

Review Article

Tetracyclines in Food and Feedingstuffs: From Regulation to Analytical Methods, Bacterial Resistance, and Environmental and Health Implications

Fabio Granados-Chinchilla¹ and César Rodríguez²

¹*Centro de Investigación en Nutrición Animal (CINA), Universidad de Costa Rica, San José, Costa Rica*

²*Centro de Investigación en Enfermedades Tropicales (CIET) and Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica*

Correspondence should be addressed to César Rodríguez; cesar.rodriguezsanchez@ucr.ac.cr

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Antibiotics are widely used as growth promoters in animal husbandry; among them, the tetracyclines are a chemical group of relevance, due to their wide use in agriculture, surpassing in quantities applied almost every other antibiotic family. Seeing the considerable amounts of tetracyclines used worldwide, monitoring of these antibiotics is paramount. Advances must be made in the analysis of antibiotics to assess correct usage and dosage of tetracyclines in food and feedstuffs and possible residues in pertinent environmental samples. The tetracyclines are still considered a clinically relevant group of antibiotics, though dissemination of tolerance and resistance determinants have limited their use. This review focuses on four different aspects: (i) tetracyclines, usage, dosages, and regulatory issues that govern their food-related application, with particular attention to the prohibitions and restrictions that several countries have enforced in recent years by agencies from both the United States and the European Union, (ii) analytical methods for tetracyclines, determination, and residues thereof in feedstuffs and related matrices with an emphasis on the most relevant and novel techniques, including both screening and confirmatory methods, (iii) tetracycline resistance and tetracycline-resistant bacteria in feedstuff, and (iv) environmental and health risks accompanying the use of tetracyclines in animal nutrition. In the last two cases, we discuss the more relevant undesirable effects that tetracyclines exert over bacterial communities and nontarget species including unwanted effects in farmers.

1. Introduction

Some compounds of veterinary relevance deserve particular attention from an economic and health standpoint, especially because of their widespread use. Among these compounds, the fluoroquinolones, β -lactams, sulfonamides, and tetracyclines are included [1].

Feed and environmental antibiotic residues are a determinant factor that is usually omitted and is not entirely understood when public health or food and feed safety are discussed. Epidemiological, toxicological, and chemical information regarding tetracyclines is presented to assess acute and chronic consequences of human and animal health as the result of their usage [2]. As such, a multifaceted and

complex issue is better addressed with a one health approach [3].

Antibiotics are extensively utilized in productive animals with therapeutic, prophylactic, metaphylactic, growth promoting, and food effectiveness enhancing ends [4]. Antibiotic usage for growth promotion is the mainstream application in animals and is the subject of regulatory efforts to decrease antibiotic consumption in livestock, poultry, and aquaculture [4]. Growth promotion and nutrient efficacy are considered to be accomplished with relatively small doses of antibiotics mixed with the feed by the manufacturer or the farmer [4]. Growth promotion has no counterpart in human medicine, and for this particular practice, antimicrobials do not require veterinary prescription [4]. However, it is

essential to pursue prudent use of antimicrobials to minimize resistance and ensure their continued effectiveness and availability of these products and curtail impacts on human health [5]. Implementation of security measures on farms, proper monitoring and registration, animal identification, more fitting management practices, implementation of the right antibiotic and dosage, and promoting the judicious and prudent use of antibiotics may extend the life of antibiotics despite their frequent, persistent, and increasing use during the last decades [4].

Tetracyclines are a family of compounds frequently employed due to their broad spectrum of activity as well as their low cost, compared with other antibiotics. Currently, there are over 20 tetracyclines available; however, tetracycline, chlortetracycline, oxytetracycline, and doxycycline are the most common ones in veterinary medicine [6]. In addition to therapeutic purposes, in many other countries, tetracyclines are often incorporated into livestock feed at subtherapeutic doses as growth promoters for swine and poultry and in aquaculture. For years, the use of antibiotics as growth promoters has been linked with beneficial aspects (especially increase in nutrient uptake efficiency and commercial revenue for farmers), though there is data that supports the fact that this exercise promotes bacterial resistance, allergic reactions in humans and animals, and changes in environmental microflora and bacterial populations among other detrimental effects [7, 8].

This review is centered in tetracycline antibiotics considering that they are among the most frequently used and in more quantities (dosage-wise) in livestock and poultry worldwide [9]. Global antibiotic consumption in livestock was conservatively estimated at $63\,200 \pm 1\,560$ tons in 2010 [10], accounting for nearly two-thirds of the worldwide antibiotic production [11], and is projected to rise. Specifically, by 2012, the estimated usage of tetracyclines was of 5 954.36 and 113.2 tons in the United States and the European Commonwealth, respectively. These stats indicate that the issue of overuse of these antibiotics is of particular significance in countries such as United States, China, and India [10] rather than in Europe, where consumption figures are much lower due to the ban of antibiotics as growth promoters [10]. Amounts represent a total of ca. \$500 million USD of a group of antibiotics still categorized as critically important in human medicine [12]; only 9 out of 27 classes of antibiotics are exclusively used in animals [13]. A thorough outlook of antibiotic usage may be found in a recent paper written by Van Boeckel et al. [10].

2. Tetracyclines Usage, Dosages, and Regulatory Aspects That Govern the Food-Related Application of Tetracyclines

2.1. Approved and Recommended Uses. Tetracyclines have several therapeutic indications dealing with infections in food-producing animal and pets. In food-producing species, including horses, usually, the first-generation tetracyclines are used, while in pets the second-generation tetracyclines are chosen. Therapeutic indications in animals comprise respiratory infections, dermal and soft tissue infections, peritonitis,

metritis, and other enteric infections as well as the treatment infections in aquatic species and honeybees. In food animals, for easier administration, the antibiotics are administered to groups simultaneously through the drinking water or feed to treat or prevent disease [14]. Tetracyclines have also been used for growth promotion, but apprehension related to emerging bacterial resistance has led to a removal of the utilization of these antibiotics in this capacity, especially in European countries [15, 16]. Till this day, the use of tetracyclines as growth promoters is still allowed in many countries [9, 10]. However, beginning from 1 January 2017, tetracyclines will no longer be allowed for use as growth promoters in USA [4]. The use will be restricted to therapeutic use only and subject to a veterinary feed directive (VFD) [4]. Tetracyclines have been active against *Mycoplasma*, *Chlamydia*, *Pasteurella*, *Clostridium*, *Ornithobacterium rhinotracheale*, and some protozoa. Examples of commercially available tetracyclines include chlortetracycline (Aureomycin® and ChlorMax®) and oxytetracycline (Terramycin®).

Of particular concern are some tetracyclines of second and third generation such as doxycycline and tigecycline. Doxycycline is a semisynthetic tetracycline derivative. As a hyclate salt, doxycycline is presented as an injectable solution (intramuscular and intravenous), water-soluble or lactodispersable powders, and tablets and capsules (for pets). Doxycycline hyclate is indicated in cattle, pigs, poultry, turkeys, and pets for the treatment of bacterial infections, susceptible to this antibacterial, at a dose of $10\text{--}20\text{ mg kg}^{-1}$ body weight per day, for 3–5 days. Doxycycline is not to be used in lactating cattle and layers. In contrast to animal therapy, this antibiotic has a long history of use in human medicine. Doxycycline was previously assessed by the Committee for Medicinal Products for Veterinary Use and an “acceptable” level of daily intake of $3\text{ }\mu\text{g kg}^{-1}$ body weight, that is, $180\text{ }\mu\text{g}$ per person, was recognized. Currently, doxycycline is comprised in the Commission Regulation (EU) number 37/2010 of 22 December 2009 [17].

On the other hand, there are currently no authorized tigecycline-containing products for veterinary use in the European Union. However, according to the Cascade rule (a legislative provision that allows a veterinary surgeon to prescribe unauthorized medicines that would not otherwise be permitted), tigecycline could be used for pets [18] especially since multidrug-resistant organisms in dogs, cats, and horses are being found with increasing frequency. The extent of use of tigecycline in veterinary medicine due to this rule is unknown. However, some countries such as Finland have barred the use of this antibiotic for animal use [19]. In Europe, since 1998, cattle and broiler chicken industries voluntarily stop the utilization of all antibiotic growth promoters, and in 1999 the swine industry followed [20]. An EU ban on all antibiotics as growth promoters went into effect in 2006 [21].

Notwithstanding, in the United States, oxytetracycline (OTC) and chlortetracycline (CTC) are licensed to promote weight gain and improve feed efficiency rates [22] (Table 1). Doxycycline, by contrast, is reserved for periodontal disease in pets [22] (Table 1).

TABLE 1: Approved applications of tetracyclines in medicated feed for different food-producing animal species and their respective withdrawal time frames.

