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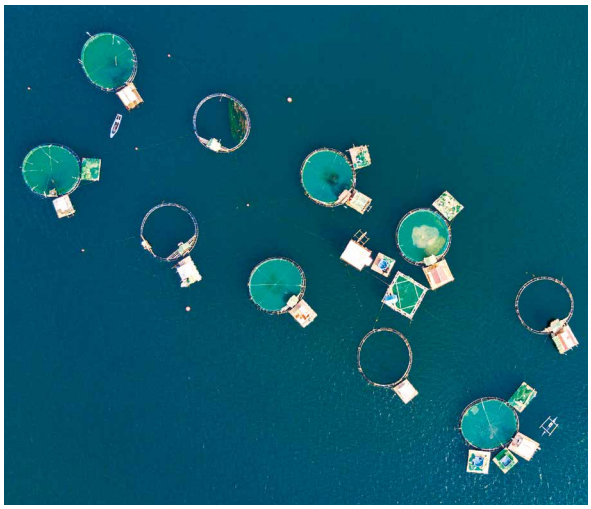

THE IMPACT OF MICROPLASTICS ON THE GUT MICROBIOME AND HEALTH

A FOOD SAFETY PERSPECTIVE

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
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ABBREVIATIONS AND ACRONYMS

DEHP	di-(2-ethylhexyl) phthalate
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nations
IFN	interferon
JRC	Joint Research Center
KEGG	Kyoto Encyclopedia of Genes and Genomes
MP	microplastics
NO	nitric oxide
NP	nanoplastics
OTU	operational taxonomic units
PAE	Phthalate esters
PAH	polycyclic aromatic hydrocarbon
PBDE	polybrominated diphenyl ethers
PCB	polychlorinated biphenyls
PE	polyethylene
PET	polyethylene terephthalate
POP	persistent organic pollutants
PP	polypropylene
PS	polystyrene
PUR	polyurethane
PVC	polyvinyl chloride
ROS	reactive oxygen species
TDI	tolerable daily intake
WHO	World Health Organization

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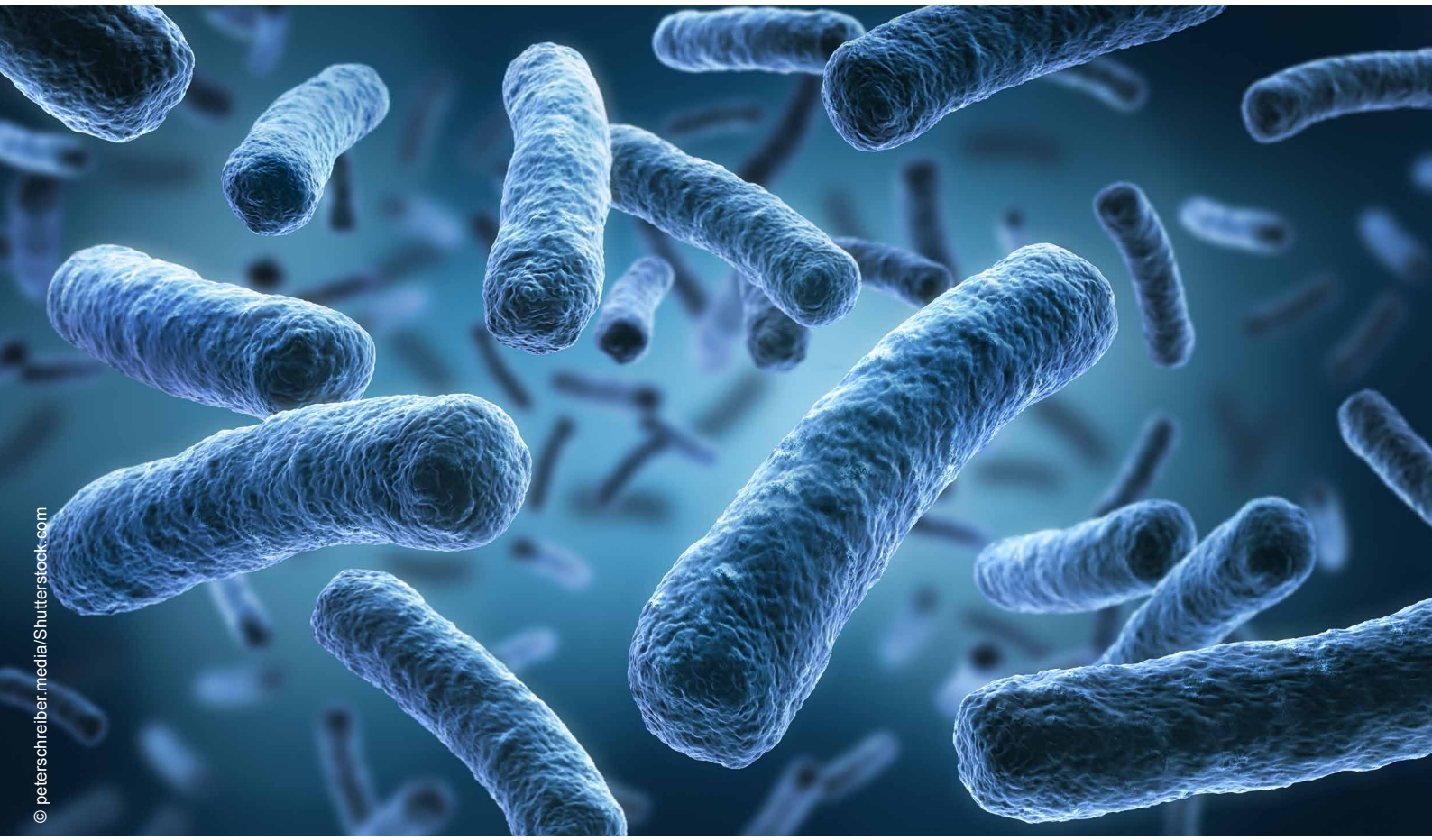

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EXECUTIVE SUMMARY

Microplastics (0.1 to 5 000 µm) and nanoplastics[1] (0.001 to 0.1 µm) are ubiquitous contaminants of emerging interest due to their potential effects on the environment, animals and human health. Their capacity to release plastic additives or adsorb, transport and release environmental contaminants (e.g. heavy metals and organic pollutants), and therefore their capacity to modify the exposure and toxicity of these contaminants, have not been well studied. Further research is required to understand if and how microplastic exposure or co-exposure with other chemicals affects the host and the gut microbiome.

