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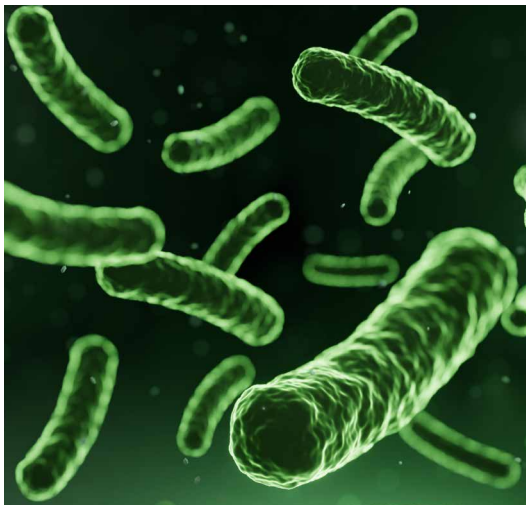

THE IMPACT OF PESTICIDE RESIDUES ON THE GUT MICROBIOME AND HUMAN HEALTH

A FOOD SAFETY PERSPECTIVE

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
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ACRONYMS AND ABBREVIATIONS

ADC	aldicarb
ADI	acceptable daily intake
ARfD	acute reference dose
CAR	constitutive androstane receptor
CAS	chemical abstracts service
CBZ	carbendazim
cRfD	Chronic reference dose
CPF	chlorpyrifos
DDT	dichlorodiphenyltrichloroethane
DLM	deltamethrin
DWEL	drinking water equivalent level
DZN	diazinon
ENS	endosulfan
EPX	epoxiconazole
ERW	electrochemically reduced water
FAO	Food and Agriculture Organization of the United Nations
FDA	United States Food and Drug Administration
GLY	glyphosate
GWI	Gulf War illness
HCH	hexachlorocyclohexane
IARC	International Agency for Research on Cancer
ICR	Institute of Cancer Research
IMZ	imazalil
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint Meeting on Pesticide Residues
MAIT	mucosal-associated invariant T-cells
MCP	monocrotophos
MLT	malathion
MRL	maximum residue limits
NOAEL	no observed adversed effect level
PCR	polymerase chain reaction
PERM	permethrin
PIC	prior informed consent
PMB	propamocarb
PND	postnatal day
PNZ	penconazole
POP	persistent organic pollutants
SCFA	short-chain fatty acids
TDI	tolerable daily intake
TMDI	theoretical maximum daily intake
USDA	United States Department of Agriculture
WHO	World Health Organization
WT	wild type

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EXECUTIVE SUMMARY

The gut microbiome is the microbial community composed of bacteria, viruses, fungi and archaea co-habiting in the gastrointestinal tract of animals and interacting with the host in several physiological functions, including digestion and the immune response. The gut microbiome is highly dynamic and sensitive to numerous physico-chemical factors, including pH, oxygen pressure, and diet composition. Such factors influence the diversity, composition and function of the microbiome, which can impact the health status of the microbiota and the interactions with the host. Although there are no consensus definitions for the related terms "healthy microbiota" and "gut dysbiosis", they are commonly used when explaining the potential role of the gut microbiome in health and disease, respectively.

Since dietary composition strongly influences the microbiome, there is a concern about the effects of chronic exposure to pesticide residues on the microbial community and consequently the impact on human health and non-communicable diseases. This systematic review collected existing research on this topic between September 2019 and May 2020, analysed the evidence linking pesticide residues–gut microbiome–human health and evaluated the potential use of microbiome data reported in these studies for the risk assessment of pesticide residues.

Considering the high number of existing pesticides, only a few have been evaluated in the gut microbiome, with glyphosate and chlorpyrifos receiving most attention. The majority of studies were conducted *in vivo* using rodent models (mice and rats) using different designs and analytical methodologies. Some *in vitro* models are also reported here. Experimental doses chosen for chronic studies were usually several times higher than the established ADIs, often using as reference health-based guidance values (e.g. NOAEL), MRLs, and environmental or occupational exposures. Such high doses tend to be of limited relevance as they are not representative of chronic dietary exposures to pesticide residues. Most of the microbiome analysis focused on the evaluation of diversity and structure by sequencing the 16S rRNA gene (typically the V3-V4 hypervariable regions), resulting in more or less pronounced changes in composition after the treatment with different pesticides. The few studies evaluating multiple doses reported dose-effect responses. The functional microbiome was only addressed in a limited number of studies, focusing primarily on the production of short-chain fatty acids, mainly acetic, propionic and butyric acids. Regarding the host, most studies focused on the evaluation of metabolism (carbendazim, chlorpyrifos, imazalil, monocrotophos, penconazole, propamocarb, p,p'-DDE), immune response (carbendazim, deltamethrin, glyphosate, diethyl phosphate), intestinal homeostasis (chlorpyrifos, glyphosate, imazalil, permethrin), or other dysfunctions (liver: epoxiconazole,

glyphosate; neurological and behavioural alterations: chlorpyrifos, glyphosate, permethrin; endocrine function: chlorpyrifos, diethyl phosphate), which – in most cases and at high doses – resulted in different degrees of alterations observed along with microbial disturbances. Studies focusing on maternal exposure reported that observed microbiome alterations early in life increased the predisposition or risk for developing disorders like type 2 diabetes or motor disabilities. The authors of most studies who discussed associations between observed health outcomes and microbiome alterations, often didn't provide mechanistic support or proof of cause-effect. Only two studies conducted fecal transplants with altered microbiota to reproduce host effects in germ-free or antibiotic-treated mice.

Although most studies report some degree of microbial disturbances and host alterations after pesticide exposure, there are important limitations that should be considered with due attention when interpreting the research outcomes and using this data for risk assessment. These include the low statistical power (small sample size), the lack of standardized models and standardized analytical methodologies, and the limited consideration and control of confounding factors, which were often not reported in the publications. All these shortcomings challenge study reproducibility and the comparison of outcomes from different studies. In addition, there are other important limitations derived from the reported research that can also delay the incorporation of microbiome data in risk assessment. These include the lack of a general discussion about the physiological relevance of observed disturbances beyond statistical significance, the lack of criteria to determine when microbiome disturbances should be considered a concern and the limited research aimed to determine causal relationships and underlying mechanisms. Another point that deserves attention is the translatability of microbiome-related outcomes observed in animals to the human context and the suitability of currently used safety factors to derive reference doses.

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CHAPTER 1
INTRODUCTION

In their publication *International Code of Conduct on Pesticide Management,* the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) define a pesticide as "any substance, or mixture of substances of chemical or biological ingredients intended for repelling, destroying or controlling any pest,[1] or regulating plant growth" (FAO and WHO, 2016, p. 6). Worldwide, many active ingredients in pesticides are used in thousands of pesticide formulations with different properties and toxicological effects (WHO, 2018).

Pesticide active ingredients can be classified by their common names, Chemical Abstracts Service (CAS) registry number, chemical type, physical state, primary use, mode of action and/or level of toxicity. Pesticides are generally classified by their common use or mode of action. For example, herbicides, also known as weedkillers, are chemical substances used to control weeds. Insecticides can help in managing and killing insect pests. Fungicides are biocidal chemical compounds used to kill parasitic fungi or spores. There are other types of pesticides such as are rodenticides and avicides, among others. Pesticides can be further classified by chemical type. Organochlorine pesticides are highly toxic organic compounds banned in several countries since the 1970s and 1980s due to their environmental persistence and capacity to bioaccumulate, thereby risking human health. Despite the ban, they are still widely detected in the environment and the human body (Tsiaoussis *et al.*, 2019; Yuan *et al.*, 2019). Organophosphate pesticides are a class of organophosphorus compounds that inhibit acetylcholinesterase, an essential enzyme for the normal functioning of the central nervous system in insects, humans and some animals. Carbamates are derived from carbamic acid and target insects similar to organophosphate pesticides, though the disruptive effect on cholinesterase is very short. Carbamates can also inhibit other esterases[2] and kill different types of pests (Struger *et al.*, 2016). Pyrethroids are organic compounds defined by their biological action, rather than their chemical structure. These compounds are commonly used as insecticides.

For many decades, pesticides have been used globally to control harmful agricultural pests and prevent crop damage and yield losses. In particular, they play an important

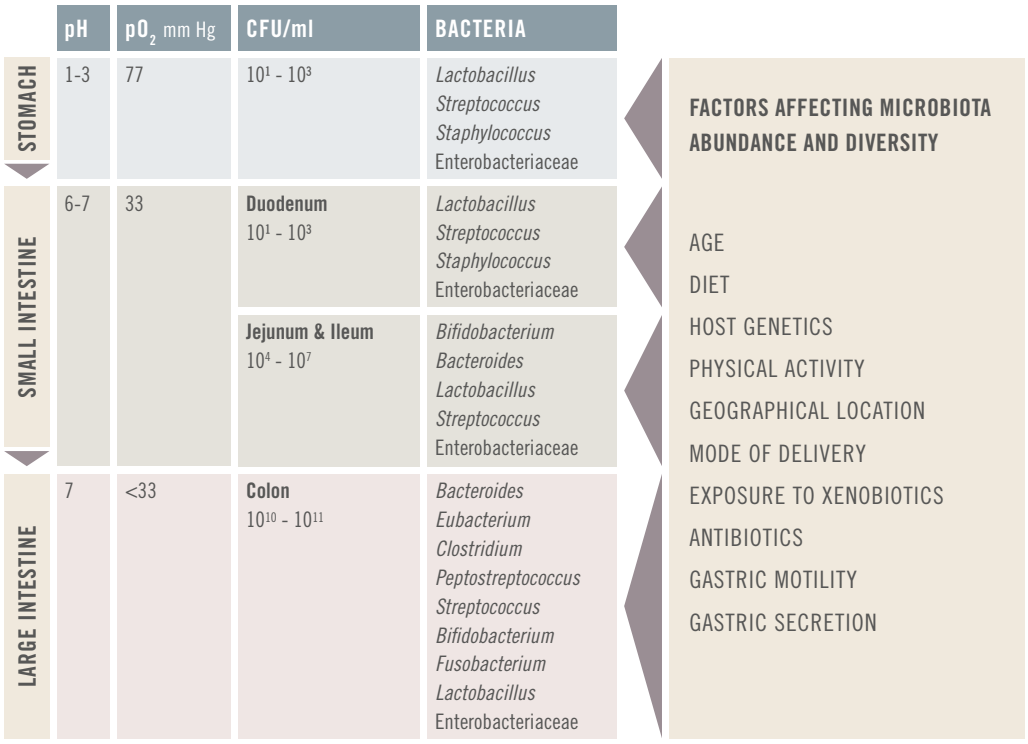
1 According to FAO and WHO (2016, p. 6), a pest is defined as: "any species, strain or biotype of plant, animal or pathogenic agent injurious to plants and plant products, materials or environments and includes vectors of parasites or pathogens of human and animal disease and animals causing public health nuisance."

2 Esterase: any enzyme that catalyses the hydrolysis of an ester into its alcohol and acid.

role in ensuring the availability of food and feed, contributing to food security to meet the needs of a growing population. Despite the positive effect of enhancing agricultural production, pesticides may also be toxic to humans. Pesticide toxicity depends on the compound function (e.g. in humans, insecticides are generally more toxic than herbicides) and other factors such as dose and route of exposure (WHO, 2018). Environmental and human health concerns have been raised since pesticide residues have been found in food, air, water and soils (Roman *et al.*, 2019; Tsiaoussis *et al.*, 2019; Yuan *et al.*, 2019), and even in human blood (Tsiaoussis *et al.*, 2019).

Health-based guidance values[3] (e.g. acceptable daily intake [ADI], tolerable daily intake [TDI], acute reference dose [ARfD]) are reference values determined for different pesticides, as well as for other chemical residues, below which there is no appreciable risk for human health (FAO and WHO, 2009). More recently, concerns are arising about the gut microbiome's[4] sensitivity to chronic exposure to low concentrations of chemical residues. The human gut microbiome is a dynamic community of bacteria, fungi, viruses, protozoa and archaea, living in a symbiotic relationship with the host (Tsiaoussis *et al.*, 2019; Yuan *et al.*, 2019) (Figure 1, Figure 2).

FIGURE 1 **GASTROINTESTINAL ENVIRONMENT AND MICROBIOTA NICHES**



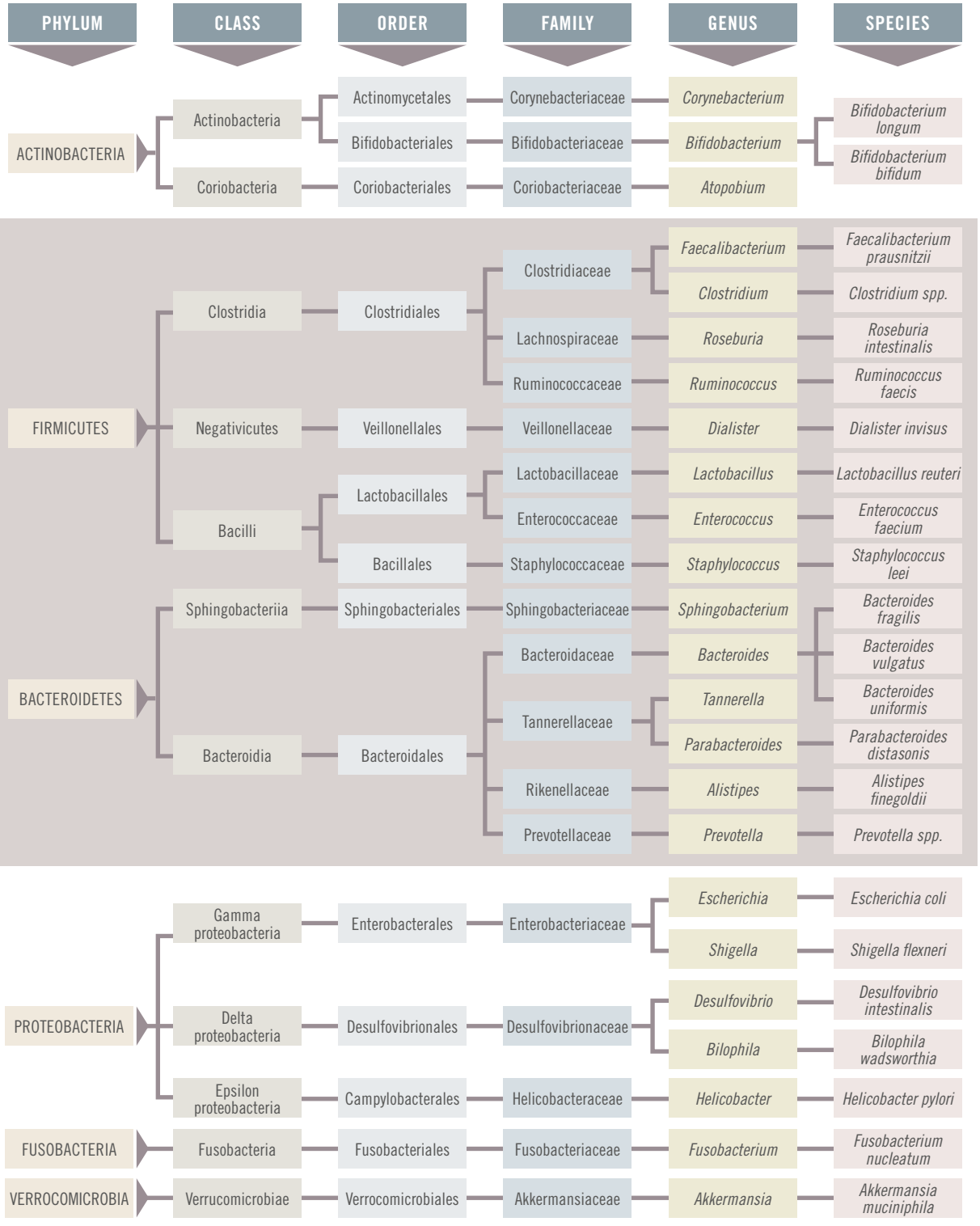
Source: **Clarke, G., Sandhu, K.V., Griffin, B.T., Dinan, T.G., Cryan, J.F. & Hyland, N.P.** 2019. Gut Reactions: Breaking Down Xenobiotic–Microbiome Interactions. *Pharmacological Reviews*, 71(2): 198. https://doi.org/10.1124/pr.118.015768

3 Health-based guidance values provide guidance on safe consumption of substances that takes into account current safety data, uncertainties in these data and the likely duration of consumption https://www.efsa.europa.eu/en/glossary/health-based-guidance-value

4 "The microbiome is defined as a characteristic microbial community occupying a reasonable well-defined habitat which has distinct physio-chemical properties." (Berg *et al.*, 2020, p. 17).

FIGURE 2 **EXAMPLES OF TAXONOMICAL COMPOSITION OF THE GUT MICROBIOTA**

Within the grey background are the predominant phyla (Firmicutes and Bacteroidetes), which constitute over 90 percent of the microbiota



Source: **Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G.A.D., Gasbarrini, A. & Mele, M. C.** 2019. What is the healthy gut microbiota composition? a changing ecosystem across age, environment, diet, and diseases. *Microorganisms*, 7(1): 14. https://doi.org/10.3390/microorganisms7010014

It is known that the gut microbiome contributes to the integrity of the host's intestinal wall, defence against pathogens, energy metabolism, fermentation of carbohydrates, and digestion of protein and peptides. The gut microbiome also participates in the bile acid metabolism and produces substances essential for the host, such as amino acids and vitamins (Tsiaoussis *et al.*, 2019). It also synthesizes short-chain fatty acids (SCFAs) such as butyrate. These compounds are physiologically relevant for the host as they can act as energy sources for enterocytes and immunomodulators, participate in the neuronal function, anti-inflammatory and metabolic processes such as gluconeogenesis and energy metabolism (Koh *et al.*, 2016; Neish, 2009).

While it has been recognized that a healthy gut microbiota contributes to the host's well-being, emerging evidence suggests that many factors like the diet, environment and exposure to chemicals, among others, may alter the composition and function of the gut microbiome (Rosenfeld, 2017). The gut microbiome imbalance is referred to as "gut dysbiosis", a term currently lacking an international consensus definition (Brussow, 2019; Perez, Dorsen and Squires, 2019). Gut dysbiosis has been linked with an increased abundance of opportunistic "pathogenic" bacteria and decreased "beneficial" species (Hooks and O'Malley, 2017). The altered microbiome may influence the host's homeostasis and potentially contributes to the development of metabolic and inflammatory disorders, endocrine imbalances and neurobehavioral alterations (Feng *et al.*, 2019; Tsiaoussis *et al.*, 2019). Pesticides have the potential to disturb the intestinal bacteria community and cause gut dysbiosis, which may affect the individuals' health (Dechartres *et al.*, 2019; Defois *et al.*, 2018; Gao *et al.*, 2019; Guardia-Escote *et al.*, 2020; Joly Condette *et al.*, 2015).

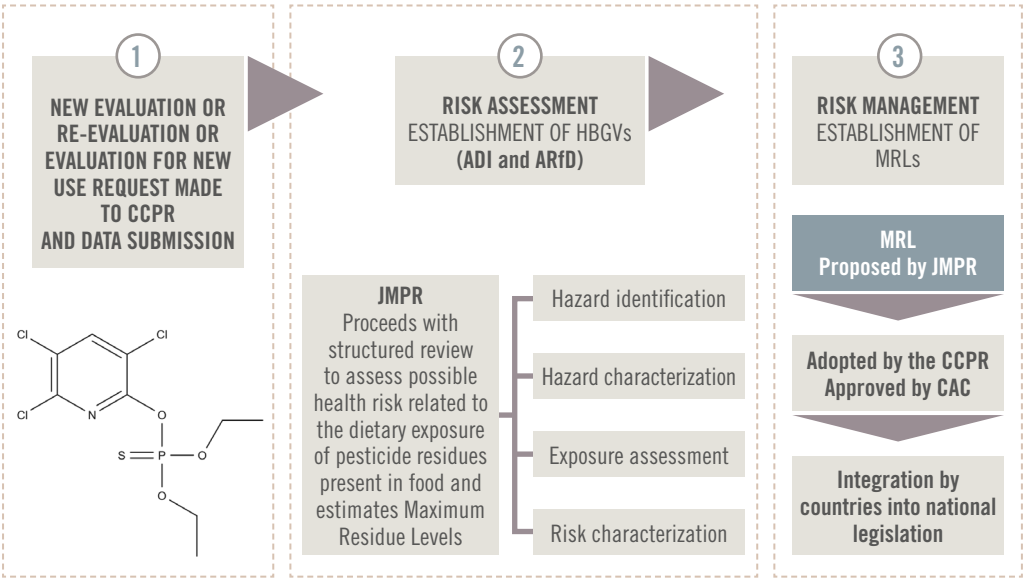
FAO and WHO have collaborated on food safety evaluations and risk assessments for over half a century. The first meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) took place in 1956. In the 1960s, this alliance was strengthened by establishing the Joint Meeting on Pesticide Residues (JMPR) to harmonize requirements and risk assessments on pesticide residues (FAO and WHO, 2009). Since the first meeting in 1963, the JMPR has met annually to conduct scientific evaluations of pesticide residues in food, providing recommendations on acceptable levels of pesticides in food. The JMPR team of experts comprises independent internationally recognized specialists to ensure transparency in the assessment procedures.

Historically, the JMPR has only evaluated active pesticide ingredients. Other potentially toxic residue compounds in pesticide formulations (e.g. solvents, emulsifiers and preservatives) have not been considered. When an active ingredient is evaluated for the first time or re-evaluated, JMPR identifies the compound by its physical and chemical properties, common name and CAS number. Ideally, the sponsors of a compound should submit all the relevant data for its evaluation. However, if data from sponsors is not submitted or is insufficient, the committee relies on available scientific literature. During this assessment, JMPR also considers aggregate,[5]

[5] Aggregate exposure is defined by FAO and WHO as "the combined exposures to a single chemical across multiple routes (oral, dermal, inhalation) and across multiple pathways (food, drinking-water, residential)" (FAO and WHO, 2009).

cumulative[6] and combined exposure[7] in addition to the individual pesticide active ingredient exposure (FAO and WHO, 2009). Therefore, pesticides undergo rigorous analysis to generate recommended health-based guidance values and propose maximum residue limits (MRLs). Proposed MRLs are then submitted to the Codex Alimentarius Commission for approval and can be used by countries to establish national MRLs. This process is summarized in Figure 3.

FIGURE 3 **RISK ANALYSIS PROCESS FOR THE EVALUATION/RE-EVALUATION OF A PESTICIDE**



Source: Authors' own elaboration.

In 2017, JMPR recommended that studies included in risk assessment evaluations should consider the effects of pesticides on the intestinal microbial community and the impact of gut bacteria on the toxicity of xenobiotic compounds. It is important to note that these interactions may be influenced by other factors such as the nutritional status of the host or the chemical metabolism before absorption (FAO and WHO, 2009). The pesticide residue assessment follows the JECFA step–wise–decision–tree approach used to establish microbiological acceptable daily intake (ADI) and/or acute reference dose (ARfD) for veterinary drugs (FAO and WHO, 2019):

> *"The decision–tree approach initially seeks to determine if microbiologically active residues are entering the human colon. If the answer is "no", a microbiological ADI is unnecessary and the toxicological or pharmacological ADI is used.*

6 Cumulative exposure is defined by FAO and WHO as "The sum of exposures to two or more food chemicals that have a common mechanism of toxicity." (FAO and WHO, 2009).

7 "Several chemicals fall into the dual-use category, i.e. used both as a pesticide and as a veterinary drug" (Arcella *et al.*, 2019). A combined exposure is an evaluation that considers exposure to mixtures of substances. "There are four types of combined effect: dose addition, response addition, synergism and antagonism" (FAO and WHO, 2009).

> *However, should potentially microbiologically active residues be present in the colon, data on the two endpoints of public health concern, disruption of the colonization barrier and increase of the population(s) of resistant bacteria, would be evaluated. During the decision–tree process, it is possible to give scientific justifications for omitting testing (i.e. the need for a microbiological ADI) for either one or both end–points"* (FAO and WHO, 2017).

Several pesticides[8] have been evaluated since assessments include microbiological data as criteria parameters. Considering the microbiome in chemical risk assessment is still an idea that needs to mature before being used as a solid parameter. As we understand it today, the gut microbiome is a complex universe by itself, even more when looking at its relationship with the host. It is a relatively novel research area that is evolving in parallel to technological developments and bioinformatics. Currently, there are several challenges to incorporating the gut microbiome in pesticide risk assessment. These include the lack of data on the exposure of the gut microbiome to pesticides, standardized models, methodological limitations and lack of guidance to evaluate microbiome-related data in chemical evaluations.

As a preliminary step towards addressing the potential use of microbiome data in risk assessments, FAO has taken the initiative to explore the status quo of pesticide impact on the gut microbiome and the possible correlation with human health by conducting a review of the existing scientific literature.