Pharmacologically active substance	Indications for use ^a	Usage level	Withdrawal time (days)
<i>Feed antibiotics for swine</i>			
<i>Chlortetracycline</i>	Increased average weight gain and feed efficiency	10–50 g ton⁻¹	Voluntary withdrawal
	Reduction of jowl abscesses	50–100 g ton ⁻¹	Voluntary withdrawal
	Control of leptospirosis in sows	400 g ton ⁻¹	Voluntary withdrawal
	Control of proliferative enteropathies (ileitis)	Body weight dosage: 10 mg lb ⁻¹ d ⁻¹	Voluntary withdrawal
<i>Chlortetracycline & penicillin & sulfathiazole</i>	Abscess abatement; treatment of bacterial enteritis; upkeep of weight gain in the presence of rhinitis	100 chlortetracycline;100 sulfathiazole;50 penicillin	7
<i>Oxytetracycline</i>	Increased average weight gain and feed efficiency	10–50	None
	Treatment of bacterial enteritis and bacterial pneumonia	Body weight dosage: 10 mg lb ⁻¹ d ⁻¹ , 7–14 days	None
	Control of leptospirosis in sows	Body weight dosage: 10 mg lb ⁻¹ d ⁻¹ , 7–14 days	None
<i>Neomycin & oxytetracycline</i>	Increased average weight gain and feed efficiency	10–50	5
	Treatment of bacterial enteritis and bacterial pneumonia	Body weight dosage: 10 mg lb ⁻¹ d ⁻¹ , 7–14 days	5
	Control and treatment of leptospirosis in breeders	Body weight dosage: 10 mg lb ⁻¹ d ⁻¹ , 7–14 days	5
	Prevention or treatment of bacterial enteritis and dysentery; maintenance of weight gain in the presence of atrophic rhinitis	50–150 oxytetracycline; neomycin body weight dosage: 35–140 mg lb⁻¹ d⁻¹	10
<i>Oxytetracycline & carbadox</i>	Treatment of bacterial enteritis and bacterial pneumonia	10–25 carbadox; oxytetracycline body weight dosage: 10 mg lb ⁻¹ d ⁻¹	42
<i>Tiamulin & chlortetracycline</i>	Control of dysentery; treatment of bacterial enteritis and bacterial pneumonia	35 tiamulin + 400 CTC (body weight dosage: 10 mg lb ⁻¹ d ⁻¹)	2
<i>Feed antibiotics for cattle (up to 700 lb)</i>			
<i>Chlortetracycline</i>	Coacting in the prevention of bacterial pneumonia associated with shipping fever complex caused by <i>Pasteurella</i> spp.	350 mg head ⁻¹ day ⁻¹	2
	Control of active infection of 2 of anaplasmoses caused by <i>Anaplasma marginale</i>	350 mg head ⁻¹ day ⁻¹ or 0.5 mg lb ⁻¹ of body weight day ⁻¹ , beef control of active infection	2
<i>Oxytetracycline</i>	Finishing cattle: to increase rate of gain and improve feed efficiency	75 mg head ⁻¹ day ⁻¹	None
	Coacting in reducing incidence and severity of liver abscesses	75 mg head ⁻¹ day ⁻¹	None
	Coacting in the prevention of bacterial diarrhea	0.1–0.5 mg lb ⁻¹ of body weight day ⁻¹	0 to 5
	Prophylaxis and treatment of the early stages of shipping fever complex	0.2–2 mg lb ⁻¹ of body weight day ⁻¹	0 to 5
<i>Chlortetracycline & sulfamethazine</i>	Feed for 28 days coacting in the maintenance of weight gain in the presence of respiratory disease such as shipping fever	350 mg head⁻¹ day⁻¹	7

TABLE I: Continued.

Pharmacologically active substance	Indications for use ^a	Usage level	Withdrawal time (days)
<i>Lasalocid</i> & <i>oxytetracycline</i>	For improved feed efficiency and increased rate of weight gain and reduction of incidence and severity of liver abscesses in cattle fed in confinement for slaughter	From 25 to 30 g ton⁻¹	None
<i>Oxytetracycline</i> & <i>neomycin base</i>	Coacting in the prevention of bacterial enteritis	From 35 to 140 g ton ⁻¹	0–7
<i>Feed antibiotics for calves (up to 400 lb)</i>			
	Increased rate of weight gain and improved feed efficiency	0.1 mg lb⁻¹ of body weight day⁻¹ or 25–70 mg head⁻¹ day⁻¹	None
<i>Chlortetracycline</i>	Treatment of bacterial enteritis caused by <i>Escherichia coli</i>	10 mg lb ⁻¹ of body weight day ⁻¹	None
	Treatment of bacterial enteritis caused by <i>Escherichia coli</i> and bacterial pneumonia caused by <i>P. multocida</i>	10 mg lb ⁻¹ of body weight day ⁻¹	Variable
<i>Oxytetracycline</i>	Increased rate of weight gain and improved feed efficiency	From 0.05 to 0.1 mg lb⁻¹ or 25–75 mg head⁻¹ day⁻¹	0 to 5
	As an aid in the treatment of bacterial diarrhea	From 0.5 to 5.0 mg lb ⁻¹ or 35 to 140 g ton ⁻¹	None
<i>Feed antibiotics for poultry</i>			
	Increased average weight gain and feed efficiency	From 10 to 50 g ton ⁻¹	None
<i>Chlortetracycline</i> & <i>oxytetracycline</i>	Control of synovitis caused by <i>Mycoplasma synoviae</i> and avian cholera caused by <i>Pasteurella multocida</i>	From 100 to 200 g ton ⁻¹	None
	To control chronic respiratory disease of the air sacs caused by <i>Mycoplasma gallisepticum</i> and <i>Escherichia coli</i>	400 g ton ⁻¹	None
	To reduce mortality due to air sac infections caused by <i>Escherichia coli</i>	500 g ton ⁻¹	1
<i>Antibiotics used in pets (companionship animals)</i>			
<i>Doxycycline</i>	Topical, to treat periodontitis. In dogs, used to treat bacterial infections and infections caused by <i>Rickettsia</i> , Canine ehrlichiosis (anaplasmosis), <i>Toxoplasma</i> , <i>Borrelia burgdorferi</i> (Lyme disease), leptospirosis, and <i>Neorickettsia helminthoeca</i> (salmon poisoning). In cats, used to treat bacterial infections and infections caused by some other organisms including <i>Bartonella</i> , <i>Hemoplasma</i> , <i>Chlamydia felis</i> , <i>Ehrlichia</i> , <i>Anaplasma</i> , and <i>Toxoplasma</i>	NA	NA

^aRows in bold font refer to growth promotion approved applications. Data based on values set in [22, 28]. In USA, tetracyclines are no longer allowed for growth promotion after 1 January 2017.

2.2. Regulatory Aspects

2.2.1. Feedingstuff. Because of its substantial implications for food and feed safety, public administrations promote integrated “farm to fork” approaches to ensure food security and verify law compliance regarding the occurrence of antibiotic residues and contaminants in feed and feed ingredients [23].

Within the EU, maximum residue limits (MRLs) of authorized veterinary drugs in foodstuffs are defined in the Council Regulation. Prior to the antibiotic ban in 2003,

Annex 1 from the Council Directive number 70/524/CEE [24] stated maximum limits for antibiotics, including tetracyclines. Three tetracyclines are listed. Tetracycline, oxytetracycline, and chlortetracycline were approved for animals bred for fur, calves, lambs, poultry, and swine with maximum contents of active ingredient of 80 mg kg⁻¹ feed. Also, according to European legislation, Good Veterinary Practices (GVP) request producers to abide by established withdrawal times for antibiotics [25]. Some countries have set still stricter measures regarding the use of antibiotics in food-producing

TABLE 2: Requirements of a medicated feed mill license.

Compound(s)	Category I		
	Type A (assay limits, %) ^a	Type B maximum, g lb ⁻¹ (200x, %)	Type B/C (assay limits, %) ^a
Chlortetracycline	85–115	40.0 (8.8)	80–115/70–130
Oxytetracycline	90–120	20.0 (4.4)	75–125/65–135
Compound(s)	Category II		
	Type A (assay limits, %)	Type B maximum, g lb ⁻¹ (100x, %)	Type B/C (assay limits, %)
Neomycin	80–120	7.0 (1.54)	70–125
Oxytetracycline	80–120	10.0 (2.2)	65–135
Sulfamethazine	85–115	10.0 (2.2)	80–120
Chlortetracycline	85–115	10.0 (2.2)	85–125/70–130

^aPercentage of labeled amount. Based on values set in [28].

animals. A definite success example of antibiotic regulation through prescription monitoring is Denmark which in 2010 adopted a “Yellow Card” system in which producers that applied more amount of antibiotic per animal are issued a warning. Failure in abiding and lowering their use would eventually imply that they would be forced to either reduce use or cut the herd size [21, 26]. They also limit the possibility of veterinarians to profit from antibiotic sales and prescription monitoring in farms [21, 26]. Application of this measurement was accompanied in the years that followed by a decrease in antibiotic usage [27].

In this regard, the FDA has established control limits for types A, B, and C animal drugs, where type A refers to pure drugs and types B and C correspond to medicated feed. US FDA medicated feed assay control limits for pure CTC and OTC (medicated articles) (Table 2). Similar limitations are established for feeds medicated with CTC and OTC [28]. On their appearance after application/withdrawal windows, feed-antibiotics can be classified into two categories. Category I antibiotics may appear in feed at the lowest use level for which no withdrawal period is required, and category II antibiotics comprise compounds for which a withdrawal period is required at the lowest use level and no residue or “zero tolerance” is allowed.

In the absence of any other antibiotics, tetracyclines can be considered as category I antibiotics. Subsequently, they can further be viewed as a type A, B, or C. A type A article is a product that consists of one or more animal drugs of consistent strength, intended exclusively for use in the production of another type A article or either a type B or a type C medicated feed. Type A medicated articles must be registered with the FDA annually (FDA-2656/e). Type B is a medicated feed that contains a type A medicated article, and it is significantly diluted with one or more nutrients to produce a type C medicated feed which is intended to be presented as a complete feed for the animal.

Before a facility can manipulate a category II, type A medicated article, it must hold an approved medicated feed mill license. Registration as a drug establishment and FDA approval of a feed mill license are required before a category II, type A medicated feed article can be purchased (Table 2).

Antibiotics in feeds, including tetracyclines, are regulated by US FDA Current Good Manufacturing Practices (cGMP)

21 CFR 225.10-115 [29] when medicated feed application is required or 21 CFR 225.120-202 where it is not [30]. They are also subjected to Part 226 of cGMP regulations [31].

When the US FDA approves new animal drugs or combination products, they obtain one of the following marketing statuses: (i) over-the-counter, (ii) veterinary prescription, or (iii) veterinary feed directive (VFD) [4]. As tetracyclines are considered medically relevant antibiotics (according to US FDA Guidance 152 [32]), tetracyclines are in need of a VFD to be incorporated in the feed. Furthermore, the US FDA Guidance 213 [33] dictates that all medically relevant drugs administered require VFD and therefore inspection and record keeping of prescription. At the time of writing, this guidance is voluntary implementation, but it will become mandatory in December 2016 [34].

While recent FDA actions have focused on production uses, there are calls in the United States also to restrict prophylactic uses as well [34, 35]. An economic analysis of benefits, costs, and perspectives of a possible ban has been analyzed in depth recently by Teillant et al. [36]. Noteworthy, jurisprudence in USA has usually been laxer than in other countries [16].

