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THE IMPACT OF VETERINARY DRUG RESIDUES ON THE GUT MICROBIOME AND HUMAN HEALTH

A FOOD SAFETY PERSPECTIVE

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
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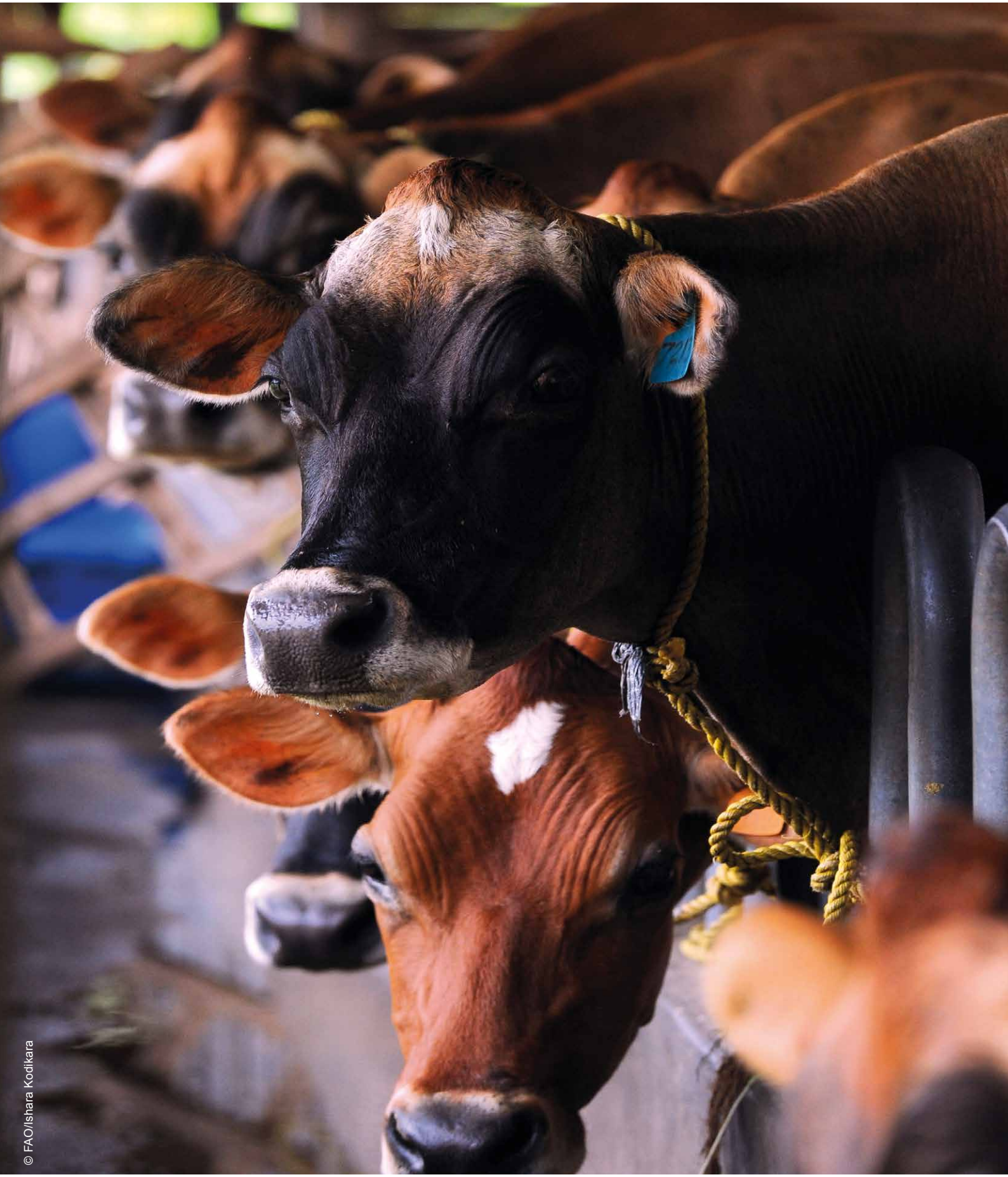
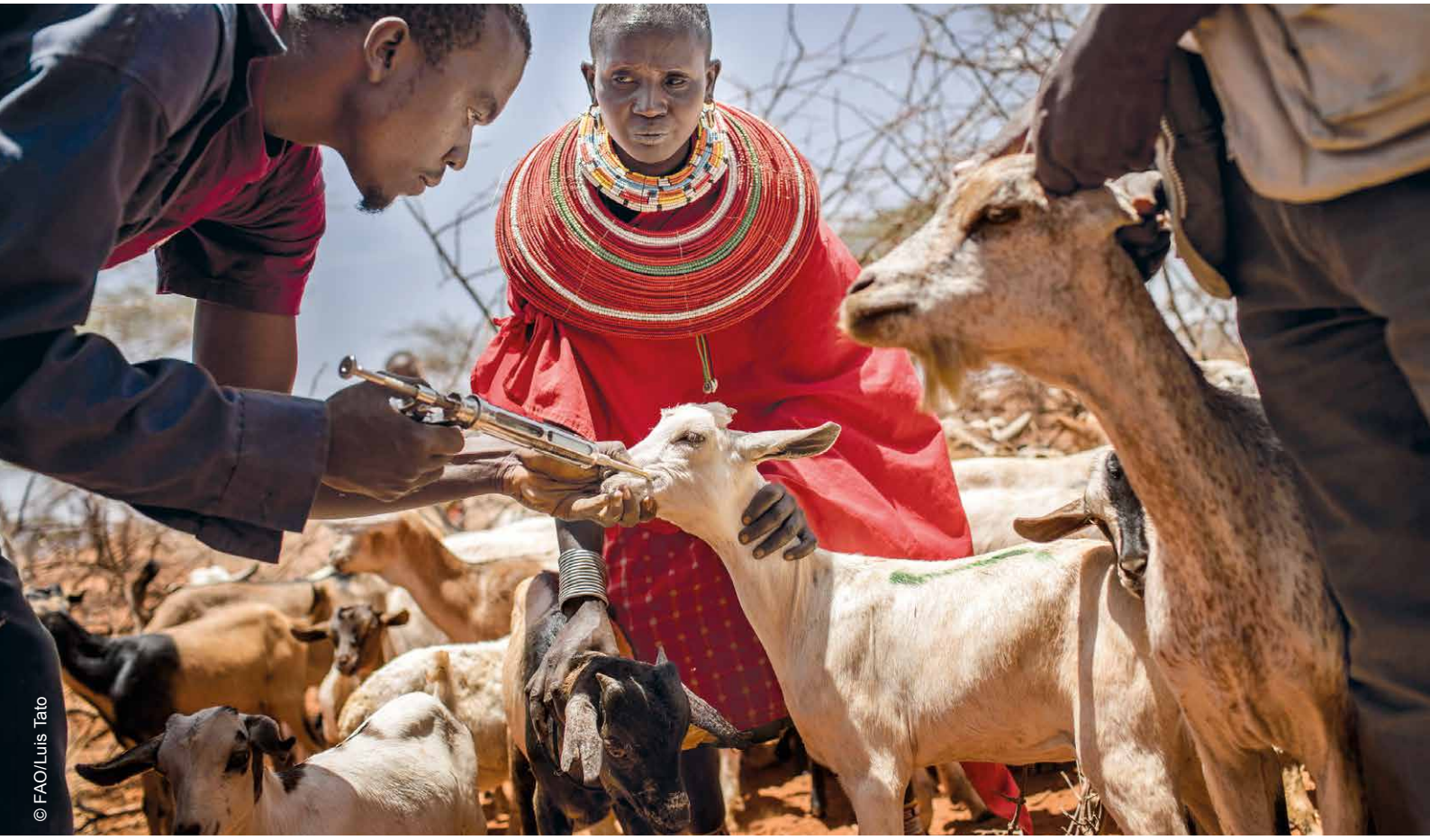


ABBREVIATIONS AND ACRONYMS

ADI	acceptable daily intake
DNA	deoxyribonucleic acid
DGGE	denaturing gradient gel electrophoresis
EMA	European Medicines Agency
FDA	United States Food and Drug Administration
GI	gastrointestinal
HFA	human flora associated
JECFA	Joint Expert Committee on Food Additives
IHMS	international Human Microbiome Standards
ITS	internal transcribed spacer
mADI	microbiological ADI
MDC	minimum disruptive concentration
MIC	minimal inhibitory concentration
mRNA	messenger RNA
NOAEC	no-observable adverse effect concentration
NOD	non-obese diabetic
NOEC	no observed effect concentration
NOEL	no observed effect level
OIE	World Organization for Animal Health
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
SCFA	short-chain fatty acids
SHIME	simulator of human intestinal microbial ecosystem
VICH	Veterinary International Conference on Harmonization
WHO	World Health Organization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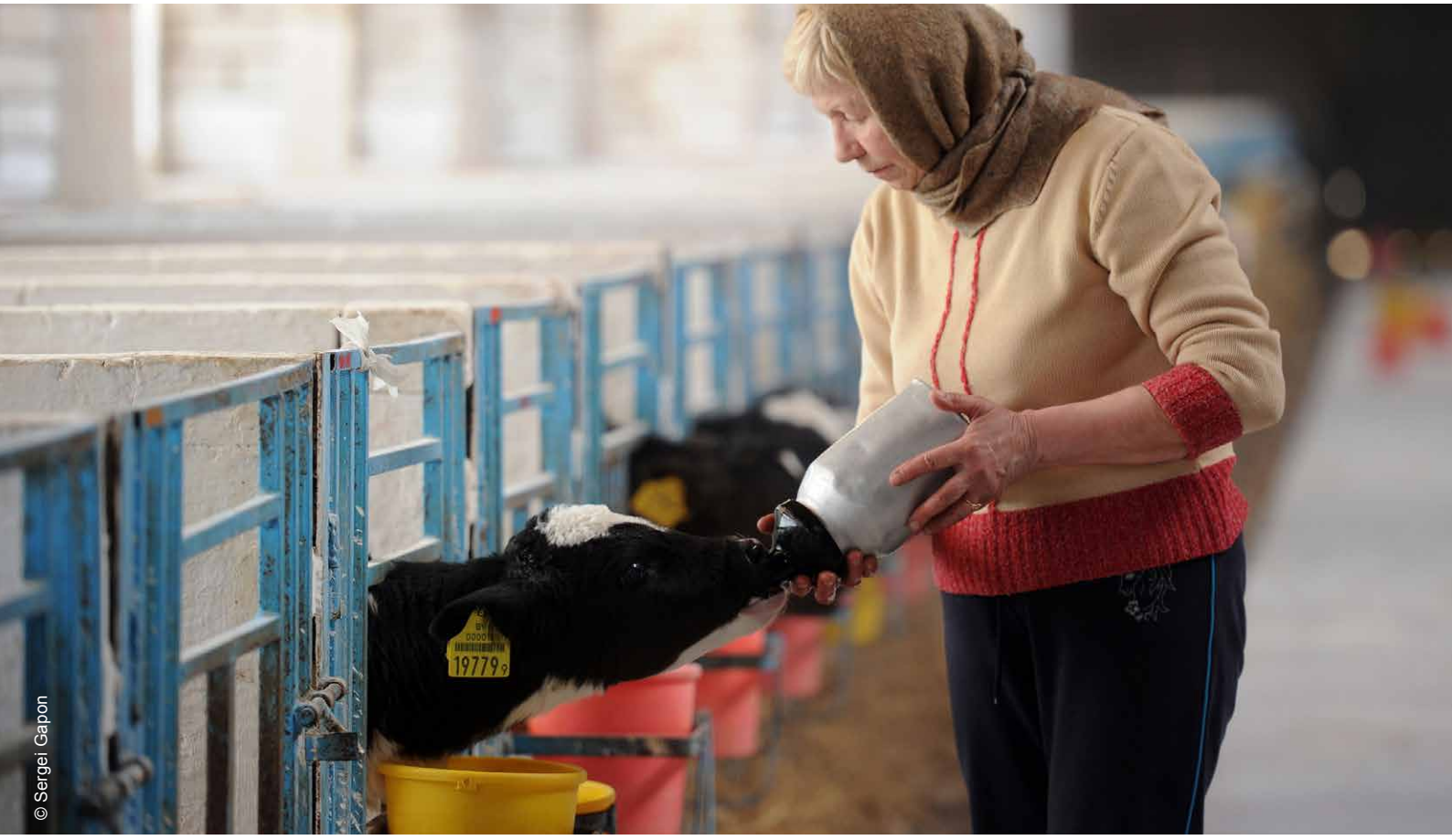
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EXECUTIVE SUMMARY

Veterinary drugs are administered to treat and prevent diseases in food-producing animals. These compounds may leave residual amounts in food products (e.g. meat, milk, eggs), especially if drugs are not used as approved (e.g. doses or dosing frequencies, off-label uses) or when clearance periods are not followed. The risk assessment of veterinary drug residues is typically conducted to evaluate their safety and determine health-based values. These assessments consider both toxicological and microbiological data. The development of omic technologies, including culture-independent analytical approaches (16S rRNA gene sequencing, shotgun metagenomics, transcriptomics, proteomics, metabolomics) has enabled the holistic evaluation of complex biological systems. These include, for example, the gut microbiome, human physiology or microbiome–host interactions. The human gut microbiome is comprised of trillions of microorganisms (bacteria, fungi, viruses and archaea), and its composition and function are highly influenced by various factors (e.g. diet, age, lifestyle, host genetics, environmental conditions along and across the gastrointestinal tract). The gut microbiome influences some physiological activities, e.g. immune system development and metabolism. However, there are concerns about the potential of residual veterinary drug in food to disturb the gut microbiome and the microbiome–host interactions, and whether these lead to short and long-term health consequences.

This review aims to evaluate the current knowledge about the effects of veterinary drug residues on the gut microbiome. It also assesses the scientific evidence on the influence of microbiome disturbances on health.

Limited research has focused on evaluating low residue levels of a few antibiotics on the faecal microbiota. These studies were primarily conducted *in vitro* and dependent on traditional bacteria cultures. They evaluated the capacity of antimicrobials to (1) disrupt the microbial barrier and the susceptibility to pathogen colonization, and (2) select for resistant bacteria. Effects were dose-dependent. All these studies, of relevance for food safety, were used to determine health-based values. However, most did not use the most modern holistic technologies (omics). Moreover, these research studies were microbe-centric and lacked consideration of host parameters.

However, most research on drugs and the gut microbiome is clinically relevant, as they evaluate treatment regimens (single therapeutical or subtherapeutic doses, schedule and duration) and drug combinations most commonly used in human medicine. Human clinical studies were not considered in database queries. Contrary to the research using low residue levels, most research evaluating therapeutical or subtherapeutic doses is conducted *in vivo* in rodents. The interest in early exposure is also reflected by the numerous research studies on this topic. Based on study conditions, most of the findings report microbial alterations and increased risk

for the development of metabolic disorders. Another common research focus is the increased susceptibility to gastrointestinal infections following microbiota disturbances caused by antimicrobial treatments.

In general, the microbiota effects reported are very diverse – in some cases contradicting – because the studies are designed differently (e.g. drugs, doses, exposure periods, models) and analytical methodologies are very heterogeneous. For these reasons, assay reproducibility inter-study comparability cannot be assessed. The lack of methodology standardization is a common observation in microbiome research. Moreover, the relationship between microbiome disturbances and health effects is associative or speculative in all the cases included in this review. In the absence of confirmed causality and mechanisms showing how the gut microbiome modulates health disorders, it is very difficult to incorporate microbiome data in risk assessments.


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CHAPTER 1
INTRODUCTION

Veterinary drugs include a large class of chemical agents defined in the Codex Procedural Manual as "any substance applied or administered to any food-producing animal, such as meat or milk-producing animals, poultry, fish or bees, whether used for therapeutic, prophylactic, or diagnostic purposes, or for modification of physiological functions or behavior" (Codex Alimentarius, 2018a). Hundreds of different drugs are used in veterinary medicine for treating and managing food-producing animals. The Joint Expert Committee on Food Additives (JECFA) evaluates the safety of veterinary drug residues in food, grouped into 13 functional classes based on their functional activity (Table 1). Some veterinary drugs may fall into several classes. For example, an adrenoreceptor agonist may also be classified as a production aid, or an antimicrobial may also have antiprotozoal properties (Codex Alimentarius, 2018b).

TABLE 1 **JECFA VETERINARY DRUG FUNCTIONAL CLASSES**

Adrenoceptor agonist	Antiprotozoal agent	Production aid
Beta-adrenoceptor blocking agent	Glucocorticosteroid	Tranquilizing agent
Anthelminthic agent	Growth promoter	Trypanocide
Antifungal agent	Insecticide	Veterinary drug, unclassified
Antimicrobial agent		

Source (italics): **Codex Alimentarius.** 2018b. Codex Veterinary Drug Residue in Food Online Database. In: *Codex Alimentarius*. Rome. Cited September 2019. https://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/vetdrugs/en

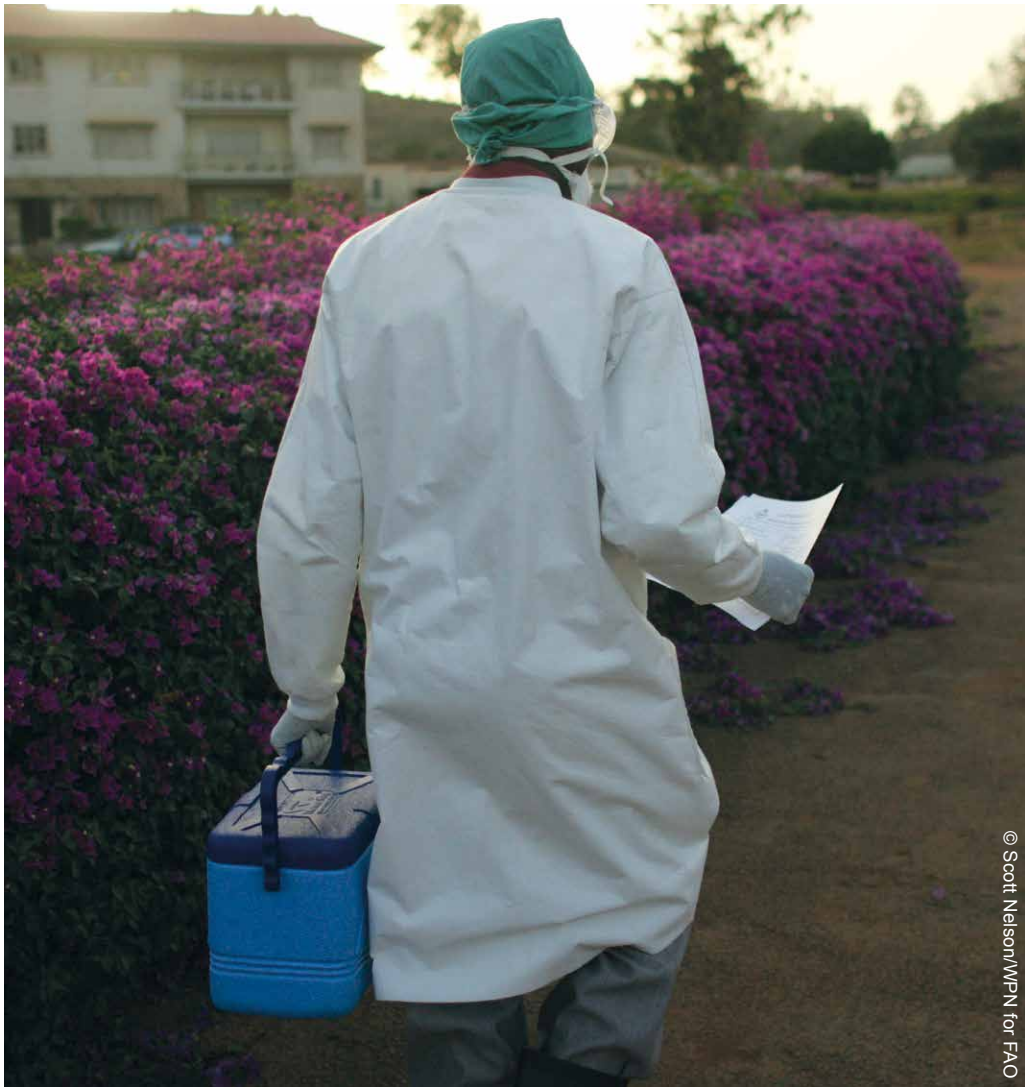
Veterinary drugs may be administered orally, including as a supplement to feed and water, injected intravenously or intramuscularly, intramammary, subcutaneously, by aerosol, applied topically on the skin, or in the case of fish, via immersion. Drugs can reach the environment via the disposal of human or animal waste (including manure) or water run-off. In addition, some antimicrobial agents, such as antibiotics (e.g. gentamycin, tetracyclines, oxalinic acid) and anti-fungal compounds, are also applied to fruits, vegetables, grains and pulses to control plant diseases. Therefore, terrestrial and aquatic animals and plants may be unintentionally exposed to drugs from environmental sources such as grazing on contaminated pastures, water or soil contamination. Environmental exposure in food-producing animals is not specifically considered or discussed in this review but is important as a consideration in the One Health paradigm.

Depending upon the pharmacokinetic properties of a specific drug, the drug preparation, and the route of administration, the drug is absorbed from the administration site and distributed systemically throughout the tissues of the animal's body. Such tissues include but are not limited to muscle, fat, organs (e.g. kidney, liver and lungs) and animal products such as milk, dairy products, eggs and honey. Drug residues may concentrate in certain parts of an animal's body following administration; for example, certain fat-soluble drugs may be sequestered in adipose tissue or concentrated in the liver or kidneys, where they are metabolized and eliminated. Notably, injection sites may have higher concentrations of drug residues than surrounding skeletal muscle. Eventually, drugs are metabolized to variable extents and eliminated from the food animal. For fish, the environmental temperature may also impact the metabolism and excretion rates. The relationship between the time of the last administration of a particular drug and the amount of drug residue present in any tissue depends upon multiple factors, including the dose and route of administration of the drug, the drug pharmacokinetics, the animal species and the health status of the animal. The withdrawal period, from the last drug administration until slaughter, is often established by governmental authorities to avoid the risks that drug residues may pose to humans.