Research on this topic has increased over the last two years, but only a limited number of studies have evaluated the impact of microplastics and nanoplastics on the gut microbiome. Although most of the studies have been conducted on aquatic animals, as they are considered sentinels of microplastic contamination, only a handful have been conducted on mammals (mice). Most of the studies investigated the effects of microplastics, and nanoplastics to a lesser extent, on the gastrointestinal tract, leading in some cases to an alteration of the intestinal structure and function (permeability, inflammatory and immune response), oxidative stress and gut dysbiosis. Although it is difficult to compare studies due to differences in model animals, type, size and concentration of microplastics, as well as exposure times, there is some indication that higher concentrations and non-spherical microplastic shapes increase the severity of effects. Most studies used micro-sized plastics and the limited research conducted at the nano-scale (some comparing the impacts of microplastics vs nanoplastics) suggest that the type of alterations is size-specific. The evaluation of the gut microbiota[2] was mainly limited to investigating changes in its composition and diversity.[3] In this sense, there is a need to study further if and how microplastics can also alter the function of the microbiome, and if alterations are a direct effect of the microplastics or a consequence of the host's response to the particles.

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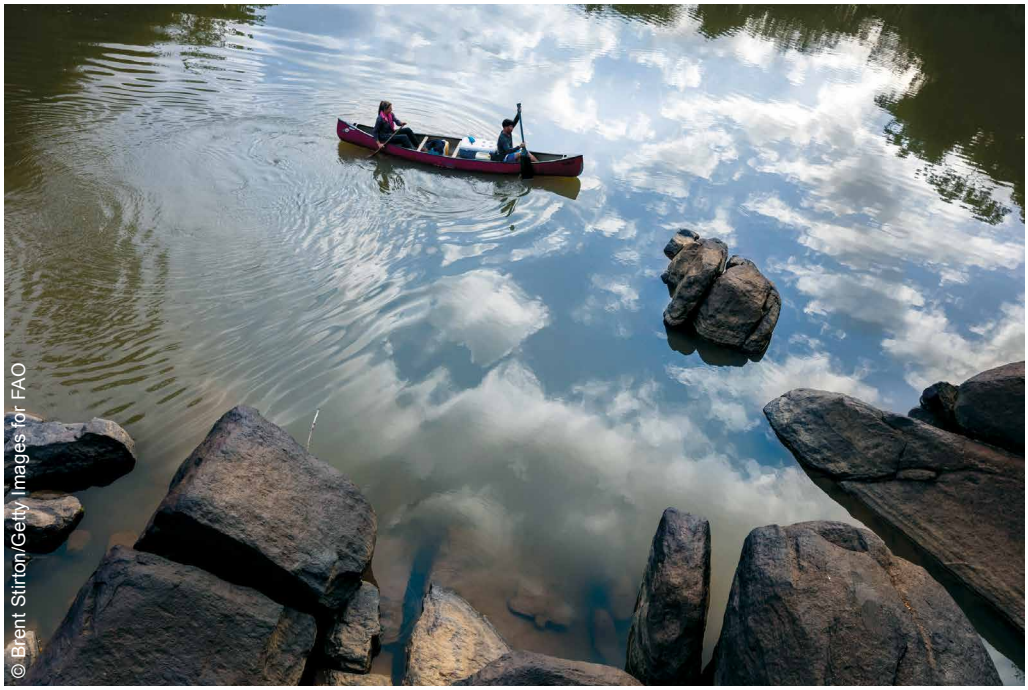
1 In this report, microplastics and nanoplastics refer only to primary or secondary small plastic particles/fragments. Engineered microparticles or nanoparticles (e.g. silver or gold nanoparticles) are not included in this work.

2 This report includes two related terms: microbiota and microbiome. In general, microbiota refers to the collection of microbial individuals. Microbiome is a more holistic term incorporating the overall genetic composition and function of the microbiota.

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

The studies included in this review corroborate the research gaps identified by the scientific community, i.e. the need for definitions of microbiome, microplastics and nanoplastics, as well as the need for a method of harmonization and for reference materials for microplastics and nanoplastics. Experiments should be conducted with great care to avoid cross-contamination. Additional research would help provide further insights on the toxicity and kinetics of smaller microplastics and nanoplastics, and it would provide data on the occurrence in food.



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CHAPTER 1
INTRODUCTION

Microplastics are ubiquitous environmental pollutants that can accumulate in organisms across the food web. Moreover, they can carry other chemical and microbiological contaminants, which may amplify the implications of plastic pollution on living organisms, including those entering the human food supply chain. This review evaluates the impact of microplastics[1] (0.1 µm to 5 mm) and nanoplastics (≤ 100 nm) on the gut microbiome of animal models – including those mimicking humans – and it evaluates the potential consequences for the host's health.

Plastics are light, strong and versatile materials made through chemical processes from a wide range of organic polymers (e.g. polyethylene, polyvinyl chloride, nylon). The most commonly known types of plastic are high- and low-density polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyurethane (PUR) and polyethylene terephthalate (PET) (Geyer, Jambeck and Law, 2017). When heated, plastics can be moulded into different hard or flexible shapes. Since the Second World War, plastics have become a convenient material used in nearly every industry and in our daily lives. In the last decades, the use of plastics has grown exponentially from 2 million tonnes in 1950 to almost 368 million tonnes in 2019 globally (Plastics Europe, 2020). About 8.3 billion tonnes have been produced to date. If the production trend continues, 26 billion tonnes of waste are estimated to be manufactured by 2050, of which 12 billion tonnes are expected to end up in the environment (Geyer, Jambeck and Law, 2017). When plastics are exposed to ultraviolet radiation, friction and other physical, chemical and biological processes in the environment, they break down into smaller fragments and eventually form smaller particles known as microplastics and nanoplastics[2] (Wayman and Niemann, 2021). The size, shape and chemical composition of these particles can vary. Microplastic pollution was not recognized as a problem until the scientific community raised the issue in the early 1970s (Carpenter and Smith, 1972). Plastics have become a major environmental concern because they degrade very slowly. This means that any plastics produced or used in the past still persist in the environment today, and most plastics manufactured now will also remain for hundreds or thousands of years. This is also true for microplastics and nanoplastics, either manufactured or degraded particles.