Despite being considered a global reference point for consumers, food manufacturers, and processors, food control agencies, and the international food trade, the *Codex Alimentarius* does not have a particular task force concerning animal feed issues [37]. The primary output of the once ad hoc Codex Intergovernmental Task Force on Animal Feeding was the Codex Code of Practice on Good Animal Feeding [38]. Regarding antibiotics in feed, it only states that antibiotics should not be used in feed for growth promoting purposes when public health safety assessment is lacking.

Other texts relevant to animal feeding have been developed by other Codex Committees such as those on Food Additives, Contaminants in Foods, Meat Hygiene, Residues of Veterinary Drugs in Foods, Pesticide Residues, and Food Labelling. Analogously, the FAO in partnership with the International Feed Industry Federation (IFIF) has issued a Manual of Good Practices for the Feed Industry. Good Manufactory Practices demand feed and feed ingredients to be free of pests and chemical, physical, and microbiological contaminants during their production, management, storage,

TABLE 3: Legally bound threshold concentrations established for food commodities or particular animal tissue.

Pharmacologically active substance	Species	Tissue	Maximum residue limit ($\mu\text{g kg}^{-1}$)	
Chlortetracycline/oxytetracycline/tetracycline	Cattle	Milk ($\mu\text{g L}^{-1}$)	100	
		Muscle	200	
		Liver	600	
		Kidney	1200	
		Muscle	200	
	Poultry	Liver	600	
		Kidney	1200	
		Milk ($\mu\text{g L}^{-1}$)	100	
	Sheep	Muscle	200	
		Liver	600	
	Swine	Kidney	1200	
		Muscle	200	
		Liver	600	
		Kidney	1200	
		Muscle	200	
		Turkey	Liver	600
			Kidney	1200
Fish (i.e., salmonids)		Muscle	200 ^a	
Lobster		Muscle	200 ^a	
Giant prawn (<i>Penaeus monodon</i>)		Muscle	200 ^a	
	Eggs	400		
	Honey	300		
Doxycycline	Cattle, swine, poultry	Muscle	100	
		Skin and fat	300	
		Liver	300	
		Kidney	600	

^aApplies only to oxytetracycline. Based on values set in [40, 41].

and transportation [39]. However, they still do not include resistant bacteria in the feed as a critical risk.

2.2.2. Food Commodities Destined for Human Consumption.

In related matrices, such as tissues from food-producing animals, statutes (set during the 38th Session of the Codex Alimentarius Commission, celebrated in July 2015) are more straightforward. [40]. On the other hand, both the United States Food and Drug Administration (US FDA) and the European Commission have established acceptable daily intake values by toxicological data and the performance of the current analytical technology. Other official documents regulating veterinary drugs in food have been put in place to protect consumers. For example, European Commission Regulation [61] lays down rules and procedures to determine (i) the MRLs (concentrations) of a pharmacological substance which may be permitted in the food of animal origin. Explicit MRLs have been set for the tetracyclines in Regulation 37/2010 [41]; here the optimal limit for pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin inside the European Union is established (Table 3).

Furthermore, the European Commission has also established guidelines for method validation of assays to be used in the evaluation of official samples and their performance [62] and specifies standard criteria for the construing of analytical results of official control laboratories for such specimens. Companyó et al. [63] published a review which summarizes legislation and regulations binding drug residues in food of animal origin.

2.2.3. Other Strategies toward Prudent Antimicrobial Usage.

To tackle a complex problem such as the usage and regulation of antibiotics and its implications, conjoint force must be applied among countries. In 2011, responding to the mounting threat of antimicrobial resistance, the Transatlantic Taskforce on Antimicrobial Resistance (TATFAR) was established. The objective of this task force is to identify urgent antimicrobial resistance issues that could be better addressed by the cooperation between the United States and the European Commonwealth regarding the following crucial areas: (i) suitable therapeutic use of antimicrobial drugs in the medical and veterinary communities, (ii) prevention of both healthcare- and community-associated drug-resistant

infections, and (iii) policies for improving the pipeline of new antimicrobial drugs [64].

3. Analytical Methods for Tetracyclines Determination and Residues Found in Feedstuffs and Related Matrices

Proper antibiotic monitoring and assessment guarantee that the correct doses and antibiotics are administered. Hence, guideline-based and prudent antibiotic use helps in reducing antibiotic consumption and in turn its residues and bacterial resistance. To this end, accurate and reliable analytical methods must be implemented to collect exact and precise quantitative data. Significant advances in this course have been made in the last few years as regards the detection and quantification of tetracyclines, degradation products, and metabolites in feeds and related matrices. Conventional screening tests for antimicrobial agents are divided into analytical methods, microbial inhibition assays, enzymatic tests, and immunological tests. Analytical methods are usually superior in specificity, selectivity, and sensibility. However, they tend to use expensive equipment that is not always available in every laboratory. Inhibition assays are relatively fast and cost-effective and may be utilized as a screening test. In turn, certain enzymatic and immunological assays can be applied as confirmatory tests. Another approach to monitoring drug residue levels is based on microbial whole cell sensors constructed using recombinant DNA technologies.

3.1. Sample Preparation and Clean-Up. Sample preparation is one of the crucial issues in food analyses because it can be a source of inaccuracy and a limitation for the development of high-throughput methods [65]. Though multiclass determination is a way to improve cost-effectiveness, food matrices are very complex and heterogeneous, and there are no universal methods for extraction of antibiotics characterized by different physicochemical characteristics. Moreover, tetracyclines are usually among the antibiotics with lower recoveries during multiresidue analyses.

An analytical method typically comprised five steps: sampling, sample preparation, separation, detection, and analysis. Usually, sampling and sample preparation are the crucial components of any analytical assay. The extraction of tetracyclines from food and feed is still a challenge as matrix effects are a major problem to be circumvented during the analyte extraction and sample treatment must be robust and reproducible (i.e., independent of disparities between matrices). A typical sample preparation and clean-up procedure for the determination of tetracyclines in foodstuffs can be accomplished by employing several techniques, but all methods include at least two steps: (i) the removal of potential interferences and (ii) the concentration of an analyte. In some cases, additional steps are deemed necessary; for example, an analyte may be changed into a more suitable chemical form prior analysis [42, 66]. In a recent paper, Li et al. [43] used a ridge analysis of the response surface, single factor experiments, and Box–Behnken designs

to optimize three critical conditions during tetracycline extraction in manure (i.e., pH, which determines tetracyclines' chemical species and extraction solution volume and temperature that will impact overall solubility). Though the majority of authors have used McIlvaine/EDTA buffer to extract tetracyclines, other aqueous buffers (e.g., Britton–Robinson) have been assayed with excellent results [44]. The same authors utilized an unusual two-phase method which involves centrifuging at -20°C organic solvent while an aqueous phase retains matrix interference such as proteins [44].

Using three cycles of ultrasound, they obtained good recoveries, precision, and sensitivity (quantification limits ranging from 1.75 to 2.35 mg kg^{-1}). Since sorbent breakthrough may lead to reduced recoveries, Liu et al. [45] applied a pressurized extraction of tetracyclines from egg, fish, and shrimp in a speedy manner; this kind of treatment is not common, since tetracyclines are considered thermally sensitive. Despite using HPLC–UV detection, very low limits were reached for minocycline, oxytetracycline, tetracycline, demeclocycline, chlortetracycline, metacycline, and doxycycline. Ibarra et al. [46] used a phenyl silica adsorbent covered with magnetite to pretreat magnetically and afterward analyzed four different compounds, tetracycline, oxytetracycline, chlortetracycline, and doxycycline, in milk. The authors reported that these magnetic particles might be a reusable technology. Considering that this is a relative inexpensive sample preparation technology, an adapted version of this approach may very well, in the future, be suited as a food or water treatment to remove undesired tetracycline residues. However, possible costs may hinder their application. On the other hand, Xu et al. [47] applied a chitosan modified multiwalled graphite nanotubes solid phase extraction (SPE) sorbent when analyzing tetracyclines in milk and honey which resulted in high sensitivity and excellent recoveries. The former is yet another example of how there are still advances to be made in SPE sorbents chemistry and structure to improve sample preparation.

Yu et al. [67] developed a multiresidue assay for banned in-feed drugs, including sulfonamides, fluoroquinolones, and tetracycline, in swine tissue using matrix solid phase dispersion (MSPD) as a sample pretreatment method. MSPD is based on several physicochemical principles, involving forces (e.g., abrasive, shearing, and grinding) applied to the sample by mechanical combination to produce whole sample disruption and the interactions of the sample matrix with a solid support bonded phase or the surface of other support materials [68]. Induced disruption of the sample's building, in conjuncture with an adequate solvent, ensures a complete sample separation or dispersion over the surface of the bonded phase support material. Chemical interactions produce an exclusive mix phase for the isolation of the target analyte from relevant solid, semisolid, and highly viscous food and biological matrices, skirting any difficulty encountered by employing the classical SPE approach.

One substantial problem that the quantitative analysis of pharmaceuticals in biological samples presents is related to the fact that the analyte is usually bound to proteins and peptides, with the consequent need for cleavage of

TABLE 4: Proficiency parameters for sample pretreatment methods reviewed.