Drugs are used to treat, control or prevent diseases. They are also used as growth promoters. For example, antibiotics have been used at subtherapeutic levels to promote animal growth, although this practice is strictly controlled or banned in many countries. When drugs are not used as approved (e.g. in different species of animals, at different doses or dosing frequencies, or at different administration rates for off-label treatment of diseases), residue levels present in tissue can be different than expected. Drugs may be used for purposes other than approved or prescribed for several reasons: a genuine lack of awareness of the proper use by some farmers, deliberate deviation from the intended use (e.g. unavailability of approved drugs), as well as a lack of regulation or monitoring oversight by government authorities. Such practices may be of concern in developing countries (Muaz *et al.*, 2018). When used in food-producing animals, these factors may result in residues in food for human consumption. Veterinary drug residues have been found not only in different products of animal origin (e.g. milk, meat, eggs, organ tissues, fish, shrimps) but also in vegetables (Chen, Ying and Deng, 2019). Residues of veterinary drugs in food may frequently exceed national or international standards (Bacanli and Basaran, 2019). National monitoring programmes are in place to survey compliance with regulatory limits for veterinary drug residues and to verify the effectiveness of veterinary drug management and best practices. The latest reports from the United States of America (USDA, 2019), the European Union (EFSA, 2021) and Australia (Australian Department of Agriculture Water and the Environment, 2020) indicate compliance in over 99.6 percent of samples. However, the frequency of veterinary drug residues found in food may be higher in developing countries due to inappropriate use of antimicrobials in the veterinary sector and the lack of strict regulatory and enforcement frameworks (Ayukekbong, Ntemgwa and Atabe, 2017).

Veterinary drug residues ingested through food products (meat, milk, dairy, eggs, etc.) that are not absorbed in the gastrointestinal tract may remain in contact with the human gastrointestinal microbiota. Moreover, drug residues ingested and absorbed can be metabolized by the host and released back to the intestine, where they can further interact with the gut microbiome. The physico-chemical and pharmacokinetic properties of a drug are factors that will determine how the drug will affect the human gastrointestinal microbiome.

This review addresses the current status of the human gastrointestinal microbiome in the context of human health and risk assessment of veterinary drug residues. It will discuss definitions, tools and methodologies used to evaluate the microbiome. It also includes published *in vitro* or *in vivo* studies aimed at assessing the exposure of the human gut microbiome to veterinary drug residues. The effect of veterinary drugs on the gut microbiota of food-producing animals is out of the scope of this document. The impact of pharmaceuticals used at therapeutic doses on the human gut microbiome is briefly discussed.

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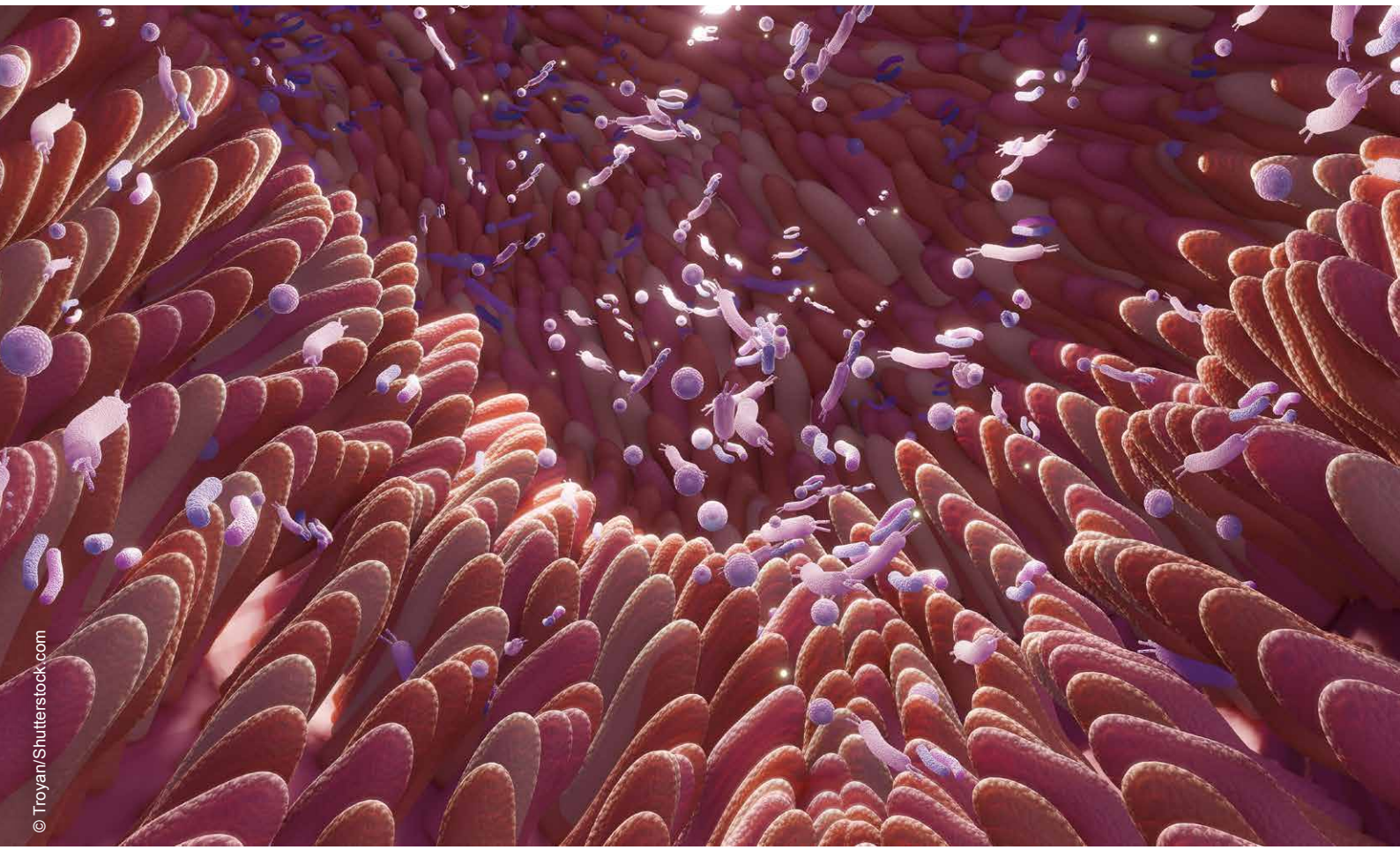
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CHAPTER 2
WHAT IS THE GUT MICROBIOME?

The gut microbiome is a dynamic microbial network composed of bacteria, fungi, viruses, protozoa and archaea living in a symbiotic relationship with the host (Durack and Lynch, 2018). Microbiota is another term that also refers to microbial populations. Microbiome and microbiota are terms commonly used interchangeably due to the lack of consensus definitions. In general, microbiota refers to the group of individual microbes within the microbial community and its taxonomical structure. The microbiome is a more complex entity that, in addition to the notion of microbiota, also encompasses the function and dynamics within this population. The most popular definition describes the microbiome as the collective microbial genomes that live at specific body sites, e.g. skin and gastrointestinal tract (Turnbaugh *et al.*, 2007). A more recent proposal defines a microbiome as "a characteristic microbial community occupying a reasonable, well-defined habitat with distinct physio-chemical properties" (Berg *et al.*, 2020, p. 17). It is essential to understand the microbiome as a population within a defined functional ecosystem and not only the sum of different individual microbes.

Most research on the gut microbiota focuses on the bacterial population. The most abundant phyla are Firmicutes and Bacteroidetes, accounting for over 90 percent of this microbial group (Almeida *et al.*, 2019; Cani and Delzenne, 2007). Minor phyla include Actinobacteria and Proteobacteria, among others less abundant (Qin *et al.*, 2010). However, less is known about other microbiota members, such as viruses and fungi, as well as their interaction and overall role within the complex microbiome network and microbiome–host relationship. The viral community, also known as the virome, outnumber the bacterial cells 10:1 and are composed of DNA and ribonucleic acid (RNA) viruses infecting bacteria (e.g. bacteriophages), archaea and eukaryotic viruses as well as retroviruses (Mukhopadhya *et al.*, 2019). Although poorly understood, gut bacteriophages are the most abundant type of viruses and are known to shape the intestinal microbial composition, drive bacterial diversity[1] and facilitate horizontal gene transfer (Sutton and Hill, 2019). The fungal

[1] Taxonomical diversity refers to *the variety and abundance of species in a defined unit of study* (Magurran, 2013). It has two components: richness (*total number of species in the unit of study*) and evenness (*relative differences in the abundance of various species in the community*) (Young and Schmidt, 2008).

community, also described as mycobiome, is present in the lower part of the gut in lower numbers than bacteria. However, it has been less studied than the bacterial community. The role of the mycobiome in the microbiome and its interaction with the host has gained interest more recently (Richard and Sokol, 2019; Santus, Devlin and Behnsen, 2021). It has been reported that the mycobiome contributes to immune homeostasis and when altered, it can contribute to chronic inflammatory disorders, such as inflammatory bowel disease (Gutierrez *et al*., 2022; Iliev and Leonardi, 2017). Limited research indicates that Archaea, another understudied microbiome component, possibly contributes to host homeostasis and inflammatory bowel disease (Houshyar *et al.*, 2021; Mohammadzadeh *et al*., 2022).

The gut microbiome starts taking shape early in life, commencing at birth upon exposure to the mother and the environment, and it continues to evolve, forming a complex ecosystem in the gastrointestinal tract (Arrieta *et al.*, 2014; Bäckhed *et al.*, 2015; Wampach *et al.*, 2017). The composition and dynamics of the gut microbiome are more highly dependent on stressors and environmental factors than on host genetics (Rothschild *et al.*, 2018). Although many reports indicate that the microbiota composition finds stability in adulthood, population-level analyses show that the microbiome remains highly dynamic (Priya and Blekhman, 2019), with high interindividual taxonomical diversity and temporal intra-individual variability (Lloyd-Price, Abu-Ali and Huttenhower, 2016; Shanahan, Ghosh and O'Toole, 2021). Also interesting is the evolution of the microbiome function compared to its composition. Studies have shown that functional stability is reached early in life and is likely to remain so for a long time afterwards (Kostic *et al.*, 2015).

The different environmental conditions along the gastrointestinal tract (e.g. pH, oxygen pressure, nutrients) determine the microbiota composition at the various sites (Figure 1). Facultative anaerobes dominate in the early segments of the intestine and, as the oxygen pressure decreases towards the colon, the abundance of strict anaerobes with high fermentative capacity increases (Kennedy and Chang, 2020). Most research studies focus on the microbiota of the colon and cecum because of their higher abundance and ease of obtaining faecal samples. Moreover, the microbiome of the large intestine is more diverse and stable than the microbial community of the small intestine, which is subject to harsher environmental conditions (low pH, enzymes, bile acids) (Kastl *et al.*, 2020; Rowan-Nash *et al.*, 2019). However, the small intestine microbiota is more dynamic due to the need to adapt to the rapidly changing environment. Absorption of nutrients and other compounds occurs primarily in the small intestine, where there is a relevant interaction between microbiota, xenobiotics and the host (Kastl *et al.*, 2020). Although most studies target faecal and caecal microbiota, the microbial community of the small intestine is the first to encounter xenobiotic compounds and is probably more responsive, with the potential for impacting the host physiology (Martinez-Guryn *et al.*, 2018; Scheithauer *et al.*, 2016).

In addition to differential longitudinal gastrointestinal ecosystems, there are also cross-sectional differences in the microbiota composition and function (Yang *et al.*, 2020). On one side, the luminal microbiota is relevant for digestion and absorbing carbohydrates. On the other side, the mucosa-associated microbiota plays an

essential protective role, e.g. maintaining the mucus layer integrity and modulating the immune function of intestinal epithelial and immune cells (Yang *et al.*, 2020).

FIGURE 1 **CONDITIONS AND PHYSIOLOGICAL ACTIVITIES IN THE GASTROINTESTINAL TRACT**

	pO_2 mm Hg	pH	CFU/ml	ACTIVITY
STOMACH	77	1-3	10^1 - 10^3	Mechanical, chemical and enzymatical digestion
SMALL INTESTINE	33	**DUODENUM** 5-7	10^1 - 10^4	Digestion (proteins, monosaccharides, SCFAs), Immunomodulation
		JEJUNUM 7-9	10^3 - 10^5	Absorption (free fatty acids, carbohydrates, small peptides, minerals, vitamins A, D, E, K)
		ILEUM 7-8	10^3 - 10^8	Absorption (vitamin V12, bile acids)
LARGE INTESTINE	<33	**PROXIMAL COLON** 5.4-5.9	10^1 - 10^{11}	Active bacterial growth High fibre and polysaccharides Carbohydrate fermentation High SCFA production
		TRANSVERSE COLON 6.1-6.4	10^{11} - 10^{12}	Substrate depletion Reduction bacterial activity Decreasing SCFA production
		DISTAL COLON 6.1-6.9	$\geq 10^{12}$	Slow bacterial growth Low substrate availability Protein fermentation Low SCFA production

FACTORS AFFECTING MICROBIOTA ABUNDANCE AND DIVERSITY

AGE
DIET
HOST GENETICS
PHYSICAL ACTIVITY
GEOGRAPHICAL LOCATION
EXPOSURE TO XENOBIOTICS
ANTIBIOTICS
ENVIRONMENT
GASTRIC MOTILITY
GASTRIC SECRETION

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The gut microbiome contributes to the host's homeostasis on three fronts (Abdelsalam *et al.*, 2020). Firstly, it assists in digesting and metabolizing food components (e.g. fermentation of complex carbohydrates) and other xenobiotic compounds (Koppel, Maini Rekdal and Balskus, 2017). The microbiome can metabolize compounds produced by the host, like intestinal bile acids into secondary bile acids, and participate in the gut-brain axis, for example, by modulating signalling processes involved in developing obesity (Schéle *et al.*, 2013). Secondly, it produces essential metabolites such as vitamins, amino acids and short-chain fatty acids (SCFAs). SCFAs result from the fermentation of carbohydrates and they are of particular interest as they are used as an energy source by intestinal enterocytes. Moreover, SCFAs can modulate metabolic pathways, neuronal and intestinal functions and participate as modulators of the host immune response (Koh *et al.*, 2016; Neish, 2009; Portincasa *et al.*, 2022).

Thirdly, the microbiome offers protection by stimulating the immune system and contributing to its maturation. Also, it participates in maintaining the intestinal barrier. The first line of intestinal defence (colonization resistance or colonization barrier) exerted by the gastrointestinal microbiota is characterized by preventing the colonization of exogenous pathogens and the proliferation of commensal opportunistic pathogens (Pilmis, Le Monnier and Zahar, 2020). The host also contributes to maintaining the colonization resistance via the intestinal immune system, for example, by modulating the production of antimicrobial peptides and mucus (Kinnebrew *et al.*, 2010; Mowat and Agace, 2014).

Both gut colonization and the protection from invasion depend on the microbial context. Stecher (2021) notes that specific strains of microbial species may be protective in the presence of a specific microbiota and refers to the mechanisms that the microbiota uses against *Salmonella enterica* Typhimurium as a model to monitor colonization resistance. These mechanisms include (1) the inhibition of gut luminal colonization by competition of key substrates, production of antimicrobial proteins or metabolites; (2) modulation of the host metabolic activity and immune response; (3) interference with the expression of virulence factors; and (4) the potential of some members of the microbiota to lower the pathogen burden caused by the proliferation of Enterobacteriaceae occurring after the inflammatory response triggered by the colonizer. SCFAs such as butyrate, propionate and acetate are microbial metabolites involved in several of these mechanisms and are commonly monitored in microbiome studies.

Although there is a substantial amount of scientific information associating the microbiome and human health and disease, there are no consensus definitions for what constitutes a healthy and an unhealthy (dysbiosis) microbiome. A major challenge in defining a healthy microbiome is the high interindividual variability within the healthy population (Wei *et al.*, 2021). Some approaches have focused on the development of population-based definitions, e.g. "core microbiota", "core microbiome" (or "core functional microbiome"), "core metatranscriptome" (or "active functional core"), referring to the "common" compositional taxa, function and translated functions of the microbiome in the population (Shetty *et al.*, 2017). In 2017, a multidisciplinary workshop was organized to explore the question: "can we begin to define a healthy gut microbiome through quantifiable characteristics"? (McBurney *et al.*, 2019). Due to the difficulties in defining a "healthy microbiome", the group suggested that research should be directed to determine factors (environmental, clinical or nutritional) that diminish symbiotic features and highlight the relevance of the holistic function of the microbiome, its diversity and activity redundancy. Activity redundancy is common in the microbiome (Louca *et al.*, 2018; McBurney *et al.*, 2019). For example, several species from different taxa groups can ferment complex carbohydrates and release SCFAs. Another example of redundancy is the capacity of several bacterial taxa to metabolize the glucocorticoid dexamethasone (Zimmermann *et al.*, 2019). The more diverse the microbial population is, the more likely the presence of functional redundancy is. Depending on the degree of disturbance, changes in microbiota composition may not be relevant if the overall function of the microbiome is not compromised. Therefore,

the study of the microbiota composition alone may not be sufficient to fully explain its function (Lozupone *et al.*, 2012) and the microbiome–host interaction. The microbiome seems functionally more stable (offering a higher discriminatory power) than its taxonomical composition (Louca *et al.*, 2016; Shanahan, Ghosh and O'Toole, 2021). Based on this, research groups have raised questions about the suitability of approaches to better interpret and understand microbiome data, e.g. characterization phenotypic traits (e.g. molecular or metabolic) *versus* taxonomical analysis alone (Martiny *et al.*, 2015; Xu *et al.*, 2014).

The imbalance of the microbiota composition and disruption of its complex structure is called dysbiosis (Petersen and Round, 2014). Unfortunately, this is another concept lacking a consensus definition (Hooks and O'Malley, 2017). Gut dysbiosis has been associated with loss of diversity and richness, changes in the Firmicutes/Bacteroidetes ratio, reduced relative abundance of beneficial bacteria, and alterations in the normal function of the microbiome (Petersen and Round, 2014; Pilmis, Le Monnier and Zahar, 2020). Dysbiosis can affect the host immune system and creates a suitable environment for minority opportunistic (e.g. *Clostridium difficile*, *Candida* spp.) or pathogenic members to proliferate (Berg *et al.*, 2020; Petersen and Round, 2014). Many of these pathogenic bacteria belong to the Proteobacteria, a low abundance phylum in the gut microbiota of healthy individuals. Within this phylum, the family Enterobacteriaceae contains potentially pathogenic species such as *Escherichia coli*, *Shigella* and *Klebsiella* spp. Proteobacteria have been proposed as a potential marker for gut dysbiosis and risk of disease (Shin, Whon and Bae, 2015). Gut dysbiosis has been associated with the disruption of the intestinal barrier function, intestinal disorders, immune-mediated and metabolic diseases (e.g. inflammatory bowel disease, obesity), as well as neurological alterations (Margolis, Cryan and Mayer, 2021; Sanders *et al.*, 2021; Zheng, Liwinski and Elinav, 2020). A recent review has collected and categorized indexes developed to determine gut dysbiosis (Wei *et al.*, 2021), primarily used as markers within the clinical context. The majority of indexes are based on parameters describing the taxonomic composition and diversity of the microbiota and illustrates the higher weight typically given to the structure of the microbial community over the functional aspect.


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CHAPTER 3
STUDY OF THE MICROBIOME

Numerous options are available to study the microbiome composition, diversity and function as well as its relationship with the host and the environment. However, there is no gold standard and the selection of the most suitable models and analytical strategies depend primarily on the purpose of the study and the questions that need to be answered.

MODELS

Since the gut microbiome–host relationship works in a symbiotic manner involving many systemic processes, using live organisms provides information that cannot be obtained by *in vitro* systems alone. However, the scientific community is under pressure to replace *in vivo* studies in animals with more humane alternatives, including *in vitro* and *ex vivo* models. Still, *in vitro* models are valuable to understand the microbiome and its dynamics in response to environmental conditions and exposure to dietary compounds.