1 Currently, there is no consensus definition for microplastics and nanoplastics.

2 For source management purposes, microplastics and nanoplastics are divided into two categories. Primary microplastics and nanoplastics are intentionally manufactured particles found in skin care products, toothpaste, molding and cosmetics; while secondary microplastics and nanoplastics are the result of the breakdown of larger plastic fragments.

Over the last few decades, research has focused on plastic pollution in oceans. Large amounts of plastics have been found either floating or submerged; these can break down, increasing the number of microparticles in marine environments. However, microplastic contamination is not limited to aquatic environments (marine, freshwater). They are also found in terrestrial environments (Dissanayake *et al*., 2022). Although it has been reported that the microplastic load in terrestrial environments is higher than in the ocean, their impact on terrestrial ecosystems and soil microbiota remains unclear (Wei *et al*., 2022). Further investigations are needed to determine the effects of soil microbiota and microplastic interactions on agriculture production.

Concerns have been raised about how these particles accumulate, how they become distributed throughout food webs and the health impact on living organisms, including microbiomes. Studies show that aquatic organisms (e.g. zooplankton, molluscs) ingest microplastics (Botterell *et al*., 2019). Microplastics and nanoplastics accumulate in the guts and gills of the marine organisms that consume them. They potentially enter the circulatory system and affect the gut microbiome of the host (Jin *et al*., 2018; van Raamsdonk *et al*., 2020). The presence of microplastics in other environments has been shown to affect microbial communities. For example, soil microbiome experts have reported that microplastic accumulation can disturb the structure and functionality of soil microorganisms (Guo *et al*., 2020). These effects can impact larger soil organisms and eventually affect entire food webs. Microplastics and nanoplastics can be transferred along the food chain and eventually reach our plates (Fackelmann and Sommer, 2019). In fact, microplastics have been found in products for human consumption (tap and bottled water, seafood, sugar, honey, beer, sea salt, tea when using plastic teabags and infant formula when mixed with hot water in polypropylene feeding bottles). They have also been found in human lung tissue, blood, placenta, meconium and faeces (Braun *et al*., 2022; Hirt and Body-Malapel, 2020; Leslie *et al*., 2022; Toussaint *et al*., 2019; van Raamsdonk *et al*., 2020).

International organizations are concerned about the growing presence of microplastics in the food web as they have the potential to induce adverse effects on human health upon consumption. Regarding food safety, the literature has mainly focused on select aquatic species, as microplastics can accumulate in the gastrointestinal tract of these organisms. While it is common to degut fish before humans consume them, some species (small fish, crustaceans, bivalves) are eaten whole, which could increase the risk of exposure. In 2016, the Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA) analysed the existing literature on the presence of microplastics and nanoplastics in food, particularly in seafood (EFSA, 2016). This panel of experts concluded that microplastics seemed unlikely to pose a health risk to humans, though more research and data are needed to confirm this hypothesis. In addition, the evaluation stated that more data on nanoplastics was necessary to determine the safety of these particles. In 2017, following the EFSA's analysis, the Food and Agriculture Organization of the United Nations (FAO) reported the presence of microplastics in 11 out of 20 of the most notable species/genera that contribute to global marine fisheries (Lusher, Hollman and Mendoza-Hill, 2017). Later, FAO conducted a study to investigate the impacts of microplastics on fish and shellfish and their relation to

food safety (Garrido Gamarro, 2020). Although it did not identify any food safety risks, the report noted that many knowledge gaps need to be addressed to allow for a complete risk assessment evaluation, especially for nanoplastics. Since 2019, the World Health Organization (WHO) has also joined with various other institutions to call for more research related to the effects of microplastics on humans.

The concern about the safety of nano- and microplastics is not only related to the particles themselves. They can adsorb organic and inorganic contaminants on their surface (Dissanayake *et al*., 2022). In addition, biofilms can form on their surface and act as carriers of pathogenic vectors and antimicrobial resistance (Kaur *et al*., 2022). However, there are many questions about whether – and to which extent – microplastics can influence the exposure and bioavailability of contaminants and pathogenic factors after consumption by living organisms.

Despite the increased interest in this topic, there are still many knowledge gaps related to consuming micro- and nanoplastics and their effects on the gut microbiome and human health. For example, there are questions concerning the gut microbiome's sensitivity to chronic exposure to microplastics and low concentrations of chemical residues, and whether microbial disturbances lead to short and long-term effects on human health. Work to characterize the risks[3] posed by microplastics and nanoplastics is ongoing. Currently, there are no recognized health-based guidance values[4] for microplastics (e.g. acceptable daily intake [ADI], tolerable daily intake [TDI], acute reference dose [ARfD]). These are reference values determined for different chemical residues (e.g. pollutants, pesticides), below which there is no appreciable risk for human health (FAO and WHO, 2009).