8 JMPR 2018: fenpicoxamid, fluazinam, mandestrobin, pydiflumetofen and pyriofenone; JMPR 2019: fidopyropen, buprofezin, pyflubumide, pyridate, tolclofos-methyl exposure, triflumuron, valifenalate.

CHAPTER 2
METHODOLOGY

SCIENTIFIC LITERATURE RESEARCH: SEARCH CRITERIA AND STRATEGY

The scientific literature was screened between September 2019 and May 2020, using English keywords, to identify peer-reviewed articles linking the potential effects of pesticides to the human gut microbiome and possible correlation with human health effects. The databases used to perform the defined queries were *PubMed* (www.ncbi.nlm.nih.gov/pubmed) and *Web of Science* (www.webofknowledge.com). *Scopus* (www.scopus.com) was occasionally used. Annex I contains methodology notes and tables with query results.

A preliminary pilot study was conducted to evaluate potential keyword combinations and to develop approaches to restrict query results (Annex I – Methodology notes, Table AI.1).

The target fields for querying the databases were the Title, Abstract and Keywords. For microbiome, the keyword combination used in the search queries went from more to less restrictive: e.g. "human gut microbiome" to "gut microbiome" to "microbiome". In the case of pesticides, it was challenging to establish a comprehensive yet feasible search strategy due to the high number of pesticides and their multiple classifications. Results from the pilot study led to the following criteria used in a cascade search approach:

1. Pesticide main use category: Keywords were identified based on the *pesticide functional class* defined by the Codex Alimentarius Commission (Codex Alimentarius, 2020), pesticide *main use* from the WHO and International Programme on Chemical Safety report (WHO, 2010), *pesticide use* from the inventory of evaluations performed by JMPR[9,10] (FAO, 2021; WHO, 2021); and *type of pest control* from the National Association of State Departments of Agriculture Research Foundation (NASDA, 2014) (Table AI.2).

2. Individual pesticides: Following the initial search on *pesticide main use category*, a second search on specific pesticides was conducted (Table AI.3).

[9] Inventory of evaluations performed by JMPR https://apps.who.int/pesticide-residues-jmpr-database (accessed 21 February 2022).

[10] JMPR Reports and evaluations https://www.fao.org/pest-and-pesticide-management/guidelines-standards/faowho-joint-meeting-on-pesticide-residues-jmpr/reports/en (accessed 21 February 2022).

3. Pesticide mixtures and co-formulants: Keywords related to pesticides mixture and pesticide formulations were also included due to the not so uncommon presence of multiple pesticide residues in agricultural and food products (EFSA, 2018; EFSA, 2020; FDA, 2020; USDA, 2020) as well as the potential negative health impact posed by pesticide co-formulants in commercial products (e.g. adjuvants)[11] (Coalova, Rios de Molina and Chaufan, 2014; Dechartres *et al.*, 2019; Mao *et al.*, 2018; Mesnage, Bernay and Seralini, 2013; Rueda-Ruzafa *et al.*, 2019). Keywords and keyword blocks identified were "pesticide formulation", "cocktail mixes", "cocktail", "pesticide mixtures", and "cocktail residues" (Table AI.4).

4. Pesticide chemical type category: A final search query approach was conducted with keywords based on the pesticide *chemical type* list from the WHO and International Programme on Chemical Safety Report (WHO, 2010), and on pesticide *chemical class* from the inventory of evaluations performed by JMPR (WHO, 2021) (Table AI.5).

The query approach was composed of two or three blocks of keywords:

1. block containing keywords related to the gut microbiome;
2. the term "Food" (optional); and
3. block containing keywords related to the pesticides.

The following is an example to illustrate the syntax used to query the databases:

("Gut microbiome" OR "Human gut microbiome" OR "Microbiome" OR "Gastrointestinal microbiome") AND "Food" AND ("Pesticides" OR "Pesticide residues" OR "keyword related to pesticide formulation" OR "keyword related to pesticide mixtures" OR "keyword related to pesticide chemical type" OR "keyword relate to pesticide use" OR "keyword related to the single active ingredient").

SCREENING OF ARTICLES AND SELECTION CRITERIA

The literature search resulted in 3 008 articles in *PubMed*, 379 in *Web of Science* and 239 in *Scopus* (Annex I – Methodology notes), including duplicate references. After removing duplicates, search information and metadata from a total of 994 articles (817 articles in *PubMed*, 147 in *Web of Science* and 30 in *Scopus*, and two articles provided by other team members) were tabulated in a master excel file (fields: searched keywords and engine, authors, title, abstract, year, volume, issue, pages and type). Additional fields were added to manage findings and facilitate further filtering, which included full-cited reference, relevance grading, comments (e.g. reason for relevance/exclusion), topic (e.g. food safety or nutrition), chemical group (e.g. pesticides, antibiotics) and chemical compounds mentioned in the article.

[11] According to FAO and WHO (2016, p. 6), a formulation is defined as: "the combination of various ingredients designed to render the product useful and effective for the purpose claimed and for the envisaged mode of application."

After removing duplicates, the title and abstract of articles were screened to categorize manuscripts by the degree of relevance as related to the topic of research "pesticides impact on the gut microbiome", i.e. "relevant", "possibly relevant", and "not relevant". The following criteria were used:

Relevant

Articles were rated *relevant* when the title or abstract included information on pesticides—independent from dose—and possible linkages or effects in the human gut microbiome. Both *in vivo* and *in vitro* studies were considered. *In vivo* studies with a focus on mammal models (ruminants excluded) were especially considered, as they share more physiological and microbiome similarities with humans, compared to other available models (e.g. fish, insects).

Possibly relevant

This category contained articles where their relevancy was uncertain after taking a glance at the title or abstract. Both *in vivo* and *in vitro* studies were considered. This category also included articles potentially relevant for our team that address the gut microbiome exposure to xenobiotic compounds other than pesticides.

Not relevant

Articles were rated *not relevant* when the title or abstract did not include any of the selection criteria used for the relevant and possibly relevant categories. Articles about pesticide trials on the gut microbiome from ruminants and non-mammal models were excluded due to their differences with human gastrointestinal physiology.

All relevant and possibly relevant manuscripts were further reviewed, resulting in a collection of articles eligible for the full-text read. Additional manuscripts were discarded after the full read.

Manuscripts used in this review were assigned a three- or four-letter code plus three numerical digits (Table 1).

TABLE 1 **MANUSCRIPT CODING**

ID REFERENCE	ARTICLE FOCUS
24D###	2,4-D
ADC###	Aldicarb
CBZ###	Carbendazim
CPF###	Chlorpyridos
DZN###	Diazinon
EPX###	Epoxiconazole
GLY###	Glyphosate
IMZ###	Imazalil

ID REFERENCE	ARTICLE FOCUS
MLT###	Malathion
MCP###	Monocrotophos
PERM###	Permethrin
PMB###	Propamocarb
REV###	Review
DTP###	Diethyl phosphate
OCP###	Organochlorine Pesticides

Source: Authors' own elaboration.

PESTICIDE DOSE NORMALIZATION RELATED TO THE ACCEPTABLE DAILY INTAKE

For comparison reasons, dose units were standardized to mg/kg body weight (bw) per day. When experimental doses were not provided as ADI units, pesticide concentrations in the food or water were converted using factors established by FAO and WHO (2009). Once normalized, doses were related to the human ADI[12] and the ARfD[13] established by JMPR.

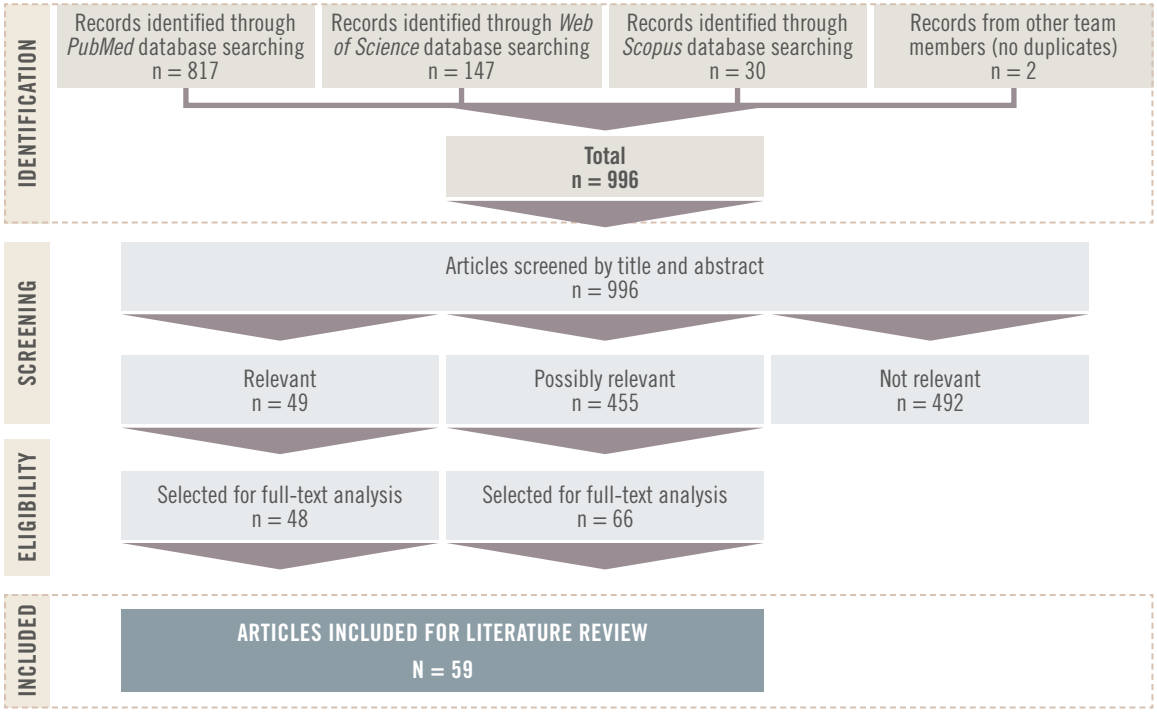
12 "The estimate of the amount of a chemical in food or drinking-water, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk to the consumer. It is derived on the basis of all the known facts at the time of the evaluation" (FAO and WHO, 2009, p. A-2).

13 "The estimate of the amount of a substance in food or drinking-water, expressed on a body weight basis, that can be ingested in a period of 24 h or less without appreciable health risk to the consumer. It is derived on the basis of all the known facts at the time of evaluation" (FAO and WHO, 2009, p. A-3).

CHAPTER 3
FINDINGS

The first electronic searches resulted in 994 unique articles, i.e. 817 articles for *PubMed*, 147 for *Web of Science* and 30 for *Scopus.* Team colleagues provided two additional manuscripts. Figure 4 displays a graphic representation of the article selection process. After screening articles by title and abstract, 98 percent of the *relevant* articles and 15 percent of the *possibly relevant* articles were included to revise the full text. About 56 percent of those eligible for full review were excluded for multiple reasons, e.g. focus on non-gut microbiome (e.g. urine, colostrum) or lack of relevant data on the impact of pesticide exposure to the microbiome and human health outcomes. A total of 59 manuscripts were included in this literature review, including 16 review articles, 36 articles on individual pesticides, 3 articles on pesticide by-products and 4 articles about pesticide mixtures. As review articles overlap with the content of the other manuscripts, they were used for discussion purposes only.

FIGURE 4 **GRAPHIC REPRESENTATION OF THE ARTICLE SELECTION PROCESS FOR LITERATURE REVIEW**



Source: Authors' own elaboration.

INDIVIDUAL PESTICIDES

2,4-DICHLOROPHENOXYACETIC ACID (2,4-D)

The herbicide 2,4-D is a legacy compound that has been widely used as the active ingredient in thousands of formulations worldwide (Tu *et al.*, 2019). The compound 2,4-D mimics the action of a natural plant hormone, indole-3-acetic acid,[14] producing uncontrolled growth in plants and eventually causing death. It was first evaluated by JMPR in 1970 and re-evaluated on several occasions. The most recent evaluation was in 2019 (FAO and WHO, 2020).

Only one manuscript was found for 2,4-D (Table AII.1). Tu *et al.* (2019), evaluated the effects of 1 ppm of 2,4-D in drinking water (~0.26 mg/kg bw/day) in male mice (C57BL/6) after 4 and 13 weeks. The dose used is 26 times higher than the ADI and 60 times lower than the no-observed-adverse-effect level (NOAEL)[15] for sub-chronic exposure in mice (15 mg/kg bw/day) (WHO, 2003). The dose was considered occupationally relevant by the authors. This study focused primarily on the evaluation of low dose exposure to 2,4-D on the microbiome and its metabolism by 16S rRNA gene sequencing, shotgun metagenomic sequencing and metabolomics from faecal samples. The findings showed reduced α-diversity and altered composition of the microbiome composition. Both metagenomics and metabolomics indicated alterations of the amino acid and carbohydrate metabolism. This observation may suggest changes in the utilization preference for these compounds, influencing the host amino acid and energy homeostasis. Host amino acid and energy homeostasis can be influenced by the microbiota's amino acid and carbohydrate metabolism (Flint *et al.*, 2008; Neis, Dejong and Rensen, 2015). Moreover, some toxic metabolites derived from protein fermentation and amino-acid metabolism are thought to have a role in colorectal cancer and chronic kidney disease (Louis, Hold and Flint, 2014; Nallu *et al.*, 2017). Acylcarnitine levels in the host plasma metabolome were also decreased. There is new evidence linking reduced levels of this compound to neurological disorders like Parkinson's and Alzheimer's disease. Although the authors could not prove the link between microbiome perturbations and low plasma levels of acylcarnitine, a clear correlation could be established between this compound and altered microbiota species.

The phyla Bacteroidetes, Chlorobi, Chloroflexi, Spirochaetes and Thermotogae were enriched. Spirochaetes is one of the phyla with increased abundance resulting from 2,4-D exposure. Species belonging to this phylum have been previously linked to the development of dementia, including Alzheimer's disease (Miklossy, 2011).

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14 Indole-3-acetic acid is defined as "a plant growth regulator that affects cell division and proliferation and its levels are maintained by a complex network of pathways" (Tampakaki, Hatziloukas and Panopoulos, 2009, p. 665).

15 "Greatest concentration or amount of a substance, found by experiment or observation, that causes no adverse alteration of morphology, functional capacity, growth, development or lifespan of the target organism distinguishable from those observed in normal (control) organisms of the same species and strain under the same defined conditions of exposure" (FAO and WHO, 2009, p. A-25).

Dehalococcoides ethenogenes was also increased, and it has been shown to play a primary role in the degradation of chlorinated hydrocarbons in contaminated environments (Adrian *et al.*, 2000; Bunge *et al.*, 2003).

ALDICARB

Aldicarb (ADC) is a carbamate insecticide used in agriculture to control mites, nematodes and aphids. It is used on registered crops such as cotton, dry beans, peanuts, soybeans, sugar beets and sweet potatoes. ADC's mode of action is cholinesterase inhibition. It has been evaluated by JMPR several times from 1979 to 2006 (FAO, 2021).

Gao *et al.* (2019) exposed 5 male mice (C57BL/6) to 2 ppm (~ 0.3 mg/kg bw/day) ADC in drinking water for 13 weeks (Table AII.2). The dose was based on the drinking water equivalent level (DWEL) for ADC (0.035 mg/L) (EPA, 2018). The dose used was below the reported equivalent NOAEL in rats (Dourson *et al.*, 1997) and 100 higher than the recommended ADI for this pesticide (0.003 mg/kg bw/day). Multi-omics approaches were used to evaluate the effects of ADC. The sequencing of the 16S rRNA gene and shotgun metagenomics sequencing analysis indicated changes in the gut microbiome structure and increased pathogenicity, respectively. Ten genera decreased, including, Christensenellaceae, which is linked to health maintenance during aging (Biagi *et al.*, 2016). Seven genera considered pathogenic increased, including Erysipelotrichaceae and *Clostridium*. Erysipelotrichaceae is linked to gastrointestinal diseases such as colorectal cancer (Kaakoush, 2015), while *Clostridium* is known to include pathogenic species such as *Clostridium difficile*. The authors reported the enrichment of gene families related to the Quorum Sensing System, which is involved in the pathogenicity of gut bacteria (e.g. virulence, adhesion and bacteriocins), induction of bacterial oxidative stress and DNA damage. Other enriched genes related to protein degradation. Moreover, the lipidomic analysis revealed alterations of lipid profiles. Brain metabolome related to energy metabolism was altered, but the causative role of the gut microbiome in the disruption of brain metabolism could not be established. This would require additional research using germ-free mice or faecal transplantation. Disruption of microbiome–gut-axis has been associated with the development of disorders, including Parkinson disease (Mulak and Bonaz, 2015; Perez-Pardo *et al.*, 2017).

CARBENDAZIM

Carbendazim (CBZ) is a systemic broad-spectrum benzimidazole fungicide,[16] widely used in agriculture to control fungal diseases in cereals and fruits and used as a preservative in agriculture and industry. It is known to act as an environmental endocrine disruptor (Adedara *et al.*, 2013). CBZ has been evaluated by JMPR on several occasions between 1973 and 2019 (FAO and WHO, 2020).

[16] Benzimidazole fungicides are a class of fungicides that include benomyl, carbendazim, thiophanate-methyl, thiabendazole and fuberidazole. They can control various fungal pathogens such as ascomycetes and basidiomycetes, but not oomycetes (Leadbeater, 2014).

Two studies looked at the impact of CBZ on the development of lipid metabolism disorder and gut microbiota dysbiosis. They also evaluated the potential influence of the gut microbiome on the host's lipid metabolism (Jin *et al.*, 2018b; Jin *et al.*, 2015) (Table AII.3). The study design in both manuscripts included mice with different genetic backgrounds, different doses and exposure times. In the first study, the research group exposed male Institute of Cancer Research (ICR) mice to high doses of carbendazim (100 or 500 mg/kg body weight per day) over 4 weeks (Jin *et al.*, 2015). These doses were 3 333 and 16 667 times higher than the ADI of 0.03 mg/kg bw/day. The second study was carried out in male C57BL/6 mice exposed to lower doses (0.1, 0.5 or 5 mg/kg bw/day) for 14 weeks (Jin *et al.*, 2018b). These experimental doses were 7, 33 and 167 times higher than the ADI (Jin *et al.*, 2018b).

Jin *et al.* (2015) observed a reduction in the richness and diversity of the caecal microbiota. CBZ exposure increased the relative abundance of phyla Firmicutes, Proteobacteria, and Actinobacteria and decreased Bacteroidetes. CBD induced an inflammatory response, and contributed to the alteration of the hepatic lipid metabolism (triglycerides and lipid accumulation in the liver and activation of genes related to triglyceride synthesis and lipogenesis). The authors postulated that the gut microbiome also contributed to the alterations observed in the host after the exposure to the unabsorbed pesticide.

At lower doses, the analysis of the transcriptome, inflammation markers and liver activity of samples collected after the chronic exposure to lower doses showed that CBZ induced alterations to the lipid metabolism, hyperlipidemia and a multi-tissue inflammatory response, considered low-grade in the intestinal mucosa (Jin *et al.*, 2018b). The intestinal imbalance was linked to alterations of the diversity and richness of the gut microbiota, characterized by reducing the relative abundance of Bacteroidetes and Verrucomicrobia and an increase of Actinobacteria. However, there was no change in the abundance of Firmicutes and Proteobacteria. Resulting from this study, the authors proposed the mechanisms connecting the microbiome imbalances and the alterations of the hepatic lipid metabolism after long-term exposure of mice to a low dose of CBZ. However, under the study conditions, the authors acknowledged that they could not prove that the gut microbiome is not a driver for the observed changes instead of a parallel event only.

Both studies suggest that pesticide exposure impacts gut bacteria. However, the hepatic metabolism disorder is a more sensitive endpoint. Hence, any potential impact of CBZ at higher doses on the microbiome may be irrelevant.

CHLORPYRIFOS

Chlorpyrifos (CPF) is an organophosphorus insecticide widely used in agriculture to control pests on fruit, vegetable crops and vineyards (Joly *et al.*, 2013). CPF acts on the insects' nervous system by inhibiting the acetylcholinesterase enzyme. This pesticide has gained interest in the research community to evaluate its toxicological risk to humans. This compound has been assessed on several occasions by JMPR from 1972 to 2006 (FAO, 2021).

Several studies reported that chlorpyrifos exposure might alter gut microbiome composition, diversity and functionality (Table AII.4). In addition to potential alterations of the gut microbiome resulting from chlorpyrifos exposure, studies have also looked at the effects on the host hepato-intestinal function, with particular focus on the lipid metabolism and the inflammatory response. The endocrine and nervous systems have also been evaluated. Diverse *in vivo* and *in vitro* approaches were used for assessing chlorpyrifos effects on the gut microbiome. Although most *in vivo* studies were conducted on Wistar rats, mice (C57BL/6 and ICR mice, ApoE-TR) were also used. Some *in vivo* studies considered different factors, including various stages of the animal's life cycle (e.g. during gestation, pups, adolescent, adults), gender, diet composition and host genetic background. *In vivo* studies were conducted at chlorpyrifos doses between 30 and 500 times higher than the recommended ADI by JMPR (0.01 mg/kg bw/day).

Four studies investigated the effects of chlorpyrifos *in vitro*. Joly *et al.* (2013) investigated the effects of chlorpyrifos in both the SHIME®[17] model inoculated with pooled human faeces (1 mg/day, 30 days exposure), and Hannover Wistar rats (1 mg/kg bw via gavage, dams between gestation and weaning day – postnatal day (PND) 21, PND 21 – and pups from PND 21 to PND 60 age), resulting in the development of gut dysbiosis in both models. This study reported an increase of potentially pathogenic *Bacteroides* spp. and a decrease of *Lactobacillus* and *Bifidobacterium* spp. *Lactobacillus* and *Bifidobacterium* spp. are commonly identified as "healthy bacteria" in the human gut microbiome (Lin and Zhang, 2017). This study did not consider the effects on the host. The microbiota evaluation was carried out using traditional microbiological techniques (selective and non-selective media, microscopy and biochemical assays). Although both *in vivo* and *in vitro* models led to gut dysbiosis, there were some differences between the two approaches, for example, *Enterococcus* spp. was more affected in the SHIME® system. Moreover, total aerobic counts were decreased in rat ileum but increased in the equivalent SHIME® reactor.

Reygner *et al.* (2016a) also used the SHIME® model inoculated with pooled human faecal microbiota to evaluate chlorpyrifos at below-threshold doses (1 mg/day) for systemic toxicity (inhibition of brain acetylcholinesterase). The SHIME bioreactor was composed of six vessels, where the last three were inoculated with the faecal material and corresponded to the ascending, transverse and descending sections of the colon. The authors reported slight and transient changes in the composition and overall diversity of the gut bacteria community by using conventional bacterial culture and molecular biology methods (polymerase chain reaction [PCR] amplification with bacteria and *Bifidobacterium* spp primers with detection by Temporal Gradient Gel Electrophoresis; and real-time quantitative PCR of the 16S rRNA gene). Such changes were SHIME-vessel specific. They also observed slightly altered fermentative activity of the gut microbiome, characterized by changes in the

[17] "Simulated human intestinal microbial ecosystem" (Molly, Vande Woestyne and Verstraete, 1993, p. 254).

profile of bacterial metabolites (SCFA). Réquilé *et al.* (2018) evaluated the effects of CPF (3.5 mg/day) in the SHIME® model (inoculated with faecal microbiota from male and female human donors). Extracts from the reactor were later added to the Caco-2/TC7 cell culture model. The authors found that chlorpyrifos has the potential to induce dysbiosis (reduced *Lactobacillus* and *Bifidobacterium* counts) and cause metabolic imbalances in the intestinal environment. The authors speculated that CPF might inhibit the growth and metabolism of lactic acid bacteria. Moreover, CPF affects the activity of the mucosal barrier, and it might potentiate the inflammation processes. This model was also used to evaluate the effect of inulin supplementation, which increased levels of SCFA and partially reversed the dysbiosis induced by chlorpyrifos. Bacterial samples from the SHIME® model were grown in culture media, and the authors acknowledged that molecular profiling of the microbiota would have provided more accurate information about chlorpyrifos and inulin exposure. Mendler *et al.* (2020) designed a study to evaluate whether CPF affects mucosal-associated invariant T-cells (MAIT)[18] cell-activating or -inhibiting bacteria. This study targeted selected non-pathogenic microbiota bacteria species (*Bifidobacterium adolescentis*, *Lactobacillus reuteri* and *Escherichia coli*) representative of the healthy microbiota. Results from this study suggest that CPF might alter the metabolism of the bacteria species evaluated, specifically riboflavin and folate biosynthesis. After CPF exposure, MAIT cell activation was increased by *E. coli* and reduced by *B. adolescentis* and *L. reuteri*. It also resulted in increased production of inflammatory cytokines by MAIT cells, which might potentially contribute to the development of inflammatory-based disorders.