In vitro models include, for example, fermentation chambers or bioreactors (Nissen, Casciano and Gianotti, 2020). These can mimic conditions of the different gastrointestinal environments. There are different types of bioreactors that vary in their degree of complexity. The simplest units (e.g. batch fermentation models) are chambers run under specific conditions and a defined medium, which is not replaced over time. For example, this system has been used to evaluate tetracycline residues using human faecal microbiota (Jung *et al.*, 2018). In continuous culture bioreactors, the medium is replaced periodically, and environmental and nutrient parameters are monitored over time. The "chemostat" bioreactor has been used to evaluate the exposure of the microbiota (e.g. pooled faecal suspensions) to veterinary drug residues, as well as colonization resistance. For example, this model has been used to study the effects of residue levels of ciprofloxacin (Carman *et al.*, 2004; Carman and Woodburn, 2001), tetracycline, neomycin, erythromycin (Carman *et al.*, 2005), cyadox (Hao *et al.*, 2013), tilcomisin (Hao *et al.*, 2015) and tulathromycin (Hao *et al.*, 2016). More modern and complex systems are composed of multiple bioreactors connected in series and mirroring the conditions of different sections of the gastrointestinal tract, including peristaltic movements (e.g. simulator of human

intestinal microbial ecosystem [SHIME®], TIM-2, SIMGI) (Guzman-Rodriguez *et al.*, 2018; Nissen, Casciano and Gianotti, 2020; Van de Wiele *et al.*, 2015). In these systems, it is possible to study the dynamics of the microbiome's composition and function (e.g. production of SCFAs, vitamins, communication signals). They have been used to evaluate the effects of diet composition, prebiotics and xenobiotics such as drugs and pesticides (Guzman-Rodriguez *et al.*, 2018; Joly *et al.*, 2013; Nissen, Casciano and Gianotti, 2020; Reygner *et al.*, 2016a). They also permit the evaluation of the microbial transformation of drugs and the potential transfer of antimicrobial resistance genes. For example, M-SHIME (mucosal SHIME), a system optimized to enable the colonization by the mucosal microbiome, has been used to demonstrate the horizontal transfer of mobile antimicrobial resistance genes from commensal *E. coli* (isolated from a broiler) to members of the human microbiota (coliforms and anaerobes) in the presence of cefotaxime (Lambrecht *et al.*, 2021). Studies in SHIME models showed the expansion of opportunistic pathogens after exposure to vancomycin (Liu *et al.*, 2020) and alterations of the human intestinal microbiota and resistome after exposure to colistin and amoxicillin (Li *et al.*, 2021).

However, none of the bioreactors can mimic all key anatomical and physiological gastrointestinal conditions (Roupar *et al.*, 2021). For example, they do not consider the impact of substances on epithelial cells of the intestinal mucosa, something that is possible with the use of epithelial cell cultures, for example, the monolayer lines Caco-2, HT29, and T84, derived from human colon cancer cells (Gokulan *et al.*, 2017; Pearce *et al.*, 2018). The latter is the best choice of the three for evaluating the epithelial barrier function because it secretes mucin, mimicking the human intestine. In addition, it expresses cell-integrity genes (e.g. claudin) and allows for measuring changes in permeability against xenobiotic substances (Gokulan *et al.*, 2017). The T84 cell line has been used to evaluate the effects of low doses of tetracycline on the barrier function of the microbiome (Gokulan *et al.*, 2017). The Caco-2 cell line has been used to develop a model to determine the minimum disruptive concentration (MDC)[2] (Wagner, Johnson and Cerniglia, 2008), an alternative to the minimal inhibitory concentration (MIC). Some of the drawbacks of cell lines are the lack of cell diversity present in the intestine, and the fact that it is not possible to culture the bacterial community. Another approach that is becoming the gold standard of *in vitro* testing is the use of reactors and cell cultures in tandem to combine the benefits of both systems (Requile *et al.*, 2018). This combination has been used to evaluate pesticides such as chlorpyrifos (Requile *et al.*, 2018) and delthamethrin (Defois *et al.*, 2018). More recent advances have permitted the development of *ex vivo* models (e.g. intestinal enteroids and organoids, organs-on-a-chip and microfluidic devices). They consist of functional live tissues with more complex cellular environments than cell cultures and resemble more closely the conditions of *in vivo* systems (May, Evans and Parry, 2017; Pearce *et al.*, 2018). These approaches, while still evolving, have many potential applications in drug discovery and

[2] MDC is the minimum concentration of an antimicrobial drug that disrupts the colonization resistance mediated by model human intestinal microbiota against a *Salmonella* invasion of Caco-2 intestinal cells (Wagner, Johnson and Cerniglia, 2008).

pharmaceutical–microbiota–host evaluations. However, their use is still limited due to their drawbacks (e.g. short-term culture, cost and difficulties in obtaining human samples, and availability) (May, Evans and Parry, 2017; Pearce *et al.*, 2018). A recent and promising development is an anaerobic intestine-on-a-chip based on mucin-producing Caco2 epithelial cells and an endothelium layer (Jalili-Firoozinezhad *et al.*, 2019). It allowed culturing of complex faecal microbiota for up to 5 days. This system has the potential to reproduce and control the environmental conditions of different sections of the gastrointestinal tract, including the incorporation of additional intestinal cell types and monitoring the intestinal barrier function. These refinements would make a complex *in vitro* system available to assess microbiome-host interactions in health and disease.

When using *in vivo* surrogate animal models to study the human gut microbiome, it is critical that they are physiological- and clinically relevant to the human context. Selecting the most suitable model depends on the study's objectives. Criteria for choosing an animal model include genetic background, baseline microbiota, or phenotypic expression of diseases (Kamareddine *et al.*, 2020). Dogs and swine have similar dominant phyla to those in humans (i.e. Firmicutes and Bacteroidetes) but differ significantly from humans at the genus level (Hoffmann *et al.*, 2015). Although non-human primates are genetically closer to humans, their gut microbiome differs significantly, making them less suitable (Amato *et al.*, 2015). The rat baseline microbiota is more similar to humans than mice (Flemer *et al.*, 2017; Wos-Oxley *et al.*, 2012). Mice have similar dominant phyla to humans but differ in several health-relevant genera absent in mice (Nguyen *et al.*, 2015). However, mice and rats have been the predominant models used to study the microbiome. Mice are genetically manipulable, e.g. to mimic human disease conditions, and have more genetic variants than rats, making them more versatile models to study mechanisms that, for example, influence microbiota composition (Turner, 2018). Germ-free mice have been valuable for testing hypotheses. They have also helped establish cause-effect relationships between shifts in the microbiome (composition and function) and physiological alterations in the host, predisposition to opportunistic infections and disease. In microbiome studies, germ-free animals are inoculated with bacterial cultures or colonized with healthy or altered microbiota from a donor. Germ-free mice can be humanized when the donor is a human (also known as human flora-associated [HFA] mice). As discussed later, HFA mice have been used, for example, to evaluate residual and therapeutic doses of tetracyclin (Perrin-Guyomard *et al.*, 2001; Perrin-Guyomard *et al.*, 2005; Perrin-Guyomard *et al.*, 2006). There are two types of germ-free mice, both having advantages and disadvantages (Kennedy, King and Baldridge, 2018). On the one hand, true germ-free mice are bred and raised free of microorganisms under rigorous environmental conditions. Germ-free animals have physiological differences from their conventional counterpart that need to be considered in the study design and when extrapolating findings. Examples include slower epithelial renewal, altered immune system, gene expression of gastrointestinal cells and decreased mucus layer (Fritz *et al.*, 2013). On the other hand, antibiotic-treated animals (near germ-free) are a less expensive alternative. Relatively high

doses of ampicillin, vancomycin, neomycin and metronidazole are commonly used to deplete the gut microbiota of mice (Kennedy, King and Baldridge, 2018; Ray *et al.*, 2021; Reikvam *et al.*, 2011). Although germ-free animals have been widely used to demonstrate causality between microbiome changes and host physiological alterations and diseases, their use to demonstrate the mediation of the microbiome on the therapeutic effect of drugs has been relatively limited (Zimmermann *et al.*, 2021).

In addition to animal type and genetic background, age is a critical factor in the study of the microbiome and the effects of dietary compounds. As mentioned earlier, the composition of the microbiome differs significantly between the early stages of life and adulthood, and alterations at earlier ages may influence the development of different disorders later in life. In particular, early antibiotic exposure has been associated with an increased risk of non-communicable diseases (e.g. metabolic and immune-mediated disorders), potentially mediated by a disturbed microbiome (Rautava, 2021).

Another consideration in animal studies is the high interindividual variability. Differences in the microbiota composition among individuals require careful attention to sample sizes to not jeopardize the statistical robustness of results.

Additional research factors to consider are the selection of doses and experimental periods relevant to the study of veterinary drug residues. The exposure of the gut microbiota to veterinary drug residues should consider doses ranging from low to therapeutic concentrations on the high end. It should also consider drug metabolites and typical drug combinations used in food-producing animals. Since the potential exposure of humans to veterinary drug residues can occur chronically, exposure periods need to be representative of chronic exposure.

ANALYTICAL CONSIDERATIONS - SAMPLING AND SAMPLE PREPARATION

A key aspect of any analytical methodology is the collection of a representative sample. Many studies evaluate microbiota from faecal material because it is cost-efficient and non-invasive. This is a practical option in longitudinal studies to monitor the microbiota evolution over time. The microbiota from different intestinal locations is also evaluated, but they are typically collected at the end of the study once the animal has been euthanized (one-time-point evaluation). However, it is important to consider that the composition of the microbiota differs depending on the location in the gastrointestinal (GI) track. Although faecal microbiota is more similar to the colonic/caecal microbiota, it may not represent the microbial population of the small intestine (Kastl *et al.*, 2020).

One of the controversial issues related to the suitability of microbiota sources is the use of pooled or unpooled material (e.g. faecal samples) from donors. The reason for using pooled material relies on interindividual variability of microbiota composition. An *in vitro* study by Jung *et al.* (2018), which will be discussed in more depth later in this document, evaluated the effect of tetracycline residues on unpooled faecal material from three individuals and reported the influence of

interindividual variability in some of the study endpoints, including the microbial community composition. Aguirre *et al.* (2014) compared unpooled and pooled faecal materials in a TIM-2 continuous bioreactor. Despite the slight differences in specific microbial groups between the microbiota from pooled and individual samples, there were no major differences in terms of diversity, and this indicated the suitability of pooled material for *in vitro* experiments in bioreactors. Pooled material offers several advantages (Aguirre *et al.*, 2014), including the availability of a standardized material that can be used in multiple experiments, which would be useful to compare results from different studies and contribute to experimental reproducibility. In addition, an optimized pooled material would result in microbial diversity representative of a specific or whole population.

Sample collection, handling, storage and processing require careful consideration to preserve microbial stability and analyte integrity (e.g. DNA and microbial metabolites) (Bharti and Grimm, 2021). For example, in the study of faecal microbiota, the dilution of faecal slurries may change the microbial composition, which may be due to the differential adhesion properties of bacterial groups to faecal particulates when compared to the liquid phase. Ahn *et al.* (2012b) observed a higher abundance of Firmicutes in 50 percent faecal suspension, compared to 10 percent and 25 percent diluted slurries. In addition, Ahn *et al.* (2012b) did not pool faecal samples from human subjects and found interindividual variation. The International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH, 2019) has published methodological recommendations describing, for example, characteristics of faecal donors, sample collection, faecal concentration and dilution of faecal slurries.

ANALYTICAL METHODS

The study of the microbiome, microbiome–xenobiotics and microbiome–host interactions has evolved rapidly over the last decade in parallel to the new advancements in omic technologies, bioinformatics and machine learning. These technical developments, e.g. sequencing, have allowed for cultivation-independent, DNA- (e.g. metagenomics) and RNA- (e.g. metatranscriptomics) based approaches to investigate the microbial community from a holistic perspective. The omic techniques (e.g. metagenomics, metatranscriptomics, metabolomics, metaproteomics) provide a unique opportunity to analyse and untangle the complex microbial ecosystem from a holistic perspective. However, although modern methods have contributed significantly to understanding the microbial community and its environment, more traditional analytical tools are also part of the toolbox to study the microbiome. Selecting the most appropriate method(s) will depend on the scientific question and hypothesis (Allaband *et al.*, 2019).

The analysis of the microbiota composition and diversity is most commonly carried out by sequencing the 16S ribosomal RNA (rRNA) gene (bacteria and archaea), 18S rRNA gene and the internal transcribed spacer (ITS) regions (eukaryotes, e.g. fungi). This method involves DNA extraction, amplification, standardization,

library construction, sequencing and bioinformatic analysis (Arrieta *et al.*, 2014). The 16S rRNA gene is highly conserved across bacteria and has been used as a reliable marker for taxonomic classification and phylogenetic analysis of prokaryotes (Yang, Wang and Qian, 2016). The gene has nine hypervariable regions (V1-V9), some of which are more conservative than others. The target region(s) will determine the taxa level of the analysis, ranging from high-level taxa (more conservative regions) to the identification of genus (less conservative regions). Due to the limited resolution of 16S rRNA gene sequencing, the identification at the species level is not always possible because the region targeted is identical among some species (Jovel *et al.*, 2016; Wang *et al.*, 2007). Different assay conditions, including sampling, genetic material extraction, selection of polymerase reaction (PCR) primers and computational pipelines, can lead to different microbiome profiles (Human Microbiome Project Consortium, 2012; Sinha *et al.*, 2017), affecting method comparison and reproducibility. Therefore, the standardization of protocols, including PCR primers, is a step forward for result consistency.

However, a comprehensive genetic microbiome analysis is possible thanks to shotgun metagenomics analysis. Unlike targeted amplicon sequencing (e.g. ITS, 16S and 18S rRNA genes), untargeted shotgun metagenomics sequences the complete genome present in a sample. This analytical approach offers a higher taxonomic range (not only bacteria but also viruses, fungi, archaea and small eukaryotes) and resolution (down to species and strain levels) than 16S rRNA gene sequencing (Allaband *et al.*, 2019). Shotgun metagenomic analysis is used to determine not only the taxonomic structure of the microbial community but also the functional potential of the microbiome. Functional profiling includes, for example, identifying genetic traits, and assessing the potential up- or down-regulation of biochemical pathways and microbiome activities. Currently, there is no consensus standard for the best sequence assembly approach (Galloway-Pena and Hanson, 2020). Although very powerful, shotgun metagenomic analysis can introduce errors and biases derived from experimental and computational factors (Bharti and Grimm, 2021) and, like 16S rRNA sequencing analysis, it is subject to reproducibility issues (Allaband *et al.*, 2019).

Genomics provides information about the presence of genes but does not indicate whether they are being expressed or not. The transcription of genes is evaluated by analysing the messenger RNA (mRNA). It provides mechanistic insights about which metabolic pathways may be up- or down-regulated. Transcriptomics techniques based on qRT-PCR or microarray are used to analyse target-specific gene transcription. Similar to metagenomics, metatranscriptomics (mRNA sequencing) targets the entire mRNA content (Shakya, Lo and Chain, 2019).

Metaproteomics and metabolomics are also analytical methods used to measure microbial function. There are different approaches to metabolomics. Targeted strategies focus on the analysis of specific groups or families of compounds (e.g. short-chain fatty acids – SCFA), while untargeted analysis aims to detect as many metabolites as possible. Metabolomics can be described using different names depending on the type of compound that is being analysed, for example,

lipidomics (lipid profiling) or volatolomics (volatile organic compounds profiling). Technologies for detection include mainly mass spectrometry, although nuclear magnetic resonance spectroscopy is also used. Altered metabolite profiles after exposure to dietary compounds may indicate changes in the normal function of the microbiome. As microbial metabolites participate in the physiological and metabolic processes of the host, changes in the microbiome's activity may potentially induce alterations also in the host. Microbial metabolites are typically analysed from caecal content or in faecal samples. However, they are also found in plasma and other tissues after being absorbed by the host. Metabolomics is usually combined with metagenomic or transcriptomic studies.

Nobody questions the potential benefits of the omic approaches to understanding microbial structures and processes. However, the omic technologies come with new challenges. They provide a vast amount of data that must be processed and translated into valuable and meaningful information. However, there is still information that cannot be interpreted due to gaps in existing knowledge. For example, some identified metabolic activities cannot be linked to genes or specific enzymes (Koppel, Maini Rekdal and Balskus, 2017). And the contrary is also true. For example, 86 percent of the faecal metagenome cannot be assigned to known metabolic pathways (Human Microbiome Project Consortium, 2012). Also challenging for metabolomics is the annotation[3] of detected new molecules or molecules modified by the microbiome or the host that do not match known compounds in reference libraries (Allaband *et al.*, 2019).

Although omics open new opportunities to understand the complexity of microbial networks and their interactions with their ecosystems, conventional and targeted analytical approaches have specific purposes and will continue to be used. For example, they can complement omics findings to, for example, characterize newly discovered microbiota members or metabolic pathways.

STANDARDIZATION AND BEST PRACTICES

The study of the microbiome requires complex studies, analytical methods and data processing. There are challenges inherent in each step of the process, e.g. experimental design, sample collection and handling, nucleic extraction, sequencing and computational analysis. A few published documents focus on best practices to improve reproducibility and avoid or minimize bias (Bharti and Grimm, 2021; Bokulich *et al.*, 2020; Knight *et al.*, 2018). Also, several initiatives aim to standardize methodologies used to study the microbiome. For example, the Human Microbiome Project[4] developed standardized methods and protocols for metagenomic analysis (The Human Microbiome Project Consortium, 2012). The International Human

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[3] Here, metabolite annotation means "tentative identification of a metabolite." Also related is ion annotation referring to the "assignation of different metabolic features (adducts, charges, and losses) into a single value" (Godzien *et al.*, 2018).

[4] The Human Microbiome Project, funded by the United States of America National Institute of Health. hmpdacc.org and commonfund.nih.gov/hmp

Microbiome Standards (IHMS),[5] funded by the European Commission, set operating procedures to optimize data quality and allow inter-study comparability. The United States National Institute of Standards and Technology (NIST) is working with stakeholders on several initiatives to educate scientists and standardize analytical methodologies.[6]

5 International Human Microbiome Standards (IHMS) www.microbiome-standards.org/index.php

6 National Institute of Standards and Technology (NIST) – Work on microbiome www.nist.gov/mml/bbd/primary-focus-areas/microbiome (accessed on July 18, 2022).

CHAPTER 4

GUT MICROBIOME, HUMAN AND PHARMACEUTICALS INTERACTIONS

Oral drugs encounter and interact bidirectionally with different microbiota populations along the various sections of the gastrointestinal tract. Drugs can alter the composition and function of the microbiome, and the microbiome can metabolize drugs. Moreover, the host also participates in this interplay, creating a triad of drug–microbiome–host interactions. The host can be affected as a result of the drug–microbiome interaction. Moreover, oral drugs and drugs administered via non-oral routes (e.g. intravenous) can be metabolized by the host and released to the intestine, where they can further interact with the gut microbiome. The new holistic research area that evaluates the interactions between the microbiome and pharmaceuticals is called pharmacomicrobiomics (Weersma, Zhernakova and Fu, 2020). However, as this review focuses on veterinary drug residues, the effect of drugs administered via non-oral routes of exposure is beyond the scope of this report and will not be further discussed.

EFFECTS OF THE MICROBIOME ON DRUGS

The gut microbiome plays a role in xenobiotic transformation, although most genes and enzymes involved in this activity are unknown (Koppel *et al.*, 2018). The microbial enzymatic repertoire includes many enzyme classes (e.g. hydrolases, lyases, oxidoreductases and transferases), which are widely present in gut microorganisms (Koppel, Maini Rekdal and Balskus, 2017). The processes involved in the microbial biotransformation of drugs include hydrolysis, removal of a succinate group, dihydroxylation, acetylation, deacetylation, cleavage of N-oxide bounds, proteolysis, denitration, deconjugation, thiazole ring-opening, deglycosylation and demethylation (Claus, Guillou and Ellero-Simatos, 2016). The biotransformation of xenobiotics by the gut microbiome and the host differs clearly and can even go in opposite directions. While oxidation and conjugation

processes dominate in the host, reduction and hydrolysis are key processes carried out by the microbiome (Spanogiannopoulos *et al.*, 2016; Wilson and Nicholson, 2017). This microbial activity is pharmacologically and toxicologically relevant. Microbial transformation processes can activate pro-drugs, inactivate drugs, alter the pharmaco- or toxicokinetic of chemicals, modify their bioavailability, and increase or decrease their bioactivity, efficacy and toxic potential (Claus, Guillou and Ellero-Simatos, 2016; Spanogiannopoulos *et al.*, 2016; Weersma, Zhernakova and Fu, 2020). Zimmermann *et al.* (2019) evaluated microbial genes and drug products metabolized by human gut bacteria. They found that about 65 percent of the 271 tested human oral drugs are metabolized by at least one of the 76 bacteria strains included in the study. The authors pointed at the microbiome as a contributor to the interindividual variability in the response to pharmaceuticals.

EFFECT OF DRUGS ON THE MICROBIOME

Pharmaceuticals typically have two targets: (1) specifically against organisms (e.g. pathogenic bacteria, viruses, fungi, parasites) by affecting their metabolism, proteins or other components, or (2) targeting the host. In both cases, the microbiome can suffer "collateral damage" resulting from drug exposure (Zimmermann *et al.*, 2021). For example, Maier *et al.* (2018) screened *in vitro* the effect of over 1 000 commercial drugs on 40 human gut bacterial strains. About 78 percent of antibacterial drugs, 53 percent of other antimicrobials and 24 percent of human-targeted drugs (including compounds from all therapeutic classes) inhibited the growth of at least one bacteria strain. The study also revealed that antibiotic-resistant strains were generally more resistant to human-targeted drugs, which might indicate the overlap of resistance mechanisms (Zimmermann *et al.*, 2021). In addition to direct effects, pharmaceuticals can also affect the microbiome indirectly by altering environmental conditions, for example, the gastrointestinal pH, partial pressure of oxygen (pO_2) or increasing gut motility (Zimmermann *et al.*, 2021).

The specific effects posed by pharmaceuticals are diverse and dependent on several factors. The impact of antimicrobial drugs on the microbiome is dose-dependent. It is influenced by the type of drug, length of treatment, activity spectrum, mode of action, drug pharmacokinetics and pharmacodynamics, as well as product formulation (e.g. syrup vs tablets). Drugs do not only alter the microbiome's taxonomical composition and diversity (Table 2 and Annex I), gene expression, protein activity, the overall microbial metabolism and functionality but also affect the selection of resistant genes (Francino, 2016). Consequently, the disturbed microbiome may result in, for example, the alteration of the protective function (e.g. colonization resistance), production of key metabolites, bloom of opportunistic commensal pathogens and selection of antimicrobial-resistant microorganisms.