Treatment	Conditions	Limit of detection	Reproducibility (RSD, %)	Recovery (%)	Detection system	Reference
Ultrasound	40°C, pH 7.15	0.03– 0.05 $\mu\text{g mL}^{-1}$	<4.1	81.89– 92.42	LC-UV	[42]
SPE	Britton-Robinson buffer, 0.008 mol L ⁻¹ , pH 10	4.4– 12 $\mu\text{g kg}^{-1}$	<11.0	76.5–95.5	LC-DAD	[43]
Pressure	3 min, 60°C, 65 bars	10.0– 15.0 $\mu\text{g kg}^{-1}$	<8.4	75.6– 102.9	LC-UV	[44]
MSPE	pH 10.0	2–9 $\mu\text{g L}^{-1}$	<2.7	99.7– 101.2	Capillary electrophoresis	[45]
SPE	CH ₃ CN/CH ₃ CO ₂ H (8:2)	0.61– 10.34 $\mu\text{g kg}^{-1}$	<7.3	81.5– 101.4		[46]
MSPD	C ₆ H ₁₄ , CH ₃ CN:CH ₂ Cl ₂ (1:1)	7–34 mg kg ⁻¹	<6.1	80.6–99.2	LC-DAD	[47]
MI-SPE	CH ₃ OH, 70°C, 105 bars	Not indicated	Not indicated	Not indicated	LC-ITMS	[48]
SPE	CH ₃ CN:CH ₃ OH (1:1), 0.1 mol L ⁻¹ phosphoric acid	Not indicated	Not indicated	96–98	Spectrophotometry	[49]
SPE	McIlvaine/EDTA buffer	1.5–8.0 $\mu\text{g L}^{-1}$	<3.6	70.3– 107.4	DAD	[49]

these structures before analysis. Sonication can be used in concert with other traditional sample preparation techniques to help speed up and assist the process of analytes extraction and sample clean-up as it provides an efficient contact between the solid and the extractant, which typically result in higher recovery rates of the target analytes [48]. Another methodology for analyte clean-up and preconcentration before analysis is molecular imprinting. The technique is defined as the construction of selective ligand recognition sites into a synthetic polymer where a template (in this specific context, a molecule) is employed to facilitate formation of binding sites during the covalent assemblage of the bulk phase by polymerization, with subsequent removal of some or all of the template being necessary for recognition to occur in the spaces vacated by the molecules use for modelling [49]. This procedure has been previously used to extract tetracyclines in methanolic solutions and is advantageous because various tetracyclines (TCs) and related compounds can be targeted simultaneously. Mojica et al. [50] developed an imprinted xerogel responsive to tetracycline. In a similar fashion, Udalova et al. [51] pre-concentrated tetracycline, oxytetracycline, chlortetracycline, and doxycycline (DC) with a hyper-cross-linked polystyrene based sorbent. Yang et al. [52] synthesized a zeolite imidazolate framework-8 as a novel solid phase sorbent to extract tetracyclines from water and milk samples; the sorbent exhibited high surface area, permanent nanoscale porosity, excellent stability, and tunable cavities. To date, a column based on molecular imprinting and an application note for the extraction of tetracyclines from meat are commercially available from AFFINISEP (AFFINIMIP®SPE). Summary of each treatment described herein is enumerated in Table 4.

3.2. Analytical Methods

3.2.1. Chromatographic Methods. The eight different tetracyclines that find usage in human and veterinary medicine can be determined using high-performance liquid chromatography (HPLC) in the reverse phase mode with various detection modes, such as spectrophotometry, fluorescence, and mass spectrometry [53]. Since UV detection has relatively low sensitivity and mass spectrometry requires costly instruments, methods using fluorescence detection are preferred due to their high selectivity and sensitivity.

(1) Spectrophotometry. Variable wavelength and diode array detection (DAD) are appropriate options for the analysis of tetracyclines and their epimers. This technology is available in most laboratories. However, it lacks selectivity or specificity and is subject to interferences. An early report of tetracycline measurement in food by HPLC-DAD was published by Van Wambeke [54]. This author validated a method to be employed along the broiler food chain feed, eggs, and muscle. In it, sodium 1-decanesulphonate was used as an ion pair during chromatography, reducing the sample clean-up considerably. Gajda and Posyniak [55] reported a complete separation and quantification of 4-epiTC, 4-epiCTC, and 4-epiOTC in addition to their parent compounds CTC, TC, OTC, and DC using a UV detector. The authors stressed that the main difficulty in the determination of TCs is their instability and, in chicken muscle, they found 4-epitetracycline, 4-epioxytetracycline, and 4-chlortetracycline. Recently, Patyra et al. [56, 57] validated a method to determine residues of fluoroquinolones (enrofloxacin, ciprofloxacin, sarafloxacin, and flumequine) and tetracyclines (oxytetracycline, tetracycline, chlortetracycline, and doxycycline) in animal drinking water. Sample preparation consisted in the use of two

different SPE cartridges for extraction and a reverse phase LC separation/detection method. The method was applied for the determination of four tetracyclines and four fluoroquinolones in 24 animal drinking water samples collected during official inspections; doxycycline and enrofloxacin residues were found in six and nine specimens, respectively. The last data hints toward possible contamination of water reservoirs during the regular usage of antibiotics.

(2) *Fluorescence Detection.* The use of fluorescence detectors provides much more sensitivity and selectivity than UV-DAD and VWD (Variable Wavelength Detector). Much effort has been invested in developing fluorescence-based methods coupled with liquid chromatography for the determination of tetracyclines in food and feed samples. Though liquid phase chromatography is the method of choice for the determination of antibiotics in various matrices, many substances of interest cannot be detected because they lack the intrinsic chromophores or their native fluorescence signal is too scant to be detected properly. A derivatization reaction is very often required to increase sensitivity or selectivity and can be achieved by a specific detection, such as fluorescence or absorption in the visible light, at a high wavelength. Derivatization is particularly important for analyses of low concentrations of analytes within complex biological samples. Reagents used for derivatization may be (i) nonfluorescent reagents with high absorption in the UV-Vis spectrum and (ii) fluorogenic reagents, molecules with highly fluorescent groups *per se* or capable of reacting with the target analytes to form conjugated fluorescent molecules. For example, Pena et al. reported a fluorometric method for the quantification and identification of anhydrotetracycline, epitetracycline, tetracycline, and 4-epianhydrotetracycline in salmon muscle using optimized solid phase extraction to improve recoveries and reproducibility and magnesium acetate in borate buffer at pH 9 to limit secondary adsorbent interactions [58]. Other authors have compared different types of metal chelators (Mg^{2+} , Al^{3+} , and Zr^{4+} , among others) as derivatization agents during chromatographic separations of chlortetracycline, tetracycline, doxycycline, and oxytetracycline [59]. The same authors recommend maintaining a pH of 9.0 during the derivatization to prevent precipitation in the HPLC tubing and tetracycline deprotonation to favor metal-TC interaction [59]. For the quantification of tetracyclines in egg yolk, egg white, and hen plasma, Zurhelle et al. [60] quantified isochlortetracycline (ICTC) and ICTC-derived compounds by fluorescence after adjustment of the HPLC eluate to pH 12.0. Pena et al. [69] also reported an analytical methodology for the determination of TC and OTC in honey by fluorescence detection in the presence of magnesium ions. Several buffers and pH conditions have been used to improve the fluorescence of tetracyclines chelates. For example, Spisso et al. [70] achieved more strong signals for ETC, OTC, TC, CTC, and DC using *N,N*-dimethylformamide (DMF) instead of an aqueous boric acid buffer. In this regard, the high solubility of magnesium acetate tetrahydrate in DMF increased the range of concentrations that can be detected without risk of precipitation in the mobile phase. Also, the interaction between TCs and magnesium ions is guaranteed by the

alkaline nature of magnesium acetate. In a similar manner, Granados-Chinchilla et al. [71] developed a green chemistry method for extraction, chromatography, and derivatization of first-, second-, and third-generation tetracyclines and epimers from feed samples. In this method, DMSO was used in conjunction with magnesium acetate to avoid precipitation of the antibiotic derivatives in the HPLC tubing. From the data collected herein, it is concluded that magnesium salts are superlative chelators for tetracycline detection and as such are widely used for this purpose. Interestingly, Abbasi et al. [72] reported a fluorescence-based method to detect tetracyclines in milk using shorter wavelengths without any reference of derivatization. The mechanism that the authors are exploiting is unclear; however, native tetracycline fluorescence is very low. Method fitness parameters of the assays described in the preceding subsections can be found in Table 5.

(3) *Mass Spectrometry.* In residue analysis of food contaminants, LC-MS has been used for screening, preparative, and confirmation purposes. Hence, qualitative (identification of the antibiotic) and quantitative (concentration of the contaminant) data can be obtained by using this technique. Given that LC-MS techniques can provide information about the chemical structure of the analytes, they have been used as confirmatory methods of antibacterial residues in food [73, 74]. Perhaps the more complete LC-MS method for antibiotic analysis in feed established to date was published by Boix et al. [75] who analyzed 116 antimicrobials simultaneously. More recently, Sun et al. [76] performed a rapid screening of 73 analytes in animal feeds using FT-Orbitrap coupled to a UPLC using a chromatographic run of just a few minutes; oxytetracycline, chlortetracycline, metacycline, doxycycline, and tetracycline were included as part of the tetracycline class of antibiotics. Comparable LC-MS methods include those developed by Gavilán et al. [77], Borràs et al. [78], and Gómez-Pérez et al. [79], including 50 antimicrobials from 13 different families or over 300 analytes including antimicrobials and pesticides using Orbitrap high-resolution mass spectrometry, respectively [75, 76]. Xu et al. [47] proposed a method that included the detection of tetracycline, oxytetracycline, chlortetracycline, metacycline, and doxycycline in milk and honey using UHPLC-Q-TOF/MS. As mass spectrometry becomes more and more available to laboratories, several research groups have exploited its capabilities and, as a result, other simultaneous residue methods have become available [80–82].

Coupled mass spectrometry in tandem using triple quadrupole (LC-QqQ-MS) in the multiple reaction monitoring (MRM) mode is currently the topmost analytical methodology for simultaneous, unambiguous identification and quantification of antibacterial residues in foodstuffs [83]. Although it is a very sensitive and selective technique, there is a technical limit to the number of target compounds that can be scrutinized by MRM-type experiments, hindering the utility of this approach. So, for multiresidue determination, there is an emerging trend to the use of certain mass full-scan MS techniques (e.g., time of flight mass spectrometry, TOF-MS) that allow simultaneous determination of hundreds of different compounds in complex matrices [84], resulting in

TABLE 5: Performance and conditions of several chromatographic methods developed for tetracycline analysis.