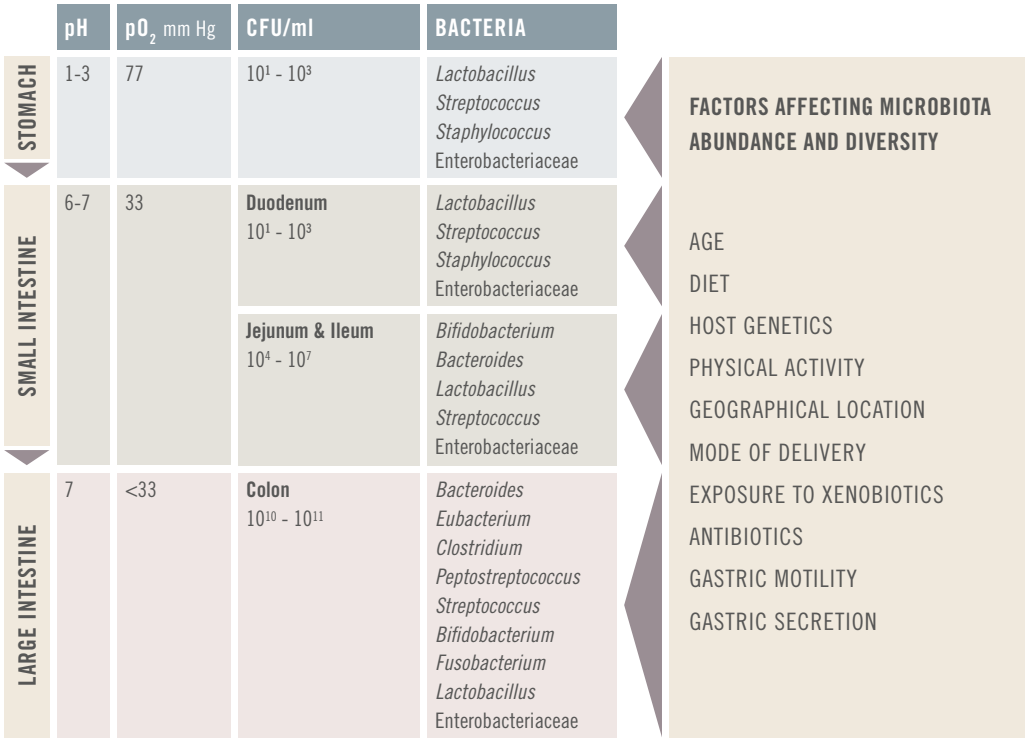
The human gut microbiome is a dynamic community of bacteria, fungi, viruses and archaea, living in a symbiotic relationship with the host (Rosenbert, 2021). Since there is no consensus definition for the microbiome, Berg *et al*. (2020, p. 17) proposed that it is "a characteristic microbial community occupying a reasonable well-defined habitat which has distinct physio-chemical properties." The different anatomical and environmental conditions along the gastrointestinal tract are responsible for differences in microbial diversity between the small and large intestines (Figure 1, Figure 2). An increasing body of evidence demonstrates that the microbiome present in living organisms contributes to maintaining their homeostasis. The gut microbiome participates in the gut barrier, host immunity, energy metabolism, fermentation of carbohydrates, and digestion of protein and peptides (Human Microbiome Project Consortium, 2012; Morais, Schreiber and Mazmanian, 2020; Neish, 2009; Tsiaoussis *et al*., 2019). The gut microbiome also participates in bile acid metabolism and produces substances essential for the host, such as amino acids and vitamins (Nicolas and Chang, 2019). Microbes also synthesize short-chain fatty acids (SCFAs) such as butyrate. These acids are physiologically relevant for the host

[3] Risk: A function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard(s) in food. Codex Alimentarius (2007).

[4] 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 www.efsa.europa.eu/en/glossary/health-based-guidance-value

as they can (1) act as energy sources for enterocytes and immunomodulators, and (2) participate in the neuronal function, anti-inflammatory and metabolic processes such as gluconeogenesis and energy metabolism (Morrison and Preston, 2016; Portincasa *et al*., 2022; Silva, Bernardi and Frozza, 2020).

FIGURE 1. **GASTROINTESTINAL ENVIRONMENT AND MICROBIOTA NICHES**



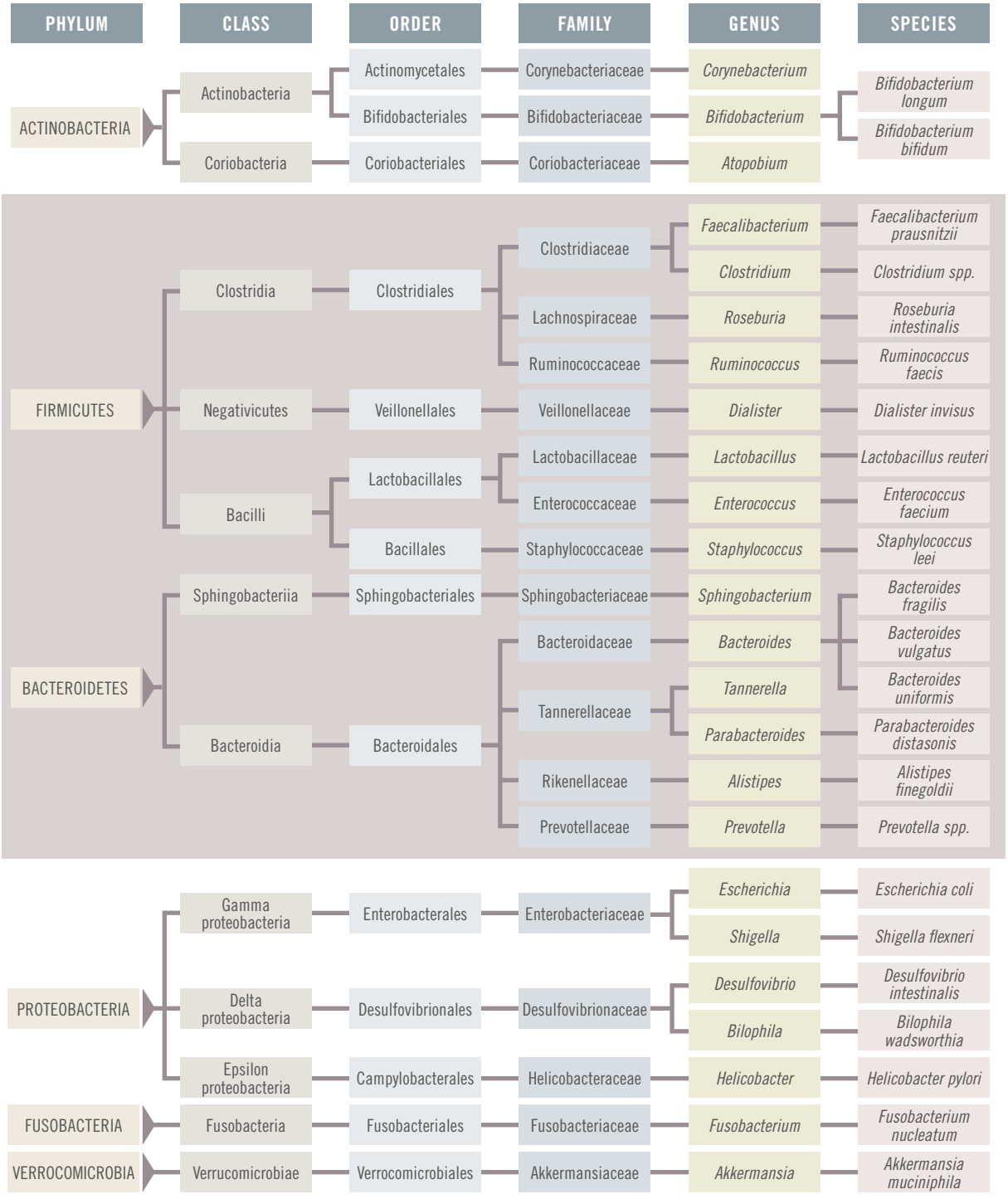
Source: **Clarke, G., Sandhu, K.V., Griffin, B.T., Dinan, T.G., Cryan, J.F. & Hyland, N.P.** 2019. Gut Reactions: Breaking Down Xenobiotic–Microbiome Interactions. *Pharmacological Reviews*, 71(2): 198. https://doi.org/10.1124/pr.118.015768