The effect of therapeutic doses of antimicrobial substances on the human gut microbiome has been previously reviewed by Zimmermann and Curtis (2019). The authors examined studies investigating the faecal microbiota from human subjects and focused on changes in the microbiota composition and diversity, production of

TABLE 2 **EFFECT OF SELECT ANTIBIOTICS ADMINISTERED ORALLY ON THE GASTROINTESTINAL MICROBIOTA**

	AMPICILLIN	CLINDAMYCIN	METRONIDAZOLE	NEOMYCIN	VANCOMYCIN
Spectrum	Gram+, Gram-, Anaerobes	Gram+, Anaerobes	Anaerobes	Gram-, Aerobes	Gram+, Aerobes
Intestinal absorption	Moderate (40–60%)	High (61–100%)	High (61–100%)	Minimal	Minimal
Absorption site	Small intestine	Small intestine	Small intestine	-	-
Impact on microbial diversity	Long-term changes	Long-term changes	Short-term changes	Long-term changes	Long-term changes

Source: Adapted from **Kim, S., Covington, A. & Pamer, E G.** 2017. The intestinal microbiota: Antibiotics, colonization resistance, and enteric pathogens. *Immunological Reviews*, 279(1): 90–105. https://doi.org/10.1111/imr.12563

SFCA and antimicrobial resistance (Annex I and Annex II). A general observation derived from this systematic analysis of the existing scientific literature was that antimicrobials decreased the abundance of beneficial commensal bacteria and increased pathogenic bacterial populations. However, some specific effects were antimicrobial-dependent. Moreover, the impact of therapeutic doses of antibiotics on the human gut microbiota was shown to be dose-dependent, with more significant effects at higher doses (Zimmermann and Curtis, 2019).

Such microbiota shifts after the therapeutic administration of antibiotics are not unexpected. But it is difficult to interpret the implications of microbiota alterations during treatment and changes in the composition after treatment cessation, especially in the absence of symptoms. There are individual capacities to restore the microbiota composition to the baseline. Dethlefsen and Relman (2011) studied the effects of ciprofloxacin on human individuals, and suggested the possibility for the microbiota to recover to an alternative stable state, with unknown consequences. They also suggested that the microbiota functional redundancy in many gastrointestinal microbial strains might explain the absence of gastrointestinal symptoms in the studied subjects.

ANTIMICROBIAL RESISTANCE

The use of antimicrobials in humans and food-producing animals has raised public concern due to the risk of developing antimicrobial resistance, as drugs can promote the selection of resistant bacteria and increase the expression of genes involved in antibiotic resistance (Kim, Covington and Pamer, 2017; Maurice, Haiser and Turnbaugh, 2013). This has led to the development of international, regional and national strategies and monitoring programmes and the publication of guidelines for the proper use of antimicrobials in humans and food-producing animals, e.g. the World Health Organization (WHO) (Aidara-Kane *et al.*, 2018; WHO, 2015), and the World Organisation for Animal Health (OIE) (OIE, 2020). Moreover, antimicrobial resistance is one of the priority topics addressed by One Health (McEwen and Collignon, 2018).

The high microbial density in the gastrointestinal tract, especially in the large intestine, makes it highly susceptible to the transfer of genetic material. It has been

reported that the gene transfer rate in intestinal microbiota is 25 times higher than in other environments (Smillie *et al.*, 2011). Moreover, the gut microbiota has been described as a reservoir of antimicrobial resistance (Hu and Zhu, 2016). Every day, the gastrointestinal tract is exposed to new bacteria from the environment and food, which may carry and potentially transfer antimicrobial resistance genes to the gut microbial population (Economou and Gousia, 2015; Penders *et al.*, 2013).

The susceptibility of bacteria to antimicrobials can be innate or acquired. In the latter, antimicrobial resistance can be developed *de novo* (e.g. mutation) or acquired through the horizontal transfer of genetic material (e.g. plasmids, integrons, and transposons, integrative conjugative elements and genomic islands) from other bacteria via transformation, bacterial conjugation or transduction (Cheng *et al.*, 2019; Hu, Gao and Zhu, 2017). Although the Firmicutes, Bacteroidetes, Actinobacteria are known to be carriers of antibiotic resistance genes, these genes are enriched in Proteobacteria, especially *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Enterobacter cloacae* (Hu *et al.*, 2016). While several studies have pointed out bacteriophages as vectors of antimicrobial resistance genes, evidence indicates that these genes are rarely encoded in phages (Enault *et al.*, 2017). Systematic reviews and meta-analyses evaluating the presence and prevalence of antimicrobial resistance genes have been carried out in humans (Zimmermann and Curtis, 2019) and food-producing animals (Nobrega *et al.*, 2021). Zimmermann and Curtis (2019) concluded that exposure to antibiotics could not only lead to changes in the microbiota composition and diversity but also to the enrichment of the antibiotic resistance trait.

Although the development of antimicrobial resistance has been attributed to the exposure of bacteria to antibiotics, there is evidence that non-antimicrobial substances used in agri-food systems (biocides, antiseptics, preservatives, heavy metals such as copper and zinc) also contribute to antimicrobial resistance, which can be transmitted via the food chain to humans (Wales and Davies, 2015).

Methods to determine the antimicrobial resistance of the gut microbiota have traditionally been based on selective culturing and isolation of specific microorganisms, typically in indicator gut bacteria, followed by antibiotic exposure (Penders *et al.*, 2013). Molecular analyses have employed the targeting of antimicrobial resistance genes in target organisms by PCR. Breakthroughs in next-generation sequencing have made possible the holistic analysis of the resistome[7] using culture-independent and high-throughput analysis, either using targeted PCR permitting the identification of several genes and gene families or holistic metagenomics. Metagenomic analysis can be applied to studying plasmids (plasmidome[8] or mobilome[9]) relevant to antimicrobial resistance. As mentioned previously, metagenomic analysis depends on the abundance of information in

7 Resistome: the repertoire of antimicrobial-resistance genes (Kim and Cha, 2021, p. 301).
8 Plastidome: overall plasmid content in a given environment (Walker, 2012, p. 379).
9 Mobilome: collection of all types of mobile genetic elements (Kim and Cha, 2021, p. 305).

metagenome libraries. In 2019, Hendriksen *et al*. (Hendriksen *et al.*, 2019) identified 47 freely available bioinformatics resources to detect antimicrobial resistance determinants in DNA or amino acid sequence data. They also highlighted that all tools have advantages and disadvantages regarding sensitivity and specificity. The authors also discussed the need to standardize databases.

The study of the resistome using metagenomics is expanding very quickly and attracting much interest due to its benefits (Hendriksen *et al.*, 2019). For example, studying the resistome allows for expanding the currently limited definition of multidrug resistance.[10] It also brings a holistic approach to surveillance programmes. With the support of bioinformatics, it is possible to identify the prevalence and trends of antibiotic resistance genes in a population, the co-resistance to antibiotic and non-antibiotic compounds, co-carriage of specific genes leading to different multidrug resistance patterns, the potential for horizontal transfer, and its distribution by source (Feng *et al.*, 2018; Hendriksen *et al.*, 2019). Moreover, although it is still developing, the application of machine learning to genome sequencing data will enable to predict the antimicrobial resistance as susceptible or resistant and potentially predict the MIC of an antimicrobial (Boolchandani, D'Souza and Dantas, 2019; Hendriksen *et al.*, 2019). A holistic approach to monitor and evaluate antimicrobial resistance in microbiomes would align with recommendations proposed to address the WHO action plan for antimicrobial resistance (WHO, 2015), i.e. establishing or improving systems to monitor antimicrobial use (Magouras *et al.*, 2017).

HEALTH IMPLICATIONS DERIVED FROM DRUG-INDUCED MICROBIOME DISTURBANCES

Most current research and knowledge about the triad interplay of drug–microbiome–human health is mainly derived from sub- or therapeutic drug use in clinical settings. In addition, studies focused primarily on the effects of antibiotics, as they are expected to significantly impact the microbial population (Dethlefsen and Relman, 2011). As discussed later in this review, the long-term implications of veterinary drug residues in food are currently understudied. As mentioned earlier, the gastrointestinal microbiome plays a relevant role in maintaining gut homeostasis and barrier function. Disruption of the gut microbiome caused by antibiotic exposure can decrease colonization resistance. Loss of protection increases the host's susceptibility to infections caused by external pathogens or the overgrowth of opportunistic indigenous pathogens in the microbiota, e.g. *Clostridium difficile* infection (Becattini, Taur and Pamer, 2016; Francino, 2016). Although microbiome disturbances caused by antibiotics have been correlated with transient and long-term biological effects in the host, the clinical implications are mostly unknown (Zimmermann and Curtis, 2019). Antibiotics have been associated with an increased risk of atopic (e.g. asthma, allergy), inflammatory (Crohn's and inflammatory bowel

[10] Multidrug resistance is defined as non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos *et al.*, 2012).

diseases) and autoimmune (e.g. necrotizing enterocolitis) disorders (Zimmermann and Curtis, 2019). There are also reports correlating microbiome disturbances caused by antibiotic exposure with metabolic alterations, increasing the risk of developing metabolic syndrome, obesity, and type 2 diabetes (Francino, 2016). However, additional research is needed to demonstrate causality and to investigate the underlying mechanisms.

How the gut microbiome is shaped early in life seems to play an essential role in the development of physiological processes and the immune system with potential long-term health implications (Salminen *et al.*, 2004). Antibiotic exposure during infancy, especially if applied intermittently, generates concerns about long-term effects later in life, as they may increase the risk for several disorders such as obesity, diabetes and cardiovascular diseases (Queen, Zhang and Sears, 2020; Singer-Englar, Barlow and Mathur, 2019). Different *in vivo* rodent studies have evaluated the effects of antibiotic exposure in pups as early as conception. For example, vancomycin, streptomycin, penicillin, colistin, ampicillin, tylosin (alone or in combination) have been shown to disturb the microbiota and lead to metabolic and immunologic alterations, increasing the risks of obesity and type 1 or type 2 diabetes (Candon *et al.*, 2015; Cho *et al.*, 2012; Cox *et al.*, 2014; Livanos *et al.*, 2016; Mahana *et al.*, 2016). Li *et al.* (2017) suggested that florfenicol and azithromycin may pose a risk for childhood obesity as these antibiotics altered the microbiome and promoted adipogenesis in mice. Neonatal exposure to streptomycin and vancomycin has been correlated with susceptibility to allergic asthma (Russell *et al.*, 2012). Higher risk of bone fractures and alterations to the bone structure have been associated with the potential disruption of the microbiota–gut–bone axis in experiments on neonatal mice treated with a cocktail of antibiotics (vancomycin, neomycin and ampicillin) (Pusceddu *et al.*, 2019).

CHAPTER 5

STUDY OF VETERINARY DRUG RESIDUES AND THE MICROBIOME

The vast majority of studies designed to evaluate the impact of pharmaceuticals on the human gut microbiome have focused on therapeutic doses. Although most antibiotics used in human medicine are also employed as veterinary drugs, some antimicrobials are exclusively used in humans and some only in animals. The impact of drugs on the microbiome of food-producing animals is out of the scope of this report. To date, very few studies have evaluated the potential impact of low concentrations of veterinary drug residues on the human gut microbiome. Most research has been conducted *in vitro*, which investigated a limited number of antimicrobials (enrofloxacin, ciprofloxacin, tetracycline, neomycin, erythromycin, cyadox, tilcomisin, tulathromycin).

IN VITRO STUDIES

Carman's team used a chemostat fermenter in two studies to evaluate residue levels of the fluoroquinolone ciprofloxacin in the human gut microbiota (Carman *et al.*, 2004; Carman and Woodburn, 2001). Both studies used pooled faecal material from healthy human individuals. In the earlier study, the microbiota was exposed to ciprofloxacin concentrations of 0.43, 4.3 and 43 µg/ml (equivalent to human intakes of 0.48, 4.8 and 48 mg/day) for 7 days. Bacterial counts in different selective media were used to evaluate the microbiota. The most relevant finding was the dose-dependent counts of *E. coli*, with reductions starting at the lowest concentration, and *Bacteroides fragilis*, with count reductions at mid and high ciprofloxacin concentrations. No changes in Enterococci counts and the production of SCFA were observed. The study was expanded and complemented later in 2004 with concentrations of 0.1, 0.43 and 5 µg/ml (Carman *et al.*, 2004). The lowest dose was based on the MIC for *Salmonella* Kedougou, used in the study to evaluate the colonization resistance. While there was no colonization of *Salmonella* Kedougou at the lowest dose, the medium dose had the most marked effect on the growth and counts. The authors speculated that the reduction in the counts of *E. coli* by ciprofloxacin observed in the previous study might contribute to the colonization of *S.* Kedougou.

Carman *et al.* (2005) used the chemostat fermenter model of the human colonic ecosystem inoculated with a pooled human faecal suspension to evaluate the NOEL[11] of tetracycline, neomycin, and erythromycin. The doses tested corresponded to faecal concentrations of the antibiotics after oral ingestion of 0, 1.5, 15 and 150 mg/60 kg person/day. The lower values of tetracycline and neomycin were equivalent to the United States Food and Drug Administration acceptable daily intake (ADI) (1.5 and 0.36 mg/60 kg person, respectively, 21CFR 556.720 and 556.430). Microbial counts were carried out in anaerobic culture and specific media. Other microbial parameters evaluated were production of SCFA, bile acid metabolism and enzymatic activity, as well as antimicrobial resistance, tested on sentinel bacteria (*Bacteroides fragilis*, *E. coli* and Enterococci). Changes observed were dose-dependent and not significant at the lowest concentration. Increased resistance of sentinel bacteria was limited, with an apparent increase in *E. coli* at the highest dose of tetracycline. Changes in metabolic activity included reduced bile acid metabolism by neomycin and erythromycin and increased propionate by neomycin. The resulting NOEL values were determined to be 15 mg/60 kg person/day for tetracycline and erythromycin, and 1.5 mg/60 kg person/day for neomycin based on these studies.

Hao *et al.* (2013) treated pooled human faecal material from healthy donors in a chemostat bioreactor with cyadox (a quinoxaline) at 16, 32 and 128 µg/ml for 7 days. The doses selected were based on a preliminary study consisting of the determination of antimicrobial susceptibility (MIC) on four dominant bacterial species (*E. coli*, Enterococci, *Bifidobacterium* and *B. fragilis*). Cyadox metabolites were also included in the preliminary research but not tested in the bioreactor as they did not show antibacterial activity. The impact of cyadox on select cultured microbiota bacteria was dose-dependent, with no effect at the lower dose (16 µg/ml). The higher doses led to increases in resistant *E. coli* and *Enterococcus*. Microbial SCFA were not affected by cyadox. The evaluation of colonization resistance was carried out with *Salmonella* Typhimurium, which could grow at the two highest doses. The *E. coli* strains isolated carried the efflux pump gene (*oqxAB*), which has been associated with antibiotic resistance. The authors used the low dose (16 µg/ml), which was the no observed effect concentration (NOEC), to derive the microbiological ADI (mADI) 1552.03 µg/kg day.

The same research group conducted additional studies to evaluate tilmicosin (Hao *et al.*, 2015) and tulathromycin (Hao *et al.*, 2016). The macrolide tilmicosin was evaluated *in vitro* for 7 days in the chemostat model of the human large bowel ecosystem inoculated with pooled human faecal material. The experimental concentrations were based on levels set by the European Medicines Agency (EMA) ADI and JECFA ADI, 0.436, 4.36 µg/ml, respectively. The highest dose (43.6 µg/ml) was one hundred-fold higher than the lowest. Select bacterial counts (*Escherichia coli*, *Enterococcus*, Bifidobacteria and *Bacteroides fragilis*) were evaluated in

[11] NOEL: No observed effect level.

culture media. Mid- and high doses increased counts of *Bacteroides* and decreased Enterococci. The lowest dose had a marked effect on resistant Enterococci but not on *E. coli*. Only long-term exposure to the high dose tilmicosin led to the upregulation of the macrolide resistance gene *ermB*. SCFAs were also evaluated with no apparent alteration, even at the highest dose of the antibiotics. In light of the results, the authors questioned the safety of the ADI recommended by JECFA (4.36 µg/ml) and EMA (0.436 µg/ml) for the human microbiota.

Later, Hao's group also evaluated the macrolide tulathromycin (Hao *et al.*, 2016). Again, pooled human faecal material from healthy individuals was the source of microbiota used to populate the chemostat. The 7-day exposure to the antibiotic was followed by another 7 days without tulathromycin to evaluate the colonization resistance against *Salmonella* Typhimurium. The low and intermediate doses used (0.1, 1 and 10 µg/ml) were based on the ADI recommended by the United States Food and Drug Administration (FDA) (50 µg/kg bw/day), EMA (10.99 µg/kg bw/day), and the Australian Pesticides and Veterinary Medicines Authority (APVMA) (5 µg/kg bw/day). A higher dose of 100 µg/ml was also included in the study. As in the two previous studies, viable cell counts of the same predominant bacteria were used to evaluate the effects of tulathromycin on the microbiota. Levels of 1 µg/ml and below didn't affect the microbiota and production of SCFAs and were regarded as the no-observable adverse effect concentration (NOAEC) used to derive the mADI (14.66 µg/kg bw/day) according to the Veterinary International Conference on Harmonization VICH GL-36 Guideline (VICH, 2019). The results suggested that only the high concentration of the antibiotic (100 µg/ml) could disrupt the colonization resistance of the gut microbiota and had a positive selection of resistant *E. faecalis*. The majority of isolated strains of *E. faecalis* carried the resistant gene *ermB* (macrolide–lincosamides–streptogramins resistance), the transferable transposon element Tn*1545* and virulence determinant genes *esp* (surface protein), *cyIA* (hemolysin activator) and *ace* (collagen-binding protein). One of the strains with high pathogenicity was identified to increase the risk of horizontal transfer.

Another *in vitro* study in a chemostat bioreactor was conducted to evaluate the effects of 8-day exposure of enrofloxacin residues (of 1.25, 12.5 and 125 µg/ml) on human faecal microbiota (Chen, Li and Wei, 2014). Bacterial counts of select isolates and microbial diversity were the parameters used to monitor the antimicrobial effects. Diversity was evaluated by amplification of the V3 region of the 16S ribosomal RNA gene, followed by denaturing gradient gel electrophoresis (DGGE). A dose-response effect was observed in the count of select bacteria (only affecting *Bifidobacterium* at the lowest enrofloxacin concentration), while diversity changed at all doses. At the end of the exposure period, the high dose led to changes in all bacteria evaluated, with an increase in *E. coli*, total aerobic and total anaerobic bacteria, and decreased *Lactobacillus*, *Bifidobacterium*, Enterococci, and *B. fragilis*. The decrease of only *Bifidobacterium* counts at the lowest concentration tested does not seem to be sufficient to explain the changes in microbial diversity. This illustrates the limited use of specific bacterial isolates to represent the entire microbiota in the evaluation of drug residues. Perrin-Guyomard *et al.* (2021) didn't find total bacterial counts

useful to determine alterations of the intestinal microbiota due to the variability and lack of sensitivity of this parameter. Chen, Li and Wei (2014) also evaluated the susceptibility of bacterial isolates from the chemostat to the enrofloxacin metabolite ciprofloxacin (1, 2 and 4 and 4, 16 and 32 µg/ml). Results showed that increasing enrofloxacin concentrations boosted the microbial resistance to ciprofloxacin. Colonization resistance was tested with *Candida albicans*, which diminished with the low and high enrofloxacin concentrations. No other microbial functional parameters were evaluated in this study.

Kim *et al.* (2012) also evaluated enrofloxacin, using a wider dose range than Chen, Li and Wei (2014), from low residue levels to therapeutical concentrations (0.1, 0.5, 1, 5, 10, 15, 25, 50 and 150 µg/ml). This study differed from the other studies discussed so far because it was carried out under anaerobic culture conditions for only 18 hours and used non-pooled suspensions of faecal microbiota from three healthy individuals. The evaluation by DGGE and pyrosequencing of the V3 region of the 16S rRNA gene showed interindividual variation and dose-dependent effects on the composition of the gut microbiota, which was more apparent at doses over 15 µg/ml. In general, there was a decrease in the abundance of members of the phyla Bacteroidetes and Proteobacteria, while Firmicutes increased. The authors acknowledged the difficulty in estimating the potential health effects of the microbiota disruption observed *in vitro*. Also, the experimental concentrations of enrofloxacin in the *in vitro* model would be lower in the colon due to food processing bioavailability, absorption and metabolism. No functional microbial parameters were evaluated in this study.

Ahn *et al.* (2012a) investigated the effects of enrofloxacin (0.06, 0.1, 1 and 5 µg/ml) on pooled human faecal suspensions (25 percent w/v cultured under anaerobic conditions for 24 and 48 hours) and microbial metabolic profiles. The microbiota was evaluated using viable counts of select bacteria (*Bacteroides* sp., *Bifidobacterium* spp, *Fusobacterium* spp and *E. coli*) and sequencing of the 16S rRNA (V3) gene amplicon. No changes in the microbiota were observed at enrofloxacin concentrations of 1 µg/ml or below. The authors suspected that the fraction of enrofloxacin available to the gut microbiota was significantly lower due to adsorption of the antibiotic to faecal material, which has been shown to be about 50 percent in previous studies (Ahn *et al.*, 2012b). The metabolomics study by NMR spectroscopy, combined with multivariate statistical analysis, seemed to be more sensitive than the techniques mentioned above, with significant differences at 1 µg/ml enrofloxacin, affecting primarily the SCFA 2-oxovalerate and the amino acids leucine, proline and phenylalanine (Ahn *et al.*, 2012a).