Sample clean-up	Summary chromatographic conditions	Column	Wavelength(s) (nm)	Limit of detection	Reproducibility (RSD, %)	Recovery (%)	Detection system	Reference
<i>Spectrophotometry</i>								
Liquid-liquid extraction/ $\text{CH}_3\text{CN}/\text{CH}_2\text{Cl}_2$ petroleum ether	$0.02 \text{ mol L}^{-1} \text{ H}_3\text{PO}_4/0.01 \text{ mol L}^{-1}$ sodium 1-decanesulphonate and CH_3CN	PLRP-S, $5 \mu\text{m}$, $150 \times 4.6 \text{ mm}$	355	2.2– 28.6 ng g^{-1}	<15.5	54–88	Ion pair-diode array detector (DAD)	[50]
SPE/Strata-X® ($33 \mu\text{m}$, 100 mg, 6 mL)	CH_3CN , oxalic acid, CH_3OH	C_{18} , $3 \mu\text{m}$, $250 \times 4.6 \text{ mm}$	355	9.8– $27.2 \mu\text{g kg}^{-1}$	<12.6	55.4–86.3	UV	[51]
SPE/Oasis® HLB (100 mg, 3 mL)	CH_3CN , oxalic acid, CH_3OH	C_{18} , $5 \mu\text{m}$, $250 \times 4.6 \text{ mm}$	350/360/370	1.64– $4.08 \mu\text{g L}^{-1}$	<9.6	83.5–108.6	DAD	[52]
SPE/Oasis HLB (100 mg, 3 mL)/Bond Elut® (500 mg, 3 mL)	Trifluoroacetic acid (TFA), CH_3CN , CH_3OH	XDB C_{18} , $150 \times 4.6 \text{ mm}$, $5 \mu\text{m}$	330	3.5–6.5 $\mu\text{g L}^{-1}$	<14.4	82.1–114.7	DAD	[53]
<i>Fluorescence</i>								
SPE/Oasis HLB (60 and 200 mg)	CH_3CN and oxalic acid 0.01 M (pH 2.0).	C_8 ($100 \times 3 \text{ mm}$, $5 \mu\text{m}$)	$\lambda_{\text{ex}} 385/\lambda_{\text{em}} 500$	$50 \mu\text{g kg}^{-1}$	<7.2	85.0–92.8	Fluorescence detector (FLD)	[54]
SPE/Oasis HLB (30 mg)	oxalic acid, CH_3CN	PLRP-S $5 \mu\text{m}$, $150 \times 4.6 \text{ mm}$ Supersphere	$\lambda_{\text{ex}} 374/\lambda_{\text{em}} 499 \text{ nm}$	0.6–1.2 $\mu\text{g L}^{-1}$	<9.0	84.0–110.0	FLD	[55]
Trace enrichment cartridge	CH_3CN and oxalic acid	RP-8 $250 \times 4.0 \text{ mm}$, $3 \mu\text{m}$ Nucleosil C_{18}	$\lambda_{\text{ex}} 350/\lambda_{\text{em}} 420 \text{ nm}$	11–15 $\mu\text{g kg}^{-1}$	<5.8	90.0–97.0	UV/FLD/MS	[56]
SPE/Oasis HLB (200 mg)	CH_3CN and oxalic acid	Nucleosil C_{18} column ($5 \mu\text{m}$, $250 \times 4 \text{ mm}$)	$\lambda_{\text{ex}} 385/\lambda_{\text{em}} 500$	$50 \mu\text{g kg}^{-1}$	<8.0	86.0–95.0	FLD	[57]
SPE/Oasis HLB (60 mg)/Sep-Pak® C_{18} (500 mg)/Bond Elut Certify®	CH_3CN and 0.01 mol L^{-1} oxalic acid	Symmetry Shield™ RP $_8$, $150 \times 4.6 \text{ mm}$	$\lambda_{\text{ex}} 385/\lambda_{\text{em}} 500$	5.1–34.7	<12.7	61.0–115.0	FLD	[58]
SPE/Oasis HLB (30 mg)	20 mmol L^{-1} TFA, CH_3CN	Zorbax SB C_{18} column ($3 \mu\text{m}$, $250 \times 4.6 \text{ mm}$)	$\lambda_{\text{ex}} 399/\lambda_{\text{em}} 473$	0.2– $500 \mu\text{g kg}^{-1}$	<5.0	59.0–97.0%	FLD/UV	[59]
SPE/Oasis HLB (60 mg)	$\text{CH}_3\text{OH}/\text{CH}_3\text{CN}/50 \text{ mmol L}^{-1}$ oxalic acid	Luna $5 \mu\text{m}$ C_{18} , $250 \times 4.6 \text{ mm}$	$\lambda_{\text{ex}} 255/\lambda_{\text{em}} 365$	2.2 ng g^{-1}	<5.0	75.3–82.5	FLD	[60]

the gathering of more data per sample. These methods are particularly attractive because they allow for identification of unknown species during routine analysis and permit the quantification of species even when a complete chromatographic separation is not possible and multiple residue analysis of species that do not share structural relationship is possible. During feedstuff analysis, these features are useful especially when studying antibacterial residues in the nonmedicated feed emerging due to cross contamination of medicated and nonmedicated feed (i.e., carry-over) [85, 86]. Though MS equipment has improved and is becoming more available, this technology is not without drawbacks [73, 86, 87]. Indeed, difficulties such as analyte suppression, the formation of solid particles, coeluent competition for applied energy, and the formation of undesired ions, to name a few, are unique to this type of detection. Moreover, these methods, in particular those using electrospray (ESI) or atmospheric pressure chemical ionization (APCI) sources, are highly susceptible to matrix interferences [88] such as those arising through enhancement or suppression of the analyte signal by coextracted and coeluted matrix compounds. Calibration with matrix-matched standards or isotopic labeled internal standards can be applied to overcome matrix effects, yet they are expensive and not always commercially available. Alternatively, Cappiello et al. [73, 86, 87] have recommended using a LC-MS interface based on direct electron ionization, as a universal detector, for small molecules such as veterinary compounds. So far, most favorable reports have used positive electrospray ionization (ESI⁺) for detection of TCs [80, 89, 90].

(4) Micellar Chromatographic Methods. The narrow structural relatedness of the tetracyclines challenges the achievement of satisfactory results in parameters such as retention factor (k), selectivity (α), efficiency, tailing factor, and resolution. To tackle this issue, minimizing sample pretreatment steps, Caballero et al. [91] used the surfactant dodecyl sodium sulfate as the mobile phase. The authors obtained good signal resolutions ($R_s > 1$) and average recoveries (79–95%) even when epimers are included and demonstrated that other antibiotics and conventional feed ingredients do not interfere with the analysis conveying high specificity. Patyra et al. [92] also developed an HPLC-DAD method that involves the use of 1-butanol, dodecyl sodium sulfate, and citric acid during the chromatographic separation step and applied this method to analyze tetracyclines in poultry and swine feeds with excellent recoveries (80.4 to 100.2%). This approach may be suited to be coupled with other detectors (e.g., FLD) to improve selectivity and sensitivity or to circumvent matrix issues in screening (nonchromatographic) methods.

3.2.2. Capillary Electrophoresis. Compared to HPLC, capillary electrophoresis (CE) uses smaller quantities of organic solvents and increases separation efficiencies. However, CE is not applied in routine feedstuff analysis on account of the small sample injection volumes and therefore low sensitivity inherent to this method. In spite of this limitation, Hsiao et al. [93] reported a CE-based methodology for the qualitative and quantitative determination of tetracycline in agriculture

formulated products. Furthermore, Miranda et al. [94] implemented a CE method with UV detection for the detection of tetracycline, oxytetracycline, and doxycycline in poultry muscle. These authors have used two different approaches for sample clean-up with similar results, namely, a reverse phase cartridge and an ion exchange resin. Analytes were distinguished at 360 nm in less than 12 min with limits of detection ranging from 61 $\mu\text{g kg}^{-1}$ to 89 $\mu\text{g kg}^{-1}$.

3.2.3. Microbial Methods. Usually, these types of assays consist in the inhibition or growth of a strain sensitive to a particular antimicrobial. These kinds of methods are usually cost-effective, easy to apply, and fast. Nevertheless, positive samples require chromatographic analyses for confirmation and quantification, and they are only applicable if a single antibiotic is present in the sample because they cannot discriminate between antibiotics of the same class.

Pikkemaat et al. [95] compared the performance of the Nouws antibiotic test (NAT) and two other microbial screening methods, STAR (screening test for antibiotic residues) and Premi®Test, on routine monitoring. Analysis of 591 muscle tissue samples from slaughter animals [porcine (423), veal (143), bovine (18), sheep (5), and horse and goat] yielded four MRL violations. Three of them concerned tetracyclines that were only detected with the NAT and the STAR method.

The NAT test comprises four test plates to enable antibiotic group-specific identification, for example, a *Bacillus cereus* plate specific for tetracyclines residues. Plates were individually optimized on the microorganism, agar medium, pH, incubation temperature, and synergistic components to provide optimal sensitivity on the MRLs in the kidney [95]. On the other hand, in the STAR protocol, each plate was favorably sensitive for one or two families of antibacterials (specifically, the plate *Bacillus cereus* for tetracyclines). The method was initially validated for use in milk and muscle. Finally, R-Biopharm's PremiTest 25 is based on the inhibition of the growth of *Bacillus stearothermophilus* and an acid-base indicator which veers when the antimicrobial is present. This protocol applies to meat (beef, pork, and poultry), fish, shrimps, eggs, liver, kidney, urine, and feed for the residues of β -lactams, cephalosporins, macrolides, tetracyclines, sulphonamides, aminoglycosides, quinolones, amphenicols, and polypeptides. However, in a 2014 note, Gondová et al. [96] using 142 slaughterhouse tissue samples stated that the STAR method and Total Antibiotics test (a EuroClone S.p.A. kit containing test tubes with *Bacillus stearothermophilus* var. *calidolactis*) yielded better results identifying positive samples against PremiTest and NAT tests. In this particular case, 39 samples produced a positive result in one or more tests (i.e., 4 samples in four tests, 14 samples in three tests, 13 samples in two tests, and eight samples in one test). The tests' predicting capabilities in descending order were as follows: STAR, Total Antibiotics, PremiTest, and NAT.