While it has been recognized that a healthy gut microbiota contributes to the host's well-being, emerging evidence suggests that many factors, e.g. chemical contaminants, may alter the composition and function of the gut microbiome (Rosenfeld, 2017). The imbalance of the intestinal microbiome is referred to as "gut dysbiosis", a term currently lacking an international consensus definition (Brussow, 2020; Perez, Dorsen and Squires, 2019). Historically, gut dysbiosis has been linked with an increased abundance of opportunistic "pathogenic" bacteria and decreased "beneficial" species (Hooks and O'Malley, 2017). Certain alterations of the microbiome may influence the host's homeostasis and potentially contribute to the development of metabolic and inflammatory disorders, endocrine imbalances and neurobehavioral alterations (Feng *et al*., 2019; Tsiaoussis *et al*., 2019).

This literature review aims to collect all the available scientific information on the potential effects of microplastics and nanoplastics on the gut microbiome to better understand the possible impact on human health.

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

Within the grey background are the predominant phyla in humans (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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CHAPTER 2
METHODOLOGY

LITERATURE REVIEW

The scientific literature was screened using English keywords between May and September 2020 to identify peer-reviewed original research articles evaluating the effects of microplastics on the gut microbiome and the possible correlation with human health effects. The databases used to perform these defined queries were PubMed (www.ncbi.nlm.nih.gov/pubmed) and Web of Science (www.webofknowledge.com). Scopus (www.scopus.com) was occasionally used.

An initial search using general query terms such as "Microplastics" AND "Human gut microbiome" led to 53 articles in PubMed and 3 on Web of Science.[5] The terms were searched in the Title, Abstract and Keyword fields. The Boolean queries used in the literature search are listed in Table 1. The first column includes query search terms and combinations, whereas the last two columns show the number of articles found in each search engine without removing duplicates.

TABLE 1. **QUERY SEARCH TERMS AND RESULTS FROM PUBMED AND WEB OF SCIENCE**

	TOTAL ARTICLES FOUND IN SEPTEMBER 2020 WITH	
Search terms	PubMed	Web of Science
Microplastics AND Human microbiome	71	3
Microplastics AND Microbiome	80	26
(microplastics OR nanoplastics) AND (Microbiome OR Microbiota)) AND Intestin*	15	-
([Polyethylene] AND [Microbiome OR Microbiota]) AND Intestin*	38	-
([Polypropylene] AND [Microbiome OR Microbiota]) AND Intestin*	1	-
([Polystyrene] AND [Microbiome OR Microbiota]) AND Intestin*	13	-
([Polyester] AND [Microbiome OR Microbiota]) AND Intestin*	8	-
Gut microbiome AND Polyethylene	-	38
Gut microbiome AND Polypropylene	-	-

continues

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

	TOTAL ARTICLES FOUND IN SEPTEMBER 2020 WITH	
Search terms	PubMed	Web of Science
Gut microbiome AND Polystyrene	-	17
Gut microbiome AND Polyester	-	1
Microplastics AND Microbiome AND Bioaccumulation	4	-
Microplastics AND Microbiome AND Accumulation	16	6
Microplastics AND Microbiome AND Metabolism	32	7
Microplastics AND Microbiome AND Human health	15	2
Microplastics AND Microbiome AND Offspring	1	-
Microplastics AND Microbiome AND Maternal exposure	1	-
Microplastic exposure AND Offspring	17	14
TOTAL (including duplicates)	**312**	**114**

An asterisk in the search (e.g. Intestin) brings up all the possible combinations such as intestine, intestinal, intestines, etc.

Source: Authors' own elaboration.

SCREENING OF ARTICLES AND SELECTION CRITERIA

The literature search yielded 312 articles in PubMed and 114 on the Web of Science. After removing duplicate articles, 146 articles published in English were placed in an Excel file with all the relevant information (searched terms and database, authors, title, abstract, year, volume, issue, pages and type).

Although many articles report the effects of microplastics in various species commonly consumed by humans, this literature review focuses specifically on *in vivo* research studying the gut microbiome. We used the following criteria to categorize articles as *relevant*, *possibly relevant* and *not relevant* to the purpose of this review:

> *Relevant*: Articles were rated *relevant* when the title or abstract included information on microplastic exposure and its effects on the gut microbiome.

> *Possibly relevant*: This category included articles where the title or abstract was uncertain to be relevant at first glance. Under this category, we also included articles that were not relevant for this review but were relevant for some of our other research areas related to the human gut microbiome (e.g. veterinary drugs, food additives, nutrition, soils).