Three studies investigated the influence of a high-fat diet on the effects that CPF exposure could exert on the gut microbiome (Fang *et al.*, 2018; Li *et al.*, 2019; Liang *et al.*, 2019). All studies were conducted on male rodents. The treatments on Wistar rats were 0.3 mg/kg bw/day for 20 weeks in adults and 25 weeks in pups (Li *et al.*, 2019), and 0.3 and 3 mg/kg bw/day for 9 weeks (Fang *et al.*, 2018). These two doses correspond to ~1/500 and 1/50 of LD50 for chlorpyrifos (Mansour and Mossa, 2010; Wang *et al.*, 2009). The third study was carried out on C57BL/6 and CD-1 male mice with a dose of 5 mg/kg bw/day for 12 weeks (Liang *et al.*, 2019). These three doses are 30, 300 and 500 times higher than the ADI recommended by JMPR for CPF. Fang *et al.* (2018) observed diet-dependent changes in the gut microbiota composition, with more relevant alterations at both doses combined with the high-fat diet. In general, there was an increase in the abundance of opportunistic pathogens, SCFA-producing bacteria, and bacteria associated with neurotoxicity, obese and diabetic phenotypes. In the host, CPF effects were dose- and diet-dependent, with the low dose and non-fat diet leading to more remarkable metabolic changes. The authors indicated that fat intake might influence the effects

[18] There is evidence that microbial-derived riboflavin and folate regulate their activity (Mendler *et al.*, 2020). They have been found in the inflamed tissues of patients with Crohn's disease, multiple sclerosis, reumatoid arthritis and asthma (Carolan *et al.*, 2015; Chiba, Murayama and Miyake, 2018; Lezmi and Leite-de-Moraes, 2018; Serriari *et al.*, 2014).

of CPF on glucose and lipid metabolism and promote the development of type-2 diabetes. However, CPF might have anti-obesity effects in rats fed with the high-fat diet. Li *et al.* (2019) concluded that CPF effects on the microbiome were more apparent in the high-fat diet and at early exposure (starting at weaning). Alterations of the microbiome affected SCFA-producing bacteria, testosterone-related genera, pathogenic bacteria and bacteria related to inflammatory processes. These bacteria seem to be involved in the regulation of the endocrine system, immune response and gut barrier. Disturbed endocrine and immune response observed in the rats after early exposure to chlorpyrifos seemed to be reverted by a high-fat diet.

Liang and colleagues (2019) reported that CPF altered the gut microbiota, affected the intestinal integrity and induced low-grade inflammation, which was aggravatated in animals fed HFD. Liang's team confirmed the gut microbiome involvement in the development of CPF-induced fat deposition and insulin resistance by recolonizing near-germ free mice (treated with antibiotics) with CPF-altered microbiota. In addition to diet, Liang *et al.* (2019) also considered the genetic background of mice C57BL/6 and CD-1 (ICR) in the evaluation of CPF. However, the differences were limited.

Another study investigating the genetic background found that changes in the gut microbiome composition and functionality were dependant on the host's genetic (apoE-TR mice - ε3 and ε4 allele, which express different isoforms of apoE, and C57BL/6 mice) and exposure to CPF (Guardia-Escote *et al.*, 2020). Postnatal exposure (PND 10-15) to 1 mg/kg bw/day of CPF was associated with the affected cerebral fatty acids synthesis in the host. This alteration may have implications for the host's cognitive function and behaviour. The dose used in this study was 100 times higher than the recommended ADI.

Two studies investigated gender as a variable in the evaluation of CPF exposure in rats (Perez-Fernandez *et al.*, 2020; Reygner *et al.*, 2016b). Researchers found that CPF can induce gut dysbiosis and gender-specific alterations in newly weaned rats. Perez-Fernandez *et al.* (2020) exposed Wistar rats to 1 mg/kg bw/day CPF for 6 days (PND 10-15). Although they observed gender-related differences in the gut microbiota, with alterations at the genus and species level, they could not link the identified bacteria species to the GABAergic system[19] or observed changes in GABA production. Moreover, the species that have been associated with the GABA system in previous studies were not altered in this one. Another study investigated the effects of 30-day exposure to 1 mg/kg bw/day of CPF in male adult KM mice, by using high-throughput sequencing and nuclear magnetic resonance-based metabolomics (Zhao *et al.*, 2016). They found a high correlation between changes in the gut microbiome and altered metabolic profiles, which the authors

[19] GABA (γ-aminobutyric acid) is defined as "an amino acid with mostly inhibitory functions in the mammalian central nervous system. Structures involved in releasing or binding GABA as a neurotransmitter constitute the GABAergic system. The GABAergic system is involved in the regulation of vigilance, anxiety, muscle tension, epileptogenic activity and memory functions" (Rudolph, 2008, p. 515).

linked to intestinal inflammation and abnormal intestinal permeability observed in the host. Joly Condette *et al.* (2015) exposed female Wistar rats to a daily dose of 1 or 5 mg/kg CPF during pregnancy until weaning and evaluated effects in male pups at PND 21 and 60. A targeted microbial evaluation was conducted using culture and molecular methods from intestinal digesta (ileum, caecum, colon) and faecal samples. The authors concluded that CPF exposure in mothers could affect the pup's intestinal development, influencing nutrient absorption, mucosal barrier, stimulation of immune system and microbial dysbiosis. Although microbial alterations varied depending on the intestinal location, mouse age and dose, the impact seemed higher at the PND 21. For example, aerobic and anaerobic bacteria counts increased primarily in the ileum, *Clostridium* spp. and *Staphylococcus* spp. increased in samples from all intestinal segments tested (ileum, caecum, colon), while *Bifidobacterium* spp. decreased only in the ileum. *Lactobacillus* spp. counts decreased in all segments at both ages, but a limited effect was shown by qPCR. Also, bacterial proliferation and invasion were stronger at PND 21 than PND 60, which, according to the authors, may be related to the less mature immune system and mucosal barrier in the younger mice.

One additional study evaluated the prebiotic inulin to possibly alleviate the effects of perinatal exposure to CPF (1 or 3.5 mg/kg bw/day) in Wistar rats dams and pups (Reygner *et al.*, 2016b). The low CPF dose had a higher effect on the microbial parameters tested. For example, it reduced the abundance of Firmicutes and the Firmicutes/Bacteroidetes ratio. On the contrary, the high dose induced more substantial effects on metabolic parameters (glucose and lipid metabolism) and body weight. It was observed that inulin supplementation could partially reverse the effects caused by the CPF treatment, including the reduced ratio Firmicutes/Bacteroidetes (associated with disorders like obesity [Tremaroli and Backhed, 2012]). Moreover, inulin also increased the intestinal concentration of the short-chain fatty acids, known to be energy substrates for gut cells and contributors to epithelial integrity (Guilloteau *et al.*, 2010; Morrison and Preston, 2016).

DELTAMETHRIN

Deltamethrin (DLM) is a synthetic pyrethroid ester insecticide, widely used in agriculture and as a home pest control agent. It acts by disrupting the function of the insects' nervous system. This compound has been evaluated several times by JMPR from 1980 to 2016 (FAO, 2021).

Only one study analysed the effects of deltamethrin on the gut microbiome (Table AII.5) (Defois *et al.*, 2018). The authors designed an *in vitro* study in a continuous bioreactor inoculated with faeces from a single human donor. They exposed the microbiota to a dose of 21 µg/mL deltamethrin for 24 hours, which is higher than the expected daily consumption. Supernatants from the fermenter were then transferred to an intestinal epithelial Caco-2/TC7 cell culture and incubated for four hours to evaluate the potential cell inflammatory response.

Metatranscriptome and microbial volatolome[20] analyses were used to study the microbiome's function (the microbiota composition was not evaluated in this study). After deltamethrin exposure, the authors found an enrichment of the microbial volatolome, especially sulphur compounds. They also observed functional dysbiosis associated with altered metabolic pathways. Deltamethrin induced an inflammatory response in TC7 cells, as evidenced by the increased cytokine IL-8 release. The authors denoted that "human biotransformation enzymes, may also take into account gut microbial processes, leading to more or less toxic compounds and/or microbial pro-inflammatory molecules. Depending on the pollutant and the intensity and frequency of exposure, gut microbiota could either protect host cells or enhance toxic and inflammatory responses" (Defois *et al.* 2018, p. 8).

DIAZINON

Diazinon (DZN) is an organophosphorus insecticide used in agriculture and veterinary medicine as an efficient insecticide. Its active biological metabolite, known as diazoxon, inhibits cholinesterases activity. Residues in food are more commonly found in edible crops. Residues in animal products (e.g. meat, offal) generally arise from its veterinary use as a drug rather than pesticide use. This compound has been evaluated several times by JMPR since 1963 and more recently in 2016 (FAO, 2021).

Gao conducted two studies on diazinon (Gao *et al.*, 2017a; Gao *et al.*, 2017b) (Table AII.6). Both studies were carried out in C57BL/6 mice exposed to DZN at a dose of 0.6 mg/kg bw/day for 13 weeks. This dose is 120 times higher than the recommended ADI (0.005 mg/kg bw/day). Gao *et al.* (2017b) focused on evaluating the effects of DZN on the microbiome composition and its metabolic functions in both genders by using omics approaches based on 16S rRNA gene sequencing, metagenomics sequencing and metabolomics analysis. Alterations observed on the microbiome structure, functional metagenome and metabolic profiles were more prominent in males than females. For example, after DZN exposure, Bacteroidetes increased and Firmicutes were reduced only in males. The prevalence of pathogenic bacteria was only observed in males, including the Burkholderiales order, which contains species involved in human disorders, including Crohn's disease (Sim *et al.*, 2010). Also, genes involved in the synthesis of neurotransmitters and signalling molecules, known to be associated with neurotoxicity (Bjørling-Poulsen, Andersen and Grandjean, 2008), were specially altered in males. The decreased abundance of Lachnospiraceae family, a relevant SCFA-producing group, was observed in both males and females. The authors also reported a possible link between gut dysbiosis induced by DZN and neurotoxicity. However, they could not establish the causative role of gut microbiome disturbances in the sex (male)-specific neurotoxicity of DZN. Gao *et al.* (2017a) studied the effects of DZN on the gut metatranscriptome. They reported that DZN modulates the Quorum Sensing System.

[20] "Volatolomics focuses on the study of volatile metabolites reducing the complexity of the analysis. This method has proven to be a promising omic approach to diagnose metabolism changes in response to physiological stresses induced by pathology or xenobiotic exposure" (Defois *et al.*, 2018, p. 2).

This system regulates cell-to-cell communications within the bacterial population and its behaviour. Specifically, DZN activated pathways related to bacterial motility and cell wall elements, which contribute to bacterial pathogenicity and systemic inflammation in the host. In addition, the metatranscriptomics analysis also showed the role of DZN in activating the stress response pathways and impairing the energy metabolism of gut bacteria.

ENDOSULFAN

Endosulfan (ENS) is an organochlorine pesticide widely used in agriculture as an insecticide and acaricide. This compound has been evaluated on several occasions by JMPR from 1965 to 2010 (FAO, 2021). JMPR established the ADI (0-0.006 mg/kg bw) and ARfD (0.02 mg/kg bw) for this pesticide in 1998. The Codex Alimentarius Commission also established pesticide MRLs for ENS on several commodities (0.01-10 mg/kg) between 2003 and 2011 (Codex Alimentarius, 2020). It is important to note that in 2011, ENS was added to the Annex A of the Stockholm Convention list for its extensive use and persistent organic pollutants (POPs) characteristics. The Stockholm Convention list is an international environmental treaty created to protect human health and the environment from POPs. Annex A prohibits the use or production of chemicals under this list, with specific exemptions (Stockholm Convention, 2020).

Zhang and colleagues (Zhang *et al.*, 2017) evaluated potential metabolic perturbations and subacute toxic effects induced by endosulfan exposure (0.5 and 3.5 mg/kg bw) in male ICR mice for two weeks (Table AII.7). The doses were derived from the NOAEL for acute neurobehavioural toxicity in rats (0.7 mg/kg bw) (Silva and Beauvais, 2010) and previous hepatotoxicity and reproductive toxicity studies in mice (5 mg/kg bw) (Guo *et al.*, 2016; Uboh, Asuquo and Eteng, 2011). The experimental doses of ENS were 83 and 583 times higher than the recommended ADI. This study did not evaluate the gut microbiota composition. The metabolome analysis revealed specific metabolites related to the gut microbial metabolic activity, i.e. decreased hippurate in both treated groups. Choline metabolism also seemed affected, as shown by the increased levels of choline, dimethylamine and trimethylamine N-oxide. According to the authors, these observations suggest alterations of the gut microbiome. However, no relationships were made between these findings and changes found in the host after ENS exposure (i.e. liver injury, disruption of amino acid, lipid, energy metabolism).

EPOXICONAZOLE

Epoxiconazole (EPX) is a broad-spectrum fungicide from the azoles class used to protect crops in agriculture by stopping the production of new fungal spores and interrupting the fungal cell membrane synthesis. JMPR has not evaluated this pesticide, and, therefore, there are no international recommended health-based guidance values (ADI, ARfD) or MRLs for this compound.

One study investigated the effects of EPX (4 and 100 mg/kg bw/day) for 90 days (~13 weeks) on female Sprague-Dawley rats (Xu *et al.*, 2014) (Table AII.8). The low dose was lower than the reported NOAEL (5 mg/kg/day), and the high dose higher than the LOAEL (15 mg/kg/day) (EPA, 2006). A clear disruption to the gut microbiome was reported after exposure to both doses, although more significant at the high dose. The phyla Bacteroidetes and Proteobacteria were increased, and Firmicutes decreased, a sign of microbiota dysbiosis. The most affected families were Enterobacteriaceae and Lachnospiraceae, where both increased. The latter is involved in carbohydrate fermentation into SCFAs (e.g. butyrate), which are relevant for maintaining the gut barrier integrity and modulation of gastrointestinal, immune responses (Cotta and Forster, 2006; Meijer, de Vos and Priebe, 2010). Biochemical alterations were limited to increased glucose levels and decreased serum levels of total bilirubin, with no microscopic liver abnormalities. Because EPX effects are observed first in the microbiome, the authors proposed it as an early indicator to monitor the host's health risks.

GLYPHOSATE

Glyphosate (GLY) is a non-selective systemic herbicide. Since the 1970s, the volume of substances containing glyphosate as an active ingredient has increased significantly, and it is widely used in combination with glyphosate-resistant genetically modified plants. Today glyphosate is one of the most used herbicides worldwide. Glyphosate's mode of action is distinctly different from other organophosphates and very specific to this herbicide. It inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS),[21] a specific enzyme found in plants and some bacterial species (Zhi *et al.*, 2014) but not in animals.

This compound has been evaluated by JMPR several times since 1986 (FAO, 2021). In 2004, the compound was re-evaluated due to public health concerns raised by the International Agency for Research on Cancer (IARC) and many research studies. After the re-evaluation in 2004, health concerns associated with cancer continued. For this reason, JMPR re-evaluated glyphosate in 2016 with a special focus on genotoxicity, carcinogenicity, reproductive and developmental toxicity. The group also considered epidemiological studies related to cancer. A review of published scientific literature was also conducted during the same evaluation to assess glyphosate's capacity to bioaccumulate or affect the human gut microbiome. The committee did not find any specific studies associated with adverse effects on the mammalian gut microbiome (e.g. mouse, rats, rabbit, humans), and concluded that several studies (e.g. pharmacokinetic, toxicokinetic and bioavailability) had demonstrated the poor absorption of glyphosate after oral administration.

Several research studies have investigated the impact of glyphosate, alone or as part of commercial formulations (e.g. Roundup® and Glyfonova®), on the gut microbiome

[21] 5-enolpyruvylshikimate-3-phosphate synthase is a key enzyme of the shikimate pathway responsible for the biosynthesis of aromatic amino acids in plants (Boocock and Coggins, 1983).

and the host (Table AII.9). They include *in vitro* and *in vivo* approaches. Only one study investigated the effects of glyphosate *in vitro* on select cultured riboflavin- and folate-producing bacteria species, *Bifidobacterium adolescentis*, *Lactobacillus reuteri*, and *Escherichia coli* (Mendler *et al.*, 2020). They also evaluated the potential of these bacterial species to activate MAIT cells. The authors concluded that exposure to GLY, and to a lesser extent than chlorpyrifos (also studied here), has the potential to alter bacterial metabolism and favour the pro-inflammatory immune response in the host.

The following scientific publications describe studies conducted *in vivo*, most using Sprague-Dawley rats (Dechartres *et al.*, 2019; Lozano *et al.*, 2018; Mao *et al.*, 2018; Nielsen *et al.*, 2018; Tang *et al.*, 2020b). Only one study used Swiss mice (Aitbali *et al.*, 2018). Research studies varied in purpose (effect of GLY on microbiome, host gut, host early development and behaviour) and design (doses, exposure time). Experimental doses were quite variable, mostly based on existing reference doses ranging from ADI to NOAEL. Except for one dose 25 x 10^{-7} times lower than the ADI for GLY (1 mg/kg bw), the rest were between 1.8-5 (at the low end) and 50-500 times (at the high end) higher than the ADI. Exposure times ranged from two weeks to two years.

Nielsen *et al.* (2018) found limited effects of GLY (pure and commercial formulation Glyfonova®) on the gut microbiota composition. Moreover, they did not observe physiological alterations in the organs of Sprague-Dawley rats after a two-week exposure to 2.5 or 25 mg/kg bw/day. Doses were 5 and 50 times the European Union ADI, 0.5 mg/kg bw (EFSA, 2015). The authors noted that the presence in the diet of aromatic amino acids might have contributed to preventing the antimicrobial effects of GLY. Therefore, they suggested that malnutrition might be a risk factor for glyphosate toxicity.

In a 13-week pilot study, Mao *et al.* (2018) evaluated the effects of doses lower than those from Nielsen's study (1.75 mg/kg bw/day pure GLY and GLY in the commercial product Roundup®) in the gut microbiome and the early development of Sprague-Dawley rats, from gestation to PND 125. Test substances were provided in drinking water and the dose used was described by the authors as comparable to the United States of America chronic reference dose (cRfD), 1.75 mg/kg bw/day at the time of the study.[22] Some alterations observed at postnatal day 31 (equivalent to pre-pubertal age in humans) were not apparent at postnatal day 57. Some changes to the microbiota composition were common after exposure to both GLY alone and in the commercial formulation (e.g. increased *Prevotella*, reduced *Lactobacillus*), and some were treatment-dependent (e.g. GLY: increased *Blautia*, decreased *Streptococcus*; Roundup®: increased *Parabacteroides*). Gender differences were only apparent at postnatal day 125. Effects on the microbiota were not significant in adult dams. No unusual behaviour was observed in either mothers or pups.

[22] After the new risk assessment of glyphosate, cRfD is 1 mg/kg/day https://downloads.regulations.gov/EPA-HQ-OPP-2009-0361-0068/content.pdf [Cited 30 December 2021].

Two studies resulted in altered microbiota composition and behaviour in Sprague-Dawley rat dams and male Swiss mice exposed to GLY (alone and in commercial formulation) at 5 mg/kg bw/day (Dechartres *et al.*, 2019) and to 250 and 500 mg/kg bw/day (Aitbali *et al.*, 2018), respectively. The dose used by Dechartres was 1/10 of the relevant maternal NOAEL for developmental toxicity (50 mg/kg bw/day) (EFSA, 2015), and Aitbali selected the experimental doses based on the NOAEL (500 mg/kg bw/day) for sub-chronic toxicity (EPA, 1993). Dechartres's group could not explain if GLY and Roundup® were the direct cause of the observed alterations of the central nervous system and rat behaviour (Dechartres *et al.*, 2019). However, they confirmed that GLY alone and in formulation could result in different outcomes (including the gut microbiota composition), likely due to the presence of co-formulants in the commercial product. At the phylum level, only Roundup® affected Bacteroidetes (increase) and Firmicutes (reduced). Aitbali *et al.* (2018) evaluated Roundup® only, resulting in altered abundance and diversity of the gut microbiota. Especially relevant was the decrease of *Corynebacterium*, Firmicutes, Bacteroidetes and *Lactobacillus*. The authors suggested that the intestinal dysbiosis observed after the herbicide exposure might increase the prevalence of neurobehavioural alterations, such as those observed in this study (anxiety and depression-like behaviours). However, they did not provide evidence about the mechanisms explaining the microbiome's potential role in this process.

One additional study evaluated the effects of pure GLY on the small intestine of male Sprague-Dawley rats and gut microbiota composition (Tang *et al.*, 2020b). The animals were exposed to 5, 50 or 500 mg/kg bw/day GLY for five weeks. The references for the experimental doses were the NOAEL 1 000 mg/kg/day (Williams, Kroes and Munro, 2000), and were approximately 1/1 000, 1/100 and 1/10 of the median lethal dose[23] (LD_{50}) (5 600 mg/kg) for rats (Benedetti *et al.*, 2004). Although the Firmicutes to Bacteroidetes ratio was not altered significantly, the microbiota diversity and composition changed, with decreased abundance of the Firmicutes, especially the genus *Lactobacillus*, and increased populations potentially pathogenic, especially at the highest dose of GLY. This dose of the herbicide also led to histological alterations of the duodenum and jejunum sections of the small intestine. It also shifted the indicators of oxidative stress, ion concentration and upregulation of genes related to the inflammatory response. The authors speculated that alterations of the microbiota could have caused the changes observed in the host. However, further research is needed to prove causality and determine the involved mechanisms.

Lozano *et al.* (2018) exposed male and female Sprague-Dawley rats to Roundup® (0.1 ppb, 400 ppm and 5 000 ppm in drinking water, with estimated GLY content of 50 ng/L, 0.1 g/L and 2.25 g/L) over two years. The study evaluated the impact of the herbicide exclusively on the gut microbiota. In the evaluation of samples after 673 days of exposure, the authors observed gender-specific alterations of

[23] Single oral dose that is predicted to cause death in 50% of the test animals. LD50 values are commonly presented as milligrams of compound per kilogram of body weight of the animal (Morris-Schaffer and McCoy, 2021, p. 25).

the gut microbiota of treated female groups. The authors observed alterations of the gut microbiota, e.g. increased abundance of Bacteroidetes and decreased Lactobacillaceae. Moreover, they studied the tolerance of cultivable bacteria species to the herbicide. The tolerance of *Escherichia coli* to Roundup® was confirmed by the absence of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene. The authors speculated that the gut dysbiosis observed in their research could be associated with liver dysfunction seen in other studies. As it will be further discussed later, there are several factors to consider in this study to assess the results and the conclusions: (1) the low number of individuals per treatment group (n=3), the length of the study (two years) with rats in the late phase of their life cycle); (2) no monitoring at mid-points, only in samples after 673 days exposure; and (3) no evaluation of the host. All these factors make it difficult to evaluate results in a scientifically sound manner.

Although it is outside the scope of this study, it should be acknowledged that in 2010 glyphosate was patented as an antimicrobial by Monsanto Technology LLC. Patents can only claim intellectual property rights, but they cannot prove that the application of a certain compound is efficient, effective or safe. When a new compound is available on the market, a company must register the chemical compound by submitting data supporting the use, mode of action, safety measures and concerns, risks, etc. Shehata *et al.* (2013) demonstrated in an *in vitro* study that glyphosate can act as an antimicrobial, leading to dysbiosis in poultry gut, diminishing the abundance of beneficial bacteria and causing overgrowth of pathogenic species lacking the EPSPS gene (glyphosate resistant). However, this statement is debatable since glyphosate alone is not very effective, and it needs other co-formulants to achieve antibacterial and antiparasitic activity (Lozano *et al.*, 2018). Additionally, similar to the JMPR evaluations of pesticide residues, JECFA evaluates the safety of veterinary drug residues and other compounds (e.g. food additives, contaminants). Glyphosate has not been approved as an antimicrobial drug by JECFA or any other regulating body.

IMAZALIL

Imazalil (IMZ) is a broad-spectrum fungicide widely used to protect and treat plants and animals from fungal diseases (Jin *et al.*, 2016). This compound was evaluated several times by JMPR between 1977 and 2018 (FAO, 2021).