As to the inhibition methods, *Bacillus megaterium* and *Bacillus cereus* have been used to detect $\mu\text{g L}^{-1}$ of tetracyclines in milk after short incubation periods [97, 98]. By

using six plates seeded with different bacteria under specific conditions (i.e., *Bacillus subtilis* at pH 8.0 [aminoglycosides], *Geobacillus stearothermophilus* var. *calidolactis* [beta-lactams], *Kocuria rhizophila* [macrolides], *B. subtilis* at pH 7.0 [sulphonamides], *B. cereus* [tetracyclines], and *Escherichia coli* [quinolones]), Althaus et al. [99] developed a microbiological multiresidue system for detection of the aforementioned antibiotics in ewe milk. Moreover, Hargrave et al. [100] developed a microdilution technique method for detecting oxytetracycline-resistant bacteria in salmon feed pellets and marine sediments. A common setback of microbiological methods based on inhibition is the discrimination of false negative results. To circumvent this issue, Raspor Lainšček et al. [98] included citric acid in an assay of tetracyclines in raw milk to prevent TC binding to metallic cations.

3.2.4. Immunological Methods. R-Biopharm, Bioo Scientific, and other manufacturers, such as Europroxima, Randox, Abraxis, and Cusabio®, to cite a few, have developed immunological assays for the screening of tetracyclines. All of these methods use monoclonal antibodies unique to tetracycline in a microtiter plate format, most often based on a biotin-avidin ELISA reaction. Although most antibodies cannot distinguish structurally similar compounds, some kits are capable of detecting as little as $\mu\text{g kg}^{-1}$ of the analytes in several matrices. These tests are sensitive enough, cheap, and fast, though they sometimes lack specificity [101]. This latter feature prevents their application in screening campaigns, as false negative results, compared to false positives, will not be submitted to confirmatory analyses. Cháfer-Pericás et al. [102] performed a comparison between the performance in LC-MS/MS and that in immunoassays available at the time applying both techniques in feed and fish samples. The authors found a good correlation in the tetracycline concentrations among the assays revised; some samples were found to be positive for the antibiotic, although no residues were found during LC-MS-MS test. Interestingly, the authors found lower detection limits (ca. ten times) in the immunoassays than in the MS analysis. Finally, recently, Wongtanprasert et al. [103] developed a new monoclonal antibody against oxytetracycline and applied it for OTC quantification in shrimp using an indirect competitive ELISA with recoveries of 82 to 118%. The antibody showed high cross-reactivity to rolitetracycline but no cross-reactivity to other unrelated antibiotics. Interestingly, though ELISA and screening assays based on antibodies have been developed for tetracyclines, no immunoaffinity column has been designed for this antibiotic family.

3.2.5. Whole Cell Biosensors. A biosensor can be described as a measurement system in which a biological constituent (i.e., a whole cell) is used as the recognition component. Whole cell biosensors provide information about the effect of a stimulus on a living system in contrast to traditional analytical measurement systems in which the quantity of a given substance is determined. Application-wise, some biosensors have found widespread acceptance in food research. Mungroo and Neethirajan [104] wrote a review of the main working

principles and mechanisms of biosensors finding usage in the poultry industry. Hansen and Sørensen [105] quantified tetracycline in milk with nonselective *E. coli* biosensors that included plasmid-encoded fusions of the tetracycline-inducible promoter P_{tet} and the regulatory gene *tetR* with different reporter gene systems (*lacZ*YA, *luxCDABE*, or *gfp*). Hence, these bacterial strains responded to low levels of tetracyclines by producing β -galactosidase and light or green fluorescent protein [105]. A similar approach was introduced to a novel methodology in 2007 by Moeller et al. [106] who constructed a biosensor using the tetracycline resistance gene *tetO* and TetR protein as a regulator. Bahl et al. [107] created an extended range whole cell tetracycline biosensor by inserting *tetM*, encoding a ribosomal protection protein, into a plasmid that contains a transcriptional fusion between a tetracycline-regulated promoter and the *gfp* gene. Korpela et al. [108] also developed a bioluminescent biosensor for the specific detection of tetracyclines *in vitro*; tetracycline, metacycline, oxytetracycline, chlortetracycline, doxycycline, demeclocycline, and minocycline efficiently induced the biosensor. In this case, the sensor plasmid contained five genes from the bacterial luciferase operon of *Photobacterium luminescens* [108]. A similar approach was followed by Pellinen et al. [109] to quantitate oxytetracycline in rainbow trout tissue. Using the same sensor, Virolainen et al. [110] analyzed doxycycline, tetracycline, chlortetracycline, and oxytetracycline in poultry meat and through sensitization of the sensor with chelating agents EDTA and polymyxin B reached sensitivities as low as five ng g^{-1} . These researchers also showed that tetracycline 4-epimers were also capable of inducing luminescence and possessed antibacterial capacity [110]. These compounds were previously thought to be non-active. Later on, Pikkemaat et al. [111] used a *lux* biosensor to determine tetracyclines in poultry muscle. This cell biosensor assay was far more accurate in testing suspect samples compared to traditional inhibition tests [111]. A review of the mechanisms involved in the construction applications of these biosensor constructs based on this technique was written by Guo [112], and Reder-Christ and Bendas [113] wrote a review regarding applications of biosensors in antibiotic research field.

Recent papers have demonstrated that, among the various tetracycline species, including both metal-complexed and unbound variants, the zwitterionic tetracycline species are the ones that most readily pass through cell membranes to elicit activation of resistance genes. Hence, TC speciation should be taken into consideration in biological drug analyses [114]. Similarly, Guerra et al. [115] have explored data regarding the ability of the tetracyclines to chelate metals and how this impacts their biological activity. Zhang et al. [116] demonstrated that organic acids in water samples enhance tetracycline uptake and bioavailability. Chen et al. [117] using the same reporter also showed that adsorption of humic acid derived dissolved organic matter by bacterial cell surfaces inhibited tetracycline diffusion into the bioreporter cells. Freely dissolved tetracycline fraction was responsible for the rate and magnitude of antibiotic resistance genes expressed [117]. Likewise, our research has demonstrated that in-feed crude protein and calcium concentrations have a dramatic

effect on the bioavailability of 14 different tetracyclines in shrimp, swine, fish, and poultry compound feed [118]. Using two different biosensors, we demonstrated that the bioavailable tetracycline fraction is inversely related to the protein and calcium content of the feed. This data is in line with a previous report regarding binding constants of oxytetracycline to animal feed divalent cations [119].

3.2.6. Novel Approaches

(1) *Plasmon Resonance*. Some authors have developed methods for detection of tetracyclines based on the unique optical properties exhibited by metallic nanoparticles (e.g., silver or gold). Amjadi et al. [120] developed a photometric analysis for tetracycline based on the reduction of AgNO_3 in alkaline medium. The nanoparticles obtained were identified by surface plasmon resonance absorption (411 nm) and transmission electron microscopy imaging. The method reached a detection limit of 0.013 mg L^{-1} for pharmaceutical products [120]. Back in 2010, Andree et al. [121] used the same optical properties to produce a surface biosensor. In this case, an indirect competitive method using the recombinant regulatory TetR protein and a sensor and a sensor chip was developed. Detection was performed using fluorescence (λ_{ex} 295 and λ_{em} 337 nm). This approach has several advantages, as nearly all molecules can be analyzed by choosing a suitable binding partner and exhibit high specificity due to the use of tetracycline-specific receptor protein. Using conjunct technologies, Verma and Gupta [122] developed an optical fiber sensor for tetracycline using surface plasmon resonance of silver metal and molecular imprinting using oxytetracycline and tetracycline as scaffolds. This sensor enjoys several advantageous features such as low cost, ease of handling, a miniaturized probe, fast response, high selectivity, reusability, and the possibility of online monitoring and remote sensing [122].

(2) *Quantum Dots (QDs)*. QDs have recently become an important aid in the quantification of feed additives, residues, and contaminants due to their refined sensibility and specificity. For example, Imani-Nabiyyi et al. [123] used Cd/Te QDs capped with luminol/L-cysteine and a periodate system to determine several tetracyclines in water samples, pharmaceutical formulations, and honey. Similarly, García-Fernández et al. [124] screened for traces of these antibiotics in muscle tissue using an immunoprobe based on OTC-bovine serum albumin-QD. In this case, a binary response was achieved facilitating assay interpretation and used previously to a confirmatory technique. Recently, Li et al. [125] recently described a novel photosensor for OTC biosensing based on a signal “switch off-on” strategy, conjugating a hairpin DNA probe and Cd/Te QDs immobilized on the ITO/ TiO_2 electrode by sulfur bonds.

4. Antibiotic Resistance and Antibiotic-Resistant Bacteria in Feedstuff

Through beneficial responses such as improved animal growth and feed efficiency, feed-additive antibiotic usage

has contributed over 50 years to the intensification and expansion of the animal production industry [8]. However, this extensive use of low-level antibiotics has irremediably exerted a selective pressure on animal and farm bacteria and thereby aided the development of antibiotic-resistant bacteria (ARB) with a potential to compromise animal and human health [126]. Historical examples of this cause-effect relationship include the following: the application of avoparcin in poultry and swine production and the emergence of glycopeptide-resistant enterococci [127], the use of virginiamycin in the same species and the development of quinupristin-dalfopristin resistance [128], and the usage of tylosin with the concomitant dissemination of erythromycin-resistant bacteria in Scandinavian countries [129], to cite just a few examples.

To ensure the innocuity of animal feed, ingredients and finished products should be maintained free from pests and chemical or microbiological contaminants during production, handling, storage, and transport [130]. Nonetheless, this microbial vigilance is often restricted to pathogens and hence disregards the hazard linked to the colonization of medicated feed with ARB which may persist in livestock, transfer their resistance traits to the pathogens that farmers intend to control [130], and reach consumers [131]. Furthermore, the antibiotic resistance genes (ARG) and mobile DNA molecules that have emerged as a consequence of the use, misuse, and abuse of antibiotics should be regarded as xenogenetic pollutants that replicate rather than degrade when released to the environment [132].