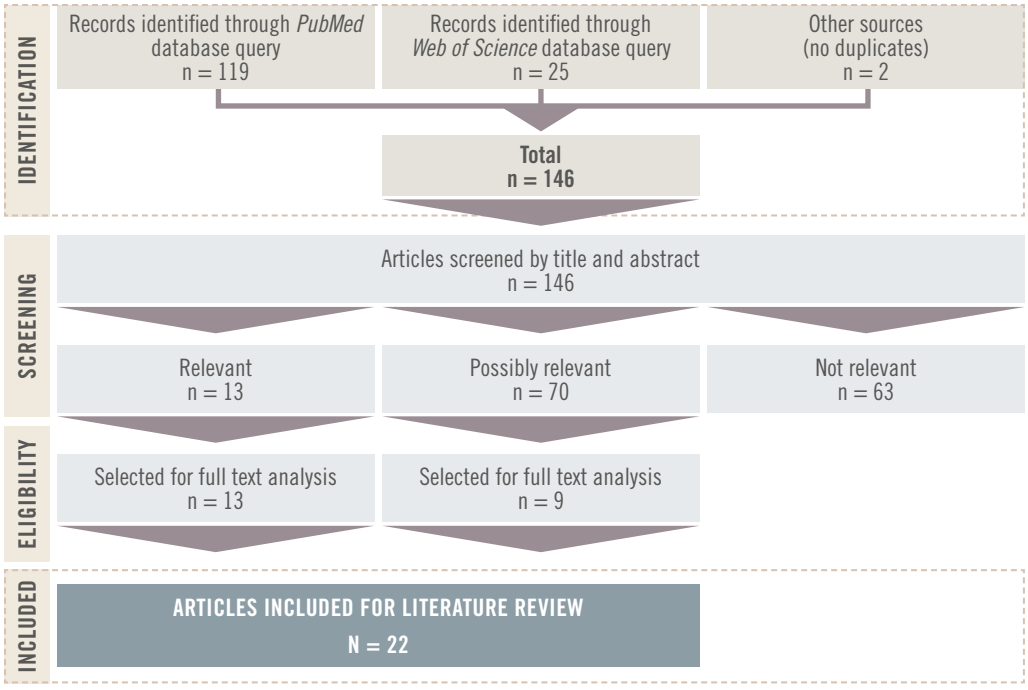
> *Not relevant*: Articles were marked as *not relevant* when the title or abstract did not meet the selection criteria mentioned above. For example, we excluded articles evaluating microplastic occurrence/effects but did not have microbiome data.

CHAPTER 3
FINDINGS

The electronic searches resulted in 119 articles for PubMed and 25 for Web of Science (excluding duplicate articles). After screening articles by title and abstract, 100 percent of the *relevant* articles and 13 percent of the *possibly relevant* articles were included for full-text reading. The remaining 87 percent of the possibly relevant articles were marked to re-direct references to other microbiome projects (e.g. nutrition, soils, plants) or other food safety topics of research (e.g. heavy metals, contaminants). A total of 22 articles were eligible for full-text analysis. The reasons to exclude articles included: the lack of gut microbiome data, focus on other microbiomes (e.g. soils, plants), articles with a good overview of the gut microbiome but with no data to analyse microplastic exposure, and manuscripts with no relevant data about microbiome or microplastics. Figure 3 displays a graphic representation of the article selection process.

The selected manuscripts are presented in groups organized by plastic type, i.e. polystyrene, polyethylene and others (polyamide or nylon, PVC).

FIGURE 3. **GRAPHIC REPRESENTATION OF THE ARTICLE SELECTION PROCESS FOR THE LITERATURE REVIEW**



Source: Authors' own elaboration.

POLYSTYRENE

Polystyrene (PS) is a synthetic plastic made from the polymerization of styrene. Hardness and stiffness make this plastic popular in the food service industry (e.g. hard trays, disposable dishes and cutlery).[6]

A group of scientists conducted two studies to investigate the effects of polystyrene microplastic spheres, administered in drinking water, on the gastrointestinal tract of adult ICR mice (Jin *et al.*, 2019; Lu *et al.*, 2018b). The mice were continuously exposed for five (Lu *et al.*, 2018b) and six weeks (Jin *et al.*, 2019). Both studies used the same concentration of beads (weight/volume) but differed in particle size. It should be noted that for a given concentration, the number of particles is inversely proportional to the particle size. Lu *et al.* (2018b) used 0.5 and 50 µm polystyrene spheres at concentrations of 100 and 1 000 µg/L (about 1.456×10^{10} particles/L for 0.5 µm and 1.456×10^{4} particles/L for 50 µm), respectively, while Jin *et al.* (2019) used 5 µm pristine polystyrene spheres at concentrations of 100 (approximately 1.456×10^{6} particles/L) and 1 000 µg/L (approximately 1.456×10^{7} particles/L). The groups used two different methods to study the microbiota, qPCR with phylum-specific primers, and 16S rRNA gene sequencing (only tested at the high concentration in caecal content), which provided contradictory information regarding the relative abundance of some phyla, e.g. Proteobacteria and Actinobacteria. Both studies reported changes in the microbiota composition at phylum and genus levels. The study of the 16S rRNA gene resulted in a reduction of the relative abundance of Firmicutes, Bacteroidetes and Verrucomicrobia, and an increase of Actinobacteria with 0.5 µm particles (Lu *et al.*, 2018b). On the contrary, Actinobacteria was reduced when dosing with 5 µm spheres (Jin *et al.*, 2019). Jin *et al.* (2019) also predicted functional differences using the Kyoto Encyclopedia of Genes and Genomes (KEGG)[7] metabolic pathways database. The analysis showed that microplastics (MP) influenced functional genes of main microbial metabolic pathways (e.g. pyruvate and tyrosine metabolisms, fatty acid biosynthesis and genes related to bacterial invasion of epithelial cells). In the host, both studies focused on the potential of MP to induce metabolic disorders. Jin *et al.* (2019) observed an alteration of the metabolism related to the amino acids arginine (ARG) and tyrosine (TYR) as well as hepatic bile acids. Lu *et al.* (2018b) focused on the hepatic metabolism and observed a dose-dependent reduction of triglyceride and total cholesterol in the treatment groups. Both studies reported MP accumulation and decreased mucin secretion. In addition, Jin *et al.* (2019) reported that the expression of some ion-transport-related proteins was down-regulated, suggesting intestinal barrier dysfunction. This situation could facilitate the accumulation of MP in the intestinal tissue, their transport beyond the gut and the abnormal microbial colonization (supported by the increase of genes related to the bacterial invasion

6 Polystyrene chemical compound. Britannica. www.britannica.com/science/polystyrene

7 KEGG (www.genome.jp/kegg). The evaluation of predicted differences in the KEGG is helpful in understanding the differences in the metabolic pathways of functional genes in the microbial community between treatment and control groups (Jin *et al.*, 2019).

of epithelial cells, as mentioned above). Lu *et al*. (2018b) only speculated about the potential role of MP-induced dysbiosis on the development of hepatic lipid alterations through an unknown mechanism.