Two studies from Jin's group have investigated the effects of IMZ on the gut microbiome and the integrity and function of the intestine (Table AII.10). The earlier study exposed male ICR mice to high doses of IMZ (25, 50 and 100 mg/kg bw/day) for four weeks, resulting in gut dysbiosis (Jin *et al.*, 2016). It was characterized by reduced richness and diversity of the caecal and faecal microbiota. There was an increase of pathogenic bacteria, i.e. Deltaproteobacteria and *Desulfovibrio*, which are sulphate-reducing bacteria that can alter the intestinal barrier function (Pitcher and Cummings, 1996; Roediger, Moore and Babidge, 1997). There was also a decrease in beneficial bacteria, i.e. *Lactobacillus* and *Bifidobacterium*, which are known to be involved in modulating the gastrointestinal immune and inflammatory processes

(Cani *et al.*, 2007; Sanz, Nadal and Sanchez, 2007). Effects were dose-dependent and more prominent at higher doses. The exposure to 100 mg/kg IMZ reduced the Firmicutes/Bacteroidetes ratio, which might cause or aggravate, directly or indirectly, colon inflammation. The authors recognized the need to consider health risks associated with exposure to environmentally relevant pesticide concentrations (Jin *et al.*, 2018a). Thus, the follow-up study design considered the WHO maximum allowable residue levels of IMZ in citrus fruits (5 mg/kg) and bananas (2 mg/kg). Based on this information, the authors exposed C57BL/6 male mice to lower doses of IMZ (0.1, 0.5 and 2.5 mg/kg) for 2, 5 and 15 weeks. Results showed that IMZ exposure induced gut dysbiosis (more significant in the 2.5 mg/kg IMZ dose for 15 weeks). IMZ also reduced the mucus secretion and altered the intestinal ion translocation through proposed mechanisms, ultimately affecting the structure and functional integrity of the mouse intestine. It is important to note that the authors found that 45 days after the study conclusion and without exposure to imazalil, the gut bacteria composition recovered partially. However, some of the adverse effects in the colon were not recovered. The doses used in these studies were 833, 1 667, 3 333 (Jin *et al.*, 2016) and 3, 17, 83 (Jin *et al.*, 2018a) times higher than the recommended JMPR ADI (0.03 mg/kg bw/day).

MALATHION

Malathion (MLT) is a non-systemic organophosphorus insecticide used to control insects on agricultural crops, stored commodities and is a vector control agent. It acts by inhibiting cholinesterase activity. JMPR has evaluated this compound on several occasions since 1965 (FAO, 2021). In 2016, it was re-evaluated due to public health concerns raised by IARC and available scientific studies. The re-evaluation considered toxicological and epidemiological studies with cancer-related outcomes. An extensive literature search was also conducted to identify potential adverse effects on the gut microbiome, or to evaluate whether the gut microbiome has the capacity to metabolize this compound. However, at this time, nothing was found.

The study by Gao *et al.* (2018) investigated the impacts of MLT on the gut microbiome only, specifically on the Quorum Sensing System (Table AII.11). This system is relevant because it can modulate intra- and interspecies gene expression and coordinate bacterial population responses, including virulence and motility (Gao, *et al.*, 2018). Male C57BL/6 mice received a daily dose of ~0.6 mg/kg bw/day (below the threshold for neurotoxicity) in drinking water for 13 weeks. It included an initial sample collection after four weeks of exposure. The dose was twice the recommended ADI by the JMPR (0.3 mg/kg bw/day). The authors observed alterations of the gut microbiome. Potentially pathogenic bacteria increased (*Clostridium*, Mogibacteriaceae), and beneficial bacteria were either decreased (*Akkermansia muciniphila*) or depleted (*Blautia, Roseburia,* Christensenellaceae and Planococcaceae). MLT also affected the Quorum Sensing System, resulting in an increased abundance of virulence and pathogenicity-related genes, e.g. those involved in motility. The authors provided a potential mechanistic explanation for the role of the microbiome Quorum Sensing System in the toxicity of MLT.

MONOCROTOPHOS

Monocrotophos (MCP) is an organophosphate insecticide. This compound has been evaluated by JMPR on several occasions from 1972 until 1994 (FAO, 2021). Monocrotophos is part of the Prior Informed Consent (PIC) procedures because "it is a highly toxic pesticide that is likely to cause problems under conditions of storage, transportation and use in developing countries" (FAO/UNEP, 1997). The PIC procedures apply to all chemicals in Annex III of The Rotterdam Convention.[24] "The PIC procedure is a mechanism for formally obtaining and disseminating the decisions of importing Parties as to whether they wish to receive future shipments of those chemicals listed in Annex III of the Convention and for ensuring compliance with these decisions by exporting Parties" (Rotterdam Convention, 2010b).[25]

Velmurugan *et al.* (2017) identified monocrotophos as the most frequently used organophosphate insecticide in a survey conducted on individuals from rural India. This population showed a high prevalence of diabetes. Also, the study aimed to evaluate the role of gut microbiota on the development of organophosphate-induced hyperglycemia. The research was carried out in female BALB/c mice (Table AII.12) treated with 28 µg/kg bw/day MCP for 180 days in drinking water. The dose chosen corresponds to 10x the theoretical maximum daily intake (TMDI) for MCP (0.17 mg/day) (Bhushan, Bharadwaj and Misra, 2013). The TMDI was calculated following WHO recommendations based on the MRL for selected grains and cereals. The dose was 47 times higher than the recommended JMPR ADI (0.0006 mg/kg bw/day). This study did not investigate changes in the gut microbiome composition but the expression of bacterial genes by metatranscriptome analysis. Faecal SCFA were also evaluated in addition to several host parameters and indicators of metabolic activity. All evidence led the authors to conclude that the gut microbiome is involved in gluconeogenesis via microbial SCFAs resulting from the degradation of the organophosphate. This contributes to the development of glucose intolerance and increases the risk of diabetes. The involvement of the microbiota in this process was further confirmed by faecal and culture transplantation, where animals receiving faecal microbiota from MCP-fed mice demonstrated significant glucose intolerance compared to animals receiving control faecal microbiota.

PENCONAZOLE

Penconazole (PNZ) is a systemic triazole fungicide widely used to control fungal diseases in fruits and vegetables. It inhibits fungal growth by interfering with the biosynthesis of sterols in cell membranes. This compound has been evaluated four times by the JMPR: 1992, 1995, 2015 and 2016 (FAO, 2021).

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[24] The Rotterdam Convention is a multilateral environmental agreement to promote shared responsibilities related to certain hazardous chemicals among countries or regional economic integration organizations (Rotterdam Convention, 2010a) http://www.pic.int/TheConvention/Overview/Howitworks/tabid/1046/language/en-US/Default.aspx (accessed 21 February 2022).

[25] http://www.pic.int/en-us/procedures/picprocedure.aspx (accessed 21 February 2022).

Meng *et al.* (2019) looked into the effects of penconazole and its enantiomers[26] on the gut microbiome (Table AII.13). Some pesticides, like penconazole, have a unique chiral structure.[27,28] ICR male mice were given 30 mg/L PNZ in drinking water for 28 days. The dose is 150 times higher than the recommended JMPR ADI (0.03 mg/kg bw/day). The authors studied the composition of the caecal microbiota and the serum metabolome. Gut dysbiosis and altered metabolic profiles related to the tryptophan, glucose and lipid metabolism were observed after exposure to all three enantiomers studied, i.e. (−)-Penconazole; (+)-Penconazole and (±)-Penconazole. However, there were differences among them, being (−)-Penconazole the enantiomer with more pronounced effects. The authors concluded that PNZ perturbs the gut microbiome, which might participate in metabolic disorders. Meng *et al.* (2019) also suggested the need for additional research to evaluate the potential toxicological effects of PNZ and its enantiomers.

PERMETHRIN

Permethrin (PERM) is a non-systemic pyrethroid insecticide that targets the nervous systems of insects and mites. It is an effective chemical against a wide range of pests in agriculture, animal and human health. This compound was evaluated on several occasions by JMPR before 2000. The first evaluation took place in 1979 and the last toxicological assessment was in 1999 (FAO, 2021).

Two related studies have investigated the effects of PERM on the gut microbiome (Bordoni *et al.*, 2019; Nasuti *et al.*, 2016) (Table AII.14). The design for these two studies were almost identical. Both investigated the effects of 34 mg/kg bw/day permethrin exposure in Wistar male rat pups from PND 6 to 21. The dose used was slightly higher than the NOAEL for PERM (25 mg/kg bw/day). This dose is 680 times higher than the recommended ADI (0.05 mg/kg bw/day). Nasuti *et al.* (2016) evaluated the effects of PERM on selected microbiota species and on the production of SCFA (butyric, propionic and acetic acids). The bacterial groups were further monitored without exposure to PERM from weaning through adulthood (total 135 days). Changes induced by PERM exposure, i.e. the abundance of selected bacteria and SCFA, reverted practically to baseline after stopping the treatment. The only exception was the abundance of *Bacteroides, Prevotella, Porphyromonas* species that remained reduced at the end of the study. Motor disabilities were also observed. However, causality was not established between the observed microbiome alterations and the mouse health issues. The authors indicated that approaching

[26] "One of a pair of molecular entities which are mirror images of each other and non-superposable" (IUPAC, 2019) http://dx.doi.org/10.1351/goldbook.E02069 (accessed 21 February 2022).

[27] "The geometric property of a rigid object (or spatial arrangement of points or atoms) of being non-superposable on its mirror image; such an object has no symmetry elements of the second kind (a mirror plane, σ=S1, a centre of inversion, i=S2, a rotation-reflection axis, S2n). If the object is superposable on its mirror image the object is described as being achiral" (IUPAC, 2019) https://doi.org/10.1351/goldbook.C01058 (accessed 22 February 2022).

[28] "In achiral environments, chiral pesticide enantiomers show similar physical and chemical properties. However, upon entering the chiral environment including natural environments and organisms, enantiomers exhibit selective environmental and toxic effects" (Meng *et al.*, 2019, p.8303).

the microbiome as a whole would have provided more accurate results and suggested that it might be used as a biomarker to detect diseases. The antimicrobial activity of PERM (as minimal inhibitory concentration) was also evaluated *in vitro* on select species and showed that beneficial bacteria (*Bifidobacterium* and *Lactobacillus paracasei*) were more sensitive than potentially pathogenic species (e.g. *Staphylococcus aureus* and *Escherichia coli*). Bordoni *et al.* (2019) used the same rat model (for Parkinson's disease) to investigate the effects of permethrin on the composition of faecal microbiota, gut permeability and potential hepatic injury. Like in Nasuti's study, the PERM exposure ended at PND 21, and the microbiome and mice were further monitored at PND 60. PERM exposure altered the intestinal permeability and induced hepatic inflammation, which authors linked to the altered gut microbiota. PERM also reduced the levels of dopamine. This study included an additional experimental group co-exposed to PERM and electrochemically reduced water (ERW). Due to the positive results observed in this treatment group, the authors speculated that ERW could create a favourable environment for the fermentation process and counterbalance the gut alterations induced by PERM. Bordoni suggested using germ-free mice to further characterize the mechanisms by which the microbiome influences the host physiology.

PROPAMOCARB

Propamocarb (PMB) is a systemic carbamate fungicide used in several edible crops to control diseases caused by *Oomycetes* species. This compound was evaluated by JMPR several times between 1984 and 2018 (FAO, 2021).

Wu and colleagues conducted two studies investigating the effects of propamocarb on the gut microbiome and the host (Table AII.15) (Wu *et al.*, 2018a; Wu *et al.*, 2018b). Wu *et al.* (2018a) assessed PMB at 3, 30 and 300 mg/L (~ 0.5, 5 and 50 mg/kg bw/day) on male ICR mice for four weeks. The second study (Wu *et al.*, 2018b) was conducted in male C57BL/6J at lower PBM doses (1, 3 and 10 mg/L or ~0.15, 0.45 and 1.5 mg/kg bw/day) and longer exposure time (10 weeks). According to the authors, the doses were chosen based on the highest residue from the European Union MRLs (European Commission, 2020) and the NOAEL for long-term toxicity in rats (29 mg/kg bw/day) (EFSA, 2006). Doses are 1.3, 13 and 125 (Wu *et al.*, 2018a) and 0.4, 1.1 and 3.8 (Wu *et al.*, 2018b) times higher than JMPR ADI (0.4 mg/kg bw/day). The first study – high doses (Wu *et al.*, 2018a) – evaluated the gut microbiota composition in faecal samples collected weekly, showing that the relative abundance of certain phyla fluctuated over time. The microbiota composition was also evaluated in caecal content at the end of the treatment, only in the high PMB dose group, showing differences with respect to faecal samples. Results also included altered microbial metabolites and gut dysbiosis in mice, which were influenced by exposure to the highest dose tested. The authors associated these effects with hepatic metabolic disruptions. In the second study, after 70 days of exposure, Wu *et al.* (2018b) found that the gut microbiome composition and functionality in mice (tested at the highest dose only) were affected at the phylum, family and genus levels.

Metabolic and transcriptional alterations were dose-dependent, mostly observed at the high dose. The authors associated these effects with enterohepatic metabolism disorders and increased risk of cardiovascular disease. These studies show that, during pesticide exposure, the composition of microbiota populations varies over time, and it is dependent on the sampling location (faeces or caecal content).

BY-PRODUCTS

Pesticide by-products or their metabolized products should also be considered when analysing pesticide effects on the gut microbiome. For example, it has been reported that three-quarters of organophosphorus pesticides are metabolized in the human body, and resulting products have the potential to affect the gut microbiota (Yang *et al.*, 2019). Three studies investigated the effects of pesticide by-products exposure on the gut microbiome (Table AII.16 and Table AII.17).

Yang *et al.* (2019) exposed male Wistar rats to 0.08 or 0.13 mg/kg bw/day diethyl phosphate (a non-specific metabolite of organophosphorus pesticides) for 20 weeks. This exposure disturbed the gut microbiome structure and altered serum hormone levels (linked to the increased abundance of butyrate-producing *Alloprevotella* and *Intestinimomas*). It also induced a pro-inflammatory response characterized by reduced IL-6, associated with the enrichment of opportunistic pathogens, i.e. *Paraprevotella*, *Parabacteroides*, *Alloprevotella* and *Helicobacter*. These alterations were more pronounced at the higher dose. The authors hypothesized that the endocrine-disrupting effects of organophosphate pesticides are related to specific metabolites. Also, the non-specific diethyl phosphate should not be used as a biomarker to evaluate the impact of parent organophosphate pesticides on the endocrine system.

Liu *et al.* (2017) investigated the effects of the major breakdown products of the organochloride pesticides dichlorodiphenyltrichloroethane (DDT) and hexachlorocyclohexane (HCH), i.e. p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE) and β-hexachlorocyclohexane (β -HCH), respectively, on the gut microbiome of mice. DDT and HCH are known to be persistent chemicals that can accumulate in the human body and threaten the host's health. In fact, both pesticides were banned in the 1970s and 1980s. However, residues and by-products of these pesticides are still widely detected in the environment. DDT is included in Annex III of The Rotterdam Convention, and it has been evaluated by JMPR on several occasions since 1966, and most recently in 2000 (FAO, 2021). Liu *et al.* (2017) carried out an eight-week exposure study of 1 mg/kg/bw (p,p'-DDE) or 10 mg/kg/bw (β-HCH) in male C57BL/6 mice, which resulted in gut dysbiosis affecting all bacterial taxonomic levels. Bile acid metabolism was also altered, with the gut microbiota being a likely contributor. The gut microbiota is intimately involved in the metabolism of bile acids by converting intestinal primary bile acids into secondary bile acids through conjugation and dihydroxylation reactions. The abundance of *Lactobacillus*, one of the main intestinal species with bile salt hydrolase activity, was increased after

chronic exposure to p,p'-DDE and β-HCH. The authors suggested the abnormal gut microbiome as one of the factors influencing hepatic metabolism and enteric bile acid profiles, potentially leading to the possible development of metabolic disorders.

Zhan *et al.* (2019) used p,p'-DDE (2 mg/kg bw/day during eight weeks) to induce obesity in male C57BL/6J mice. This model showed a reduced abundance of the genus *Bacteroides*, body weight gain, dyslipidemia and insulin resistance. This obese model was used to evaluate whether pectin supplementation, both during and after DDE exposure, could revert the disorder. Considering the results, the authors suggested that pectin might help reverse the p,p'-DDE-induced metabolic alterations and obesity by modulating the gut microbiome. Pectin also reduced the accumulation of p,p'-DDE.

PESTICIDE MIXTURE AND MULTI-RESIDUE EXPOSURE

Humans are exposed to a broad range of chemicals (human-made or naturally occurring) through food, including mixtures of various substances (e.g. food additives, veterinary drug residues) and pesticide mixtures. JMPR evaluates single compounds by aggregate, cumulative and combined exposure, but the assessment of pesticide mixtures remains mostly unaddressed.

One study investigated the effects of a cocktail of six pesticides commonly used in France to treat apple orchards (Lukowicz *et al.*, 2018). Male and female C57BL/6J wild type (WT) and constitutive androstane receptor (CAR)[29] deficient mice (C57BL/6J background) were exposed to a 52-week treatment of boscalid, captan, chlorpyrifos, thiofanate, thiacloprid and ziram at their ADIs (European Commission, 2020), 0.04, 0.1, 0.01, 0.08, 0.01, 0.006 mg/kg bw/day, respectively (Table AII.18). This study did not assess the microbiota composition, but the metabolome analysis of urine revealed increased microbial-related metabolites in WT females compared to controls (i.e. indole-derivative 3-indoxyl sulphate, and phenyl-derivatives phenylacetylglycine and p-cresol glucuronide). Because these metabolites were detected after 48 weeks of pesticide exposure, following the observed metabolic alterations, the authors suggested that the gut microbiota's alterations could be the consequence of host disturbances and not the cause. Obesogenic and diabetogenic effects differed between the two genders. According to the authors, mice's exposure to non-toxic doses of a defined pesticide cocktail (based on human TDI levels) induced a metabolic disruption consistent with diabetic status. However, the authors indicated that the potential diabetogenic role of gut microbiota should be further evaluated.

Three other studies analysed the effects of pesticide mixed with non-pesticide compounds on the gut microbiome for short periods (< 7 days). Zhan *et al.* (2018) evaluated the influence of antibiotics (ampicillin alone or in combination with neomycin, gentamicin, and metronidazole vancomycin) and the gut microbiome

[29] CAR: modulator of the expression of genes involved in xenobiotic and energy homeostasis (Evans and Mangelsdorf, 2014).

on the bioavailability of triazine herbicides (2 and 20 mg/kg bw/day of each simazine, atrazine, ametryn, terbuthylazine and metribuzin) in male Sprague-Dawley rats. As expected, the antibiotic exposure decreased bacteria counts, which affected especially Ruminococcaceae, Lachnospiraceae and *Anaerotruncus*. The treatment increased the bioavailability of triazine herbicides. The microbiome's participation in triazine bioavailability was confirmed by microbiota transfer to a microbiota-deficient model. According to the authors, the altered microbiota might induce changes in intestinal absorption and liver metabolism, therefore contributing to the increased pesticide bioavailability.

Seth *et al.* (2018) and Alhasson *et al.* (2017) treated wild type male C57BL/6J and TLR4 gene knock-out mice (mouse model of Gulf War illness [GWI]) with a combination of the pesticide permethrin (200 mg/kg) and pyridostigmine bromide (2 mg/kg).[30] These are some of the compounds responsible for symptoms of GWI. The authors postulated that the altered microbiome might be associated with the disorder, and relate the microbial SCFA butyrate as a compound of interest in the treatment of GWI symptoms (by attenuating the pro-inflammatory environment in the small intestine). After a three-time exposure in seven days, both studies resulted in gut dysbiosis in mice. In Seth's study (2018), both butyrate-producing bacteria, *Lactobacillus* and *Bifidobacterium,* were decreased, and mice developed systemic inflammation. In addition to intestinal inflammation, Alhasson *et al.* (2017) also reported neuroinflammation in mice, a common health effect seen in patients who suffer from GWI. The authors noted that these alterations probably resulted from gut leakiness and endotoxemia induced by the altered microbiome (correlated with abundant *Coproccocus* and *Turicibacter*). Since mice were co-exposed to permethrin and pyridostigmine bromide, their individual contribution to the observed gut dysbiosis and negative health effects in the host is not clear.

[30] A reversible cholinesterase (ChE) inhibitor (carbamate compound): "Is a drug used during the Gulf War as a pretreatment to protect troops from the harmful effects of nerve agents" (Fulco, Liverman and Sox, 2000).

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CHAPTER 4
DISCUSSION

There are many active pesticide ingredients used in thousands of pesticide formulations. JMPR has conducted toxicological and residue evaluations for approximately 407 pesticide active ingredients (FAO, 2021), and Codex Alimentarius has established around 230 pesticide MRLs (Codex Alimentarius, 2020). Considering the numerous pesticides available worldwide, only a small fraction has been included in microbiome research. Some belong to major groups of pesticides, such as carbamates, organochlorines and organophosphates. Moreover, most studies have selected "controversial pesticides", such as glyphosate and chlorpyrifos.

This review gathers recent scientific publications involving the study of the gut microbiome in both *in vivo* and *in vitro* models exposed to pesticides. Perhaps, the first observation noted is the interchangeable use of the terms *microbiome* and *microbiota*. Although there are no consensus definitions for either term, it may be helpful to clarify the difference for the purpose of this document. Microbiota typically refers to the taxonomical diversity of organisms. The microbiome is a more complex concept that also considers the overall genetic composition and function of the microbiota. In the case of the gut microbiome, it relates to the microbial community of the gastrointestinal tract. A recent proposal defined the microbiome as a "characteristic microbial community occupying a reasonable well-defined habitat which has distinct physio-chemical properties" (Berg *et al.*, 2020, p. 17).

There are two overarching target areas in the report investigating the pesticide exposure on both the microbiome and the host. One of the areas focuses on evaluating the gut microbiota composition (abundance and taxonomical diversity). The second target area of investigation is more functional and includes functional microbial genomics, metabolic activities, intra- and inter- microbial signalling and behaviour, as well as the host's metabolism, physiological functions and histopathological observations. In the context of microbiota structure vs function, it has been reported that the functional microbial genomic diversity across individuals is more similar than the microbiota composition (Lozupone *et al.*, 2012). In other words, microbiotas are different, but microbiomes are similar. This point is relevant because studies with a main focus on the microbiota structure may not provide an accurate description of the microbiome's functional role in pesticide-induced alterations in the host or the development of non-communicable diseases (NCD).

Not all studies reported here have the same purpose, the same experimental design or evaluate the same endpoints. For example, studies differ in the pesticides studied,

alone or in mixtures, pure or as part of commercial formulations, doses, exposure periods and model type (*in vivo* and *in vitro*). These differences and the lack of standardization challenge potential comparisons and the identification of relevant common points. The majority of studies evaluated the effects of pesticides on the microbiota composition (Annex II – Findings). They typically described changes in the diversity and abundance of specific taxonomical groups resulting from exposure to pesticides. Some add the functional component of the microbiome by evaluating genes or gene expression and the production of microbial metabolites (e.g. SCFAs, secondary bile acids). For example, carbendazim (Jin *et al.*, 2018b), chlorpyrifos (Requile *et al.*, 2018; Reygner *et al.*, 2016a; Reygner *et al.*, 2016b), and permethrin (Nasuti *et al.*, 2016) have been shown to alter the production of microbial SCFAs. Host assessments focused primarily on the enterohepatic function and related metabolic activities (i.e. lipid and glucose metabolism, often in connection with obesity and diabetes), as well as the intestinal- or systemic immune response. A reduced number of studies also evaluated the brain, neurobehaviour and endocrine system. The pesticide impact on the microbiome is evaluated in parallel to the host or in connection with it. Many authors speculate on the potential contribution of the microbiome in the pesticide-induced alterations in the host. However, only a few cases evaluate causality or try to identify the mechanisms involved in this relationship. Some studies aimed to elucidate the mechanisms underlying the microbiome's contribution to metabolic alterations and the development of chlorpyrifos-induced obesity (Liang *et al.*, 2019). The microbiome has also been linked mechanistically to the development of neurobehavioural alterations induced by pyridostigmine bromide and permethrin. Mechanisms have also been established to explain the microbiome involvement in the bioavailability of triazine herbicides (Zhan *et al.*, 2018). Research also showed how exposure to diazinon and malathion influence the pathogenic behaviour of the gut microbiome by altering the Quorum Sensing System (Gao *et al.*, 2017a; Gao *et al.*, 2018). Other studies aimed to investigate the effectiveness of dietary interventions (e.g. diet supplementation with pectin and the prebiotic inulin) to revert some of the effects (e.g. metabolic alterations and gut dysbiosis) caused by p,p'-DDE and chlorpyrifos (Requile *et al.*, 2018; Reygner *et al.*, 2016b; Zhan *et al.*, 2019).

It is important to recognize that while all studies consider the bacterial population only, the microbiome contains other microorganisms such as viruses and fungi. How these subpopulations are affected by pesticide exposure or how much they contribute to microbiome-host interactions are questions completely unaddressed in the studies. Previous research investigations have shown that the mycobiota is modulated by the diet and is involved in several human diseases (Mims *et al.*, 2021; Nagpal *et al.*, 2020). The viral component of the microbiome, or gut virome (endogenous retroviruses, eukaryotic viruses and bacteriophages), has not been well studied. Recent research has identified about 142 000 unique gut phage genomes (Camarillo-Guerrero *et al.*, 2021). About 36 percent of phage viral clusters can infect multiple bacterial species. This is a relevant aspect of the virome because they facilitate gene flow among phylogenetically distinct microbial species comprising the gut microbiome. The human gut virome has also been associated with diseases (Mukhopadhya *et al.*, 2019; Santiago-Rodriguez and Hollister, 2019).