As regards the load of animal feed with ARB and the identity and level of resistance of these ARB, Kerry et al. [133] detected 10^3 – 10^4 CFU of oxytetracycline-resistant bacteria g^{-1} in 8 out of 16 commercial fish feed samples. The resistance frequencies in these samples ranged from 7 to 65%. Depaola [134] found a 100% resistance in bacteria (governed mostly by *Enterobacter agglomerans*) in catfish feed samples. Miranda and Zemelman [135] found multidrug-resistant bacterial strains, mainly from the genus *Acinetobacter*, in pelletized feed for salmon marketed in Chile. In a similar study, the same authors found that 0.39–50% of the 10^4 – 10^6 bacteria colonizing Chilean salmon feed were resistant to $30 \mu\text{g mL}^{-1}$ of oxytetracycline [136]. Later on, Miranda and Rojas [137] detected 10^3 florfenicol-resistant bacteria g^{-1} in pelletized feed for salmon in one of two farms. Martins da Costa et al. [138] investigated 1137 enterococci and 163 *Escherichia coli* strains recovered from 23 samples of commercial broiler feed and 66 samples that were derived from raw feed ingredients. Among the enterococci recovered from feed ingredients, resistance to rifampicin, erythromycin, nitrofurantoin, tetracycline, and ciprofloxacin was found in ca. 60%, 22%, 21%, 18%, and 7% of the isolates, respectively. A considerable proportion of the enterococci isolates obtained from broiler feed displayed resistance to tetracycline (69%) [138]. Yang et al. [139] found around 10^6 CFU mL^{-1} and 10^8 CFU mL^{-1} of bacteria resistant to $8 \mu\text{g mL}^{-1}$ of tetracycline or $4 \mu\text{g mL}^{-1}$ of ceftiofur, respectively, in unused feed, collected in Colorado farms. Two of the 91 feed samples analyzed in this work gave positive signals for class I integrons, which are markers of

anthropogenic pollution [140] and often include ARG [141]. Also in the USA, Graham et al. [142] isolated multidrug resistance enterococci and staphylococci from flies caught near confined poultry feeding operations. In surveillance of *Salmonella* contamination in 2 058 samples of animal feeds, feed ingredients, pet foods, pet treats, and supplements for pets, Li et al. [143] recovered 257 bacterial isolates, 54 of which were resistant to at least one antimicrobial agent (21%). The largest proportion of isolates was resistant to tetracycline and sulfisoxazole [143]. Though in lower numbers, Molina et al. [144] also found tetracycline-resistant *Salmonella* in animal feed samples collected in Costa Rica. In another study from Costa Rica, Granados-Chinchilla et al. [145] observed between 10^1 and 10^6 CFU of OTC-resistant Gram-positive bacteria classified as *Staphylococcus* and *Bacillus* g^{-1} . These strains were recovered from samples of tilapia, poultry, and swine feed and were characterized by $MIC_{50} > 192 \mu g mL^{-1}$. These authors noted a clear correlation between OTC dosage and colonization with OTC-resistant bacteria in medicated feed for fish. Nonetheless, some unmedicated feed for fish, swine, and poultry contained considerable populations of OTC-resistant bacteria, suggesting that raw materials and manufacturing processes may also impact the carriage of OTC-resistant bacteria in animal feed [145]. Carballo et al. [146] recovered a total of 63 isolates from manure samples collected from Spanish cattle farms; in this study *Escherichia coli* and *Comamonas testosteroni* accounted for 25% and 19.6% of the species recovered, respectively. The most common antimicrobial resistance documented in Gram-negative bacteria was toward tetracycline (67%).

On the other hand, only a few studies have addressed the occurrence or quantification of ARG in animal feed or feed ingredients. In this regard, Lu et al. [147] found abundant bacterial DNA in animal feed-grade avoparcin. This DNA included a cluster of *van*-like genes for glycopeptide resistance closely related to that of *Amycolatopsis orientalis* and *Streptomyces toyocaensis* and a 16S rDNA sequence from the avoparcin-producer *Amycolatopsis coloradensis*. Hence, they demonstrated that antibiotic preparations might act as delivery systems for their cognate ARG. Just et al. [148] found bacitracin-resistance, erythromycin-resistance, and tetracycline resistance genes in bioaerosols from poultry operations, particularly in floor-housed farms. Also, genes associated with erythromycin and quinupristin-dalfopristin resistance, including *ermB*, *ermA*, *msrC*, and *msrA/B*, as well as mobile genetic elements related to the conjugative transposon *Tn916*, have been found in bacterial isolates recovered from flies collected at poultry feeding operations [142]. More recently, McEachran et al. [149] demonstrated that wind-dispersed particulate matter from feed yards harbors tetracycline-resistant bacteria and up to 4 000 times more copies of six different tetracycline resistance genes than particulate matter collected upwind of these feed yards.

Altogether, these investigations demonstrate that animal feed delivers antibiotics along with antibiotic-resistant bacteria with mobile resistance genes to animals and farms, jeopardizing the use of these drugs in the treatment of bacterial diseases and the health of the ecosystem.

5. Health and Environmental Risks Associated with the Use of Tetracyclines in Animal Nutrition

In recent years, the occurrence and fate of antibiotics in the environment, including surface water, groundwater, and soil, have drawn increased attention because of the serious environmental challenges that antibiotics may pose in environmental compartments.

5.1. Tetracycline Fate and Degradation. The acid-base chemistry of the tetracyclines is rather complex and impacts their behavior in environmental matrices severely. Different pH conditions affect the ionizable groups present in the molecules of tetracycline to various degrees (e.g., carboxylic acids, enols, and amines). For instance, 4-epimers such as 4-epitetracycline (4-TET), 4-epioxytetracycline (4-EOTC), and 4-epichlortetracycline (4-ECTC) can be reversibly formed under mildly acidic conditions (pH 2–6). Strong acidic conditions (pH < 2), in turn, facilitate the formation of anhydrotetracyclines that could transform to their corresponding epimers. Xuan et al. [150] have even described degradation products for anhydroxytetracycline, which is a molecule considered to be relatively stable. Other conditions that influence the environmental behavior of the tetracyclines are exposure to luminous radiation and binding to divalent cations. Hydrolysis of oxytetracycline in aqueous media generates 4-epitetracycline and α/β -apotetracycline by photolysis with relative ease (first-order kinetics with a degradation constant of $3.61 \pm 0.06 \text{ day}^{-1}$) and chelation of this antibiotic with Ca^{2+} accelerates its degradation [150]. This behavior can be expected from other members of this class of antibiotics.

It is important to note that some tetracycline degradation products exhibit biological activity. For instance, Hsieh et al. [151] demonstrated that the degradation products generated by heat treatment of certain tetracyclines increase the minimum inhibitory concentration (2–1 024 times) and the number of mutagenic revertants (2–6 times) of *Salmonella*. Although degradation products of tetracyclines are not conceived as active as their precursors, several products have exhibited equal or higher activities on sludge and soil bacteria than their parent compound at comparable concentrations [152]. A singularity of tetracyclines is that they can, under some conditions, revert from degradation products to their parent compounds [153].

The quinolones, tetracyclines, ivermectin, and furazolidone are usually considered antibiotics of rapid degradation with half-life initially reported from <1 h to 22 days. However, the behavior of tetracycline in relevant environmental matrices seems to be variable. For example, Wang et al. [154] calculated a half-life of 16.12 h for oxytetracycline in a *Penaeus chinensis* farm. Halling-Sørensen et al. [155] found that the average degradation half-life of CTC varied from 25 to 34 days in two Danish soils, and Samuelsen et al. [156] reported no degradation for OTC in marine sediment after six months of incubation. The tetracyclines may persist in soil for 10 to 180 days with a 0–50% degradation [157]. Residues of

tetracyclines ranging from $\mu\text{g kg}^{-1}$ to g kg^{-1} have been found in animal tissues and waste products, including manure [158–161] to cite a few. Residual concentrations of tetracyclines from animal wastes have been reported to range from 11 to $880 \mu\text{g kg}^{-1}$ [162]. Research published by Li et al. [163] evaluated the residues of several veterinary antibiotics in animal feces and found that tetracyclines were the predominant antibiotic with a maximum level of 56.81 mg kg^{-1} , mostly detected in pig feces.

Several researchers have proven that composting is an efficient approach for the removal of antibiotics in animal manure. For example, Arikian et al. [164] demonstrated that, within the first six days of bovine manure composting, levels of OTC in the mixture achieved a 95% reduction. Another research team [165] demonstrated that the concentration of CTC during manure composting experienced a 99% reduction in less than ten days. Wu et al. [166] examined the degradation products of TET, CTC, and OTC during a pilot scale swine manure composting. Decomposition rates for the parent compounds followed first-order kinetics and removal was calculated to be 74%, 92%, and 70%, with calculated half-lives of 8.2, 1.1, and 10.0 days, respectively [166]. In these experiments, these authors reported the emergence of tetracycline degradation products such as 4-ETC, 4-EOTC, 4-ECTC, DEM, and ATC [166].

The arguments developed above, along with the fact that TCs find extensive usage in veterinary medicine and for growth promotion and the high hazard quotients calculated for TCs in hazard assessments [167], strongly support the notion that TC represents an increasing risk to human health and ecosystem safety [168, 169].