In a later study, the same research group evaluated the effects of maternal exposure to microplastics during gestation and lactation and monitored F1 and F2 generations (Luo *et al.*, 2019). They used the same ICR mouse model and the doses 100 and 1 000 µg/L of 5 µm pristine polystyrene in drinking water. In dams, a 42-day exposure to microplastics during pregnancy and lactation induced microbial dysbiosis, with increased α-diversity, changes at phylum (Actinobacteria and Epsilonbacteraeota increased in high dose group) and genus levels, and intestinal barrier dysfunction. These effects were also associated with metabolic disorders. Microplastic exposure did not induce microbiome changes in pups. However, they led to alterations in gene transcription (hepatic transcriptome and serum metabolite). The authors speculated about the potential risk of metabolic disorders in F1 and F2 after long-term microplastic exposure in F0.

Several studies have been carried out in non-mammal models, including fish (mostly zebrafish, but also guppy fish and yellow croaker), crustaceans (Chinese mitten crab) and molluscs (mussels). Five studies investigated the short-term effects of polystyrene microplastic in adult zebrafish (Gu *et al.*, 2020b; Jin *et al.*, 2018; Qiao *et al.*, 2019a; Qiao *et al.*, 2019b) and one in zebrafish larvae (Wan *et al.*, 2019).

Similar to the mouse study by Lu *et al.* (2018b), zebrafish *Danio rerio* (AB strain) were exposed to the same particle size (0.5 and 50 µm polystyrene beads) size and doses (100 and 1 000 µg/L, about 1.456×10^{10} particles/L for 0.5 µm and 1.456×10^{4} particles/L for 50 µm) in the tank water (Jin *et al.*, 2018; Wan *et al.*, 2019). While Jin *et al.* (2018) studied adult males for 14 days, Wan *et al.* (2019) studied larvae for 7 days. Both studies observed a decreased richness and changes in the diversity of the gut microbiota (at phylum and genus levels), mainly at the higher concentration (1 000 µg/L) and larger particle size (50 µm), characterized by the decrease of the relative abundance of Bacteroidetes and Proteobacteria, and increase of Firmicutes. Jin's group observed that, after the two-week exposure of adult male zebrafish to polystyrene spheres of different sizes (0.5 and 50 µm), the volume of the intestinal mucus layer was reduced, a sign pointing to the disruption of the gut barrier function. They also reported the possible development of intestinal inflammation due to increased levels of mRNA transcription for the interleukins IL1α, IL1β and interferon (IFN), and the levels of these cytokines in the gut. These have been suggested as biomarkers of inflammation in zebrafish (Jin *et al.*, 2018). In the young zebrafish (Wan *et al.*, 2019), the analysis of metabolite and gene expression showed alterations of the energy metabolism, specifically glucose and lipid metabolisms, with stronger effects with larger particle size (50 µm). Some metabolites related to inflammatory and neurotoxic responses and oxidative stress were also altered. Wan *et al.* (2019) speculated that metabolic disturbances may have resulted from reduced food intake because MP could lead to satiety, as previously reported in crabs (Watts *et al.*, 2014). In addition, the authors indicated that the altered microbiome could have also influenced the alterations observed in the larval zebrafish.

Another study used two doses (50 µg/L and 500 µg/L) of 5 µm polystyrene beads in adult zebrafish for 21 days (Qiao *et al.*, 2019b). The doses selected were based on environmentally relevant concentrations determined in surface water (0.2–4 137.3 particles/L) (Zhao *et al.*, 2014) and on the reported toxicity of MP on zebrafish (1–1 000 µg/L) (Jin *et al.*, 2018; Lei *et al.*, 2018). The exposure resulted in reduced diversity and changes to the gut microbiota composition. At the phylum level, significant changes were observed at the high concentration only, where Proteobacteria decreased, and Fusobacteria increased. The families contributing to the microbiota alterations belonged to the phyla Proteobacteria and Actinobacteria. The treatment, especially at the higher concentration, also resulted in the accumulation of particles, histological changes, alterations of lipid metabolism, oxidative stress, and intestinal inflammation. Qiao *et al.* (2019a) evaluated the bioaccumulation and toxicity of different shapes of polystyrene MP in adult zebrafish. They produced MP fibres (diameter: 20 µm; length: 20 to 100 µm) and fragments (4 to 40 µm, peak at 15 µm) from commercial pristine polystyrene beads (15 µm). Zebrafish were exposed to 10 µg/L (beads and fragments: ~5 400 particles/L; fibres: ~680 particles/L) for 21 days. Doses were based on concentrations of microplastics detected in different aquatic environments (3–7 ug/L) (Eriksen *et al.*, 2013; Lacerda *et al.*, 2019) and also on previous studies (Au *et al.*, 2015; Rochman *et al.*, 2014; Ziajahromi *et al.*, 2017). Although all particle shapes accumulated in the gut, the intestinal alterations observed were clearly more severe after exposure to fibres, followed by fragments, and then beads to a lesser extent. Such alterations included mucosal damage, increased intestinal permeability, inflammation, metabolic disturbances, and microbial dysbiosis. Dysbiosis was characterized by reduced diversity and altered composition after exposure to the different shapes of microplastics, with higher impacts caused by non-spherical particles. At the phylum level, all shapes increased the relative abundance of Proteobacteria (more pronounced with fibres), and only MP fibres and fragments reduced Actinobacteria. Fibres affected specific bacteria genera. Fragments caused a decrease in *Pseudomonas* and *Aeromonas*, while all three shapes increased the relative abundance of *Gordonia*. It has been reported that *Gordonia* catabolizes substances used in the manufacture of plastics, such as plasticizers (Drzyzga, 2012). This is the only study reported in this review that analyses the different shapes of microplastics.