DOSES AND EXPOSURE

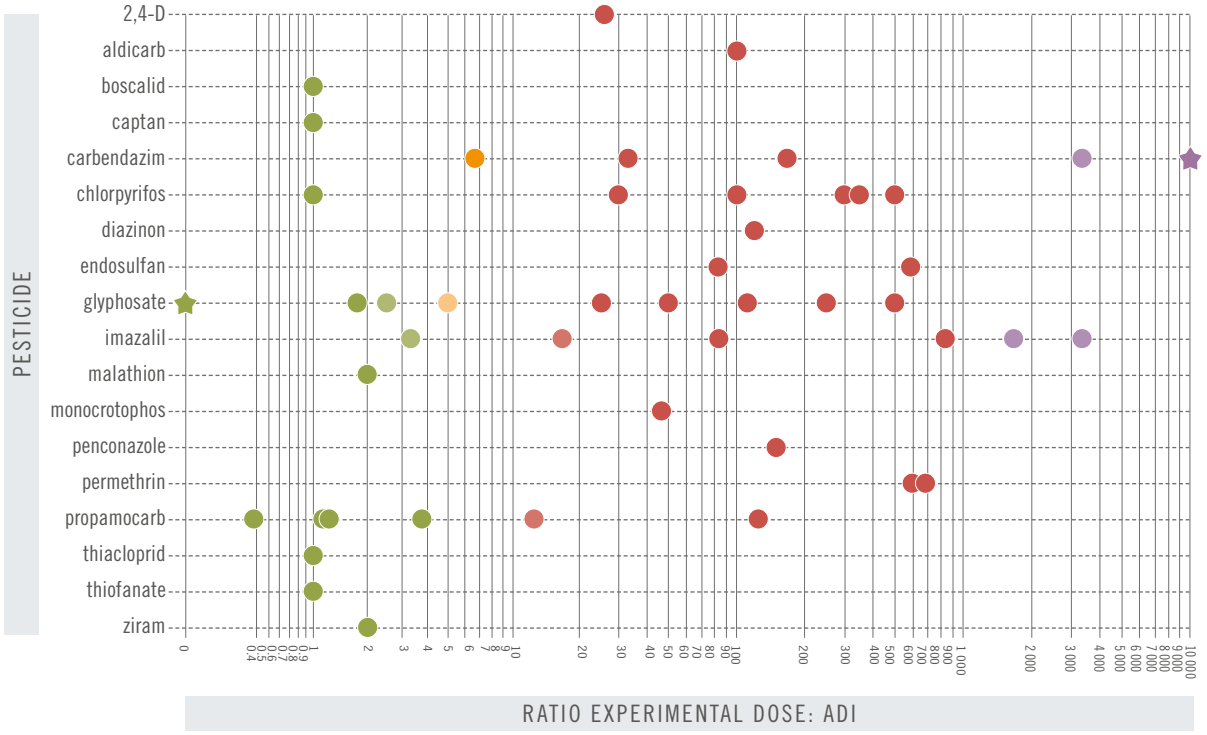
Despite best agricultural and food manufacturing practices and the efforts to minimize the environmental pesticide contamination, it is currently challenging to avoid exposure to low pesticide levels. Moreover, POP compounds will continue to be problematic as they have accumulated in the environment. A growing amount of data shows that humans are exposed to very low levels of pesticides through the food supply. For example, in the European Union, about 40 percent of food products analysed by member states during 2018 contained quantifiable pesticides below regulatory MRL levels (EFSA, 2020). To compare conventional vs organic food samples collected between 2013 and 2015, the European Food Safety Authority reported measurable levels of pesticide below MRLs in 42.8 percent vs 6.3 percent of food samples, respectively (EFSA, 2018). In the United States of America, approximately 56.2 percent of agricultural samples analysed in 2019 as part of the Pesticide Data Program run by the United States Department of Agriculture (USDA) contained detectable pesticide residues below the established MRLs (USDA, 2020). Similarly, the United States Food and Drug Administration (FDA) reported detectable pesticides below tolerance levels (or MRL) in 49.6 percent of human and animal food samples tested in 2018 (FDA, 2020).

Considering the occurrence of low levels of pesticide residues in crops and food, it is relevant that studies involving the gut microbiome and host include doses and exposure periods equivalent to real exposure conditions, i.e. chronic exposure to low pesticide concentrations. The ranges of doses and exposure periods used in the studies included in this document are quite variable (Annex II). After normalizing most experimental pesticide doses to the corresponding ADI, doses were between 0.4 and 3 333 times higher than the ADI, except one dose of glyphosate 25 x 10^{-7} times below the ADI and the highest concentration of carbendazim, 16 667 times higher than the ADI (Figure 5). About 67 percent of these studies (23 out of 34) used doses lower than 100 times the ADI. Five studies have evaluated pesticides around their corresponding ADI value, i.e. glyphosate (Lozano *et al.*, 2018; Mao *et al.*, 2018), propamocarb (Wu *et al.*, 2018a; Wu *et al.*, 2018b), and the cocktail containing chlorpyrifos, thiacloprid, boscalid, thiofanate and captan (Lukowicz *et al.*, 2018).

Reference values used as the basis for setting experimental doses were mostly NOAELs and MRLs. However, some authors have based their doses on reference values as high as LD50 and as low as ADI and DWEL. In the context of the gut microbiome, it is relevant that reference doses are related to oral exposure. For example, Tu *et al.* (2019) evaluated the effects of 2,4-D on the gut microbiome at occupational-relevant concentrations (professional turf applicators). Establishing the selection of oral doses based on concentrations relevant for other routes of exposure (skin and lungs) may not be a best practice in the study of the gut microbiome.

The majority of studies (26 or 61 percent) evaluated single doses, while 23 percent used two experimental doses and about 18 percent used three different pesticide concentrations. Seven of the studies evaluating multiple doses used ranges of concentrations between 10 and 100-fold difference and only one study on Roundup® evaluated a wider range (10^{10}-fold) (Lozano *et al.*, 2018).

FIGURE 5 **EXPERIMENTAL PESTICIDE DOSES USED IN THE DIFFERENT STUDIES RELATIVE TO THEIR CORRESPONDING ADI**



Starred icons are truncated values above or below the X-axis limits used in the chart

Source: Authors' own elaboration.

Exposure periods were also variable, ranging from 24 hours in *in vitro* models to a two-year *in vivo* study. However, most exposure studies were conducted either between 2–4 weeks or 8–20 weeks. The length of exposure to low doses of pesticide was dependent on the purpose and target age of the study. Short exposures to low doses of the test substance do not represent a real scenario, i.e. the chronic exposure to low levels of pesticide residues. However, short exposure periods can be used to determine the pesticide ARfD. The absence or presence of limited alterations after short exposures may raise the question of whether the exposure period was too short to see potential effects, or if the pesticide has indeed no effect at the experimental dose. This point is illustrated by one study where the authors only saw a limited impact on rats' gut microbiome after a two-week exposure to a relatively low concentration of glyphosate (2.5 mg/kg bw/day, 2.5 times the glyphosate ADI,) (Nielsen *et al.*, 2018).

MODELS

Since the gut microbiome–host relationship is complex and works in a symbiotic manner, using living organisms provides information that cannot be obtained by *in vitro* systems alone. However, the scientific community is under pressure to replace animal *in vivo* studies with more humane alternatives.

When using surrogate animal models to study the human gut microbiome and its interaction with the host, it is critical that they are physiologically and clinically relevant as well as fit for purpose. The selection of the most suitable model depends on the study's objectives. Criteria for selection include, for example, genetic background, baseline microbiota, or phenotypic expression of the diseases (Kamareddine *et al.*, 2020). Dogs, swine and humans have similar dominant phyla (i.e. Firmicutes and Bacteroidetes) but differ significantly at the genus level (Hoffmann *et al.*, 2015; Xiao *et al.*, 2016). 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 as humans but differ in several health-relevant genera absent in mice (Nguyen *et al.*, 2015). However, mice are genetically manipulable (e.g. mimic human disease conditions) and have more genetic variants than rats, making them more versatile models to study, for example, the mechanisms that influence microbiota composition (Turner, 2018).

This review included research studies conducted primarily *in vivo* using rodents (mice and rats), being almost limited to Wistar and Sprague-Dawley rats and C57BL/6 mice. These are the most popular rodent strains. They are commonly used in biomedical research (Johnson, 2012) and diet-induced models of metabolic syndromes (Wong *et al.*, 2016). Both Wistar and Sprague-Dawley backgrounds have been the subject of studies to evaluate the effect of diet on stress and gut–brain axis dysfunction (Bassett *et al.*, 2019). Tu *et al.* (2019) justified using their mouse C57BL/6 model to assess the impact of 2,4-D because it was used in previous studies evaluating microbiome–xenobiotics interactions. The basis for selecting C57BL/6 mice in Jin's carbendazim study was its metabolic background and fattening feature (Jin *et al.*, 2018b). Mice with other genetic backgrounds, i.e. ICR, CD-1, Swiss, KM and BALB/c, have also been employed in the studies included in this review, but to a much lesser extent. Liang *et al.* (2019) used two different mouse strains on their chlorpyrifos study, the inbred C57BL/6 because they are genetically similar and facilitate reproducible data, and the outbred strain CD-1 mice, which is a non-homogeneous population with high genotypic and phenotypic variance, being more representative of the human population. Both chlorpyrifos and glyphosate have received special attention considering the high number of studies involving the gut microbiome compared to the other pesticides. The majority of studies investigating the impact of these two pesticides were carried out predominantly in rats. However, only Wistar rats were used in chlorpyrifos studies, while only Sprague-Dawley were used in the glyphosate research discussed here.

There were also genetically manipulated models and models of disease in the literature reviewed, all using both the wild type C57BL/6 or C57BL/6J and their corresponding altered genotype variants. Such variants express a specific gene or, in the case of knockout mice, are manipulated to inactivate or delete genes. These variants allow the study of the mechanisms involved in biological processes. Guardia-Escote *et al.* (2020) used mice with the apolipoprotein E (apoE) gene replaced with two alleles of human apo ε3, apo ε4 to study their influence on the microbiota composition when exposed to chlorpyrifos. They concluded that the apoE mice exposed early in life to chlorpyrifos showed changes in the gut microbiota and the production of SCFA, with potential implications for cognitive function. A constitutive androstane receptor-deficient (CAR-/-) mouse model was used to evaluate the chronic exposure (one year) to a mix of six pesticides (boscalid, captan, chlorpyrifos, thiofanate, thiacloprid and ziram) at doses equivalent to their respective ADIs (Lukowicz *et al.*, 2018). It resulted in alterations of the microbiome in wild-type female mice. However, the involvement of the microbiome on the obesogenic and diabetogenic effects observed was not clear. A toll-like receptor 4 (TLR4) knockout mouse – a GWI model – has been used to investigate mechanisms to identify and treat alterations (gastrointestinal inflammation and hepatic metabolic abnormalities, neuroinflammation) caused by compounds involved in the GWI (permethrin -PERM- and pyridostigmine bromide -PB-), which have been associated with the microbiome dysbiosis (Alhasson *et al.*, 2017; Seth *et al.*, 2018).

Pesticides have also been used to develop models of disease. For example, permethrin has been used to induce Parkinson's disease in rats, characterized by intestinal permeability, liver inflammation and brain alterations linked to changes in the gut microbiota (Bordoni *et al.*, 2019). This same model was used to evaluate the efficiency of ERW to lessen the microbiome-related alterations caused by permethrin (Bordoni *et al.*, 2019).

Germ-free laboratory animals, most commonly mice and rats, play a crucial role in the study of the metabolic capacity of the gut microbiota and in evaluating the causal contribution of the intestinal microbiome to host homeostasis. Germ-free mice can be obtained in two different ways, both having advantages and disadvantages (Kennedy, King and Baldridge, 2018). True germ-free mice are bred and raised free of microorganisms under stringent environmental conditions, and antibiotic-treated animals are a less expensive alternative. In microbiome research studies, they are inoculated with bacterial cultures or recolonized with healthy or altered microbiota from a donor. Antibiotic-based germ-free mice have been used to evaluate and confirm the influence of the microbiome on alterations observed in the host upon exposure to chlorpyrifos and a mixture of different triazine herbicides (Liang *et al.*, 2019; Zhan *et al.*, 2018).

In addition to the species and genetic background, gender and age are also important considerations when designing the experimental study. About two-thirds of the studies were conducted in male adults only. The remainder of the studies included females alone, female and males, and/or pups. Zhang *et al.* (2017) indicated that

they chose male mice because of their sensitive response to xenobiotic exposure in environmental research.

The microbiome starts developing at birth, reaches its maturity in adolescence, remains practically stable during adulthood and becomes compositionally unstable and less diverse in the elderly (Lynch and Pedersen, 2016). How the microbiome develops at early stages will determine its composition and function later in life, and it may influence the host's predisposition to diseases. For this reason, there is special interest in the study of the microbiome exposure to xenobiotics in young individuals, from gestation to near adulthood. Exposure of pups to chlorpyrifos (Guardia-Escote *et al.*, 2020; Joly *et al.*, 2013; Joly Condette *et al.*, 2015; Li *et al.*, 2019; Perez-Fernandez *et al.*, 2020; Reygner *et al.*, 2016b), and permethrin (Bordoni *et al.*, 2019; Nasuti *et al.*, 2016) affected gut bacteria abundance and composition of the pups. As observed for adults, male pups are again the gender most studied. Reygner *et al.* (2016b) concluded that pre- and postnatal chlorpyrifos exposure might be a factor affecting the onset of the normal metabolism regulation in adulthood. Such alterations seem to be alleviated in adulthood by supplementation with inulin. The authors acknowledged the need for additional research to provide insights on the cross-talk mechanisms between microbiota and host, by which the microbiota alleviates metabolic alterations induced by chlorpyrifos. Early exposure to chlorpyrifos also seems to affect the composition of microbiome-related SCFA with implications for cognitive function (Guardia-Escote *et al.*, 2020).

Sample size, i.e. the number of animals per treatment group, was between six and eight in about 75 percent of the studies. Considering the high interindividual variability in microbiota composition, the sample size should be large to be representative, therefore ensuring robust results and meaningful interpretations (Turner, 2018). Besides, it is essential to ensure that the number of animals is adequate in chronic exposures to account for potential casualties occurring during long-term studies that may jeopardize the statistical significance of the results. The longest study mentioned in this review (two years) had the smallest sample size (three rats per treatment group exposed to Roundup®) (Lozano *et al.*, 2018). Although none of the rats died during the experimental period, the results are questionable due to the low statistical power resulting from the small sample size.

In vitro studies are also very useful for studying gut microbiota composition, microbial interactions, and related metabolic activities in the presence or absence of xenobiotics. They are also used to elucidate certain mechanisms in an isolated and controlled environment. *In vitro* methods overcome several limitations of *in vivo* systems, e.g., they can be exposed to a large variety of pollutants and contaminants, and offer the possibility to evaluate the microbiome in a non-invasive manner. Unlike *in vivo* studies, *in vitro* research does not require approval by an ethics committee. Obviously, these systems are disconnected from the host, and therefore limited for evaluating microbiome–host interactions. They do not replace *in vivo* models but complement them. A significant advantage of *in vitro* systems is the possibility to assess the human gut microbiota, typically from faecal material.

Existing *in vitro* models differ in complexity, from bioreactors or fermenters to intestinal cell cultures to more traditional bacterial cultures. There are different types of bioreactors that have been used to study the gastrointestinal microbiome, from simple batch units (containing non-replenishable media) to continuous bioreactors (continuous replacement of media) composed of either single or multiple vessels (Guzman-Rodriguez *et al.*, 2018). Complex bioreactors, for example, the modular SHIME®, are fermentation chambers built to simulate the environmental conditions of the different sections of the human gastrointestinal tract, including the intestinal peristaltic movement. They offer the advantage of evaluating the microbiome in a more physiological environment. These systems are inoculated with gut microbiota from animals or human donors. They can be used to evaluate the effects of nutrients, probiotics, prebiotics and other xenobiotics in the gut microbiome. Bioreactors have been used to study the effects of chlorpyrifos and deltamethrin on the gut microbiota (Defois *et al.*, 2018; Joly *et al.*, 2013; Requile *et al.*, 2018; Reygner *et al.*, 2016a). However, one limitation of this type of system is that they do not consider the impact of substances on epithelial cells, which is possible with the use of cells cultures. They facilitate the evaluation of cellular and molecular mechanisms that lead to structural lesions. Some of the most common intestinal cell lines are Caco-2, HT-29 and the Caco-2-derived TC7 cells (Aguilar-Rojas, Olivo-Marin and Guillen, 2020; Hu *et al.*, 2004; Turco *et al.*, 2011). The Caco-2 cell line has been used in the past to evaluate the effects of chlorpyrifos, which resulted in perturbations affecting cell junctions, and causing loss of barrier effect and increased permeability (Tirelli *et al.*, 2007). The tandem fermenters and cell lines, like SHIME® and Caco-2 cells, is becoming a gold standard for *in vitro* studies, and allows combining the benefits of both systems (Requile *et al.*, 2018). This tandem model has been used to show that chlorpyrifos induces dysbiosis and metabolic imbalance in the SHIME® environment, and samples transferred to the Caco-2/TC7 cell culture affected the activity of the mucosal barrier, with the potential to induce inflammation (Requile *et al.*, 2018). The tandem system has also been used to study the effects of deltamethrin (Defois *et al.*, 2018). The microbiome exposure to the pesticide in the reactor resulted in changes in the bacterial volatolome, and microbiota-free supernatants transferred to Caco-2/TC7 cell lines led to metabolic pathways alterations and biochemical changes.

STUDY OF THE MICROBIOME AND MICROBIOME HOST RELATIONSHIP - METHODS

SAMPLING

Although it is widely understood that the microbiota is stable in adulthood, it remains dynamic within and between individuals due to changes in habitat conditions (Fisher, Mora and Walczak, 2017). It has been shown that 60 percent of the main microbiota phylotypes within a healthy individual remain consistent over three years (Lozupone *et al.*, 2012). Moreover, the genomic composition of the microbiome varies continuously in response to environmental factors, including

diet and exposure to xenobiotics (Clarke *et al.*, 2019). Therefore, long exposure studies to xenobiotics would benefit from monitoring the gut microbiome and host response at different time points (e.g. in faeces and blood serum). Establishing a sampling frequency plan to monitor both the microbiome and the host would provide a strategy to better understand the dynamics of the microbiome over time and the evolution of different experimental parameters during long-term exposure to pesticides, and whether changes are transient or permanent. For example, exposure to propamocarb was evaluated at different time points, allowing the observation of trends such as the abundance of certain microbial populations (Wu *et al.*, 2018a; Wu *et al.*, 2018b). Sampling frequency would also allow the evaluation of factors that modulate the host/microbiome overtime, such as age, hormonal and immune status. Moreover, it would also be useful in establishing the sequence of events taking place after pesticide exposure, for example, to help determine if gut dysbiosis appear before or after host alterations. To illustrate this point, in a 52-week exposure to a pesticide cocktail (boscalid, captan, chlorpyrifos, thiofanate, thiacloprid and ziram), alterations of microbial metabolite profiles were observed at week 48 following metabolic alterations in the host (Lukowicz *et al.*, 2018). This suggests that microbiota alterations could be the consequence of host disturbances and not the cause.

Research studies included in this document showed that time point evaluations were typically conducted in young animals at key ages (birth, weaning, young adulthood). However, test samples were collected only at the end of the experimental study in many adult models. The longest study in this review exposed rats to a broad range of Roundup® doses for nearly two years, with samples collected after 673 days of exposure (Lozano *et al.*, 2018). Alterations of the microbiome were limited to females only. Rats at this age are at the end of their life cycle. The low statistical power of the study (three rats per group) increases the likelihood that statistically significant differences result in false positives (Dumas-Mallet *et al.*, 2017), and without monitoring the evolution of the microbiome for such a long treatment period, it is challenging to attribute the findings to the pesticide exposure alone. Many other factors may influence the outcome and result in variabilities like the immune and hormonal status of the old female rats.

The point of sample collection influences the composition of the microbiota. It has been reported that the microbiota composition of faecal and caecal samples may differ (Tanca *et al.*, 2017). Wu and colleagues (Wu *et al.*, 2018a; Wu *et al.*, 2018b) reported differences in the microbiota composition of faecal and caecal samples after propamocarb exposure. Such differences may have implications in the interpretation of findings (Tang *et al.*, 2020a). The advantage of faecal samples is their non-invasive nature, which facilitates monitoring the microbiome and other functional parameters (e.g. metabolites) over time. Samples from the gastrointestinal tract are difficult to obtain from live animals, which are usually collected at the end of the study when animals have been sacrificed. The sampling location(s) could be determined by the pesticide toxicokinetics, if it is metabolized in the gut and absorbed, where these activities takes place.

ANALYTICAL CONSIDERATIONS

Studying the microbiome, microbiome–host interactions and effect of xenobiotics on these systems requires holistic analytical approaches conducted by a multidisciplinary team of scientists. Evolving bioinformatics and the latest technological advances have enabled omics analysis, which, used alone or in combination with traditional analytical approaches, has made it possible to make a holistic evaluation of complex biological structures and functions. Selecting the most appropriate method(s) depends on the scientific question and hypothesis (Allaband *et al.*, 2019). This section will describe the omics approaches used to study the microbiomes.

The microbiome composition and function can be studied using targeted or untargeted analytical methodologies to analyse the microbial DNA, mRNA, proteins or metabolites of different chemical natures. The analysis of the microbiota composition (abundance and diversity), designed to respond to the questions "who is there?" and "how many?", is typically carried out by culture-independent molecular tools. The most commonly used is the sequencing of the 16S ribosomal RNA (rRNA) gene amplicon. The 16S rRNA gene target has been used as a reliable marker for the 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). The identification at the species level is not always possible, partly due to the fact that some species are identical in this region (Wang *et al.*, 2007). The regions V1, V2 and V6 contain the broadest intraspecies diversity (Coenye and Vandamme, 2003) and regions V4-V6 have been found optimal for primer design due to superior phylogenetic resolution for bacterial phyla (Yang, Wang and Qian, 2016). Different PCR primer sets can lead to different microbiome profiles (Human Microbiome Project Consortium, 2012). Moreover, other considerations affecting this method can add bias to the result, e.g. sequencing alignment and statistical methods (Pollock *et al.*, 2018). These are all variables that affect method comparison and reproducibility. Most of the studies cited here targeted the regions V3-V4, and to lesser extent V4-V5, which are useful in identifying the taxa levels: phylum, class, order, family and genus. Lozano *et al.* (2018) targeted all hypervariable regions of the 16S rRNA gene except V1 and V5 in his Roundup® study. Non-bacterial members of the microbiome require other analytical targets. For example, the 18S rRNA gene or the internal transcribed spacer regions are used to evaluate eukaryotes (e.g. fungi).

However, a truly comprehensive analysis of the microbiome, including bacteria, viruses, fungi and small eukaryotes, is possible thanks to shotgun metagenomics analysis. Unlike the targeted 16S rRNA gene sequencing, shotgun metagenomics captures the entire DNA. It does not only determine the phylogenetical composition of the microbiota, but also provides functional information (functional metagenomics). With shotgun metagenomics it is possible to identify the presence of genetic traits and determine the abundance of genes involved in metabolic pathways

and microbiome activities. However, the metagenomic analysis relies on databases that depend on the quality of information they contain and their completeness. Shotgun metagenomic was used to evaluate the exposure of the gut microbiota to 2,4-D, showing functional perturbations as indicated by the increased abundance of pathways and gene families related to amino acid and carbohydrate metabolism (Tu *et al.*, 2019). It also made it possible to determine the increased abundance of genes related to the Quorum Sensing System (e.g. motility and pathogenicity) after malathion exposure (Gao *et al.*, 2018). Shotgun metagenomics also helped identify potential mechanisms explaining the gut microbiome's role on the neurotoxicity of diazinon and its gender-specific effects (Gao *et al.*, 2017b).