Jung *et al.* (2018) evaluated tetracycline (0.15, 1.5, 15, and 150 µg/ml) using an *in vitro* batch culture of unpooled faecal slurries from three healthy individuals. The lowest dose was equivalent to the United States Food and Drug Administration ADI (25 µg/kg bw/day). Exposure to tetracycline resulted in interindividual variation of the ratio Firmicutes/Bacteroidetes and slight variation in the microbiota composition, affecting the genus *Bacteroides* at all doses tested (as shown by evaluating the regions V1–V3 of the 16S rRNA gene). *Clostridium* was only elevated in one individual.

Four resistance genes (*tetO*, *Q, W, X*) were detected in control and samples, with a variable and slight increase depending on the dose and exposure duration. The authors concluded that tetracycline could have minimal or subtle effects at or below the mADI. However, because of interindividual variability, similar studies will require a higher number of faecal samples to increase the statistical robustness. In a previous study, the team showed that tetracycline could disrupt *in vitro* the integrity of epithelial cells (T84) starting at 1.5 µg/ml (Gokulan *et al.*, 2017). Moreover, they observed the translocation of labelled bacteria from apical to basal compartments, which was interpreted as a sign of intestinal barrier disruption.

Other studies have used pooled faecal material from healthy donors as starting material to isolate specific bacteria in selective media to determine the MIC. For example, Jeong *et al.* (2009) isolated the 10 predominant bacteria (10 strains each *B. fragilis*, other *Bacteroides* spp., *Fusobacterium* spp., *Bifidobacterium* spp, *Eubacterium* spp., *Clostridium* spp., *Peptococcus, Peptrostreptococcus* spp., *Enterococcus* spp., *Lactobacillus* spp. and *E. coli*) to determine the NOEC and the ADI of 4 antimicrobials. According to the most susceptible bacteria species, the observed NOEC and ADI, respectively, were: (1) 0.008 µg/ml and 0.15 µg/kg bw/day for ciprofloxacin (most susceptible species: *E. coli*); (2) 0.25 µg/ml and 1 µg/kg bw/day for flavomycin (most susceptible species: *Fusobacterium* and *Lactobacillus*), (3) 0.125 µg/ml and 3 µg/kg bw/day for olaquindox (most susceptible species: *Eubacterium* and *Fusobacterium*); and (4) 1.0 µg/ml and 7 µg/kg bw/day for colistin sulfate (most susceptible species: *E. coli*).

Due to the nature of the *in vitro* studies described above, the models cannot consider interactions with the host. The models used in these studies are basic, batch culture and chemostat. More complex *in vitro* bioreactors (e.g. SHIME® or M-SHIME®) alone or in combination with cell cultures (e.g. T84) would allow investigating into other relevant parameters as these models are designed to mimic, with limitations, the different environmental conditions of the gastrointestinal tract. The inclusion of factors like the digesta volume and passage time, drug sorption to the intestinal content, and drug abiotic or biotic degradation would affect the concentration of the drug under normal *in vivo* conditions, and therefore the degree of exposure of the microbiota to those compounds. In addition, most studies still rely on classical microbiological methods, e.g. monitoring bacterial growth in specific culture media, count of viable cells, and a few studies incorporate modern technologies like gene sequencing. The functional aspect of the microbiome is almost limited to the study of SCFA production. Only Ahn *et al.* (2012a) used a metabolomics approach to monitor the impact of enrofloxacin on the microbiome. These studies illustrate the lack of holistic consideration of the microbiome in the safety assessment of veterinary drug residues.

The selection of relevant antibiotic concentration ranges has permitted the demonstration of the dose-effect relationship, allowing the determination of NOEC or NOEL. From these studies, it is not possible to estimate whether the changes in the evaluated microbiota parameters are biologically relevant or not. Also, treatment periods used in these studies, with a maximum of 8 days, are not suitable to mimic

chronic exposure. The challenges and limitations of microbiome data obtained from *in vitro* testing for use in the risk assessment of veterinary drug residues will be discussed later under the section Potential of the gut microbiome in the assessment of veterinary drugs.

IN VIVO STUDIES

Although the primary purpose of this review focuses mainly on veterinary drug residues, several findings from the literature search included *in vivo* studies related to the clinical context and the use of therapeutic or sub-therapeutical doses. Because the queries did not cover terms related to human medicine, the number of references discussed in this section is not comprehensive. Still, we will use the studies as examples to illustrate the impact of drugs on the gut microbiome and their potential implications on the host's health. Since our focus is on residues of veterinary drugs, we have excluded studies of drugs used exclusively in human medicine. All *in vivo* studies included in this section were conducted in rodent models (rats and mice). Tables summarizing *in vivo* studies are contained in Annex III (antimicrobials, glucocorticosteroids production aids) and Annex IV (insecticides).

ANTIMICROBIALS

Two studies conducted by Perrin-Guyomard's research team evaluated the impact of tetracycline (Perrin-Guyomard *et al.*, 2001) and ciprofloxacin (Perrin-Guyomard *et al.*, 2005) residues in a human flora-associated mice model. This model consists of a germ-free mouse strain that receives a transplant of pooled human faecal microbiota. Because of differences in the physiological conditions of the intestinal tract between humans and mice, it is important to monitor the human faecal microbiota for potential changes that may occur during its establishment in the mice' gastrointestinal tract. The antibiotic exposure (*ad libitum* in drinking water) commenced once the microbiota was established. Both studies evaluated changes in culturable bacteria isolated from faecal material as well as the MIC, metabolic parameters (enzymes and SCFAs) and barrier effect. In the earlier study, Perrin-Guyomard *et al.* (2001) evaluated tetracycline in two trials (six and eight weeks) at 1, 10 and 100 mg/L. The low dose corresponds to 0.125 mg/kg bw/day, while the high dose is approximately half the human therapeutic dose (24 mg/kg bw/day). Positive selection of resistant bacteria (Gram-positive anaerobes, *Bacteroides fragilis*, Enterobacteria and Enterococci) was observed at the two higher doses, while similar effects in a slight and transient manner were observed in female mice at 1 mg/L. Perrin-Guyomard indicated the usefulness of the emergence of resistant bacteria as a sensitive endpoint. Resistance to the colonization by *Salmonella* Scharzendrung was affected only at the highest dose. Tetracycline did not alter the metabolic parameters. The NOEL for tetracycline was less than 1 mg/L (0.125 mg/kg bw/day). The same doses (0.125, 1.25 and 12.5 mg/kg bw) and endpoints were used to evaluate ciprofloxacin in the second study, although the treatment period was slightly shorter (five weeks) and all mice were females

(Perrin-Guyomard *et al.*, 2005). In this case, all doses tested reduced aerobic bacteria, mainly Enterobacteriaceae, and selected resistant *Bacteroides fragilis* at the highest dose. Colonization resistance against *Salmonella* Typhimurium was disturbed by ciprofloxacin treatments. Like in the previous study, metabolic parameters were not affected at the doses tested. The NOEL for ciprofloxacin was less than 0.125 mg/kg bw. Similar results were observed in a human-flora-associated rat model (germ-free Sprague-Dawley) exposed to daily doses of 0, 0.25, 2.5 and 25 mg/kg bw ciprofloxacin for five weeks (Perrin-Guyomard *et al.*, 2006). All doses reduced aerobic populations, while only the highest concentration depleted Enterobacteriaceae, reduced Bifidobacteria, and led to the selection of resistant *B. fragilis*. Colonization by *Salmonella* Typhimurium was observed at the highest dose of 25 mg/kg bw. Microbiota alterations were reversed after the treatment stopped. None of these studies monitored host-related parameters.

The rest of the studies in this section were designed to assess antibiotics in the clinical context and not antimicrobial residues of relevance for food safety. The following studies evaluate mostly single therapeutic or sub-therapeutic doses and include host parameters. These studies were conducted primarily in C57BL/6 mice, but other mouse strains and rats were also used. Several of these studies evaluate the impact of early antibiotic exposure as a risk factor for developing immune and metabolic disorders such as diabetes and obesity. Moreover, antibiotic cocktails are also investigated, with particular attention paid to the development of *Clostridium difficile* infection. Other studies also assess the impact of antibiotics on colonization resistance.

As long-term health effects have been associated with early exposure to antibiotics, including the development of non-communicable diseases, several studies have evaluated the potential involvement of the gut microbiome in this type of disorders. A NOD/ShiLtJ mouse model was used to assess the possible relationship between the early exposure to a continuous low dose of penicillin V (1 mg/kg bw/day) or an intermittent therapeutic dose of the macrolide tylosin tartrate (50 mg/kg bw/day) and the development of type 1 diabetes (Livanos *et al.*, 2016). While the high dose of tylosin significantly impacted the microbiota of male mice with the almost complete absence of ileal and caecal Bacteroidetes, Actinobacteria and *Bifidobacterium* and increased risk of type 1 diabetes, the microbiota composition at the low dose of penicillin V did not differ from controls. In a different study conducted in C57BL/6J mice, the same therapeutic dose of 1 mg/kg bw/day of several antibiotics (penicillin, vancomycin, penicillin plus vancomycin, or chlortetracycline) did not change the microbial census, although there was an increase in Firmicutes and bloom of Lachnospiraceae (Cho *et al.*, 2012). Antibiotic exposure also led to the perturbation of the microbiome metabolic activity as demonstrated by the substantial increase of caecal SCFA acetate, propionate and butyrate. According to the authors, such an increase could explain the induction of adiposity observed in mice. In addition, the genomic evaluation of liver samples showed alterations in the metabolic pathways of fatty acids and lipids. Cox *et al.* (2014) found that early exposure of C57BL/6J mice to 1 mg/kg bw/day penicillin for 30 days led to a transient modification of the gut microbiota composition, negatively affecting the relative abundance of *Lactobacillus,*

Candidatus Arthromitus, Rikenellaceae and *Allobaculum*. These microorganisms showed a positive correlation with markers of ileal immunity and were identified by the authors as potentially protective against weight gain. Cox *et al.* (2014) indicated that although the microbiota recovered, the microbial-induced metabolic effects and body composition remained. The differences in models, exposure time and ages, sample (ileum, caecal content and faecal pellets), and analysis of the microbiota (e.g. different target regions of the 16S rRNA gene) accounted for some of the differences observed between these studies. Like Cox *et al.* (2014), Mahana *et al.* (2016) investigated the link between the microbiome and adiposity in a study in C57BL/6 mice pups exposed to 6.8 mg/L penicillin G from gestation to the end of the 32-week study, with a high-fat diet starting at week 13. This study also showed positive associations between *Candidatus* Arthromitus and *Allobaculum* and body composition and adiposity, while different *Lactobacillus* could be positively associated with either lean or fat phenotypes. The penicillin treatment group showed increased adiposity and showed insulin resistance. Mahana *et al.* (2016) concluded that the delayed development of the gut microbiome following early-life exposure to the penicillin G is associated with an increased risk for metabolic disorders later in life, including diabetes type 2 and non-alcoholic fatty liver disease.

Hou *et al.* (2019) evaluated a human-equivalent therapeutic dose of doxycycline (15 mg/kg/day in drinking water) and a low dose (1 mg/kg/day) in male C57BL/6J mice from gestation to seven weeks of age. The sequencing of the V3 and V4 regions of the 16S rRNA gene revealed a reduced richness of the gut microbiota, with changes at genus levels, affecting *Candidatus* Saccharimonas, *Ruminococcus*, *Helicobacter* and *Anaeroplasma*, which were more evident at the high dose. The authors associated the early exposure to low doses of doxycycline with an increased risk of obesity. They also pointed out changes in the microbiota composition and potentially its metabolic activity as causes for the host's weight gain after early exposure to doxycycline. However, they didn't provide evidence to confirm this hypothesis.

C57BL/6 mice receiving 1 g/L ampicillin or erythromycin for five weeks had reduced microbial diversity in faecal samples (Bech-Nielsen *et al.*, 2012). Antibiotic exposure changed glucose metabolism (ampicillin improved glucose tolerance), which is attributed to changes in the microbiome, and showed no immunological alterations in the gut. There is an indication that improved glucose tolerance induced by early exposure to certain antibiotics may be related to the reduction of LPS, a bacterial compound that has been shown to promote insulin resistance (Rune *et al.*, 2013). Antibiotics applied early in life may increase intestinal permeability in the pre-weaning period, allowing LPS to reach the plasma in higher concentrations. This may result in increased glucose tolerance, an effect not seen when antibiotic exposure occurs later in life. Therefore, glucose tolerance would improve by reducing or eliminating LPS-producing bacteria, as Rune *et al.* (2013) hypothesized. The research group intermittently treated C57BL/6NTac mice from birth for 17 weeks with a dose of 1 g/L ampicillin and a high-fat diet. The faecal microbiota was disturbed during exposure to ampicillin, and mice showed improved glucose tolerance compared to the control group. Microbiota alterations and glucose tolerance disappeared in the absence of antibiotics.

Non-obese diabetic mice (NOD) were treated chronically with vancomycin (0.2 mg/ml) and a mixture of broad-spectrum antibiotics (5 mg/ml streptomycin, 1 mg/ml colistin and 1 mg/ml ampicillin in drinking water) from conception (via dams) through adulthood (40 weeks total) (Candon *et al.*, 2015). The 16S rRNA gene profiling showed profound alterations of the gut microbiota, with almost complete disappearance caused by the antibiotic mixture. Vancomycin led to a decrease in Clostridiales, Lachnospiraceae, Prevotellaceae and Rikenellaceae, and an increase in *Escherichia* and *Suterella* and *Lactobacillus*. Early exposure to these antibiotics increased the incidence of type 1 diabetes.

Early exposure to antibiotics has also been used to evaluate its impact on bone structure. A cocktail of vancomycin (0.5 mg/ml), neomycin (1 mg/ml) and ampicillin (1 mg/ml) was given to C57BL/6J dams in the drinking water and by gavage to pups (male and females) for 16 days (pre- and post-weaning) (Pusceddu *et al.*, 2019). The antibiotics were selected based on their high prescription frequency and poor intestinal absorption. The microbiota richness and diversity were reduced in the treatment groups. Changes to the microbiota composition were gender-dependent at the phylum level. In males, Firmicutes were increased, while Bacteroidetes and Actinobacteria disappeared. However, in females, Proteobacteria and Tenericutes were the most abundant. At the genus level, the relative abundance of *Bacteroides* and *Lactobacillus* was lower, and Paenibacillaceae and *Bacillus* were higher. The treated mice presented increased colonic permeability with the absence of inflammation. Males showed decreased bone structural features, while females presented alterations in the mineral distribution, which is associated with high bone fracture risk. The authors could only speculate about the potential role of the gut microbiome on the host alterations. They acknowledged the need for additional research to understand the potential role of the gut microbiome in bone health and disease. Such research would contribute to the knowledge about the microbiota–gut–bone axis (Sjögren *et al.*, 2012).

A similar antibiotic cocktail (vancomycin, ampicillin, neomycin, and metronidazole) was given in the drinking water to adult C57B6 mice for 2 weeks followed by a clearance period of 9 weeks or 11 weeks to monitor fungal and bacterial gut populations at different time points in faecal pellets (Dollive *et al.*, 2013). This study focused exclusively on the microbiota composition and did not monitor any other microbial functional variable or host parameter. The abundance of the bacterial population, initially more than 3 to 4 orders of magnitude larger than the mycobiome, decreased over 3 orders of magnitude after antibiotic treatment. The fungal population bloomed with the antibiotic treatment, increasing 40 times with significant structure alterations. After the treatment, bacteria richness returned to pre-treatment values, while there were some fluctuations in the bacteria community structures. Major bacteria groups recovered, although at different rates. For example, Lachnospiraceae and *Clostridium* returned to normal within a week, and Bacteroidales had not returned to normal by the end of the experimental period. Mycobial community also recovered, although, at the end of the study, *Candida* remained more abundant than before the antibiotic treatment.

Loss of colonization resistance to *Clostridium difficile* and consequent severe colitis (or even death) has been associated with alterations of the gut microbiota, dominated by Proteobacteria, after 3-day oral exposure to an antibiotic cocktail (0.4 mg/ml kanamycin, 0.035 mg/ml gentamicin, 850 U/ml colistin, 0.215 mg/ml metronidazole and 0.045 mg/ml vancomycin) or 10 days of 0.5 mg/ml cefoperazone in drinking water in a C57BL/6 mouse model (Reeves *et al.*, 2011). Animals received an intraperitoneal dose of clindamycin before the challenge with *C. difficile*. Proteobacteria dominated in clinically ill animals (primarily the cefoperazone group), showing severe colitis. Although the microbiota of these animals recovered after the treatment, the structure remained different from the baseline. The microbiota composition of animals exposed to the cocktail that remained clinically well (with less severe colitis) appeared to return to baseline structure. The microbiota of these animals was more similar to the control group than to the severely ill mice, with Firmicutes as the predominant phylum. Kim, Wang and Sun (2016) also evaluated the colonization of *C. difficile* using the same antibiotic cocktail in C57BL/c mice but added another treatment group receiving a combination of the antibiotic cocktail with dexamethasone (100 mg/L in drinking water). Both drug types have been identified as risk factors for *C. difficile* infection. Both treatments affected the diversity of faecal microbiota, with a remarkable decrease in the relative abundance of *Lactobacillus* and increased *Parabacteroides*. After treatment cessation, the proportion of both genera reverted. The microbial diversity increased within days post-treatment, but was delayed in the group also exposed to dexamethasone. The authors speculated the role of the immunosuppressive drug in the slower microbiota recovery, which might explain the more pronounced severity of infection observed in this group after being challenged with *C. difficile*.

O'Loughlin *et al.* (2015) evaluated the colonization resistance against *Campylobacter jejuni* in ampicillin-treated adult female CBA/J mice (0.2 mg by oral gavage, 24 and 48 hours prior to inoculation). Inoculation with *C. jejuni* led to colonization (recovered from the colon, mesenteric lymph nodes and spleen). The authors identified *Enterococcus faecalis* as a major member of the microbiota capable of inhibiting the pathogen colonization. Treatment led to a decrease in Firmicutes and a shift of the microbiota towards Bacteroidetes, which was correlated with a disruption of colonization resistance against *C. jejuni*. The authors also determined the "core microbiota" composition shared by all non-treated animals (9 genera, including *Clostridium*_XIVa and _XVIII, Lachnospiracea and *Roseburia*) or all ampicillin-treated animals (5 genera, including *Lactobacillus*, *Clostridium*_XIVa and *Enterococcus*).

Exposure of young C57BL/6 mice to 5 mg/kg/day florfenicol or azithromycin for 4 weeks reduced the richness and diversity of the colonic microbiota and increased the ratio of Firmicutes/Bacteroidetes (Li *et al.*, 2017). Firmicutes were higher in males than females in both antibiotic treatments. Both antibiotics reduced the relative abundance of genera *Alistipes*, *Desulfovibrio*, *Parasutterella* and *Rikenella*. However, other changes were antibiotic-specific. Florfenicol increased the abundance of phyla Verrucomicrobia and reduced the abundance of Deferribacteres and genera

Christensenella, *Gordonibacter* and *Anaerotruncus*, while azithromycin reduced Bacteroidetes, Proteobacteria and the genus *Lactobacillus*. The production of overall SCFA and secondary bile acids, both important microbial metabolites, decreased with both antibiotics. This study did not evaluate any mouse parameters other than body weight and body fat, which were higher in the treatment groups and males. Based on their findings, the authors suggested a risk for obesity in children exposed to both florfenicol and azithromycin.

Another study with florfenicol was conducted to evaluate the effects of florfenicol on the intestinal barrier of the jejunum in adult KM mice at a 100 mg/kg bw (equivalent to the prophylactic dose used in chickens) for 7 days (Yun *et al.*, 2020). Although microbiota diversity was not affected, exposure to florfenicol induced changes in the microbial community structure, with a reduced relative abundance of phylum Firmicutes. At the genus level, *Lactobacillus* and *Allobaculum* were reduced, while *Bacteroides*, *Alistipes*, *Alloprevotella*, among others, increased. These changes were observed parallel to the severe epithelial damage and altered expression of proteins involved in maintaining tight junctions and cytokines involved in maintaining gut homeostasis. These findings were indicative of decreased intestinal barrier function and compromised intestinal immunity.

McCracken *et al.* (2001) evaluated the effect of a 14-day exposure to 25 mg/L cefoxitin in drinking water and different diet types (standard vs low residue, no fibre) on the faecal microbiota from C57BL/6NHsd mice. Analysis of the V3 region of the 16S rRNA gene by PCR-DGGE revealed no change in the microbial diversity and richness. However, the microbiota composition was altered by the antibiotic in all treatment groups. The diet itself had higher impact on the microbiota with the low fibre diet leading to more pronounced effects than the standard chow. No other parameters were evaluated either in the mouse or the host.

Zhang *et al.* (2018) evaluated relatively high doses of the macrolide roxithromycin (30 mg/kg bw) for 14 days in a Sprague-Dawley rat model. The evaluation of the microbiota from the small intestine and the cecum by 16S rRNA gene sequencing (regions V3 to V4) showed reduced diversity of the caecal microbiota. The microbiota composition, especially Gram-positive bacteria, was affected at both intestinal locations with a decreased relative abundance of *Bifidobacterium* and *Clostridium* species. However, there were also location-specific findings. In the cecum, the relative abundance of Gram-negative bacteria, *Bacteroides* and Enterobacteriaceae was increased, while *Streptococcus* and *Prevotella* were inhibited. In the small intestine, Gram-negative, Gram-positive and the relative abundance of *Enterococcus* was increased. The gene expression analysis of colonic epithelial cells showed the down-regulation of genes related to the metabolism of xenobiotics by P450, which may indicate the decreased metabolism of roxithromycin and prolonged exposure of the microbiota. Other genes related to the immune and healing responses were also altered, with the potential for increased risk of fibrosis. However, it was not possible to prove whether any changes in gene expression were due to the altered microbiota or the direct effect of roxithromycin in the host.