5.2. Undesired Effects on Bacterial Communities. The use of antibiotics in animal feed as growth promoters appears to boost the emergence of antibiotic-resistant strains. An early report by Jacobs and Chenia [170] showed 78% of TC resistance in species of *Aeromonas* associated with South African tilapia, trout, and koi aquaculture systems. Single and multiple Tet determinants were observed in 27% and 48.7% of isolates, respectively, with *tetA* being the most prevalent *tet* gene [170]. Miranda et al. [171] compiled evidence linking resistance emergence in human pathogens to TC applications in agroecosystems. Furthermore, a great variety of resistance determinants has been found in aquaculture environments, including several resistance mechanisms coded within mobile elements [172]. In this regard, bacterial plasmids isolated from soil bacteria have repeatedly been reported to carry resistance determinants for antimicrobials of different chemical classes, including tetracycline [173, 174]. Forsberg et al. [175] showed that multidrug-resistant bacteria found in soil possess genes that highly resembled those observed among human pathogens. Tetracycline resistance genes have been detected in bacteria associated with fresh produce from the United States [176] and lettuce from Costa Rica [177] and Nigeria [178]. Li et al. [179] characterized bacteria populations in surface water receiving effluent from an oxytetracycline production plant and upstream river. The authors isolated 341 bacterial strains and found 23 tetracycline (*tet*) resistance

genes; >94% of the isolates harbored these genes, with *tetA* being the most common (67.0%), followed by *tetW*, *tetC*, *tetJ*, *tetL*, *tetD*, *tetY*, and *tetK*. Yang et al. [180] examined the presence of antibiotic-resistant commensal bacteria in cattle operations, city locations, and a national park. Compared to water samples from other environments, wastewater samples from the cattle operations were characterized by higher tetracycline-resistant bacteria counts and numbers of copies of tetracycline resistance genes [180]. The tetracycline resistance genes *tetB*, *tetC*, *tetW*, and *tetO* were detected in all types of tested samples, except in soil samples from the national park. Tetracycline resistance gene pools containing *tetO* and *tetW* genes were significantly more extended than pools with *tetB* and *tetC* in fecal and water samples. In another study, Resende et al. [181] found that Gram-positive cocci isolated from water from an aquaculture system exhibited high resistance to tetracycline as well as a high tolerance for heavy metals. Both parameters were positively correlated. Furthermore, Harnisz et al. [182] demonstrated that a fish farm influenced water quality by increasing the diversity of tetracycline resistance genes. Isolates of *Aeromonas* sp. and *Acinetobacter* sp. were able to transfer 6 out of 13 tested *tet* genes into *Escherichia coli*, which can promote the spread of antibiotic resistance in the environment. Out of the 105 bacterial isolates, 85 (81%) and 20 (19%) were Gram-negative or Gram-positive, respectively. In line with these results, a 2015 Nigerian study [183] analyzed 105 bacteria isolates from the water. Twenty-nine isolates carried at least one of the targeted tetracycline resistance genes, including strains of *Aeromonas*, *Alcaligenes*, *Bacillus*, *Klebsiella*, *Leucobacter*, *Morganella*, and *Proteus*; *tetA* was the most common gene (16/29) followed by *tetE* (4/29) and *tet30* (2/29). Furthermore, a recent study made by Huang et al. [184] encountered, during the screening of tetracycline resistance genes, a new determinant designated as *tet47* in *Providencia* spp. from fish intestines. Hence, fish and byproducts were shown to possibly disseminate resistance determinants through food, feed, and environmental contacts. Considering the added mobility that water provides, bacteria from water systems carrying such resistance genes exhibit a higher probability of dissemination. Further research has demonstrated as well the impact of productive activities on environmental resistance proliferation and spread [185, 186].

More recently, the role of biofilm formation and resistance in environmental niches has been studied in depth, and several studies have found evidence linking tetracycline resistance determinants with said biofilms. For example, Engemann et al. [187, 188] found that the abundance of six genes conferring resistance to tetracycline was reduced at different rates in the water column (from a cattle feedlot lagoon), and some genes, particularly *tetW*, readily migrated into biofilms. The evidence was considered by the authors to be one of the contributing factors explaining reduction rates of *tet* genes from a planktonic compartment [187, 188]. Furthermore, the first-order loss coefficients (k_d) for the sum of the resistance genes were calculated and, at the same time, demonstrated that gene disappearance rates were always highest when the light was present, irrespective of OTC level [187, 188]. As a hint toward a practical approach,

the authors suggest that maximizing radiation over receiving waters could accelerate resistance gene loss rate after lagoon water is released. Zhang et al. [189] observed that, in a swine waste lagoon, bacteria with *tet* genes swiftly migrate into biofilms, where they can persist longer than in surface waters. Borjesson et al. [190] found a high proportion of genes encoding resistance to aminoglycosides and tetracyclines in biofilm samples collected at a wastewater treatment plant. Finally, Salcedo et al. [191] described in *E. coli* and *P. aeruginosa* biofilms that subinhibitory concentrations of tetracycline and cephradine induced biofilm formation, enhancing the transfer rate of the plasmid pB10 among the biofilm biomass faster than without antibiotic treatment. As biofilm formation is a common trait among most bacterial pathogens, we need a better understanding of the sources and mechanisms that contribute to the emergence and spread of antibiotic resistance. A review on this topic was written recently by Balcázar et al. [192].

Moreover, Alali et al. [193] conducted a 3-year study integrating a vertical swine and human agrifood system. The relationship between the prevalence of antibiotic resistance commensal bacteria (*Escherichia coli*) isolated from human wastewater and swine fecal samples was examined. The authors contrasted the resistance resulting from swine workers versus non-swine workers, swine production group, and season [193]. The authors encountered, for example, that the relative odds of encountering oxytetracycline and chlortetracycline resistance among isolates were significantly increased in medicated feed as compared to the control group. The authors found higher odds of resistance in swine workers in contact with swine medicated feed, which commonly contains significant amounts of subtherapeutic chlortetracycline.

In a recent review, Chen and Jiang [194] described microbiological safety issues regarding the use of chicken litter or fertilizers based on this byproduct. In this scenario, integrons with ARG have been found in *E. coli* isolates recovered from poultry compost in which at least 50% of the isolates showed resistance to tetracycline [195]. In this case, all isolates were resistant to at least seven different antibiotics.

On the other hand, normal environmental microbiota may be adversely affected by the antibiotics as a result of the accumulation of rather high concentrations of these substances in the environment secondary to anthropic activities such as agriculture, aquaculture, livestock production, and animal farming. As a result, TC parent compounds, metabolites, and byproducts that emerge during the degradation of these antibiotics have an effect on nontarget bacterial populations. A recent paper by Chessa et al. [196] investigated the effect of the microbial community of tetracycline on clay and sandy soil. They showed that tetracycline caused transient effects on the activity and structure of the microbial life particularly in the short term and at the highest concentrations tested. These authors also concluded that microbial communities with increased metabolic activity characterize soils treated with cow manure. Our research group demonstrated that when a sediment community is exposed to both tetracycline and 4-epitetracycline, not only are TC-tolerant microorganisms selected but also the composition and

function of the community are altered. In this regard, we have also demonstrated that parent compounds and epimers do not exert similar effects on the structure/function of microbial assemblages [197]. More recently, Fernández et al. [198] studied the effect of environmentally significant concentrations of oxytetracycline on sediment sulfate-reducing bacteria from a tropical Tilapia pond. In this study, a single exposure of OTC triggered dramatic functional and structural changes that quickly became evident and persisted for a month. This type of bacteria, which is relevant in geochemical cycles, is seldom examined for the effects exerted by antibiotics. A comprehensive review published by Brandt et al. [199] highlighted the overall impact of antibiotics on bacterial communities and the approaches that can be followed to appraise such impact.

Less research has been devoted to evaluating the direct effects that antibiotics and specifically tetracyclines exert directly on human and animal health. Earlier research by Hamscher et al. [200] demonstrated that 90% of the dust samples collected during two decades from a swine production facility exhibited up to five different antibiotics, including tylosin, and various tetracyclines, sulfamethazine, and chloramphenicol, in total amounts up to 12.5 mg kg^{-1} dust. These authors recognized dust as an additional health hazard and as a new entry route for veterinary drugs in the environment. Other researchers have focused their efforts on similar experiments but with different antibiotic classes, for example, isolating bacteria resistant to tylosin from workers from a swine feeding operation which had been exposed to airborne concentrations [201] or isolation of multidrug-resistant coagulase-negative staphylococci from pharmaceutical workers as a result of occupational exposure [202]. Dysbiosis has been reported as a result of antibiotic exposure for growth promotion purposes [8]; gut microbiota has been linked as a reservoir of the antibiotic resistance gene pool [203]. Sapkota et al. [204] described several feed ingredients and their health impacts toward animals and humans including both nontherapeutic antibiotics and antibiotic-resistant bacteria. At least one paper has focused on evaluating potential treatments to reduce antibiotic-resistant bacteria [205].

Recently, a research group has evaluated directly effects of OTC over livestock wastewater [206]. The authors found that this antibiotic reduced chemical oxygen demand (COD) removal efficiency, CH_4 production, and bacterial diversity in the wastewater [206]. Continuous evaluation of environmental samples (e.g., wastewater and soil) is imperative. A recent study using shotgun metagenomics demonstrated a decrease in diversity in resistome during feedlot (verifying selective pressure) [207]. Furthermore, tetracycline resistant determinants are conserved during the feedlot period (arrival to exit) and though meat processing seems to diminish the possibility of the determinants subsequently passing the food chain upward [207], it does pose an increased environmental burden when exposed to the feedlot resistome [207]. Additionally, Jung et al. [208] made a compelling case that the structure and diversity of an aquaculture bacterial community treated with OTC exhibited the same behavior as a bacterial community treated with red clay instead. The

authors go as far as this to suggest the use of red clay as an attractive substitute to OTC in a particular example of the search for alternatives.

Finally, Chatzisprou et al. [209] stated in a recent review that mitochondria are affected by tetracycline antibiotics. The authors demonstrated that doxycycline disturbs mitochondrial proteostasis and metabolic activity and induces widespread gene expression changes [209]. Furthermore, mitochondrial modulation by doxycycline has been shown to have consequences in cancer research [209].

6. Conclusions, Future Perspectives, and Recommendations

Though some authors have used AAFCO check samples, there is a need for new, certified reference materials (CRMs) and matrix blank materials to improve validation and check the performance of analytical methods for antibacterial residues in foodstuffs. The rate of inclusion of new antibiotics in human medicine is slow at best. Hence, novel antibiotics must be developed, and the rational and justified use of current antibiotics should be advocated. Substitutes to antibiotics have been studied and suggested. However, antibiotic replacement by correct hygiene practices and overcrowding avoidance in farms remains a controversial issue.

Scientific publications still debate over the benefits versus the perils of the extensive use and application of antibiotics as growth promoters. In addition to immediate adverse effects, the long-term effects linked to exposure to low levels of antibacterial residues are still unknown.

Gaps in regulatory matters and research approaches must be tackled to manage and control the emergence of resistance in the environment. We agree with this asseveration and urge compilation of information of qualitative and quantitative nature to facilitate proper decision-making and the correct design of contingency plans, policies, or regulations aiming to ameliorate the illegal use of antibiotics and the burden of antibiotic resistance.

Competing Interests

The authors declare that there are no competing interests.

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