Genomics provides information about the presence of genes but does not indicate whether they are active or not. The expression of genes is evaluated by analysing the messenger RNA (mRNA). It provides mechanistic insights about which metabolic pathways are up or down regulated. Transcriptomics techniques based on qRT-PCR have been used to analyse target-specific gene expression from faecal or tissue samples resulting from pesticide exposure. In the reviewed literature, most mRNA transcriptome analysis has been done in the host tissue (it depends on the study purpose, but most commonly from the liver and intestine) and, to a lesser extent, applied to the study of the microbiome. Metatranscriptomics, which is the untargeted method considering the entire mRNA, has been applied to evaluate the effects of diazinon on the microbiome Quorum Sensing System (Gao *et al.*, 2017a). It has also been used, in combination with microbial volatolome, to assess the influence of deltamethrin on the microbiome metabolism (Defois *et al.*, 2018). Microbial metatranscriptomics also revealed the upregulation of the metabolic routes responsible for the biodegradation of monocrotophos (Velmurugan *et al.*, 2017).

Metabolomics allows the detection and identification of metabolite profiles. The metabolome analysis is another way to evaluate the activity and function of the microbiome or the host. Microbial metabolites are typically analysed from caecal content or in faecal samples. Moreover, they are also found in other tissues and organs after being absorbed and distributed systemically. Some microbial metabolites are known to participate in the physiological and metabolic processes of the host, and therefore changes in microbiota metabolites may impact the normal functions of the host. SCFAs, particularly butyrate, are of particular interest as they are used as an energy source by intestinal enterocytes. Moreover, SCFAs can interact with the energy metabolism, neuronal and intestinal functions and participate as modulators of the host immune response (Koh *et al.*, 2016; Neish, 2009). SCFAs have been shown to reduce the risk of certain diseases, including colon cancer (Koliarakis *et al.*, 2018). Besides, the microbiome can metabolize compounds produced by the host, like intestinal bile acids into secondary bile acids. It also has the potential to metabolize xenobiotics, and resulting metabolites can influence the host physiology (Koppel, Maini Rekdal and Balskus, 2017). There are different analytical methodologies to analyse microbial metabolites, from targeted approaches focusing on the analysis of specific groups or families of compounds to untargeted approaches, that are optimized to cover as many metabolites as possible. Detection technologies

typically include mass spectrometry and nuclear magnetic resonance spectroscopy. Analytical methods involving these technologies have allowed the identification of microbial metabolic imbalances caused by 2,4-D (Tu *et al.*, 2019), chlorpyrifos (Reygner *et al.*, 2016a; Reygner *et al.*, 2016b), deltamethrin (Defois *et al.*, 2018) and diazinon (Gao *et al.*, 2017b). Metabolomics also showed the disturbed host's lipid and brain metabolisms by aldicarb exposure (Gao *et al.*, 2019), as well as altered enterohepatic metabolism by endosulfan (Zhang *et al.*, 2017), monocrotophos (Velmurugan *et al.*, 2017), penconazole (Meng *et al.*, 2019), propamocarb (Wu *et al.*, 2018a) and pesticide mixtures (Lukowicz *et al.*, 2018).

Although some metabolites are known to be produced by the microbiome, such as SCFA and secondary bile acids, it is challenging to distinguish if many other metabolites are produced by either the host or the gut microbiome (Gao *et al.*, 2019).

Nobody questions the benefits of the omics methods to understand the structures and processes of complex organisms. However, the omics also come with new challenges since they address the genetic composition and function of the organisms from a holistic perspective. They provide a vast amount of information that cannot be fully interpreted yet with current knowledge. For example, by analysing the human metagenome, Pasolli *et al.* (2019) identified 3 795 new species-level clades from the body-wide human microbiome that are waiting for a name. Many of the identified metabolic activities cannot be linked to genes or specific enzymes (Koppel, Maini Rekdal and Balskus, 2017). But 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). In addition, there have been many new analytical methodologies developed, but the lack of standardization, validation and best practice guidance make it challenging to reproduce studies and compare results from similar investigations.

PESTICIDE MIXTURE, PESTICIDE FORMULATIONS AND CO-EXPOSURE WITH OTHER XENOBIOTICS

As can be deduced from this review, the number of studies involving pesticides and the microbiome is limited and mainly focused on the evaluation of individual pesticides. However, the reality is that pesticides are used as part of formulations containing other substances such as surfactants and adjuvants. Concerning existing microbiome studies in this review, glyphosate has been the only pesticide evaluated alone and as part of a commercial formulation (e.g. Roundup®, Glyfonova®). Glyphosate and glyphosate-based commercial herbicides are among the most controversial pesticide products. A substantial amount of contradictory scientific and pseudo-scientific information has been published, creating confusion about the safety of this pesticide (Mesnage and Antoniou, 2017). Since many of the glyphosate safety assessments are about 30 years old, there have been recommendations for research and re-evaluation of glyphosate, including commercial formulations and pesticide mixtures (Vandenberg *et al.*, 2017). In fact, the glyphosate studies mentioned in this review demonstrated that commercial formulations have a higher

impact on the overall gut microbiome composition and diversity than glyphosate alone (Dechartres *et al.*, 2019; Mao *et al.*, 2018; Nielsen *et al.*, 2018). Moreover, as previously reported, adjuvants or other components in the formulation may increase the potentially toxic effects of glyphosate, either in an additive or synergistic manner (Coalova, Rios de Molina and Chaufan, 2014; Mesnage, Bernay and Seralini, 2013; Williams, Kroes and Munro, 2000). This observation calls for more attention to co-formulants in pesticide products.

Due to agricultural practices and aspects related to hygiene and other activities along the supply chain, it is not uncommon to find multiple pesticide residues in the same food sample. For example, the latest EFSA report on pesticide residues in food, which gathers data from official national control activities of the European Union Member States, showed that 29.1 percent of samples contained more than two quantifiable pesticides, of which 50 percent had between two and three pesticides (EFSA, 2020). Similarly, the Annual Summary of the United States of America Pesticide Program reported that 16.6 percent of all samples tested in 2019 contained one detectable pesticide, and 40.9 percent had two or more detectable pesticides (USDA, 2020). The evaluation of the combined effects of pesticides or food contaminants adds additional complexity to the already intricate microbiome–host system. The interpretation of findings resulting from combined pesticide exposure is especially challenging when there is no previous information on the effects of individual compounds. Choosing pesticide combinations and their proportions should be relevant. Recently, EFSA has published a "Guidance Document on the scientific criteria for grouping chemicals into assessment groups for human risk assessment of combined exposure to multiple chemicals" (EFSA Scientific Committee *et al.*, 2021). There are a limited number of studies involving the microbiome response to pesticide combinations or mixtures of pesticides and other substances. Lukowicz *et al.* (2018) selected six pesticides (boscalid, captan, chlorpyrifos, thiofanate, thiacloprid and ziram) commonly used in France and mixed them at their corresponding ADI. Zhan *et al.* (2018) selected a group of environmentally relevant triazine herbicides (simazine, atrazine, ametryn, terbuthylazine and metribuzin) widely used in agriculture and frequently detected in food, water and soil. The compounds used in this study were mixed in equal parts of 2 mg/kg bw. Two additional studies selected two compounds, the pesticide permethrin and the drug pyridostigmine bromide, focusing on their role in the Gulf War illness etiopathogenesis rather than on their environmental relevance (Alhasson *et al.*, 2017; Seth *et al.*, 2018).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CHAPTER 5
CHALLENGES

RELEVANCE OF ALTERATIONS TO THE MICROBIOME AND HOST CAUSED BY PESTICIDE EXPOSURE

Living organisms, including the complex microbiomes, always look to maintain homeostasis. Since they are constantly exposed to environmental agents, there are mechanisms that are continuously being activated or deactivated to counteract the effects of the exposure until stability is reached. Within established limits, these ups and downs are considered part of a physiological baseline. We usually rely on a combination of symptoms, predictors or markers outside a range of normal values that help us anticipate or determine the development of disease or toxic effect. Moreover, when defence mechanisms are sustained over time and are not enough to fully or partially reverse the effects of the offending substance or pathogen, chronic disorders start developing. For example, a long-standing inflammatory response (the defence mechanism to eliminate foreign substances or microorganisms and stimulate healing) can lead to the development of pathologies such as bowel disease and metabolic disorders (Kaser and Tilg, 2012). So, as a word of caution, alterations *per se* do not necessarily translate into a health disorder or negative health outcome. They need to be placed in context considering other influencing variables.

The determination of dysbiosis has been the standard microbiome parameter evaluated in the majority of studies, especially related to its taxonomical composition. Different functional aspects have also been evaluated but in fewer studies. Almost all the studies included in this review have reported some degree of disturbance in the microbiome or/and the host, which include one of several of the following: gut dysbiosis, change in gene abundance and expression, up or down-regulated metabolic paths, and alteration of metabolite and marker profiles. In some cases, these alterations have been observed in the absence of histopathological changes in the host. Evaluating and interpreting the information resulting from these studies has to be done with caution to avoid formulating inaccurate conclusions. The dimension, quantification and meaning of effects and their physio-pathological meaning require further discussion. There is the need to determine if changes observed in the microbiome result from physiologically normal adaptation processes or if they are indeed alterations of concern. So, the questions to ask are: Are the observed alterations within normal or "healthy" ranges? Are the alterations physiologically relevant or not? Are they transient or permanent?

CAUSALITY

To determine if a significant alteration of the microbiome has a relevant impact on the host's health, it is necessary to establish causality and the mechanisms involved. Several research groups and scientists have emphasized the need to be more rigorous when interpreting and communicating experimental results when causality cannot be proven (Fischbach, 2018; Li *et al.*, 2020; Wade and Hall, 2020; Walter *et al.*, 2020). In general, most scientific research can only support associations or correlations between the microbiome and health outcomes. Although many studies shown here suggest that pesticide-induced disturbances lead to alterations in the host, very few studies are designed to confirm the causality. For example, many authors speculate about the microbiome's influence in the development of host alterations or diseases after observing changes in the abundance of certain pathogenic and beneficial bacteria, which have been previously linked to the development of certain health disorders. Establishing causality is challenging as many variables and confounding factors influence the microbiome, the host and their relationship. Causality is typically confirmed by transplanting microbiota in germ-free mice. Such approaches made it possible to establish the influence of the aldicarb-disrupted gut microbiome in the altered brain metabolism (Gao *et al.*, 2019).

Another remark about causality is related to its direction. Although many authors suggest and propose that the observed microbiome disturbances can induce physiopathological changes in the host, there is a need to evaluate if such microbial disturbances are the result of host responses to the pesticide. Lukowicz *et al.* (2018) suggested that gut dysbiosis could have resulted from the host response to a mix of pesticides. Here, microbial disturbances appeared late in the study, while alterations in the host were reported earlier. This point reinforces the need to introduce check points analysis during experiments with long exposure periods to determine the sequence of events and avoid potential misinterpretations of findings.

Establishing the relevance of findings and establishing causality after short-term or long-term exposure to pesticides requires discussion. In the process, it will be necessary to identify and validate quantifiable variables or biomarkers and to determine limits to distinguish the healthy from the unhealthy microbiome. Limits should also be set for microbiome-related physiological vs pathological responses in the host. However, defining what constitutes a healthy microbiome is the first step in the process. This is challenging given the different factors involved in shaping it, including dietary patterns or environmental conditions (Hills *et al.*, 2019). Some activities have already been conducted to identify parameters, research gaps and limitations in establishing a definition for a healthy microbiome (McBurney *et al.*, 2019).

GUT MICROBIOME IN PESTICIDE RISK ASSESSMENT

From the chemical risk assessment perspective, there are two relevant aspects to consider related to the exposure of the human gut microbiome to pesticides. One is the potential of the pesticide to perturb the microbiome and the eventual health

implications in the host. And the second is related to the microbiome's role in modulating the toxicity of the pesticide (or any other xenobiotic compound).

A solid foundation for risk assessment relies on well-defined and robust endpoints. However, microbiome-related endpoints still need to be defined. To achieve this goal, it is necessary to address the points described above to (1) clarify what constitutes a healthy microbiome, define microbiome-relevant alterations and microbiome-derived adverse effects, and identify suitable biomarkers; (2) establish conditions under which biomarker values are considered either normal or abnormal; and (3) establish causality and related mechanisms.

The potential use of the microbiome – understood as a complex, diverse and functional network or microorganisms living in a symbiotic state with the host or environment – as a component of risk assessment is being considered by several organizations. For example, EFSA has published a report indicating the potential relevance of microbiomes in future chemical risk assessments and predictive risk models, but also the need to address gaps, limitations, including questions relative to data interpretation and method standardization, for example (Merten *et al.*, 2020).

One of the research gaps observed in the studies cited in this review is the lack of standardization of *in vivo* and *in vitro* studies and analytical methodologies. Standardization is crucial to improving experimental reproducibility and data comparability. *In vitro* observations should be validated *in vivo* in models chosen. To minimize the effect of the variability observed in exposure studies, Licht *et al.* (2019) suggested selecting animal models with high bacterial diversity, standardized microbiota and routinely checking its microbial composition. Causality should be confirmed by microbiota transfer to defined germ-free models. The limitations of these models should be clearly identified and acknowledged to account for uncertainties related to differences between animals and humans.

There is also a need for experimental designs more suitable for evaluating pesticide residue levels, including chronic exposures, with ranges of experimental pesticide doses permitting the determination of dose–response curves.

To ensure a consistent, transparent and proper evaluation of studies involving the gut microbiome for pesticide risk assessments, the assessors must be guided in the interpretation of data derived from the microbiome research studies, including those resulting from omics analysis.

Some attempts have provided frameworks for the toxicological risk assessment of chemicals involving the gut microbiome. Velmurugan (2018) proposed a workflow for assessing the gut microbiota for known and new drugs and chemicals. It focuses on two aspects: (1) The focus is on identifying changes in the structure of microbial communities, relating disturbed species to those previously linked to diseases. However, this point is of limited use as long as no causal relationship is demonstrated between microbiome disturbances and host alterations. (2) The focus is on identifying and assessing compounds resulting from the microbial metabolism of the studied chemical.

CHAPTER 6
CONCLUSIONS

Pesticide exposure in rodent models has resulted in alterations of the gut microbiome and the animal's homeostasis in the vast majority of cases, with limited demonstration of causality. A few *in vitro* studies also showed microbial disturbances. Assessing the relevance of these findings remains challenging in the absence of a defined healthy microbiome and dysbiosis. Additional research and guidance are needed to (1) establish causality and the involved mechanisms; (2) investigate the impact of low-level pesticide residues in the microbiome; and (3) consider the gut microbiome in pesticide residue risk assessment.

RECOMMENDATIONS

- Organize a series of meetings involving risk assessors and multidisciplinary microbiome experts to:
  - provide definitions for healthy microbiome and dysbiosis in the context of risk assessment;
  - discuss the gaps and limitations of the microbiome as a potential component of chemical safety assessments and provide recommendations for research activities;
  - identify suitable microbiome-related parameters and endpoints with patho-physiological relevance;
  - update existing assessment processes and set criteria to include and evaluate microbiome-derived data, including those generated from the omics technologies; and
  - develop a guideline to support risk assessors in evaluating and interpreting gut microbiome-derived data.
- Encourage research activities that investigate:
  - the gut microbiome's involvement in the chemical transformation of pesticides, and changes in the compound toxicokinetics and toxicity;
  - chronic exposure to low-level pesticide residue;
  - pesticide co-exposure and evaluation of pesticide co-formulants; and
  - demonstration of causality and involved mechanisms.

- Join and contribute to the efforts of the scientific community that aim to:
  - establish suitable models for microbiome research;
  - standardize *in vivo* and *in vitro* methods used to evaluate the safety of pesticide residues (and other chemicals of relevance to food safety); and
  - standardize analytical methodologies, including those based on the omics technologies.
- Develop guidelines for scientists to help harmonize microbiome studies and ensure data quality. The guidelines could cover:
  - selection criteria for animal and *in vitro* models;
  - determination of appropriate sample size (i.e. number of animals per treatment group), and sampling frequency;
  - selection of suitable microbiota (human) donors, sample collection and sample handling (for *in vitro* studies);
  - criteria for selecting pesticide doses (e.g. based on existing health-based reference values) and minimum exposure times;
  - criteria for selecting primers for evaluating the 16S rRNA gene amplification; and
  - guidance for statistical analysis.

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ANNEX I
METHODOLOGY NOTES

To validate the pilot search strategy for the pesticides category, an initial search using general query search terms such as "Gut microbiome" AND "Food" AND "Pesticides", led to 34 articles in PubMed and 8 in Web of Science[31] (Table AI.1). The first three left columns used blocks of query keywords and keyword combinations, whereas the last three show the number of articles found in each search engine without removing duplicates.

In this preliminary search, after removing duplicates, 40 articles were categorized considering the scope of the study as follows: 13 relevant, 10 potentially relevant and 19 not relevant. From 13 relevant articles, 4 studies investigated two types of pesticides (3 chlorpyrifos and 1 glyphosate), and 5 were review articles. It is important to note that these review articles mentioned several pesticides in addition to chlorpyrifos and glyphosate. This initial search seemed limited as very few pesticides were reported in these articles.

TABLE AI.1 **INITIAL SEARCH QUERY TERMS AND RESULTS FROM PUBMED, WEB OF SCIENCE AND SCOPUS**

			ARTICLES FOUND WITH		
Search terms	AND	AND	PubMed	Web of Science	Scopus
"Human Gut Microbiome"	Food	Pesticides	26	5	15
"Gut Microbiome"	Food	Pesticides	34	8	18
"Gut Microbiome"		Pesticides	113˅	36˅	53
"Gastrointestinal Microbiome"		Pesticides	95*	36*	41
"Human Gut microbiome"		"Pesticide Residues"	5	1	

* "Gastrointestinal Microbiome" AND "Pesticides" resulted in duplicates of the first three query searches
˅ "Gut Microbiome" AND "Pesticides" resulted in 3 new articles for PubMed and 8 new articles for Web of Science after the first two query searches
Source: Authors' own elaboration.

After the pilot methodology, the first approach was to build a search query based on terms related to pesticide main use. Table AI.2 shows all pesticide groups within the pesticide *main use* category. The search query had three main blocks: the microbiome keywords first (e.g. Human gut microbiome, gut microbiome),

[31] Differences in search results are likely due to MeSH terms being included for searches in PubMed, and not in Web of Science (e.g. PubMed query of "Microbiome" also includes in the search "Microbiota" and "gastrointestinal").

then added or excluded "food", and finally, the pesticide *main use* (e.g. herbicide, insecticide, fungicide). As expected, using less restrictive keyword groups (e.g. "human gut microbiome" vs "gut microbiome"; inclusion or exclusion of "food") resulted in a higher number of articles.

TABLE AI.2 **SEARCH QUERY TERMS AND RESULTS FOR PESTICIDE MAIN USE FROM PUBMED, WEB OF SCIENCE AND SCOPUS**

MAIN USE			ARTICLES FOUND WITH		
Search terms	AND	AND	PubMed	Web of Science	Scopus
ACARICIDE					
Human Gut Microbiome	Food	Acaricides	0	0	
Gut Microbiome	Food	Acaricides	0	0	
Gut Microbiome		Acaricides	0	0	
ALGICIDES					
Human Gut Microbiome	Food	Algicides	3	0	
Gut Microbiome	Food	Algicides	4	0	
Gut Microbiome		Algicides	20	0	
ANTIFEEDANTS					
Human Gut Microbiome	Food	Antifeedants	0	0	
Gut Microbiome	Food	Antifeedants	0	0	
Gut Microbiome		Antifeedants	0	0	
APHICIDES					
Human Gut Microbiome	Food	Aphicides	0	0	
Gut Microbiome	Food	Aphicides	0	0	
Gut Microbiome		Aphicides	0	0	
AVICIDES					
Human Gut Microbiome	Food	Avicides	0	0	
Gut Microbiome	Food	Avicides	0	0	
Gut Microbiome		Avicides	0	0	
BACTERICIDES					
Human Gut Microbiome	Food	Bactericides	6	0	0
Gut Microbiome	Food	Bactericides	18	0	0
Gut Microbiome		Bactericides	56	0	0
Gastrointestinal Microbiome		Bactericides			0
Microbiome		Bactericides			3
BACTERIOSTAT					
Human Gut Microbiome	Food	Bacteriostat	0	0	
Gut Microbiome	Food	Bacteriostat	0	0	
Gut Microbiome		Bacteriostat	0	0	
BIRD REPELLENTS					
Human Gut Microbiome	Food	Bird repellents	0	0	
Gut Microbiome	Food	Bird repellents	0	0	
Gut Microbiome		Bird repellents	0	0	

continues

MAIN USE			ARTICLES FOUND WITH		
Search terms	AND	AND	PubMed	Web of Science	Scopus
CHEMICAL CLASSES					
Human Gut Microbiome	Food	Chemical classes	33	0	1
Gut Microbiome	Food	Chemicals classes	77	1	2
Gut Microbiome		Chemicals classes	212	10	24
Gastrointestinal Microbiome		Chemicals classes			24
CHEMOSTERILANTS					
Human Gut Microbiome	Food	Chemosterilants	0	0	
Gut Microbiome	Food	Chemosterilants	0	0	
Gut Microbiome		Chemosterilants	0	0	
FUMIGANT					
Human Gut Microbiome	Food	Fumigant	0	0	
Gut Microbiome	Food	Fumigant	0	0	
Gut Microbiome		Fumigant	0	0	
FUNGICIDES					
Human Gut Microbiome	Food	Fungicides	26	0	2
Gut Microbiome	Food	Fungicides	34	0	2
Gut Microbiome		Fungicides	108	6	12
Gastrointestinal Microbiome		Fungicides			10
HERBICIDES					
Human Gut Microbiome	Food	Herbicides	26	2	0
Human Gut Microbiome		Herbicides	56	5	5
Gut Microbiome	Food	Herbicides	34	3	1
Gut Microbiome		Herbicides			14
Gastrointestinal Microbiome		Herbicides			12
HERBICIDE SAFENERS					
Human Gut Microbiome	Food	Herbicide safeners	0	0	
Gut Microbiome	Food	Herbicide safeners	0	0	
Gut Microbiome		Herbicide safeners	0	0	
INSECTICIDE					
Human Gut Microbiome	Food	Insecticides	10	0	
Gut Microbiome		Insecticides	21	9	
Gut Microbiome	Food	Insecticides	19	2	
Gastrointestinal Microbiome		Insecticides			
INSECT ATTRACTANTS					
Human Gut Microbiome	Food	Insect attractants	0	0	
Gut Microbiome	Food	Insect attractants	2	0	
Gut Microbiome		Insect attractants	2	0	
INSECT REPELLENTS					
Human Gut Microbiome	Food	Insect repellents	0	0	
Gut Microbiome	Food	Insect repellents	0	0	
Gut Microbiome		Insect repellents	0	0	

continues

MAIN USE			ARTICLES FOUND WITH		
Search terms	AND	AND	PubMed	Web of Science	Scopus
IXODICIDE					
Human Gut Microbiome	Food	Ixodicide	0	0	
Gut Microbiome	Food	Ixodicide	0	0	
Gut Microbiome		Ixodicide	0	0	
LARVICIDES					
Human Gut Microbiome	Food	Larvicides	0	0	
Gut Microbiome	Food	Larvicides	0	0	
Gut Microbiome		Larvicides	0	1	
MAMMAL REPELLENTS					
Human Gut Microbiome	Food	Mammals repellents	0	0	
Gut Microbiome	Food	Mammals repellents	0	0	
Gut Microbiome		Mammals repellents	1	0	
MATING DISRUPTERS					
Human Gut Microbiome	Food	Mating disrupters	1	0	
Gut Microbiome	Food	Mating disrupters	1	0	
Gut Microbiome		Mating disrupters	3	0	
MITICIDES					
Human Gut Microbiome	Food	Miticides	0	0	
Gut Microbiome	Food	Miticides	0	0	
Gut Microbiome		Miticides	0	1	
MOLLUSCICIDE					
Human Gut Microbiome	Food	Molluscicides	0	0	
Gut Microbiome	Food	Molluscicides	0	0	
Gut Microbiome		Molluscicides	1	0	
NEMATICIDES					
Human Gut Microbiome	Food	Nematicides	1	0	
Gut Microbiome	Food	Nematicides	2	0	
Gut Microbiome		Nematicides	0	0	
NITRIFICATION INHIBITORS					
Human Gut Microbiome	Food	Nitrification inhibitors	0	0	
Gut Microbiome	Food	Nitrification inhibitors	0	0	
Gut Microbiome		Nitrification inhibitors	0	0	
PLANT ACTIVATORS					
Human Gut Microbiome	Food	Plant activators	144	0	
Gut Microbiome	Food	Plant activators	277	1	
Gut Microbiome		Plant activators	457	1	
PLANT GROWTH REGULATORS					
Human Gut Microbiome	Food	Plant growth regulators	4	3	
Gut Microbiome	Food	Plant growth regulators	7	4	
Gut Microbiome		Plant growth regulators	16	5	

continues

MAIN USE			ARTICLES FOUND WITH		
Search terms	AND	AND	PubMed	Web of Science	Scopus
RODENTICIDE					
Human Gut Microbiome	Food	Rodenticides	26	0	
Gut Microbiome	Food	Rodenticides	34	0	
Gut Microbiome		Rodenticides	108	0	
SYNERGIST					
Human Gut Microbiome	Food	Synergists	24	0	
Gut Microbiome	Food	Synergists	56	0	
Gut Microbiome		Synergists	105	1	
VIRUCIDES					
Human Gut Microbiome	Food	Virucides	0	0	
Gut Microbiome	Food	Virucides	0	0	
Gut Microbiome		Virucides	0	0	

Source: Authors' own elaboration.

