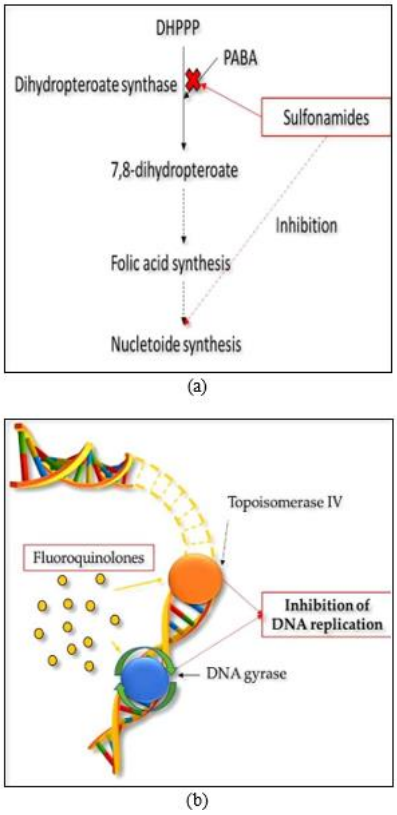

Figure 2: Mechanism of action in bacterial cells of (a) sulfonamides, such as sulfamethoxazole and (b) fluoroquinolones such as ciprofloxacin and enrofloxacin.

Ciprofloxacin (CIP) was the first fluoroquinolone that displayed significant activity not only in the urinary tract [80]. For more than 20 years, ciprofloxacin has continued to be one of the most commonly prescribed antibacterial drugs and used to treat a variety of Gram-negative and to a lesser extent, Gram- positive infections. The clinical success of ciprofloxacin spawned an array of newer-generation quinolones that displayed an even broader spectrum of activity, especially against Grampositive species [80]. A consequence of its massive use is the inclusion of CIP in the Watch List by EU (2018) [81].

Enrofloxacin (ENR) is another broad-spectrum antibiotic related to the class of fluoroquinolones [82]. It has been widely used in many countries for the treatment of a variety of poultry diseases, mainly those associated with Escherichia coli and Pasteurella multocida, but also avian mycoplasmosis. In the United States, the usage of enrofloxacin in poultry was banned in 2005. However, FQ-resistant strains are still found [83]. Enrofloxacin

and ciprofloxacin are closely related, since the latter is the main metabolite of ENR [84]. Even if CIP is not directly applied to livestock, metabolization of ENR could end in the occurrence of CIP in veterinary field. Both ENR and CIP are adsorbed to soil and their biodegradation rates are quite low, consequently significant concentrations of FQ are found in agricultural soil [40, 85]. FQ persistence in soil depends on both abiotic (e.g. light, soil organic matter) and biotic factors (bacterial populations able to degrade them) [86]. On the other hand, strong adsorption lead to less uptake of FQs residues by plants [87]. Once in the soil, the fate of this class of antibiotics in the ecosystem food web (including human one) is still to be clarified [88].

Several resistance mechanisms induced by quinolones in bacteria cells are reported [89] such as those related to a chromosomal mutation in genes encoding for topoisomerase IV or gyrase IV, or to a decrease in drug accumulation (Fig. 3). The latter can happen if bacterial efflux pumps are overexpressed (and the drug is pumped outside the cell), or when porin proteins are down-regulated, avoiding the passive diffusion of ciprofloxacin inside the cell. Moreover, so-called plasmid-mediated quinolone resistance has been recognized. It acts in different ways: the first plasmid-mediated resistance discovered was the qnr gene, encoding for a pentapeptide capable of binding chromosomal DNA and protecting it from drug action. Another plasmid-mediated resistance mechanism is the cr variant of the aac(6')-lb gene, which encodes for an aminoglycoside acetyltransferase that acetylates ciprofloxacin[90].

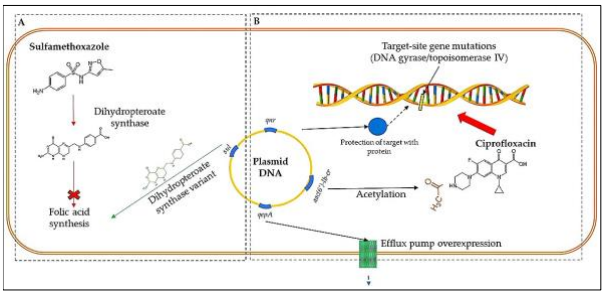

Figure 3. Resistance mechanisms for sulfonamides (A) and fluoroquinolones (B).

Within this context, the project titled "Evaluation of the presence of Antibiotics in Zootechnical waste and in the digestate of biogas plants: study of strategies for their RemOval - AZeRO antibiotics" is a research project funded by Lazio Innova (Lazio Regional Development Agency) in line with environmental sustainability, the green economy and the protection of human health and ecosystems. It is an interdisciplinary project where the microbial ecology, soil and ruminal ecosystems, environmental and fermentation chemistry and molecular biology interact in order to investigate the AD process and how antibiotics can affect it. Moreover, the environmental fate of antibiotics and ARGs is also studied.

In particular, the project aims at:

- evaluating the presence and concentration of SMX, CIP and ENR antibiotics in zootechnical waste and in the digestate of full-scale biogas digesters in the Lazio region, (Central Italy);
- assessing how SMX, CIP and ENR occurrence can influence the anaerobic digestion process;
- assessing which factors and conditions can favor antibiotic degradation in anaerobic conditions;
- Identifying ecological solutions and "best practices" for preventing or reducing the environmental spreading of residual antibiotics and ARGs by agricultural activities.
- Evaluating if the AD process is able to decrease the antibiotics and ARGs which enter biogas digesters with manure. The latter aspect is particularly important because it could make digestate more suitable for replacing chemical fertilizers and meet the European Green Deal target "From Farm to Fork" [30].

The two-year project will involve several biogas plants located in cattle farms in central Italy, where milk, meat and cheeses, including numerous PDO (Protected Designation of Origin) and PGI (Protected Geographical

Indication), are produced. Both the reactor feeds (mainly cattle manure) and digestates will be sampled in those farms monthly, over 2 year to evaluate the residual concentrations of antibiotics and the resistance genes.

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