GLUCOCORTICOSTEROIDS AND PRODUCTION AIDS

This section contains studies involving the evaluation of non-antimicrobial drugs. Wistar rats were gavaged with dexamethasone (0.01 and 0.05 mg/kg bw/day) for 7 weeks to investigate the potential effects of chronic glucocorticoid treatment on the host physiology and the microbiota (Wu *et al.*, 2018). Caecal microbiota was evaluated by sequencing the regions V3 to V4 of the 16S rRNA gene. It showed that treatment decreased microbial abundance and diversity, with a decreased abundance of phyla Firmicutes, Bacteroidetes α-Proteobacteria, γ-Proteobacteria, and Actinobacteria, as well as the lower order Clostridiales and *Lactobacillus*. The effects observed in parallel in the host included decreased mucus secretion in the colon and increased expression of antimicrobial peptide genes. Dexamethasone also slowed weight gain, reduced feed intake, increased fat accumulation, and altered the circadian rhythm, glycolipid and energy metabolism.

Javurek *et al.* (2016) evaluated ethinyl estradiol in two generations (F0 and F1) of male and female California mice. Only F0 received a daily dose of 0.1 µg/kg ethinyl estradiol in the diet starting 2 weeks before breeding (through gestation and lactation) until postnatal day 30 (weaning). Contrary to the mice used in other studies (e.g. C57BL/6), California mice are outbred. In addition, they are monogamous and biparental. According to the authors, they selected this mouse model because all these features are representative of the majority of human societies. This study focused exclusively on faecal microbiota composition, evaluated by 16S rRNA (region V4) gene sequencing. Exposure to ethinyl estradiol changed the microbiota composition in gender and generational-dependent manner. However, the control group also showed generational and gender-dependent changes. The microbial function was predicted based on the 16S rRNA gene data using the PICRUSt[12] tool and the KEGG[13] pathway database, which are used to correlate the relative abundance of genera with their predictive metabolic function. However, the authors acknowledged that they could not link microbiota changes to phenotypic or molecular alterations. They also discussed the need to test several hormone doses in future research.

INSECTICIDE RESIDUES

Several pesticides are used in veterinary medicine as insecticides that can end up as residues in food products of animal origin, e.g. meat, eggs and milk (Dallegrave *et al.*, 2018; LeDoux, 2011). The information contained in this section has been reported in more detail in the FAO review "The impact of pesticide residues on the gut microbiome and human health. A food safety perspective". Findings are summarized in the table of Annex IV.

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12 Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.

13 Kyoto encyclopedia of genes and genomes [KEGG] https://www.genome.jp/kegg/ (accessed on July 25, 2022).

The effect of chlorpyrifos and deltamethrin on the gut microbiota has been assessed *in vivo* and *in vitro* models. Chlorpyrifos has been evaluated *in vitro* in SHIME bioreactors or combined with Caco-2/TC7 cell cultures at concentrations of 1 or 3.5 mg/day for periods ranging between 15 and 30 days (Joly *et al.*, 2013; Joly Condette *et al.*, 2015; Requile *et al.*, 2018; Reygner *et al.*, 2016a). All studies reported a decrease in the relative abundance of *Bifidobacterium* and *Lactobacillus,* and most observed an increase in *Bacteroides*. Deltamethrin (21 mg/ml) was also evaluated *in vitro* using a fermenter (24 hours), followed by the evaluation of fermenter supernatants in Caco-TC7 cell culture (4 hours) (Defois *et al.*, 2018). In this case, the microbiota composition was not evaluated, but its function by analysing the microbial volatolome and metatranscriptome, which showed functional dysbiosis. Exposure of cell culture to chlorpyrifos or deltamethrin led to pro-inflammatory responses (Defois *et al.*, 2018; Requile *et al.*, 2018). Chlorpyrifos also altered the mucosal barrier activity (Requile *et al.*, 2018).

Although chlorpyrifos has been evaluated primarily on Wistar rats, mice (C57BL/6 and KM) have also been used as model animals. The various studies differed in many aspects, including doses, exposure periods, gender and age. They were designed to address different aspects concerning the host, i.e. early development, endocrine function, behaviour and metabolism. Doses ranged from 0.3 to 5 mg/kg bw/day and exposure periods between 6 and 25 weeks. All studies reported alterations in the microbiota composition. Although different studies reported differences in the affected microbial groups, most of them aligned with the findings from *in vitro* studies (increased relative abundance of *Bacteroides* and decreased *Bifidobacterium* and *Lactobacillus*). Some authors suggested the potential role of chlorpyrifos-induced dysbiosis in host alterations, which may result in increased risk for inflammatory and metabolic diseases (e.g. diabetes and obesity) (Fang *et al.*, 2018; Liang *et al.*, 2019; Reygner *et al.*, 2016b), altered intestinal function (Joly Condette *et al.*, 2015; Zhao *et al.*, 2016), altered endocrine function (Li *et al.*, 2019), and neurological disorders (Li *et al.*, 2019; Perez-Fernandez *et al.*, 2020).

CHAPTER 6
GUT MICROBIOME AND HEALTH EFFECTS

Of all *in vivo* studies presented above, only three of them, carried out by the same research group (Perrin-Guyomard *et al*., 2001; Perrin-Guyomard *et al*., 2005; Perrin-Guyomard *et al*., 2006), were designed to evaluate veterinary drug residues (tetracycline and ciprofloxacin) in the context of food safety. As such, they evaluated the chronic exposure to ranges of doses, including therapeutical levels at the high end, making possible to show dose-dependent effects on microbiota composition, selection of resistant bacteria species and disruption of the colonization barrier by pathogenic *Salmonella* strains. Such studies did not evaluate host parameters, and microbial metabolic activity (SCFA and enzymes) changed slightly or not at all.

The rest of the studies were designed to respond to clinical questions, which entailed evaluating one or two therapeutical or sub-therapeutical doses of individual antimicrobials or commonly used mixtures of antibiotics and administered continuously or intermittently. Considering the concern about the implications of early exposure to antibiotics in the maturation of the gut microbiota and long-term health effects, it is not unexpected to see a high number of studies targeting this topic. Nine of these studies focused on evaluating the impact of early exposure to antimicrobials on the microbiota and immune or metabolic alterations, primarily focusing on the development of type 1 and 2 diabetes and obesity (Bech-Nielsen *et al.*, 2012 ; Candon *et al.*, 2015 ; Cho *et al.*, 2012; Cox *et al*., 2014; Hou *et al*., 2019; Livanos *et al*., 2016; Mahana *et al*.,2016; Pusceddu et al., 2019; Rune et al. 2013). Although most studies report more or less profound alterations of the microbiome, their contribution to the adverse effects observed in the host, without clarifying the mechanisms involved, is speculative. In any case, the authors of those investigations reported that early antibiotic exposure increased the risk of metabolic type 1 diabetes (Candon *et al*., 2015; Livanos *et al*., 2016), metabolic disorders (Mahana *et al*., 2016, in the context of a high-fat diet), obesity (Hou *et al*., 2019), and risk of bone fracture in females (Pusceddu *et al*., 2019). However, the outcomes are not always negative. Rune *et al*. (2013) observed improved glucose tolerance in mice fed with a high-fat diet, suggesting a reduction in bacterial LPS as a possible cause.

The impact of early exposure to non-antimicrobial drugs (glucocorticoids, insecticides) has also been evaluated. Chronic exposure to oral dexamethasone reduced microbial diversity and richness and altered host parameters related to

energy metabolism, with increased fat accumulation in spite of reduced feed intake. Maternal exposure (gestation and lactation) of pups to chlorpyrifos has also been shown to induce bacterial disturbances and alterations of the intestinal barrier (Joly Condette *et al*., 2015), lipid dysregulation and insulin dysregulation, potentially increasing the risk of diabetes mellitus (Reygner *et al.*, 2016b), and motor and cognitive dysfunction (Guardia-Escote *et al*., 2020; Perez-Fernandez *et al*., 2020).

The rest of the studies evaluating antimicrobials mainly used mice models to study single doses of several antimicrobials or drug cocktails and are designed to address different questions. For these reasons, these studies cannot be compared. The risk of developing obesity after antibiotic treatment (florfenicol or azithromycin) has also been reported in adult mice by Li *et al.* (2017). Florfenicol has also been shown to alter the microbiota located in the jejunum and compromise the intestinal barrier and immune function (Yun *et al.*, 2020). However, the involvement of the microbiota has not been clarified by the authors of these two studies. Dollive *et al*. (2013) showed that the reduction of bacterial populations by a broad-spectrum antimicrobial cocktail could lead to overgrowth of the fungal co-habitants. However, more research is needed to assess the cause-effect proposed by the authors. The increased susceptibility to fungal infections (e.g. candidiasis) after broad-spectrum antibiotic treatment, especially in vulnerable patients, has been linked to impaired gut immune response (Drummond *et al*. 2022). Alteration of the microbiota by short-term treatment with antibiotics (cocktails or ampicillin) can jeopardize the colonization resistance and increase the risk of infection by *Clostridium difficile* (Kim, Wang and Sun, 2016; Reeves *et al*., 2011) and *Campylobacter jejuni* (O'Loughlin *et al*., 2015). The severity of the infection seemed to be influenced by the individual's microbiota composition (Reeves *et al*., 2011) or co-treatment of antibiotics with immunosuppressive drugs (dexamethasone [Kim, Wang and Sun, 2016]). In all the studies evaluating pathogen colonization, the microbiota components returned to baseline, although at different speeds, or in severe infections, the microbiota returned to a different baseline.

The effect of diet has also been evaluated as a confounding factor, and shown to have a higher impact on the microbiota composition than cefoxitin treatment (McCracken *et al*., 2001). Although most studies evaluate faecal or caecal microbiota, Zhang *et al.* (2018) observed different effects of roxithromycin on the microbiota composition in samples from the cecum and the small intestine. The authors also reported the downregulation of the P450 pathway, involved in xenobiotic metabolism. If confirmed, this finding could illustrate how reduced antibiotic metabolism could increase the exposure time of the microbiota to the antibiotic.

The authors investigating the effects of the insecticide chlorpyrifos suggested that microbial alterations observed in rodents, might result in increased risk for inflammatory and metabolic diseases (e.g. diabetes and obesity) (Fang *et al.*, 2018; Liang *et al.*, 2019; Reygner *et al.*, 2016b), altered intestinal function (Joly Condette *et al.*, 2015; Zhao *et al.*, 2016), altered endocrine function (Li *et al.*, 2019), and neurological disorders (Li *et al.*, 2019).

Although many of these studies report alterations of the gut microbiota and host effects after oral exposure to drug at levels usually higher than those found in food, more research is needed to evaluate and confirm if the gut microbiome modulates the interaction between diet and development of host health effects and potentially non-communicable diseases.

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CHAPTER 7

THE MICROBIOME IN VETERINARY DRUG RESIDUE RISK ASSESSMENT

Policy recommendations based on conservative risk assessments have been put forward to minimize the risk associated with veterinary drug residues in food (Cerniglia, Pineiro and Kotarski, 2016; Piñeiro and Cerniglia, 2020). Risk assessments have always required a strong toxicological focus. However, as drugs can also affect the gut microbial population, and potentially impact gut homeostasis, new microbiological data are being considered in risk assessments. JECFA follows a step-wise, decision-tree approach to establish the mADI, showed in the formula include in page 45 (VICH, 2019). The first question is to determine if the compound is microbiologically active against bacteria representative of the human intestinal microbiota (*E. coli*, and species of *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Eubacterium*, *Collinsella*, *Fusobacterium*, *Lactobacillus*, *Peptostreptococcus/Peptococcus*), which is typically determined by the MIC.[14] This list of target bacteria can be expanded to include other relevant microbiota members by considering recent research using molecular and metagenomic methods (WHO, 2018). If the compound is active against any of the species listed above, the next steps aim to answer whether residues enter the human colon and remain microbiologically active. If the drug residue does not reach the colon or is microbiologically inactive, then the toxicological or pharmacological ADI is used. But if the compound shows antimicrobial activity against the representative bacteria, the mADI is established based on two endpoints of concern: disruption of the colonization barrier[15] and increase in antimicrobial-resistant[16] bacteria.

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[14] MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. It is different from the minimum bactericidal concentrations (MBCs) as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media (Andrews, 2001).

[15] Colonization barrier is a function of the normal microbiota of the colon (VICH, 2019).

[16] Resistance is defined as the "*increase of the population(s) of bacteria in the intestinal tract that is (are) insensitive to the test drug or other antimicrobial drugs*" (VICH, 2019, p. 5).

The first endpoint is the disruption of the colonization barrier. This endpoint addresses the potential of the veterinary drug residue to induce changes in the bacterial community that can lead to decreased colonization resistance, therefore offering a window of opportunity for exogenous pathogens or opportunistic commensal pathogens to colonize the colon. Data can be obtained from *in vitro* studies, which differ in complexity from cultures of bacterial isolates (used to calculate the MIC_{50})[17] to more complex systems (e.g. bioreactors) used to evaluate bacterial populations of faecal inocula from healthy individuals. The closer the microbial composition of a sample is to the gut microbiota, the more robust the testing system and the more appropriate and relevant the NOAEC[18]. Data can also be obtained *in vivo* using animal models. Complex *in vitro* and *in vivo* systems allow performing challenges with test drug-resistance pathogens to evaluate disruptions to the colonization resistance. These systems also allow for monitoring bacteria function (e.g. production of short-chain fatty acids).

Another *in vitro* model, an alternative to the MIC, used to calculate the mADI of antimicrobials used in food-producing animals has been developed to evaluate the colonization resistance of the intestinal microbiota. The bioassay measures the minimum disruptive concentration (MDC)[19] of the drug (Wagner, Johnson and Cerniglia, 2008). The microbiota model consisted of a mixture of 33 obligate and facultative bacteria strains (obtained from the American type culture collection [ATCC]) present in both the ileum and colon. The authors observed differences when comparing ADIs derived from MIC (CVMP-VICH Safety Working Group, 2004) and MDC. For example, erythromycin, lincomycin and tylosin were higher using the MDC method, while ADIs for apramycin, bacitracin, neomycin, novobiocin, penicillin G, streptomycin, tetracycline and vancomycin were higher using the MIC method. The authors suggested that this model could be used along with animal and bioreactor models to calculate ADIs for antimicrobial drug residues.

The second endpoint considers the potential increase of antimicrobial-resistant bacteria, assessed using *in vitro* or *in vivo* test systems. The increase in antimicrobial-resistant bacteria can originate from acquired resistance or a relative increase in the proportion of organisms that are less sensitive to the tested antimicrobial drug.

Until now, there have not been reports linking changes in the proportion of antimicrobial-resistant bacteria of the normal human microbiota and health effects (VICH, 2019). Although the concept of antimicrobial resistance is defined in the context of the gut microbial population (microbiota), the majority of studies revised by the Joint Committee on Food Additives (JECFA) only consider one

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17 MIC_{50} is the concentration of an antimicrobial compound at which 50 percent of the tested isolates within a relevant genus are inhibited.

18 NOAEC: The highest concentration that was not observed to cause any effect in a particular study. It is derived from the lower 90 percent confidence limit for the mean NOAEC from the *in vitro* systems.

19 MDC is defined as the minimum concentration of an antimicrobial drug that disrupts the colonization resistance mediated by model human intestinal microbiota against *Salmonella* invasion of Caco-2 intestinal cells (Wagner, Johnson and Cerniglia, 2008).

species, *Escherichia coli* (WHO, 2018). In spite of conservative assumptions used throughout the establishment of the mADI (Anadón *et al.*, 2018; Cerniglia, Pineiro and Kotarski, 2016; VICH, 2019), there is evidence that sub-inhibitory levels of antibiotics, a few hundred-fold below the MIC, have the potential to select resistance bacteria and increase the development of antimicrobial resistance through mutations, recombination and horizontal gene transfer (Andersson and Hughes, 2012; Andersson and Hughes, 2014; Liu *et al.*, 2011). Based on these findings, Subirats *et al.* (2019) assessed whether human exposure to antibiotics (based on maximum residue limits [MRLs], ADIs or published concentrations in foods) of different classes (tetracycline, oxytetracycline, ciprofloxacin, sarafloxacin, erythromycin, spiramycin, tilmicosin, tylosin and lincomycin) could exceed the minimal selective concentration (MSC).[20] Based on several assumptions, including the effects of cooking on the drugs, they concluded that estimated concentrations of antibiotic residues in the colon could potentially select for resistant bacteria in the gut microbiota, and recommended the revision of current ADI and MRLs.

The derivation of the mADI from *in vivo* data is obtained by dividing the no-observed-adverse-effect level (NOAEL) by the uncertainty factor, which will depend on the compound class and other factors related to the *in vivo* study (VICH, 2019). The following formula is used to calculate the mADI from *in vitro* data, which is based on NOAEC:

CALCULATION OF mADI FROM *IN VITRO* DATA

$$\text{mADI} = \frac{\text{NOAEC x Mass of colon content (500 ml/day)}}{\text{Fraction of oral dose available to microorganism x 60 kg person}}$$

Source: **VICH.** 2019. *VICH GL36 Studies to evaluate the safety of residues of veterinary drugs in human food: General approach to establish a microbiological ADI - Revision 2.* https://www.ema.europa.eu/documents/scientific-guideline/vich-gl36r2-studies-evaluate-safety-residues-veterinary-drugs-human-food-general-approach-establish_en.pdf

[20] MSC estimates of the lowest concentration of antibiotic that provide resistant bacteria an advantage over susceptible bacteria (Subirats, Domingues and Topp, 2019).

CHAPTER 8

POTENTIAL OF THE GUT MICROBIOME IN THE ASSESSMENT OF VETERINARY DRUGS

The application of risk assessment to identify and manage risks is a tool used to protect public health. Such tools are currently used by WHO/FAO to assess the risk of different compounds, and in the same way, governmental authorities from different countries at the time of the approval of those products. Risk assessment is based on the evaluation of available scientific information, which is quite challenging. It has to deal with new areas of research (e.g. microbiome), data obtained with novel technologies (e.g. omics) and the uncertainty derived from incomplete data sets. Risk assessments and evaluation procedures are dynamic as they evolve with scientific development. For all these reasons, it is a common practice to re-evaluate compounds as new data become available.

The omics revolution has made it possible to tackle microbial ecosystems from a holistic perspective. We are starting to define the membership of large microbial communities and deconvolute the microbiome's complex interactions with its ecosystem. However, it is essential to acknowledge that as omic technologies are relatively new and rapidly evolving, so are their applications to the microbiome. Moreover, the causal role of microbiome disturbances caused by veterinary drug residues on the onset or progression of diseases is either unconfirmed or poorly understood. So, the applicability of the microbiome in chemical safety assessments is still very premature. The reasons will be further discussed in this section.

FROM MICROBIAL ISOLATES TO MICROBIOTA

The determination of the MIC is the most popular *in vitro* test to determine the susceptibility of representative gut individual bacterial isolates to different drugs because it is easier to implement and more cost-effective than other complex *in vitro* or *in vivo* models. Representativeness is challenging to assess. The selection of gut bacteria at the species level may not be sufficient to assess the impact of veterinary

drug residues, since it has been reported that drug metabolism and drug sensitivity is a strain-specific characteristic (Koppel *et al.*, 2018). To this end, the VICH GL36 Guideline recommends including 10 isolates per genus, not per species (VICH, 2019). Microbiota or bacterial isolates for drug residue assessment should be obtained from healthy individuals to avoid bias in the baseline microbiota due to the potential dysbiosis in unhealthy individuals. Moreover, unhealthy individuals may have received treatment, which could also influence the composition and function of the microbiota. Gut bacteria isolates are also available from public collections, e.g. ATCC. However, collections should be further expanded to incorporate species representative of, for example, diet preference (e.g. vegetarian, vegan), geographies, ethnicities, gastrointestinal (GI) locations, etc., and include rare species and strain diversity (Zimmermann *et al.*, 2021). Considering the ongoing efforts in this field, it is expected that microbial collections will continue to expand to incorporate more strains and species that are currently unknown.

One critical point is whether the response of bacterial isolates to drugs is mirrored by the gut microbiota in an *in vivo* system. Currently, it is not clear if a bacterial response to drugs in isolation would differ and how much in the presence of the gut microbial communities at the different environmental niches of the gastrointestinal tract (Zimmermann *et al.*, 2021). It is possible that upon drug exposure and, for example, after drug transformation, the microbial community would confer protection (e.g. collective resistance) (Vega and Gore, 2014) and cross-sensitization (i.e. collateral sensitivity)[21] (Roemhild, Linkevicius and Andersson, 2020). However, these need additional research.

Microbiota can be naturally obtained from humans, but they can also be assembled ("synthetic microbiome"), with defined and well-characterized members resourced from single or pooled material from multiple donors (Zimmermann *et al.*, 2021). Faecal material is the most common microbiota source, although it may not fully represent the microbiota from the gastrointestinal tract, especially of the small intestine or mucosa-associated microbiota (Klymiuk *et al.*, 2021; Sun *et al.*, 2021). Biobanks and stool banks are also available, e.g. OpenBiome, AdvancingBio, the Netherlands Donor Faeces Bank, Metagenopolis or HMGU Biobank (Ryan *et al.*, 2021). Also, standardized fabricated microbial communities, e.g. EcoFABs, have been developed (Zengler *et al.*, 2019). The optimal sampling, preservation and storage of microbiome samples are challenging steps in microbiome analysis, which can influence the accuracy of results. Altering the integrity of the microbiome sample could lead to losing microbial components and changing the functional representativeness.

[21] Collateral sensitivity refers to the resistance mechanisms against a specific antibiotic class that confer increased susceptibility to other antibiotic classes (Roemhild, Linkevicius and Andersson, 2020).