Following the initial search by *pesticide main use* category, a second search on *specific pesticides* was conducted (Table AI.3). In this occasion, it was expected that more relevant papers would be found by doing an additional search excluding the terms "gut", "food" and "human". However, this approach resulted in many articles related to soils, water and/or plant microbiome.[32] The search on specific pesticides resulted in 245 articles in PubMed and 101 in Web of Science.

TABLE AI.3 **SEARCH QUERY TERMS AND RESULTS FOR SPECIFIC PESTICIDES FROM PUBMED AND WEB OF SCIENCE**

PESTICIDE				ARTICLES FOUND WITH	
	Search terms	AND	AND	PubMed	Web of Science
2,4-D					
	Human Gut Microbiome	Food	2,4-D	2	0
	Gut Microbiome	Food	2,4-D	7	0
	Human Gut Microbiome		2,4-D	3	1
	Microbiome		2,4-D	17	3
ALDICARB					
	Human Gut Microbiome	Food	Aldicarb	0	0
	Gut Microbiome	Food	Aldicarb	0	0
	Gut Microbiome		Aldicarb	1	0
	Microbiome		Aldicarb	1	1

continues

[32] Shared with other team members workings on these topics.

PESTICIDE				ARTICLES FOUND WITH	
	Search terms	AND	AND	PubMed	Web of Science
CARBENDAZIM					
	Human Gut Microbiome	Food	Carbendazim	0	0
	Gut Microbiome	Food	Carbendazim	0	0
	Gut Microbiome		Carbendazim	2	1
	Microbiome		Carbendazim	10	2
CHLORPYRIFOS					
	Human Gut Microbiome	Food	Chlorpyrifos	9	0
	Gut Microbiome	Food	Chlorpyrifos	11	2
	Gut Microbiome		Chlorpyrifos	9	3
	Microbiome		Chlorpyrifos	37	16
DDT					
	Human Gut Microbiome	Food	DDT	0	0
	Gut Microbiome	Food	DDT	0	0
	Gut Microbiome		DDT	0	1
	Microbiome		DDT	8	2
DELTAMETHRIN					
	Human Gut Microbiome	Food	Deltamethrin	0	0
	Gut Microbiome	Food	Deltamethrin	0	0
	Gut Microbiome		Deltamethrin	1	0
	Microbiome		Deltamethrin	4	0
DIAZINON					
	Human Gut Microbiome	Food	Diazinon	0	0
	Gut Microbiome	Food	Diazinon	0	0
	Gut Microbiome		Diazinon	2	9
	Microbiome		Diazinon	2	9
ENDOSULFAN					
	Human Gut Microbiome	Food	Endosulfan	1	0
	Gut Microbiome	Food	Endosulfan	1	0
	Gut Microbiome		Endosulfan	1	0
	Microbiome		Endosulfan	3	1
EPOXICONAZOLE					
	Human Gut Microbiome	Food	Epoxiconazole	0	0
	Gut Microbiome	Food	Epoxiconazole	0	0
	Gut Microbiome		Epoxiconazole	1	1
	Microbiome*		Epoxiconazole	1	1
GLYPHOSATE					
	Human Gut Microbiome	Food	Glyphosate	1	2
	Gut Microbiome	Food	Glyphosate	2	4
	Human Gut Microbiome		Glyphosate	9	4
	Microbiome		Glyphosate	51	27

continues

PESTICIDE				ARTICLES FOUND WITH	
	Search terms	AND	AND	PubMed	Web of Science
HEXACHLOROCYCLOHEXANE (HCH)					
	Human Gut Microbiome	Food	HCH	1	0
	Gut Microbiome	Food	HCH	1	0
	Gut Microbiome		HCH	2	1
	Microbiome		HCH	12	2
IMAZALIL					
	Human Gut Microbiome	Food	Imazalil	0	0
	Gut Microbiome	Food	Imazalil	0	0
	Gut Microbiome		Imazalil	3	0
	Microbiome		Imazalil	4	0
MALATHION					
	Human Gut Microbiome	Food	Malathion	0	0
	Gut Microbiome	Food	Malathion	0	0
	Gut Microbiome		Malathion	1	1
	Microbiome		Malathion	5	1
MONOCROTOPHOS					
	Human Gut Microbiome	Food	Monocrotophos	0	0
	Gut Microbiome	Food	Monocrotophos	0	0
	Gut Microbiome		Monocrotophos	0	0
	Microbiome		Monocrotophos	1	0
PENCONAZOLE					
	Human Gut Microbiome	Food	Penconazole	1	0
	Gut Microbiome	Food	Penconazole	1	0
	Gut Microbiome		Penconazole	1	0
	Microbiome		Penconazole	3	0
PERMETHRIN					
	Human Gut Microbiome	Food	Permethrin	0	0
	Gut Microbiome	Food	Permethrin	0	0
	Gut Microbiome		Permethrin	2	1
	Microbiome		Permethrin	5	3
PROPAMOCARB					
	Human Gut Microbiome	Food	Propamocarb	0	0
	Gut Microbiome	Food	Propamocarb	0	0
	Gut Microbiome		Propamocarb	2	1
	Microbiome		Propamocarb	3	1

Source: Authors' own elaboration.

Some articles analysed in the first two categories (pesticide *main use* and individual pesticides) suggested that negative health effects caused by pesticide exposure are not only dependent on the active ingredient, but could also be related to adjuvants in commercial formulations. In addition, a pesticide mixture or cocktails were

also included in the search query, given the growing interest in the co-exposure to multiple pesticide residues (Table AI.4). This search included the same query approach used previously, with the addition of the term "dietary exposure". This category resulted in 314 articles in PubMed and 114 in Web of Science. Nevertheless, the majority of these articles were duplicates.

TABLE AI.4 **SEARCH QUERY TERMS AND RESULTS FOR PESTICIDE MIXTURES FROM PUBMED AND WEB OF SCIENCE**

			ARTICLES FOUND WITH	
Search terms	AND	AND	PubMed	Web of Science
Human Gut Microbiome	Food	Pesticide formulation	1	0
Gut Microbiome	Food	Pesticide formulation	1	0
Gut Microbiome		Pesticide formulation	3	1
Gastrointestinal Microbiome		Pesticide formulation	2	0
Human Gut Microbiome		Pesticide formulation	1	0
Human Gut Microbiome	Food	Cocktail mixes	0	0
Gut Microbiome	Food	Cocktail mixes	0	0
Gut Microbiome		Cocktail mixes	0	0
Gastrointestinal Microbiome		Cocktail mixes	0	0
Human Gut microbiome		Cocktail mixes	0	0
Human Gut Microbiome	Food	Cocktail	22	1
Gut Microbiome	Food	Cocktail	32	4
Gut Microbiome		Cocktail	101	61
Gastrointestinal Microbiome		Cocktail	76	13˅
Human Gut microbiome		Cocktail	45	20*
Human Gut Microbiome	Food	Pesticide mixtures	2	1
Gut Microbiome	Food	Pesticide mixtures	4	1
Gut Microbiome		Pesticide mixtures	5	2
Gastrointestinal Microbiome		Pesticide mixtures	5	2
Human Gut microbiome		Pesticide mixtures	3	2
Human Gut Microbiome	Food	Pesticide cocktail	1	0
Gut Microbiome	Food	Pesticide cocktail	1	0
Gut Microbiome		Pesticide cocktail	2	0
Gastrointestinal Microbiome		Pesticide cocktail	2	0
Human Gut microbiome		Pesticide cocktail	1	0
Human Gut Microbiome	Food	Cocktail residues	0	0
Gut Microbiome	Food	Cocktail residues	0	0
Gut Microbiome		Cocktail residues	0	0
Gastrointestinal Microbiome		Cocktail residues	0	0
Human Gut microbiome		Cocktail residues	0	0
Dietary exposure		Pesticide cocktail	4	6

˅ "Gastrointestinal Microbiome" AND "Cocktail" resulted in two new articles for Web of Science after the first three query searches
* "Human Gut Microbiome" AND "Cocktail" resulted in duplicates of the first three query searches
Source: Authors' own elaboration.

Finally, a literature search was conducted based on the *pesticide chemical type* category (Table AI.5). Search queries followed the same structure as the pesticides *main use category*. This search resulted in 141 articles in PubMed and 23 in Web of Science.

TABLE AI.5 **SEARCH QUERY TERMS AND RESULTS FOR PESTICIDE CHEMICAL TYPES FROM PUBMED AND WEB OF SCIENCE**

CHEMICAL TYPE				ARTICLES FOUND WITH	
	Search terms	AND	AND	PubMed	Web of Science
ARSENIC COMPOUNDS					
	Gut Microbiome	Pesticide	Arsenic	6	3
	Gastrointestinal Microbiome	Pesticide	Arsenic	5	0
	Gut Microbiome	Pesticide	Arsenic compounds	6	2
	Gut Microbiome	Food	Arsenic compounds	6	3
BIPYRIDYLIUM DERIVATIVE					
	Gut Microbiome	Pesticide	Bipyridylium	0	0
	Gastrointestinal Microbiome	Pesticide	Bipyridylium	0	0
	Gut Microbiome	Pesticide	Bipyridylium derivative	0	0
	Gut Microbiome	Food	Bipyridylium derivative	0	0
CARBAMATES					
	Gut Microbiome	Pesticide	Carbamates	9	2
	Gastrointestinal Microbiome	Pesticide	Carbamates	8	0
	Gut Microbiome	Food	Carbamates	5	0
COPPER COMPOUND					
	Gut Microbiome	Pesticide	Copper	2	0
	Gastrointestinal Microbiome	Pesticide	Copper	2	0
	Gut Microbiome	Pesticide	Copper compound	0	0
	Gut Microbiome	Food	Copper compound	0	1
COUMARIN DERIVATIVE					
	Gut Microbiome	Pesticide	Coumarin	0	0
	Gastrointestinal Microbiome	Pesticide	Coumarin	0	0
	Gut Microbiome	Pesticide	Coumarin derivative	0	0
	Gut Microbiome	Food	Coumarin derivative	0	0
HETEROCYCLIC					
	Gut Microbiome	Pesticide	Heterocyclic	1	0
	Gastrointestinal Microbiome	Pesticide	Heterocyclic	1	0
	Gut Microbiome	Food	Heterocyclic	10	3
MERCURY COMPOUND					
	Gut Microbiome	Pesticide	Mercury	4	2
	Gastrointestinal Microbiome	Pesticide	Mercury	2	0
	Gut Microbiome	Pesticide	Mercury compound	0	0
	Gut Microbiome	Food	Mercury compound	0	0

continues

CHEMICAL TYPE				ARTICLES FOUND WITH	
	Search terms	AND	AND	PubMed	Web of Science
NITROPHENOL DERIVATIVE					
	Gut Microbiome	Pesticide	Nitrophenol	0	0
	Gastrointestinal Microbiome	Pesticide	Nitrophenol	0	0
	Gut Microbiome	Pesticide	Nitrophenol derivative	0	0
	Gut Microbiome	Food	Nitrophenol derivative	0	0
ORGANOCHLORINE COMPOUND					
	Gut Microbiome	Pesticide	Organochlorine	3	3
	Gastrointestinal Microbiome	Pesticide	Organochlorine	2	1
	Gut Microbiome	Pesticide	Organochlorine compound	4	1
	Gut Microbiome	Food	Organochlorine compound	11	1
ORGANOPHOSPHORUS COMPOUND					
	Gut Microbiome	Pesticide	Organophosphorus	3	2
	Gastrointestinal Microbiome	Pesticide	Organophosphorus	3	0
	Gut Microbiome	Pesticide	Organophosphorus compound	22	2
	Gut Microbiome	Food	Organophosphorus compound	24	0
ORGANOTHIOPHOSPHORUS					
	Gut Microbiome	Pesticide	Organothiophosphorus	1	0
	Gastrointestinal Microbiome	Pesticide	Organothiophosphorus	1	0
	Gut Microbiome	Food	Organothiophosphorus	1	0
ORGANOTIN COMPOUND					
	Gut Microbiome	Pesticide	Organotin	0	0
	Gastrointestinal Microbiome	Pesticide	Organotin	0	0
	Gut Microbiome	Pesticide	Organotin compound	0	0
	Gut Microbiome	Food	Organotin compound	0	0
PHENOXYACETIC ACID DERIVATIVE					
	Gut Microbiome	Pesticide	Phenoxyacetic acid	0	0
	Gastrointestinal Microbiome	Pesticide	Phenoxyacetic acid	0	0
	Gut Microbiome	Pesticide	Phenoxyacetic acid derivative	0	0
	Gut Microbiome	Food	Phenoxyacetic acid derivative	0	0
PYRAZOLE					
	Gut Microbiome	Pesticide	Pyrazole	0	0
	Gastrointestinal Microbiome	Pesticide	Pyrazole	0	0
	Gut Microbiome	Food	Pyrazole	0	0

continues

CHEMICAL TYPE				ARTICLES FOUND WITH	
	Search terms	AND	AND	PubMed	Web of Science
PYRETHROID					
	Gut Microbiome	Pesticide	Pyrethroid	4	0
	Gastrointestinal Microbiome	Pesticide	Pyrethroid	4	0
	Gut Microbiome	Food	Pyrethroid	0	0
THIOCARBAMATE					
	Gut Microbiome	Pesticide	Thiocarbamate	0	0
	Gastrointestinal Microbiome	Pesticide	Thiocarbamate	0	0
	Gut Microbiome	Food	Thiocarbamate	0	0
TRIAZINE DERIVATIVE					
	Gut Microbiome	Pesticide	Triazine	2	0
	Gastrointestinal Microbiome	Pesticide	Triazine	2	0
	Gut Microbiome	Pesticide	Triazine derivative	0	0
	Gut Microbiome	Food	Triazine derivative	0	0

Source: Authors' own elaboration.

ANNEX II
FINDINGS

TABLE AII.1 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *2,4-D* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 0.01 mg/kg bw **ARfD: Unnecessary** **Use: herbicide**							
Dose reported on study	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
1 ppm in drinking water (~0.26 mg/kg bw/day)	Mouse C57BL/6 (male)	n= 5 per group	14 weeks (faecal samples taken also at W4)	> 16S rRNA (V4) gene sequencing > Shotgun metagenomic sequencing (faeces) > Metabolomic profiling (LC-MS Q-TOF) (faeces)	Perturbations to the gut microbial composition: ↑ Bacteroidetes, Chlorobi, Chloroflexi, Spirochaetes and Thermotogae; *Streptomyces coelicolor*, *Methylobacterium extorquens* and *Dehalococcoides ethenogenes* Metagenome analysis: Pathway alteration: urea degradation, amino acid metabolism and carbohydrate utilization Metabolic profiles (faeces) 6394 molecular perturbations (e.g prostaglandins, nitrogen metabolites)	-	(Tu *et al.*, 2019)

Source: Authors' own elaboration.

TABLE AII.2 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *ALDICARB* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0-0.003 mg/kg bw **ARfD: 0.003 mg/kg bw** **Use: acaricide, miticide, insecticide, nematicide**							
Dose reported on study	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
2 ppm in drinking water (~ 0.3 mg/kg bw/day)	Mouse C57BL/6 (male)	n =5 per group	13 weeks	> 16S rRNA (V4) sequencing, > Shotgun metagenomics sequencing (faeces) > Metabolomics and lipidomics (faeces, liver, brain)	↑ Erysipelotrichaceae; *Clostridium*, *Dehalobacterium*, *Coprococcus*, *Oscillospira*, *Ruminococcus* ↓ Christensenellaceae, Clostridiaceae (completely depleted), Coriobacteriaceae, Peptostreptococcaceae, *Anaerostipes*, *Roseburia*	> Altered lipid profile > Disturbed brain metabolism	(Gao *et al.*, 2019)

Source: Authors' own elaboration.

TABLE AII.3 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *CARBENDAZIM* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 0.03 mg/kg bw **ARfD: 0.1 mg/kg bw** **Use: fungicide**							
Dose reported on study	**Model and Method**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
100 or 500 mg/kg bw per day in diet	Mice ICR (male)	n= 15 per group received dose	4 weeks (7 or 8 per group killed at day 8 and 28, respectively) (faeces collected every 2 days)	> 16S rRNA (V3-V4) gene sequencing > Gene expression analysis (liver) > Faecal SCFAs > Histochemical (intestine)	> Significant reduction in richness and diversity of gut microbiota: ↑ Firmicutes, Proteobacteria and Actinobacteria; Desulfovibrionaceae, Lachnospiraceae and Ruminococcaceae ↓ Bacteroidetes; Bacteroidaceae, Christensenellaceae, Paraprevotellaceae, Porphyromonadaceae, Prevotellaceae and Rikenellaceae	Hepatic metabolism disorder: accumulation of hepatic lipid and triglycerides; and liver inflammation response	(Jin *et al.*, 2015)
0.2, 1, 5 mg/kg bw/day dissolved in acetic acid and drinking water	Mice C57BL/6 (male)	n= 8 per group	14 weeks (faeces collected every other day – first week and once per week for the remaining time)	> 16S rRNA gene sequencing > Gene expression analysis (different tissues) > Histochemical analysis (fat, liver, colon)	↑ Actinobacteria ↓ Bacteroidetes, Verrucomicrobia No change in Firmicutes and Proteobacteria	Lipid metabolism disorder, hyperlipidemia, inflammatory response	(Jin *et al.*, 2018b)

Source: Authors' own elaboration.

TABLE AII.4 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *CHLORPYRIFOS* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 0.01 mg/kg bw **Use: insecticide**			**ARfD: 0.1 mg/kg bw**				
Dose reported on study	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
1 mg/day	SHIME®		30 days	Standard microbiological techniques	↑ *Bacteroides* spp. and *Enterococcus* spp. ↓ *Bifidobacterium* spp. and *Lactobacillus* spp.		(Joly *et al.*, 2013)
1 mg/kg bw per day by 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	Standard microbiological techniques	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)	16S rRNA gene qPCR, and culture methods	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: ↑ *Clostridium leptum* (PND 60 in colon) ↓ *Bacteroides/Prevotella* (PND60 in ileum)	In 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	Methods	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	> 16S rRNA (V3-V4) gene sequencing	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 potential to disturb 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	> 16S rRNA (V4-V5) gene sequencing > Recolonization study	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	Methods	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	> 16S rRNA gene sequencing	NFD: 12 bacterial genera affected Low dose: ↑ *Allobaculum*, *Candidatus Saccharimonas*, *Coprococcus*, *Anaeroplasma*, *Roseburia* and *Sutterella* ↓ *Pseudoflavonifractor*, *Anaerosporobacter*, *Aerococcus*, *Brevundimonas* and *Trichococcus* High dose: ↓ *Pseudoflavonifractor*, *Anaerosporobacter*, *Aerococcus*, *Brevundimonas*, *Trichococcus* and *Bacteroides* HFD: 13 bacterial genera affected Both doses: ↑ *Sutterella* and *Candidatus Arthromitus* ↓ *Olsenella*, *Clostridium sensu stricto*, *Amphibacillus*, *Enterorhabdus* and *Alloprevotella* Low dose ↑ *Acinetobacter*, *Blautia* and *Oscillibacter* ↓ *Ruminococcus* and *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	> 16S RNA qPCR analysis	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	> 16S rRNA gene sequencing	↑ 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	Methods	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	> Standard microbiological techniques > SCFA > Gene expression (Caco-2/TC7 cells)	↓ *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	> Conventional bacterial culture and molecular biology methods > 16S rRNA genes using bacterial group specific primers	COMPOSITION CPF-oil exposure: ↓ Bifidobacteria population D15; and ↑ *E. coli* count D30 Plate culture techniques: ↑ *Bacteroides* spp., *Clostridium* spp. and enterobacterial populations D15 and 30; ↓ Bifidobacterial 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)	16S rRNA gene (V3-V4) sequencing	> 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 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	Methods	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)	16S rRNA gene (V3-V4) sequencing	> 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: ↑ spontaneous activity, ↑ 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)
50, 100 or 200 µM	Cultured bacteria: *Escherichia coli*, *Bifidobacterium adolescentis*, *Lactobacillus reuteri*	n = 6	16 h	> Rivoflavin and folate analysis > LC MS/MS proteomic analysis (*E.coli*) > MAIT cell activation assay and flow cytometry	> Altered bacterial metabolism > No growth inhibition	Potential inflammatory immune response	(Mendler *et al.*, 2020)

Source: Authors' own elaboration.

TABLE AII.5 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *DELTAMETHRIN* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 — 0.01 mg/kg bw **ARfD: 0.05 mg/kg bw** **Use: insecticide**							
Dose reported on study	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
21 µg/mL = 21 mg/kg	*In vitro* Tandem fermentor and Caco-TC7 cell culture	n = 5 replicates	24 hours in fermentor 4 hours in cell culture	Microbial volatolome, metatranscriptome	Microbiota composition not studied ↑ sulfur compounds ↓ ketone compound (2,2,4,4-tetramethyl-3-pentanone) > Functional dysbiosis	Pro-inflammatory intestinal response	(Defois *et al.*, 2018)

Source: Authors' own elaboration.

TABLE AII.6 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *DIAZINON* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 0.005 mg/kg bw **ARfD: 0.03 mg/kg bw** **Use: acaricide, miticide insecticide**							
Dose reported on study	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
4 ppm in drinking water Estimated: 0.6 mg/kg bw/day	Mice C57BL/6 (male)	n = 5 per group	13 weeks	Metatranscriptomic sequencing	Modulated Quorum Sensing System Stress response pathways activated Impaired energy metabolism	-	(Gao *et al.*, 2017a)
4 mg/L in drinking water Estimated: 0.6 mg/kg bw/day	Mice C57BL/6 (male and female)	n = 5 per group	13 weeks	> 16S rRNA (V4) gene sequencing, metagenomics sequencing, MS–based metabolomics	Sex-specific microbial changes, stronger response in male. Female: ↑ Lachnospiraceae (*Johnsonella*) ↓ Lachnospiraceae, Ruminococcaceae, Clostridiaceae and Erysipelotrichaceae, Male: ↑ Bacteroidaceae, *Bacteroides*, Bacteroidales; Burkholderiales, Clostridiaceae and Erysipelotrichaceae (*Coprobacillus*) ↓ Lachnospiraceae (*Johnsonella*), Bacteroidetes, completely inhibited: Lachnospiraceae (*Butyrivibrio*), Lachnospiraceae (*Shuttleworthia*), Staphylococcaceae (*Staphylococcus*)	Potential neurotoxicity	(Gao *et al.*, 2017b)

Source: Authors' own elaboration.

TABLE AII.7 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *ENDOSULFAN* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 0 .006 mg/kg bw **ARfD: 0.02 mg/kg bw** **Use: acaricide, miticide insecticide**							
Dose reported on study	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
0, 0.5 and 3.5 mg/kg/bw via oral gavage	Mice (*Mus musculus*, ICR) (male)	n = 6 per group	2 weeks	Urine metabolomics (^{1}H-NMR) Serum metabolomics (HPLC-MS/MS)	Altered gut microbiota metabolism	Not linked to microbiome: Alterations of amino acid, energy, lipid metabolism	(Zhang *et al.*, 2017)

Source: Authors' own elaboration.

TABLE AII.8 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *EPOXICONAZOLE* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

Use: fungicide							
Dose reported on study	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
0, 4 or 100 mg/kg bw/d in diet	Rats Sprague-Dawley (Female)	n= 10 per group	90 days (~13 weeks}	16S rRNA (V4-V5) gene sequencing	↑ Bacteroidetes, Proteobacteria; Lachnospiraceae, Enterobacteriaceae and Bacteroidaceae (high dose) ↓ Firmicutes; Lactobacillaceae (high dose)	Potential liver toxicity (no clear causality)	(Xu *et al.*, 2015)

Source: Authors' own elaboration.

TABLE AII.9 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *GLYPHOSATE* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 1 mg/kg bw **ARfD: not necessary** **Use: herbicide**								
Pesticide	**Dose reported on study**	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
Roundup®	0.1 ppb, 400 ppm and 5000 ppm Roundup® in drinking water (GLY content ~ 50 ng/L, 0.1 g/L and 2.25 g/L, respectively) Estimated: 0.0000025, 5, 112.5 mg/kg bw/day	Rats Sprague-Dawley (Male and female)	n = 3 per dose	> 2 years > Samples collected after 673 days (~96 weeks or 1.8 years)	16S rRNA (V2, V3, V4, V6, V7, V8, V9) gene sequencing traditional culture methods	Sex-specific alterations > Males: ↓ Firmicutes > Females: ↑ Bacteroidetes ↓ Firmicutes, Lactobacillaceae > *in vitro* growth inhibition: Bifidobacteria, *Clostridia* and *Enterococci* at 400 ppm *Lactobacilli* at 5000 ppm No growth inhibition in Coliforms	Liver dysfunction	(Lozano *et al.*, 2018)
Glyphosate and Glyfonova® (active ingredient: glyphosate)	2.5 or 25 mg/kg/day Glyphosate OR 25 mg/kg/day Glyfonova® (glyphosate acid equivalent (NOVA)) by oral gavage	Rats Sprague-Dawley	n = 20	2 weeks	16S rRNA (V3) gene sequencing SCFA (faeces, caecum)	No significant changes	Very limited impact dependent on the availability of aromatic amino acids	(Nielsen *et al.*, 2018)

continues

Pesticide	Dose reported on study	Model	Sample size (n)	Period	Methods	Impact on gut microbiota	Health outcomes	References
Glyphosate (and Roundup® (active ingredient: glyphosate)	1.75 mg/kg bw per day in drinking water	Rats Sprague-Dawley dams and pups (male and female)	GLY group 13.3 (range 11–17) RU group 13.9 (range 11–16)	GD 6 up to PND 125	16S rRNA (V3-V4) gene sequencing	Microbiome changes in mainly at PND31 ↑ Bacteroidetes (*Prevotella*), Deferribacteres (*Mucispirillum*) ↓ Firmicutes (*Lactobacillus*), Proteobacteria (*Aggregatibacter*) Roundup® ↑ Bacteroidetes (*Parabacteroides*), Firmicutes (*Veillonella*) ↑ Firmicutes (*Clostridia*, *Blautia*), Actinobacteria (Actinobacteria, *Rothia* and *Bifidobacterium*)	Exposure at early life development may shape gut microbiota	(Mao *et al.*, 2018)
Glyphosate and Roundup®	5 mg/kg/day glyphosate Roundup® with 5 mg/kg/day of glyphosate equivalent in the diet	Rats (pregnant females) Sprague-Dawley	n= 7 per dose	GD 10 to PD22 (about 34 days)	16S rRNA (V3-V4) gene sequencing	Both: ↓ Ruminococcaceae Roundup®: ↑ Bacteroidetes, Erysipelotrichaceae, *Alloprevotella* and *Turicibacter* ↓ Firmicutes, Lachnospiraceae Glyphosate ↓ *Butyricicoccus*	Maternal behaviour and neuroplasticity modulation (influence of gut microbiota not evaluated)	(Dechartres *et al.*, 2019)
Roundup®	250 or 500 mg/kg bw/ day by oral gavage	Mice Swiss (male)	n = 6 per group	6 and 10 weeks	Phoenix system identification method	↓ *Corynebacterium,* Firmicutes, *Bacteroidetes and Lactobacillus*	Neurobehavioral dysfunction	(Aitbali *et al.*, 2018)
Glyphosate	5, 50 and 500 mg/kg bw /day via gavage	Rats Sprague-Dawley (male)	n = 8 per group	5 weeks	16 S rRNA (V3-V4) gene sequencing Gene expression (intestine)	Altered gut microbial composition-significantly increased α-diversity (mainly high dose) No change on Bacteroidetes/ Firmicutes ratio ↑ Fusobacteria, *Ruminococcus*, Prevotellaceae, *Prevotella* ↓ Firmicutes, *Lactobacillus*	Potential inflammatory response, and alterations to the integrity, and function of the small intestine.	(Tang *et al.*, 2020b)

continues

Pesticide	Dose reported on study	Model	Sample size (n)	Period	Methods	Impact on gut microbiota	Health outcomes	References
Glyphosate	75, 150 or 300 mg/L	Cultured bacteria: *Escherichia coli*, *Bifidobacterium adolescentis*, *Lactobacillus reuteri*	n = 6	16 h	> Rivoflavin and folate analysis > LC MS/MS proteomic analysis (E. coli) > MAIT cell activation assay and flow cytometry	> Altered bacterial metabolism > No growth inhibition	Potential inflammatory immune responses (less than CPF)	(Mendler *et al.*, 2020)

GD: Gestational day; PND: Postnatal day; PD: Postpartum day M: males; F: females

Source: Authors' own elaboration.

TABLE AII.10 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *IMAZALIL* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 0.03 mg/kg bw **ARfD: 0.05 mg/kg bw** **Use: fungicide**							
Dose reported on study	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
25, 50 or 100 mg/kg bw per day in diet	Mice ICR (male)	n = 8 per group (25 or 50 mg/kg), or 13 per group (100 mg/kg or control)	4 weeks + 5 weeks with no treatment for a subgroup of control and highest dose)	16S rRNA (V3-V4) gene sequencing Gene expression (liver and colon)	Abundance and diversity: Differences between caecal and faecal samples. Faeces: ↑ Chloroflexi, Firmicutes, Actinobacteria and Acidobacteria ↓ Bacteroidetes, Proteobacteria, Cyanobacteria Caecal content: ↑ Clostridiales, Lachnospiraceae, Helicobacteraceae and *Helicobacter* ↓ Rikenellaceae, *Prevotella*, *Anaerostipes* and Citerobacter, *Lactobacillus*, *Bifidobacterium* and *Desulfovibrio* High dose (abundance faeces and caecal) ↓ Bacteroidetes, Firmicutes and Actinobacteria	Colonic inflammation (especially with high dose)	(Jin *et al.*, 2016)
0.1, 0.5 or 2.5 mg/ kg bw/day orally	Mice C57BL/6 (male)	n= 24-30 per treatment group (8 mice killed each time point)	2, 5 and 15 weeks	16S rRNA (V3-V4) gene sequencing Gene expression (tissue)	Caecal content and faeces: ↑ Bacteroidetes (decreased in caecal content), Clostridiales, Helicobacteraceae and *Oscillospira* ↓ Firmicutes, Actinobacteria, α-, β-, γ-Proteobacteria, *Prevotella*, Bacteroidetes and *Parabacteroides*	Metabolic disorder and intestinal barrier dysfunction	(Jin *et al.*, 2018a)

Source: Authors' own elaboration.

TABLE AII.11 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *MALATHION* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 0.3 mg/kg bw **ARfD: 2 mg/kg bw** **Use: acaricide, miticide insecticide**							
Dose reported on study	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
2 mg/L in drinking wate (~0.6 mg/kg bw/day)	Mice C57BL/6 (male)	n = 5 per group	13 weeks (checkpoint at 4 weeks, microbiome composition in faeces)	> 16S rRNA (V4) gene > Sequencing > Shotgun metagenomics sequencing	↑ *Clostridium*, Mogibacteriaceae, ↓ *Akkermansia*, *Dorea*, *Anaerostipes*, Lachnospiraceae Appeared: *Corynebacterium* Depleted: *Blautia*, *Roseburia*, Christensenellaceae and Planococcaceae	-	(Gao *et al.*, 2018)

Source: Authors' own elaboration.

TABLE AII.12 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *MONOCROTOPHOS* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 0.0006 mg/kg bw **ARfD: 0.002 mg/kg bw** **Use: acaricide, miticide insecticide**							
Dose reported on study	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
0.028 mg/kg bw/day in drinking water	Mice BALB/c (female)	n= 9 per group	180 d (~26 weeks)	> Bacterial metatranscriptomic > SCFA (faeces) > Metabolomics (tissue) > (Faecal transplant)	Modified microbiome	Risk of diabetes	(Velmurugan *et al.*, 2017)

Source: Authors' own elaboration.

TABLE AII.13 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *PENCONAZOLE* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 0.03 mg/kg bw **ARfD: 0.8 mg/kg bw** **Use: fungicide**								
Pesticide	**Dose reported on study**	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
(–)-penconazole; (+)-penconazole; (±)-penconazole	30 mg/L in drinking water Estimated: 4.5 mg/kg bw/day	Mice ICR (male)	n = 8 per group	4 weeks	> 16S rRNA (V3-V4) gene sequencing > Targeted serum metabolomics	(–)-penconazole: ↑ Bacteroidetes, Cyanobacteria, Actinobacteria, *Prevotella* ↓ Firmicutes, *Helicobacter*, Lachnospiraceae, Rikenellaceae, Erysipelotrichaceae, Bacteroidales (+)-penconazole: ↑ Bacteroidetes ↓ *Helicobacter*, Lachnospiraceae, Bacteroidales (±)-penconazole: ↓ Proteobacteria, *Bacteroides*, *Helicobacter*, Erysipelotrichaceae, Bacteroidales ↑ Rikenellaceae	Risk of metabolic disorders	(Meng *et al.*, 2019)

Source: Authors' own elaboration.

TABLE AII.14 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *PERMETHRIN* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 0.05 mg/kg bw **ARfD: 1.5 mg/kg bw** **Use: insecticide**							
Dose reported on study	**Model**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
34 mg/kg bw per day via oral gavage	Wistar rats (male pups)	n = 6 per group	Exposure to permethrin: PND 6 to PND 21 (2 weeks) Microbiome checkpoints in faeces (no exposure: PND 21 (weaning), PND 51 (adolescent), PND 81 and PND 141 (adulthood)	> Bacteria quantification by qPCR > SCFA analysis (faeces) (qPCR) and culture	↑ *Bacteroides*, *Prevotella*, *Porphyromonas* *Lactobacillus* spp. (PND 21, PND 51) ↑ Enterobacteriaceae (PND51) ↓ *Bacteroides*, *Prevotella*, *Porphyromonas* (PND 141)	Risk for motor disabilities (suggested based on alterations to targeted bacteria and SCFA)	(Nasuti *et al.*, 2016)
PERM: 34 mg/kg bw/d by gavage	Wistar rats (male pups)	n= 10 per group	Exposure to permethrin: (PND 6 to 21) PND 21 to PND 60 with no exposure	> 16S rRNA (V3) gene sequencing > Faecal SCFAs	> Altered microbiota ↑ Defluviitaleaceae ↓ *Lachnospira*	Intestinal permeability and hepatic inflammation Motor disabilities	(Bordoni *et al.*, 2019)
PERM+ Electrolysed Reduced Water (ERW): PERM 34 mg/4 mL/ kg bw/d by gavage + ERW 10 mL/kg bw twice a day					↑ Firmicutes, *Lactobacillus*, *Blautia*, Lachnospiraceae, Ruminococcaceae, *Papillibacter*, *Roseburia*, *Intestinimonas*, *Shuttleworthia*, *Oscillibacter* ↓ Bacteroidetes	Counteracts PERM effects under experimental conditions	

PND: Postnatal day

Source: Authors' own elaboration.

TABLE AII.15 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *PROPAMOCARB* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

JMPR ADI: 0 – 0.4 mg/kg bw **ARfD: 2 mg/kg bw** **Use: fungicide**							
Dose reported on study	**Mode**	**Sample size (n)**	**Period**	**Methods**	**Impact on gut microbiota**	**Health outcomes**	**References**
~0.5, 5, 50 mg/kg bw/day	Mice IRC (male)	n= 8 per group	4 weeks	> 16S rRNA (V3-V4) gene sequencing (weekly evaluation of microbiome in faeces) > Gene expression (liver, colon) > Faecal and serum metabolomics	Faecal content (measured weekly): ↓ α,γ-Proteobacteria and Bacteroidetes, β-Proteobacteria (week 1) ↑ Firmicutes (only first 3 weeks exposure at the 2 lower doses), Actinobacteria, β-Proteobacteria (week 3-4) Caecal content - High dose level: ↑ Bacteroidetes, Acidobacteria, Chloroflexi and Planctomycetes; Bacteroidaceae, Dehalobacteriaceae; Genus: *Bacteroides*, *Dehalobacterium*, *Butyricimonas* ↓ Firmicutes, Proteobacteria, Actinobacteria and Tenericutes; Ruminococcaceae, Lachnospiraceae, Rikenellaceae, Porphyromonadaceae, Desulfovibrionaceae; *Oscillospira*, *Parabacteroides*, *Desulfovibrio*, *Ruminococcus* New appeared: *Bacteroides plebeius*	High dose: Metabolic disorder (altered succinate, short chain fatty acids, bile acids and trimethylamine)	(Wu *et al.*, 2018a)
1, 3, 10 mg/L in drinking water Estimated: 0.150, 0.45, 1.5 mg/kg bw/day	Mice C57BL/6J (male)	n= 4 per group	10 weeks	> 16S rRNA (V3-V4) gene sequencing > Gene expression (host tissues) > Faecal and serum metabolomics	Caecal and faecal content: ↑ Bacteroidetes ↓ Firmicutes Faecal content: ↑ Proteobacteria, Chloroflexi, Bacteroidetes and Actinobacteria; Bacteroidia, Prevotellaceae, *Prevotella*, *Dorea* ↓ Verrucomicrobia Caecal content (high dose): ↑ Verrucomicrobia, Odoribacteraceae and Porphyromonadaceae; *Butyricimonas*, *Oscillospira*, *Parabacteroides*, ↓ Proteobacteria	Enterohepatic metabolism disorders Risk of cardiovascular disease	(Wu *et al.*, 2018b)

Source: Authors' own elaboration.

TABLE AII.16 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *DIETHYL PHOSPHATE* (NON-SPECIFC ORGANOPHOSPHORUS PESTIDIDE) ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

Dose reported on study	Model	Sample size (n)	Period	Method	Impact on gut microbiota	Health outcomes	References
0.08 or 0.13 mg/kg/bw via gavage	Wistar rats (male)	n =10 per group	20 weeks	16S rRNA (V3-V4) gene sequencing	Low Doses: ↑ *Bacteroides*, *Pectenophilus*, Adlercreutzia *Paraprevotella* Depleted: Ruminococcaceae, *Jeotgalicoccus* and *Faecalibaculum* High doses: ↑ *Lactobacillus*, *Parabacteroides*, *Alloprevotella*, *Clostridium sensu stricto* 1, *Helicobacter*, *Eubacterium ventriosum* group, *Intestinimonas* and norank f Erysipelotrichaceae Depleted: *Jeotgalicoccus*, Ruminococcaceae, *Eubacterium xylanophilum* group, *Candidatus Saccharimonas*, Defluviitaleaceae UCG-011, *Catabacter*, *Parasutterella*, norank f Christensenellaceae, Peptostreptococcacea, *Mucispirillum*, *Erysipelatoclostridium* and *Candidatus Soleaferrea*	Potential endocrine alterations and pro-inflammatory responses (higher DTP doses)	(Yang *et al.*, 2019)

Source: Authors' own elaboration.

TABLE AII.17 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *PESTICIDE METABOLITES OR BYPRODUCTS, P, P'-DICHLORODIPHENYLDICHLOROETHYLENE (P,P'-DDE) β-HEXACHLOROCYCLOHEXANE (β-HCH)* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

Dose reported on study	Model	Sample size (n)	Period (days)	Methods	Impact on gut microbiota	Health outcomes	References
p,p'-DDE 1 mg/kg bw/day OR β-HCH (10 mg/kg body weight/day via oral gavage	Mice C57BL/6 (male)	n= 8 per group	8 weeks	> 16S rRNA (V4-V5) gene sequencing > Gene expression	↑ Firmicutes and Proteobacteria; Betaproteobacteria; Verrucomicrobiales, Burkholderiales, Bifidobacteriales, Campylobacterales, Bacillales, *Barnesiella*, *Alloprevotella*, *Oscillibacter*, *Lactobacillus*, *Parasutterella*, *Akkermansia* ↓ Bacteroidetes, Verrucomicrobia, Actinobacteria, Candidatus Saccharibacteria; Bacteroidia, Bacilli; Bacteroidales, Lactobacillales, Oceanospirillales; *Parabacteroides*, *Prevotella*, *Bacteroides*, *Clostridium* XIVa, *Clostridium* IV	Metabolic-related disorders	(Liu *et al.*, 2017)
p,p'-DDE 2 mg/kg bw/day via oral gavage, supplemented with our without 2% pectin in water	Mice C57BL/6J (male)	n = 5 per group	8 weeks + 4 weeks exposure to pectin only	> 16S rRNA (V3-V4) gene sequencing > Faecal and plasma SCFA	p,p'-DDE: ↓ *Bacteroides* p,p'-DDE + pectin: ↑ Bacteroidetes, *Parabacteroides*, *Streptococcus*, *Lactococcus*, *Blautia*, *Clostridium*, *Bacteroides* ↓ Proteobacteria, Deferribacteres, Cyanobacteria Pectin (after p,p'-DDE exposure stops: ↑ Bacteroidetes, *Parabacteroides*, *Streptococcus*, *Lactococcus*, *Blautia*, *Clostridium* ↓ Firmicutes, Proteobacteria, Actinobacteria	Metabolic syndrome, e.g. hyperglycemia, insulin resistance and obesity (reduced by pectin supplementation)	(Zhan, *et al.*, 2019)

Source: Authors' own elaboration.

TABLE AII.18 **SUMMARY OF EXPERIMENTAL STUDIES REPORTING THE IMPACT OF *PESTICIDE MIXTURES* ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

Formulation or mixture	Dose reported on study	Model	Sample size (n)	Period	Methods	Impact on gut microbiota	Health outcomes	References
Mix: boscalid, captan, clorpyrifos, thiofanate, thiacloprid and ziram	Boscalid (0.04mg/kg bw/d), Captan (0.1 mg/kg bw/d), Chlorpyrifos (0.01 mg/kg bw/d), thiofanate (0.08 mg/kg bw/d), thiacloprid (0.01 mg/kg bw/d), and ziram (0.006 mg/kg bw/d) in standard chow	Wild type (WT) C57BL/6J and constitutive androstane receptor–deficient (CAR–/–) mice (male and female)	n= 4/5 per group per day	52 weeks	> Transcriptomics (liver) Metabolomics (urine, plasma, liver) > Lipidomics	Microbiome composition not studied	Metabolic-related disorders, diabetes (no clear role of microbiome)	(Lukowicz *et al.*, 2018)
STUDY A Triazine herbicides (simazine, atrazine, ametryn, terbuthylazine and metribuzin) and Ampicilin	2 mg/kg bw/d of herbicide orally + ampicillin 90 mg/kg bw/d (3 times/day)	Rats Sprague-Dawley (male)	n = 5	Total 7 days (after 3 d ampicilin + 4 d both)	> 16S rRNA (V3-V4) genes > Gene expression (liver)	↑ *Bacteroides* ↓ Ruminococcaceae, Lachnospiraceae, *Anaerotruncus*	Enhanced bioavailability of triazine herbicides, increasing exposure risk	(Zhan *et al.*, 2018)
STUDY B Triazine herbicides and antibiotic mix (ampicillin, neomycin, gentamicin and metronidazole) and vancomycin	2 and 20 mg/kg bw/d of herbicide orally + 7 mg/kg per bw cocktail mix (1.75 mg/d each and vancomycin at 0.875 mg/d by gavage)			14 d cocktail mix + after herbicide (unknown timline)		Change: Ruminococcaceae, *Anaerotruncus*		
STUDY C Transplantation		Germ-free rats				Compared to rats with normal microbiota ↓ Firmicutes; Coriobacteriia; Lachnospiraceae, Ruminococcaceae, *Oscillibacter*		
Permethrin (PERM) and Pyridostigmine bromide (PB)	200 mg/kg PERM and 2 mg/kg PB orally (mice later treated IP with corticosterone)	Mice C57BL/6J wild type and TLR4 KO (Gulf War illness)	n = 3 per group	3 times in 7 d and 5 d	16S rRNA (V3-V4) sequencing and cell culture	↓ *Lactobacillus*, and *Bifidobacterium*	Systemic inflammation	(Seth *et al.*, 2018)
Permethrin (PERM) and Pyridostigmine bromide (PB)	200 mg/kg PERM and 2 mg/kg PB orally (mice later treated IP with corticosterone)	Mice C57BL/6J wild type and TLR4 KO (Gulf War illness) (male)	n= 6 per group	3 times in 7 d	16S rRNA V4 gene sequencing	↑ Firmicutes, Tenericutes; *Allobaculum*, *Coprococcus*, *Turicibacter*, *Dorea*, *Ruminococcus* ↓ Bacteroidetes	Neuronal and intestinal inflammation	(Alhasson *et al.*, 2017)

Source: Authors' own elaboration.

ANNEX III
PESTICIDE CLASSIFICATION

COMMON NAME, CAS REGISTRY NUMBER, CHEMICAL TYPE, PHYSICAL STATE, MAIN USE, MODE OF ACTION AND/OR LEVEL OF TOXICITY

PESTICIDES	CAS REGISTRY NUMBER[a]	CHEMICAL CLASS[a]	USE[a]	CHEMICAL TYPE[b]	TOXICITY (WHO CLASS)[b]
2,4-D	94-75-7	Phenoxy	Herbicide	Phenoxyacetic acid derivative	II - Moderately hazardous
Aldicarb	116-06-3	Carbamate	Acaricide, miticide, insecticide, nematicide	Carbamate	Ia - Extremely hazardous
Carbendazim	10605-21-7	Carbamate heterocyclic	Fungicide	-	U - Unlikely to present acute hazard
Chlorpyrifos	2921-88-2	Heterocyclic organophosphorus / organothiophosphorus	Insecticide	Organophosphorus compound	II - Moderately hazardous
DDT	50-29-3	Organochlorine	Contaminant	Organochlorine compound	II - Moderately hazardous
Deltamethrin	52918-63-5	Pyrethroid	Insecticide	Pyrethroid	II - Moderately hazardous
Diazinon	333-41-5	Heterocyclic organophosphorus / organothiophosphorus	Acaricide, miticide insecticide	Organophosphorus compound	II - Moderately hazardous
Endosulfan	115-29-7	Heterocyclic organochlorine	Acaricide, miticide insecticide	Organochlorine compound	II - Moderately hazardous
Epoxiconazole			Fungicide*		
Glyphosate	1071-83-6	Organophosphorus / organothiophosphorus	Herbicide	-	III - Slightly hazardous
HCH	608-73-1[b]		Insecticide[b]	Organochlorine compound[b]	II - Moderately hazardous

continues

PESTICIDES	CAS REGISTRY NUMBER[a]	CHEMICAL CLASS[a]	USE[a]	CHEMICAL TYPE[b]	TOXICITY (WHO CLASS)[b]
Imazalil	35554-44-0	Heterocyclic	Fungicide		II - Moderately hazardous
Malathion	121-75-5	Organophosphorus / organothiophosphorus	Acaricide, miticide insecticide	Organophosphorus compound	III - Slightly hazardous
Monocrotophos	6923-22-4	Organophosphorus / organothiophosphorus	Acaricide, miticide insecticide	Organophosphorus compound	Ib - Highly hazardous
Penconazole	66246-88-6	Heterocyclic organochlorine	Fungicide		III - Slightly hazardous
Permethrin	52645-53-1	Pyrethroid	Insecticide	Pyrethroid	II - Moderately hazardous
Propamocarb	24579-73-5	Carbamate	Fungicide		U - Unlikely to present acute hazard

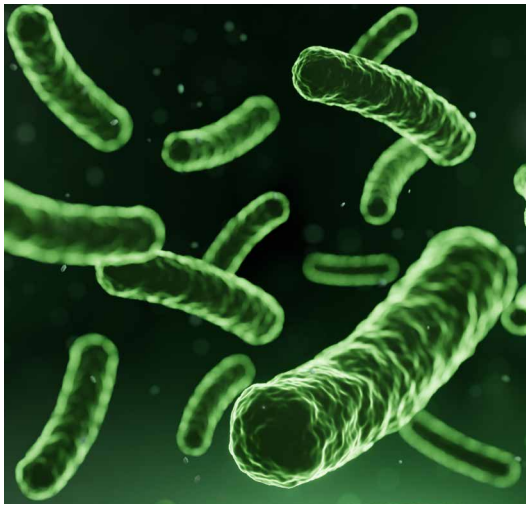
* Fungicide not included in the WHO classification.

Sources:

[a] **WHO.** 2021. Inventory of evaluations performed by the Joint Meeting on Pesticide Residues (JMPR). In: WHO. Cited 30 December 2021. https://apps.who.int/pesticide-residues-jmpr-database

[b] **WHO.** 2010. *The WHO recommended classification of pesticides by hazard and guidelines to classification 2009*. Geneva, WHO. ttps://apps.who.int/iris/handle/10665/44271





THE IMPACT OF PESTICIDE 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 pesticide 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 pesticides on the composition, diversity and function of gut microbiome using *in vivo* or *in vitro* models; (2) health implications resulting from the pesticide–microbiome interactions and underlying mechanisms; (3) establishment of causality; and (4) influence of the gut microbiome on the metabolism and bioavailability of pesticides. 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–pesticide interactions. The outcomes will provide information which can be used to improve nutritional strategies and food safety policies.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO)

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