MICROBIOME FUNCTION, GASTROINTESTINAL LOCATION AND HOST IMPACT

The gut microbiome is in a symbiotic (functional) relationship with the host. In other words, functionality is the foundation for the relationship. So, it is not only important to understand which microbes are present in the microbiota but also what they do. Looking only at shifts in the composition and diversity of the microbial population may not be sufficient to predict functional alterations of the microbiome (e.g. alterations of metabolic activity and antibiotic resistance repertoires) and their potential health impact on the host.

The current microbiological assessment of veterinary drug residues focuses primarily on alterations of the microbiota members and puts limited emphasis on the functional aspect of the microbiome. This is also reflected by the lack of functional markers in many studies evaluating the impact of veterinary drug residues on the gut microbiome. The adaptation of the microbiota to the different environmental conditions along the gastrointestinal tract determines its composition and function.

The microbiological endpoints of the veterinary drug residue assessment are limited to the colonic segment of the gastrointestinal tract. Although the colonic microbiota is more abundant, dense, and relatively easier to sample from faecal material, the microbial populations of the small intestine should not be completely neglected. The microbiome of the small intestine – more dynamic, less diverse and abundant than the colonic microbiota (Kastl *et al.*, 2020) – encounters drugs that do not reach the colon because they are absorbed earlier in segments of the intestine. Moreover, the concentration of many drugs is higher in the small intestine (although transit times are shorter).

Another aspect relative to the endpoints of the assessment is that they do not consider potential microbiome-derived effects in the host, locally or at the systemic level. However, the difficulties in clarifying and quantifying the actual role of the microbiome in the host physiology make it challenging to establish microbiome-related endpoints at this point.

ALTERATIONS OF CONCERN OR NORMAL MICROBIAL FLUCTUATION

Another challenge surrounding the study of the microbiome is the interpretation of alterations and their biological relevance. The microbiome is very sensitive to environmental changes, and it responds very quickly to adapt to new conditions. Identifying when statistical microbial alterations are biologically relevant for the microbiome and the host is challenging. It is also important to measure the dimension of a biologically relevant event. To overcome these challenges, it is necessary to:

> Define a healthy microbiome;

> Define or clarify what constitutes a microbiome-related adverse event; and

> Identify, develop and validate measurable microbiome-associated biomarkers, including limits to flag alterations of concern when evaluating the impact of drugs on the microbiome.

As shown in some of the studies included in this review (Dollive *et al*., 2013; Kim *et al*., 2016; Perrin-Guyomard *et al*., 2006; Reeves *et al*., 2011), adding a clearance period after the treatment is an important step to evaluate if alterations are transient (i.e. microbiome return fully or partially to baseline) or permanent.

FROM ASSOCIATIONS TO CAUSALITY

Studies evaluating drug effects on the gut microbiota and the host, and the potential relationship between the two, fall into one of the following categories:

> Those that do not establish associations between observations in the microbiota and the host. Both are studied independently in parallel.

> Those that establish statistical correlations (associations) between microbiome disturbances and host alterations, but do not prove the causal relationship.

> Those that determine a cause–effect relationship (causality).

It is relevant to mention that a limited number of studies are designed to establish causality (Fischbach, 2018). Most scientific work linking specific gut bacteria or the microbiome to human health outcomes is based on statistical associations.[22]

Some studies establish statistical correlations between microbial imbalances (e.g. composition, diversity or function) and host alterations (e.g. metabolic) resulting from environmental changes or exposure to xenobiotics (e.g. drugs, pesticides, food additives). However, associations do not prove the participation of the microbiome in the development of adverse effects in the host or their contribution towards no observed effects. A fundamental limitation is the lack of understanding of the mechanisms involved in the microbiome–host interactions. However, the lack of certainty about the role of a drug-altered microbiome on health effects does not eliminate the risk posed by drug exposure. It is also important to note that the microbiome–host relationship is symbiotic and bi-directional. This means that after exposure to a given substance, not only can the microbiome modulate activities in the host, but the host also influences the normal function and structure of the microbiome. In other words, gut dysbiosis could result from a host alteration. Another possibility to consider is the development of parallel effects in both the microbiome and host without one affecting the outcome of the other.

Establishing causality and the underlying mechanisms is very challenging in the context of the complex interactions between two complex systems, i.e. the microbiome and the host in a given environment. The use of germ-free mice colonized with altered or normal microbiota, or select microbiota members, has been an approach used to confirm causality. In the case of colonization resistance, causality is demonstrated when an infection is alleviated by the addition of a single bacteria strain, a group of select bacteria or a complex microbial community or microbiota (Stecher, 2021).

Although tools are being used to assess the impact and safety of veterinary drug residues (as described above), these assessments will be much improved and accurate when based on established causality and robust biomarkers of microbiome disturbance.

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22 Association here it refers to the statistical relationship between two variables.

As a note of caution, there is a significant amount of speculation surrounding the role of the microbiome in human health and disease, perhaps due to overinterpretation of scientific findings, scientific ignorance or misinterpretation of the term association as an established cause–effect. In any case, this is a sensitive issue that requires careful scrutiny when examining published scientific work, health-related reports and general public communications.

THE OMICS IN RISK ASSESSMENT

There are many approaches to the study of the microbiome. Still, the lack of methodology standardization and harmonization does not provide the consistency needed for robust risk assessments. For this reason, the incorporation of microbiome data in veterinary drug residue evaluations is difficult at this time. After performing an exploratory evaluation of the potential microbiome as a component of risk assessments, EFSA concluded that sequencing tools and multi-omic techniques require more refinement and standardisation before considering data for future chemical risk assessment (Merten *et al.*, 2020).

Frameworks for risk assessment using omics data have been proposed, such as the framework based on the adverse output pathway (AOP) (Piña *et al.*, 2018). The AOP concept originates from toxicology and ecotoxicology but can be expanded to other fields. It refers to the link between a unique molecular trigger (e.g. drug as a specific biomolecule) that escalates and affects several layers of the organization, with outcomes at the ecosystem or population level (Ankley *et al.*, 2010). The AOP framework has been useful in establishing the correlation between the initial molecular interaction and the truly adverse outcome, which is relevant to risk assessment (Piña *et al.*, 2018). Some initiatives have evaluated how high-throughput molecular-level datasets can support (chemical) risk assessments using the AOP framework (Brockmeier *et al.*, 2017).

ADDITIONAL CONSIDERATIONS

Some other aspects relevant for assessing the risk of chemical residues, including veterinary drugs, require further thoughts. In the United States of America, about 51 percent of veterinary pharmaceuticals are approved in human medicine, so 49 percent of drugs reserved exclusively for animal use could end up as residues in food (Scott *et al.*, 2020), but only a handful have been evaluated in microbiome studies. Additional research needs to investigate the influence of the microbiome in the pharmaco/toxicokinetics and pharmaco/toxicodynamics of drugs, including microbial transformation, that could affect the dose and active form of the compound. A better understanding of these microbial activities on drugs would also improve the assessment of veterinary drug residues as they can influence the hazard characterization and exposure assessment.

Another area requiring more discussion is the extrapolation of *in vivo* and *in vitro* microbiome-related findings to humans and the suitability of current approaches.

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CHAPTER 9
RESEARCH GAPS AND NEEDS

Most studies evaluating the impact of pharmaceuticals on the human gut microbiome are scoped within the clinical context, testing therapeutically relevant doses and treatment regimes. This scenario is inadequate to evaluate the effects of veterinary drug residues on the gastrointestinal microbial population, which needs chronic exposure at levels significantly lower than those used in preventive or therapeutic treatments. Also, experimental studies assessing the safety of veterinary drug residues are limited to *in vitro* models, primarily evaluating select bacteria isolated from faecal material, with a high dependency on culturing and the use of traditional microbiological and targeted analytical methods. The application of omics in the holistic evaluation of the microbiome in this field is almost non-existent. Moreover, although the evaluation of drug effects on the microbiome focuses primarily on taxonomical composition and diversity, the functional aspect of the microbiome, which has high relevance in the interaction with the host, is not always considered. So, until now, the microbiome, as a complex functional entity, has had a very limited consideration in the evaluation of veterinary drug residues.

The study of the microbiome is complex and the science surrounding it is still evolving. Some general aspects need to be addressed to have a more accurate picture about the role of the microbiome in human health in the context of chemical exposure and, therefore, its applicability in regulatory activities:

> reproducibility improvement (models, analytical tools and statistical approaches);

> use of doses and treatment periods relevant to assessing veterinary drug residues;

> identifying phenotypes, or measurable biomarkers, that are clearly and unambiguously derived from the microbiome;

> determination of biological relevance;

> expansion to incorporate other members of the microbiome (virus, fungi, etc.); and

> establishing causality and its direction (microbiome > host or host > microbiome).

Achieving these goals will require collaboration and multidisciplinary efforts to improve and optimize research activities to evaluate the impact of veterinary drug residues on the human gut microbiome and human health.

The following are more detailed needs and areas of improvement:

Revising terminology and developing consensus definitions

> Consensus definitions of healthy microbiome and dysbiosis should consider both the taxonomical and functional aspects.

> Modernizing terminology. "Flora" makes reference to the plant kingdom and has become an obsolete term. Microflora, or microbial flora, is still widely used with origins in the former classification of organisms, like bacteria, within the plant kingdom. "Microbiota" is a more current term that should replace the term "flora".

Research and methodologies

To improve assay reproducibility and allow the comparison of results from different studies, it is necessary to (1) standardize and harmonize study designs and analytical methodologies and (2) develop or improve consensus guidance and best practice guidelines. The following should be considered:

> Study objectives and design

  > *In vitro* and *in vivo* studies should use dose ranges (e.g. between residue to therapeutic concentrations) that make it possible to build dose–response curves and derive NOEC and NOAEL.

  > Provide recommendations for experimental periods relevant to chronic exposures. Longitudinal studies should also monitor the microbiome before and after the treatment period.

  > Consider multi-residue *in vivo* and *in vitro* studies of most likely combinations of drugs typically found in residue monitoring programmes.

  > Conduct *in vivo* studies aimed at establishing causality and mechanisms.

  > Conduct *in vivo* studies to validate *in vitro* results.

  > Studies should consider combining the microbiome taxonomical structure and function using omic analytical approaches.

  > Studies should aim at determining the biological/toxicological relevance of microbiome disturbances.

  > Provide recommendations for selecting the most suitable *in vivo* and *in vitro* models to evaluate veterinary drug residues.

  > Define housing conditions to minimize interferences and bias introduced by environmental factors and the grouping of animals.

  > Establish requirements for microbiota donors (e.g. healthy individuals, absence of medication in the months preceding the donation, age, geography and diet), with special consideration to confounding factors.

  > Guide to determine sample size (number of subjects, e.g. animal studies, number of faecal donors): minimum number of subjects per group and groups per treatment must be sufficient to guarantee statistical power. This is especially relevant given the high interindividual variability of microbiota composition.

  - Sampling: Studies should consider monitoring the microbiome at several points of the study, including a pre-treatment sampling to determine the baseline microbiota. Combining microbiome and host metrics in longitudinal studies is essential to assess fluctuations and trends in the dynamics of the microbial community and microbiome–host interaction (e.g. metabolome profiling). Ideally, studies should include clearance periods after treatments to evaluate whether biologically relevant microbiome alterations are transient or permanent.
  - Monitor the microbiome in the different sections of the gastrointestinal tract (duodenum, jejunum, ileum, cecum, colon) to assess the regional impact of drug residues.

- Analytical methods
  - Provide recommendations for sample collection, handling and processing conditions. These include, but are not limited to, collection site (e.g. faecal, caecal, small intestine; lumen or mucosa), individual vs pooled microbiota samples, recommended dilutions, sample storage, etc.
  - Provide guidance for genomic material extraction and handling.
  - Make recommendations for selecting regions and primers for 16S rRNA (bacteria), 18S rRNA genes or ITS (fungi) analysis.
  - Provide guidance on sequencing and computational analysis.
  - Make recommendations for selecting libraries, data processing tools and statistical treatments.

Risk assessments

- Define and validate microbiome-related biomarkers.
- Provide more accurate estimations of the drug amount not bound by the digesta and faecal material that is available to the microbiota.
- Evaluate if existing microbiological endpoints are suitable when considering the microbiome or if there is a need to define and validate more appropriate endpoints.
- Evaluate the potential relevance of the microbiome of the small intestine.
- Evaluate the need to define microbiome-derived adverse effects.
- Determine causality and underlying mechanisms.
- Evaluate whether current approaches to extrapolate *in vivo* and *in vitro* data to the human context are suitable for microbiome-related data.
- Develop guidelines and an assessment framework to assist risk assessors in evaluating microbiome- and omics-derived data.

CHAPTER 10
CONCLUSION

The study of veterinary drug residues on the gut microbiome is limited. Only a few studies address the impact of chronic exposure to low-level concentrations, most are conducted *in vitro* and are highly dependent on the traditional bacteria cultures of select or representative gut bacterial species. Although omic analytical approaches have been used to characterize changes in the composition and function of the microbiome after exposure to sub-doses or therapeutical doses of pharmaceuticals, these techniques have not been widely used to evaluate the effects of residual levels. Because of the nature of *in vitro* studies used to assess veterinary drug residues, it is difficult to evaluate the potential impact of gut microbiome disturbances on human health and non-communicable diseases. Moreover, current microbiological endpoints used to assess the safety of dietary veterinary drug residues focus on evaluating the impact of these substances on the gastrointestinal barrier and the development of resistance in the human gut microbiome. At this time, there are no endpoints defined beyond the gastrointestinal tract. In addition, most research on drug–microbiome–host physiopathological interactions has mainly shown associations, but not causality or mechanisms. In most studies, it is difficult to evaluate whether alterations of the microbiome and host physiology after drug exposure are parallel effects, if the microbiome changes induce disruptions in the host homeostasis, or if the microbiome is altered by the host's response to veterinary drug residues. Therefore, the actual contribution of the gut microbiome to health and disease – and to which extent – remains an important challenge to be addressed with more research. Further, more research is critical to investigate the potential long-term negative impact of veterinary drug residues on the human gut microbiome and the consequent influence on human health.

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ANNEX I. **MICROBIOTA MEMBERS ALTERED BY EXPOSURE TO THERAPEUTICAL DOSES OF ANTIBIOTICS**

ANTIBIOTIC	PROTEOBACTERIA	*Citrobacter* spp	Enterobacteriaceae	*Enterobacter* spp	*E.coli*	*Klebsiella* spp	BACTEROIDETES	*Bacteroides* spp.	FIRMICUTES	*Bacillus* spp	*Blautia* spp	*Doria* spp	*Clostridium* spp	*Coprococcus* spp	*Enterococcus* spp	*Eubacterium* spp	*Lachnospirum* spp	*Lactobacillus* spp	*Peptostreptococcus* spp	*Roseburia* spp	*Ruminococcus* spp	*Staphylococcus* spp	*Streptococcus* spp	*Veillonella* spp	ACTINOBACTERIA	*Bifidobacterium* spp	*Corynebacterium* spp	FUSOBACTERIA	*Fusobacterium* spp	Yeast	*Candida* spp
Penicillins																															
Penicillins																															
Amoxicillin & ampicillin			■	■	*	■		■							■	■				▬				▬		■				■	■
Amoxicillin/ clavulanate			■		■								▬													▬				■	
Piperacillin & ticarcillin													▬		■			▬													
Cephalosporins																															
1st, 2nd gen		■		■	▬	■							*		■	▬		▬								▬					■
3rd, 4th, 5th gen					*	*		*					1			■ ▬		*											▬	■	■
Carbapenems								▬					▬		■								▬	▬							■
Lipoglycopeptides			■												■	■						▬									
Macrolides and ketolides		■		■	*	■		▬					*		▬			*						▬		▬			▬		
Lincosamides																															
Clindamycin		■		■	▬	■		▬			▬	▬	▬	▬	▬	▬	▬	▬		▬	▬		▬	▬		▬					
Tetracyclines																															
Doxycycline															▬																
Quinolones		■	*	■	▬	■		*		▬			*					▬	▬		▬			▬		*	▬				
Sulphonamides					▬																										
Nitrofurantoin																															
Fosfomycin				■		■																									
Rifaximin																								▬							

■ increased ▬ decreased

* With some exceptions

[1] Except 5th gen

Source: Adapted from **Zimmermann, P. & Curtis, N.** 2019. The effect of antibiotics on the composition of the intestinal microbiota - a systematic review. *Journal of Infection*, 79(6): 471–489. https://doi.org/10.1016/j.jinf.2019.10.008

ANNEX II. GUT MICROORGANISMS FOUND TO HAVE INCREASED ANTIBIOTIC RESISTANCE

ANTIBIOTIC	PENICILLINS					CEPHALOSPORINS					CARBAPENEMS	LIPOGLYCOPEPTIDES			MACROLIDES AND KETOLIDES					
	Amoxicillin	Pivmecillinam	Mezlocillin	Amoxicillin/ Clavulanate		Cefoxitin	Cefpodoxime proxetil	Ceftazidime/ avibactam	Ceftaroline/ avibactam		Ritipenem acoxil		Vancomycin	Teicoplanin		Spiramycin	Erythromycin	Clarithromycin	Dirithromycin	Telithromycin	Solithromycin
Diverse bacteria																					■
Anaerobic bacteria																■	■				
Anaerobic cocci																					
Non-fermentative gram-negative							■														
Gram positive rods																					
PROTEOBACTERIA																					
Acinetobacter spp																					
Alcaligenes spp																					
Citrobacter spp																		■	■		
Citrobacter freundii																					
Enterobacteriaceae	■	■														■		■			
Enterobacter spp																			■		
Enterobacter cloacae			■				■														
Enterobacter agglomerans						■															
Escherichia coli	■																				
Klebsiella spp																		■	■		
Klebsiella pneumoniae																					
Proteus spp																		■	■		
Pseudomonas spp																		■	■		
Pseudomonas aeruginosa						■															
Serratia spp																			■		
Xantomonas spp																					
BACTEROIDETES																					
Bacteroides spp						■			■									■		■	
Bacteroides fragilis																					
Bacteroides thetaiotaomicron																					
Bacteroides vulgatus																					
Bacteroides ovatus																					
Bacteroides distasonis																					
FIRMICUTES																					
Clostridium spp						■		■			■						■				
Clostridium dificile																					
Enterococcus spp	■			■		■		■					■	■				■			
Enterococcus faecalis																					
Enterococcus faecium																					
Lactobacillus spp								■													
Pediococcus acidlactici														■							
Coagulase-negative staphylococci														■							
Group D streptococci																■					
ACTINOBACTERIA																					
Bifidobacterium spp																					
Corynebacterium spp																					
YEAST																	■				
Candida spp																					

continues

ANTIBIOTIC	LINCOSAMIDES	Clindamycin	TETRACYCLINES	Doxycycline	GLYCYLCYCLINE	Tigecycline	STERPTOGRAMINS	Quinupristin/ dalfopristin	OXAZOLIDINONES	Linezolid	QUINOLONES	Norfloxacin	Ciprofloxacin	Levofloxacin	Gemifloxacin	Clinafloxacin	Garenoxacin	Sitafloxacin	Trovafloxacin	TRIMETHOPRIM & SULPHONAMIDES	Sulphasomidine	Sulphalene	Trimethoprim	Trimethoprim/ sulfamethoxazole
Diverse bacteria																								
Anaerobic bacteria								■					■											
Anaerobic cocci				■																				
Non-fermentative gram-negative																								
Gram positive rods				■																				
PROTEOBACTERIA																								
Acinetobacter spp													■										■	■
Alcaligenes spp													■											
Citrobacter spp																								
Citrobacter freundii													■											
Enterobacteriaceae		1		■													■							
Enterobacter spp																								
Enterobacter cloacae						■																		
Enterobacter agglomerans																								
Escherichia coli		■				■						■	■		■				■		■	■		■
Klebsiella spp																								
Klebsiella pneumoniae						■																		
Proteus spp																								
Pseudomonas spp																							■	■
Pseudomonas aeruginosa																								
Serratia spp																								
Xantomonas spp													■											
BACTEROIDETES																								
Bacteroides spp		■		■												■								
Bacteroides fragilis						■				■														
Bacteroides thetaiotaomicron						■																		
Bacteroides vulgatus														■				■						
Bacteroides ovatus														■										
Bacteroides distasonis																		■						
FIRMICUTES																								
Clostridium spp		■																						
Clostridium dificile												■												
Enterococcus spp		■		■				■									■							
Enterococcus faecalis													■											
Enterococcus faecium													■											
Lactobacillus spp				■																				
Pediococcus acidlactici																								
Coagulase-negative staphylococci													■											
Group D streptococci																								
ACTINOBACTERIA																								
Bifidobacterium spp				■									■											
Corynebacterium spp													■											
YEAST																								
Candida spp												■												

[1] Enterobacteriaceae other than *E. coli*.

Some studies tested the following antibiotics and didn't find antimicrobial resistance: penicillin, bacampicillin, cefaclor, cefuroxime axetil, loracarbef, cefixime, ceftriaxone, cefpodoxime proxetil, ceftibuten, cefpirome, ceftaroline, ceftobiprole, meropenem, telavancin, dalbavancin, clarithromycin, roxithromycin, azithromycin, nalidixic acid, norfloxacin, ofloxacin, enoxacin, lemofloxacin, ciprofloxacin, levofloxacin, gatifloxacin, gemifloxacin, perfloxacin, tinidazole, polymyxin E, fosfomycin.

Source: Adapted from **Zimmermann, P. & Curtis, N.** 2019. The effect of antibiotics on the composition of the intestinal microbiota - a systematic review. *Journal of Infection*, 79(6): 471–489. https://doi.org/10.1016/j.jinf.2019.10.008

ANNEX III. *IN VIVO* STUDIES EVALUATING THE EFFECTS OF DRUGS ON THE GUT MICROBIOTA AND HOST HEALTH

ANIMAL	TREATMENT	MICROBIOME RESULTS	HOST RESUTLS	REFERENCE
Studies designed to evaluate veterinary drug residues				
HFA mice pooled human fecal microbiota	1, 10 and 100 mg/L tetracycline (0.125, 1.25, 12.5 mg/kg bw/day) in drinking water (ad libitum) for 6 and 8 weeks	↑ 2 highest doses: Gram-positive anaerobes, *Bacteroides fragilis*, Enterobacteria and Enterococci high dose: colonization by *Salmonella* Scharzendrung Metabolic parameters (enzymes and SCFA) not altered	Host not evaluated	Perrin-Guyomard *et al.*, 2001
HFA mice (females) pooled human fecal microbiota	1, 10 and 100 mg/L ciprofloxacin (0.125, 1.25, 12.5 mg/kg bw/day) in drinking water (ad libitum) for 5 weeks.	↑ *Bacteroides fragilis* ↓ Aerobic bacteria, Enterobacteriaceae All doses: colonization by *Salmonella* Typhimurium Metabolic parameters (enzymes and SCFA) not altered	Host not evaluated	Perrin-Guyomard 2005
GF Sprague-Dawley rats pooled human fecal microbiota	0.25, 2.5 and 25 mg/kg bw ciprofloxacin for 5 weeks	↑ *Bacteroides fragilis* ↓ All doses: aerobic populations Highest dose: depleted Enterobacteriaceae, reduced Bifidobacteria highest dose: colonization by *Salmonella* Typhimurium Alterations reverted after treatment cessation	Host not evaluated	Perrin-Guyomard 2006
Studies designed to evaluate early exposure				
NOD/ShiLtJ mice	1 mg/kg bw/day penicillin V (continuous dose) 50 mg/kg bw/day tylosin tartrate (intermitent dose)	Penicillin: no alterations Tylosin: in males almost complete absence of ileal and caecal Bacteroidetes, Actinobacteria and *Bifidobacterium*	Male: increased risk type 1 diabetes.	Livanos *et al.*, 2016
C57BL/6J mice	1 mg/kg bw/day penicillin, vancomycin, penicillin plus vancomycin, or chlortetracycline	↑ Firmicutes, Lachnospiraceae ↑ Caecal SCFA acetate, propionate and butyrate	↑ Adiposity alterations in the metabolic pathways of fatty acids and lipids	Cho *et al.*, 2012
C57BL/6J mice	1 mg/kg bw/day penicillin for 30 days	↓ *Lactobacillus*, *Candidatus* Arthromitus, *Rikenellaceae* and *Allobaculum* microbiota recovered after treatment	Metabolic effects and body composition (remained after treatment)	Cox *et al.*, 2014
C57BL/6 mice	6.8 mg/L penicillin G for 32 weeks	Altered MB composition ↑ *Candidatus* Arthromitus and *Allobaculum*	↑ Adiposity and insulin resistance increased risk for metabolic disorders later in life	Mahana *et al.*, 2016

continues

ANIMAL	TREATMENT	MICROBIOME RESULTS	HOST RESUTLS	REFERENCE
C57BL/6J mice	1 or 15 mg/kg per day doxycycline from gestation to 7 weeks of age	Dose-dependent ↓ richness, *Candidatus* Saccharimonas, *Ruminococcus*, *Helicobacter* and *Anaeroplasma*	Early exposure to low doses of doxycycline with an increased risk of obesity	Hou *et al.*, 2019
C57BL/6 mice	1 g/L ampicillin or erythromycin for 5 weeks	Reduced microbial diversity	No immunological alterations Erythromycin altered glucose metabolism Ampicillin improved glucose tolerance	Bech-Nielsen *et al.*, 2012
C57BL/6NTac mice	1 g/L ampicillin (intermittent dose), birth-17 weeks high-fat diet	Disturbed microbiota	Improved glucose tolerance	Rune *et al.*, 2013
Non-obese diabetic (NOD) mice	0.2 mg/ml vancomycin or broad-spectrum antibiotics (5 mg/ml streptomycin, 1 mg/ml colistin and 1 mg/ml ampicillin in drinking water) from conception to adulthood (40 weeks)	Profound alterations of the gut microbiota Vancomycin: ↓ Clostridiales, Lachnospiraceae, Prevotellaceae and Rikenellaceae ↑ *Escherichia, Suterella, Lactobacillus*	Increased the incidence of type 1 diabetes	Candon *et al.*, 2015
C57BL/6J mice	Cocktail of vancomycin (0.5 mg ml), neomycin (1 mg/ml) and ampicillin (1 mg/ml) for 16 days	↓ Richness and diversity Gender-dependent microbiota alterations: Males: ↑ Firmicutes; ↓ Bacteroides, Actinobacteria disappeared. Females: ↑ Proteobacteria, Tenericutes, Paenibacillaceae, *Bacillus* ↓ *Bacteroides* and *Lactobacillus*	Increased colonic permeability in the absence of inflammation Gender-dependent bone alterations: males: decreased structure features females: altered mineral distribution > associated with high bone fracture risk	Pusceddu *et al.*, 2019
Studies in adults to evaluate colonization resistance to *Clostridium difficile*				
C57BL/6	Cocktail (0.4 mg/ml kanamycin, 0.035 mg/ml gentamicin, 850 U/ml colistin, 0.215 mg/ml metronidazole and 0.045 mg/ml vancomycin) for 3 days 0.5 mg/ml cefoperazone for 10 days drinking water	Individual sensitivities to infection Less severely ill animals: Firmicutes dominate Severely ill animals: ↑ Proteobacteria Loss of colonization resistance to *Clostridium difficile* Microbiota recovered and returned to normal in less severely ill animals, but remained different than the baseline in severely-ill animals	Increased risk of severe colitis (*C. difficile* infection)	Reeves *et al.*, 2011
C57BL/c mice	Cocktail (kanamycin, gentamicin, colistin, metronidazole, vancomycin and clindamycin) with or without dexamethasone (100 mg/l) drinking water	↓ Diversity, *Lactobacillus* ↑ Parabacteroides Microbiota return to baseline, slower in the dexamethasone group Group antibiotics and dexamethasone: severe *Clostridium difficile* infection after challenge	Increased risk of severe *C. difficile* infection and colitis (antibiotics + dexamethasone group)	Kim, Wang and Sun, 2016

continues

ANIMAL	TREATMENT	MICROBIOME RESULTS	HOST RESUTLS	REFERENCE
Studies in adults to evaluate colonization resistance to *Campylobacter jejuni*				
CBA/J mice	0.2 mg ampicillin by oral gavage, 2 days	↓ Firmicutes ↑ Bacteroidetes > correlated with disruption of colonization resistance against *C. jejuni* *Enterococcus faecalis* potentially inhibits *C. jejuni* infection	Increased susceptibility to *C. jejuni* infection	O'Loughlin *et al.*, 2015
Other studies in adult animals				
C57B6 mice	Cocktail of vancomycin, ampicillin, neomycin, and metronidazole (therapeutical doses not specified) in water Two groups: 2 weeks treatment + 9 weeks clearance 11 weeks treatment (no clearance)	↓ Bacterial population 40x increase in fungal population Bacterial and fungal populations return to baseline at different speeds	Not evaluated	Dollive *et al.*, 2013
C57BL/6 mice	5 mg/kg/day florfenicol or azithromycin for 4 weeks	↓ Diversity, richness; ↑ Firmicutes/ Bacteroidetes Gender-dependent effects Both treatments: ↓ *Alistipes*, *Desulfovibrio*, *Parasutterella, Rikenella* Florfenicol: ↑ Verrucomicrobia; ↓ Deferribacteres *Christensenella*, *Gordonibacter*, *Anaerotruncus* Azithromycin: ↓ Bacteroidetes, Proteobacteria, *Lactobacillus* Reduced production of SCFA, secondary bile acids	Based on microbial findings: increased risk for obesity	Li *et al.*, 2017
KM mice	100 mg/kg bw florfenicol (prophylactic dose in chickens), 7 days	Altered microbiota (jejunum) ↓ Firmicutes, *Lactobacillus* and *Allobaculum* ↑ *Bacteroides*, *Alistipes*, *Alloprevotella*	Gut epithelial damage Compromised intestinal barrier function and intestinal immunity	Yun *et al.*, 2020
C57BL/6NHsd mice	25 mg/L cefoxitin in drinking water, for 14 days different groups exposed to different diets (standard and low fibre)	No change diversity and richness Microbiota composition altered by cefoxitin in mice with both diets Higher effects of diet on microbiota composition, more severe in low fibre-fed mice	Not evaluated	McCracken *et al.* 2001

continues

ANIMAL	TREATMENT	MICROBIOME RESULTS	HOST RESUTLS	REFERENCE
Sprague-Dawley rats	Roxithromycin (30 mg/kg bw) for 14 days	↓ Gram+, *Bifidobacterium* and *Clostridium* GI location-dependent effects: Cecum: ↓ *Streptococcus*, *Prevotella*, diversity ↑ Gram-, *Bacteroides* and Enterobacteriaceae Small intestine: ↑ Gram-, Gram+, *Enterococcus*	Downregulated P450 xenobiotic metabolism > decreased roxithromycin metabolismAltered immune response increased risk of fibrosis	Zhang *et al.* 2018
Glucocorticosteroids and production aids				
Wistar rats	Dexamethasone (0.01 and 0.05 mg/kg bw/day), gavaged, for 7 weeks	↓ Diversity, Firmicutes, Bacteroidetes α- Proteobacteria, γ-Proteobacteria, and Actinobacteria, Clostridiales, *Lactobacillus*	↓ Mucus secretion ↑ Expression antimicrobial genes Slowed weight gain, reduced feed intake, increased fat accumulation, and altered the circadian rhythm, glycolipid and energy metabolism	Wu *et al.*, 2018
California mice	Dams: 0.1 µg/kg Ethinyl estradiol in diet (during gestation and lactation)	Alterations were generational and gender-dependent	Not evaluated	Javurek *et al.* 2016

Source: Authors' own elaboration.

ANNEX IV. *IN VIVO* STUDIES EVALUATING THE EFFECTS OF INSECTICIDES ON THE GUT MICROBIOTA AND HOST HEALTH

DOSE REPORTED ON STUDY	MODEL	SAMPLE SIZE (N)	PERIOD	IMPACT ON GUT MICROBIOTA	HEALTH OUTCOMES	REFERENCES
1 mg/day	SHIME®		30 days	↑ *Bacteroides* spp. and *Enterococcus* spp. ↓ *Bifidobacterium* spp. and *Lactobacillus* spp.	Induced intestinal dysbiosis	Joly *et al.*, 2013
1 mg/kg bw per day through oral gavage	Rats Hannover Wistar (female and pups)	n = 10 per group	Pups > exposed via dams: gestation day 0 – postnatal day 21 > Gavage: postnatal day 21-60	Slight ↑ *Enterococcus* spp. ↓ *Lactobacillus* spp. and *Bifidobacterium* spp.		
1, 5 mg/kg bw/day exposed through utero and maternal milk by gavage	Rats (Hannover Wistar) pregnant female; male pups	Females n = 6 per dose and control Pups PND21: n = 10 for control and CPF1; n = 8 for CPF5 Pups PND60: n = 10 for control and CPF1; n = 9 for CPF5	From gestation through weaning (PND21) and through adulthood (PND60)	Intestinal microbial dysbiosis – most alterations found in culture, dependent on species, mouse age, location (ileum, caecum, colon), CPF dose, analytical method Culture methods: ↑ PND21: aerobic and anaerobic bacteria (ileum), *Clostridium*, *Staphylococcus* (ileum, caecum, colon) ↓ *Bifidobacterium* (PND21 in ileum, PND60 in colon), *Lactobacillus* (all ages, all intestinal segments) Molecular methods: ↑ Bacteria, *Clostridium leptum* (PND 60 in colon) ↓ *Bacteroides/Prevotella* (PND60 in ileum)	Pups: ↓ Perturbed intestinal development, with morphological alteration of the structures involved in nutrient absorption, alteration of mucosal barrier (mucin-2), stimulation of the innate immune system, and increased bacterial translocation	Joly Condette *et al.*, 2015

continues

DOSE REPORTED ON STUDY	MODEL	SAMPLE SIZE (N)	PERIOD	IMPACT ON GUT MICROBIOTA	HEALTH OUTCOMES	REFERENCES
0.3 mg/kg bw/day by gavage (normal or high-fat diet)	Rats Wistar male (weaned pups and adults)	n = 6 per group	Pups: 25 weeks Adults: 20 weeks	Adult Normal Fat diet: ↑ *Streptococcus*, *Ruminiclostridium*, Coriobacteriaceae ↓ *Romboutsia*, *Turicibacter* and *Clostridium* Adult High Fat diet: ↑ *Escherichia-Shigella* Depleted: *Ruminococcaceae*, *Oscillibacter*, *Paenalcaligenes* and *Peptococcus* Pup High Fat diet: ↑ *Faecalibaculum*, *Parasutterella*, Erysipelotrichaceae, Coriobacteriaceae, *Peptococcus*, *Brevibacterium* ↓ Christensenellaceae, Ruminococcaceae, [*Eubacterium*] coprostanoligenes group, Ruminococcaceae, Defluviitaleaceae, Lachnospiraceae, *Anaerovorax*, Coriobacteriaceae	Alteration of endocrine function and inflammation (with the potential to disturb the central nervous system) Potentially related to infertility and colitis	Li *et al.*, 2019
5 mg/kg/day via gavage (high or normal-fat diet)	Mice C57Bl/6 and CD-1 (ICR) (male)	n = 8 per group	12 weeks	Non-fat diet: ↓ Proteobacteria Bacteriodetes	> Risk of inflammatory-related disorders, obesity and diabetes > Genetic background and diet pattern have limited influence on the CPF results	Liang *et al.*, 2019

continues

DOSE REPORTED ON STUDY	MODEL	SAMPLE SIZE (N)	PERIOD	IMPACT ON GUT MICROBIOTA	HEALTH OUTCOMES	REFERENCES
0.3 or 3 mg/kg bw per day by oral gavage combined with a normal (NFD) and high fat diet (HFD)	Rats Wistar (male)	n = 6 per group	9 weeks	NFD: 12 bacterial genera affected Low dose: ↑ *Allobaculum*, *Candidatus* Saccharimonas, *Coprococcus*, *Anaeroplasma*, *Roseburia*, *Sutterella* ↓ *Pseudoflavonifractor*, *Anaerosporobacter*, *Aerococcus*, *Brevundimonas*, *Trichococcus* High dose: ↓ *Pseudoflavonifractor*, *Anaerosporobacter*, *Aerococcus*, *Brevundimonas*, *Trichococcus*, *Bacteroides* HFD: 13 bacterial genera affected Both doses: ↑ *Sutterella*, *Candidatus* Arthromitus ↓ *Olsenella*, *Clostridium sensu stricto*, *Amphibacillus*, *Enterorhabdus*, *Alloprevotella* Low dose: ↑ *Acinetobacter*, *Blautia*, *Oscillibacter* ↓ *Ruminococcus*, *Hydrogenoanaerobacterium* High dose: ↑ *Pseudomonas*	Identified potential health outcomes based on changes in microbiota diversity after exposure to chlorpyrifos > Increased risk of obesity and diabetes > Bacteria associated with Neurotoxicity, β-cell dysfunction and pancreatic Injury increased NFD-low dose: largest metabolic changes, exhibiting pro-obesity phenotype	Fang *et al.*, 2018
1 or 3.5 mg/kg/ day by gavage with/without free access to inulin (10g/L in drinking water)	Rats Wistar (Dams and male pups)	n = 5/6 per treatment group and 5 control	From gestation to (PND21) pups were exposed to CPF via dams receiving CPF Male pups received CPF in diet from PND21 until PND60	CPF ↓ Firmicutes, *Clostridium coccoides* group CPF3.5+Inulin ↑ *C. coccoides* group	> Risk of diabetes mellitus > Pups to adults: impaired metabolism leading to insulin and lipid dysregulation > CPF nor inulin affected maternal weight gain, food or water intake and no cholinergic toxicity CPF ↓ body weight (no difference food and water intake)	Reygner *et al.*, 2016b
1 mg/kg/ bw/d in corn oil	Mice, *Mus musculus* KM (male)	n= 5 per group	30 d	↑ Bacteroidetes, Bacteroidaceae ↓ Firmicutes, Lactobacillaceae	Altered metabolic profiles: intestinal inflammation and abnormal intestinal permeability	Zhao *et al.*, 2016

continues

DOSE REPORTED ON STUDY	MODEL	SAMPLE SIZE (N)	PERIOD	IMPACT ON GUT MICROBIOTA	HEALTH OUTCOMES	REFERENCES
3.5 mg/day CPF	SHIME® Caco-2/TC7 cell culture	n = 3 per sample	15 and 30 days	↓ *Lactobacillus* and the *Bifidobacterium*	Altered mucosal barrier activity and potential inflammation	Requile *et al.*, 2018
3.5 mg day CPF + 10g/day inulin					> Pro-inflammatory signal triggered by the pesticide is completely inhibited by the prebiotic.	
1 mg/day dissolved in rapeseed oil	SHIME®		15 and 30 days	COMPOSITION CPF-oil exposure: ↓ Bifidobacteria population D15; and ↑ E. coli count D30 Plate culture techniques: ↑ *Bacteroides* spp., *Clostridium* spp. and Enterobacteria populations D15 and 30; ↓ Bifidobacteria count at D30 DIVERSITY Altered total bacteria by D15; and effect on bifidobacterial population on D30 METABOLITES Altered fermentative activity	-	Reygner *et al.*, 2016a
1 mg/kg bw/day	ApoE4-TR, apoE3-TR and C57BL/6 mice – pups (Male)	n = 6 animals / group	6 d (PND 10 to PND 15)	> Changes dependant on host's genetic and environmental background > Differences between genotypes at different taxonomic levels, where apoE4 differed in microorganism proportion > Differences were found in genera belonging to phylum Proteobacteria**:** *Helicobacter*, *Escherichia*, *Enterobacter* and *Serratia*, among others ApoE4-TR: > Most susceptible on gut microbiome composition > Changes in Phylum Verrucomicrobia: (+ than other groups) species *Akkermansia muciniphila* ↑ *Rhodothermus* C57BL/6: ↓ *Streptococcus*	Genetic and environmental effects on SCFA composition in the brain with potential implications for cognitive functioning: ApoE3 SCFA increased more than others (acetic acid, butyric acid and propionic acid); ApoE4 was unchanged	Guardia-Escote, *et al.*, 2020

continues

DOSE REPORTED ON STUDY	MODEL	SAMPLE SIZE (N)	PERIOD	IMPACT ON GUT MICROBIOTA	HEALTH OUTCOMES	REFERENCES
1 mg/kg/ ml/day diluted in corn oil oral gavage	Wistar rats – pups (male and females)	n = 5 animals / group	6d (PND10 to PND15)	Dysbiosis at both genus and species levels ↑ *Anaerobranca*, *Borrelia*, *Brevundimonas*, *Butyrivibrio*, *Mogibacterium* and *Pelagicoccus* ↓ *Candidatus* Contubernalis, *Hyphomicrobium*, *Nitrincola*, *Paracoccus*, *Rhizobium* and *Vogesella*	Sexual dimorphic effects Months after exposure: ↑ motor reaction to stress (in females), hypersensitized animals to both antimuscarinic and GABAergic challenges (predominantly in females), upregulated transcription of both M2 receptor and GABA-A-α2 subunit genes in the dorsal striatum and frontal cortex, respectively	Perez-Fernandez, *et al.*, 2020

CPF: Chlorpyrifos; PND: Postnatal day; HFD: high-fat diet; SCFA: short-chain fatty acids

Source: Authors' own elaboration.

THE IMPACT OF VETERINARY DRUG RESIDUES ON THE GUT MICROBIOME AND HUMAN HEALTH

A FOOD SAFETY PERSPECTIVE

Corrigendum

03 June 2023

The following corrections were made to the PDF of the report after it went to print.

PAGE	LOCATION	TEXT IN PRINTED PDF	TEXT IN CORRECTED PDF/ NOTES
V	Paragraph 3	FAO is grateful to the experts Mark Feeley (Consultant, Canada), Silvia Pi eiro (United States Food and Drug Administration) and Heather Harbottle (United States Food and Drug Administration) for their insightful comments and recommendations to improve the draft.	FAO is grateful to the expert Mark Feeley (Consultant, Canada) for his insightful comments and recommendations to improve the draft.

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THE IMPACT OF VETERINARY DRUG RESIDUES ON THE GUT MICROBIOME AND HUMAN HEALTH

A FOOD SAFETY PERSPECTIVE

With a food safety focus, a scientific literature review was conducted to characterize the current understanding about the effects of veterinary drug residues on the human gut microbiome and potential implications on human health and non-communicable diseases (NCDs). The main aspects analysed are (1) effects of individual or combined drugs on the composition, diversity and function of gut microbiome using *in vivo* or *in vitro* models; (2) health implications resulting from the veterinary drug–microbiome interactions and underlying mechanisms; (3) establishment of causality; and (4) influence of the gut microbiome on the metabolism and bioavailability of veterinary drugs. The research was also scoped to identify current gaps, limitations and needs for the eventual consideration of microbiome-related data in chemical risk assessment.

With this work, ESF contributes to the FAO global programme on the impact of food systems on NCDs and obesity, by understanding the potential health implications of gut microbiome–veterinary drug interactions. The outcomes will provide information which can be used to improve nutritional strategies and food safety policies.

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