Gu *et al.* (2020b) evaluated a 21-day exposure of adult zebrafish to different sizes of nanoplastic and microplastic beads (0.1 µm, 5 µm, and 200 µm) at a concentration of 500 µg/L (1.1×10^{11} particles/L for 0.1 µm MP, 9.1×10^{5} particles/L for 5 µm MP, and 14 particles/L for 200 µm MP). The dose was chosen based on the concentration range of MP in the aquatic environment. The metagenomic analysis of the microbiota revealed the increased abundance of pathogenic bacteria, which correlated with the abundance of genes related to the immune response. The effects were dependent on the particle size. For example, *Aeromonas*, *Actinobacillus* and *Mycoplasma* increased in the 100 nm group, *Staphylococcus* increased in the 5 µm group, and *Vibrio*, *Acinetobacter*, *Porphyromonas*, *Haemophilus*, *Neisseria* and *Lactococcus* were more abundant in the 200 µm group. The authors suggested that exposure

to 200 µm MP increases the health risk from pathogenic bacteria. Microplastics accumulated in the intestine, causing limited toxic effects at the histopathological level, indicating low-grade intestinal inflammation. However, transcriptomics revealed alterations in intestinal cells. They included dysfunction of immune cells (lymphocytes, phagocytes) caused by all three particle sizes. Changes in enterocytes and secretory cells were more diverse with exposure to the smallest particle (0.1 µm bead). Only the nanosized particles increased mucus secretion and changed the expression of genes related to the generation of reactive oxygen species. The authors noted that cellular effects were probably due to the fact that nanoplastics are more capable of entering the systemic circulatory system than microplastics (Pesonen and Vähäkangas, 2019).

Two studies have investigated the potential effects of polystyrene microplastics beads on juvenile fish: the guppy (Huang *et al.*, 2020) and the large yellow croaker (*Larimichthys crocea*) (Gu *et al.*, 2020a). Gu *et al.* (2020a) exposed yellow croaker to three concentrations of nanoplastic polystyrene beads (100 nm): low (10 items/L, 5.50×10^{-12} mg/L), medium (10^4 items/L, 5.50×10^{-9} mg/L) and high (10^6 items/L, 5.50×10^{-7} mg/L). Exposure to the nanoplastics (NP) did not change the microbiota diversity but reduced the richness. Only the two lowest concentrations were used to evaluate the gut microbiota, with increased Firmicutes (only at the lowest dose) and Bacteroidetes and decreased Proteobacteria, at phylum level. At genus level, there was an increased relative abundance of beneficial (*Lactobacillus*, at the lowest dose) and potentially pathogenic (*Parabacteroides* and *Alistipes*) bacteria. Microbial gene functions were predicted, with two main KEGG pathways influenced by the nano-PS treatment, i.e. biosynthesis of secondary metabolites increased while those related to the circulatory system decreased. Regarding the host, results showed reduced immune and digestive enzymatic activity. The author suggested these findings as the cause of the dose-dependent negative effects on mortality and growth rates.

Huang *et al.* (2020) conducted a 28-day exposure study in juvenile guppies using microplastic polystyrene beads (32 to 40 µm) at two different doses (100 and 1 000 µg/L). The lower dose was based on MP concentrations in highly polluted marine and freshwater environments (Burns and Boxall, 2018; Goldstein, Rosenberg and Cheng, 2012; Zhao *et al.*, 2014). Previous studies were also considered to establish the two experimental doses. The authors observed the decreased activity of digestive enzymes and increased immune cytokine levels in the fish. Although the histopathology did not detect signs of inflammation, the mucus secretion by goblet cells increased in a dose-dependent manner, more pronounced at higher doses. The MP exposure also had a dose-dependent effect on the guppy microbiota, showing decreased diversity and evenness, with no apparent impact on richness. The relative abundance of Proteobacteria increased at the high dose, and Planctomycetes decreased after exposure to both MP concentrations. Both concentrations led to a reduction of the family Microbacteriaceae and an increase of Rhizobiaceae. Authors suggested that MP exposure may pose a risk for the microbiome activity, including inhibition of metabolism and repair mechanisms.

Liu *et al.* (2019) evaluated the effects of 5 µm microplastic beads on the innate immune system and the intestinal microbiota of freshwater crabs (*Eriocheir sinensis*). Crabs were exposed to four different MP concentrations in culture water (0, 0.04, 0.4, 4, and 40 mg/L) for 7, 14 and 21 days. Doses were based on published MP toxicity studies in aquatic organisms, including crabs. Although the microbial richness did not vary between control and treatment groups, the diversity decreased after exposure to MP. The microbiota was evaluated only at the highest concentration and 21-day exposure, which resulted in dysbiosis, characterized by the increased relative abundance of the phyla Cyanobacteria, Chloroflexi, Fusobacteria and Proteobacteria, while Nitrospirae, Bacteroidetes, and Firmicutes were reduced at the highest dose. Negative effects in the haemolymph and hepatopancreas (i.e. enzyme activity and gene expression related to the crab immune response) were observed at the high dose and long exposure.

One study investigated the effects of amino-modified nanopolystyrene particles (50 nm) at a dose of 10 µg/L on the immune parameters and the hemolymph microbiota composition of mussels (*Mytilus galloprovincialis* Lam.) (Auguste *et al.*, 2020). The selection of the experimental concentration and exposure time were based on previous data obtained by the research group. After 96-hour exposure, the microbiota composition changed in the NP-exposed group, with a decreased relative abundance of *Mycoplasma*, *Shewanella*, *Tenacibaculum* and *Pseudoalteromonas*, and increased *Psychrobium*, *Vibrio* and *Arcobacter*-like. The increase of *Vibrio* and *Arcobacter*-like genera suggests that exposure to NP promotes a shift toward potentially pathogenic bacteria. NP exposure also altered the innate immune response of the mussels, including decreased phagocytosis, inhibition of nitric oxide (NO) production, and increased production of reactive oxygen species (ROS) and lysozyme activity. The authors suggested that NPs potentially threaten the microbiome–host crosstalk and host health.

POLYETHYLENE

Polyethylene is the most widely used plastic in the world. It is a light and versatile chemical compound made from the polymerization of ethylene. Polyethylene is mass-produced and mainly used in packaging (e.g. bags, plastic films, containers) by many industries.[8]

The scientific research included in this section was conducted with polyethylene particles on the micro-scale, ranging from 4 to 150 µm. The next two studies were conducted in adult mice (Deng *et al.*, 2020; Li *et al.*, 2020a). In Li *et al.* (2020a), adult C57BL/6 mice were exposed to polyethylene microplastics (10 to 150 µm) in the feed at a daily dose of 6, 60, and 600 µg/day over five weeks. The exposure resulted in different degrees of intestinal inflammation, more severe at the highest MP dose, as well as changes in the composition and diversity (increase) of the gut microbiota, with most changes occurring at the two highest doses.

[8] www.britannica.com/science/polyethylene

In a more recent study, polyethylene spheres ranging from 1 to 10 µm suspended in corn oil were given to adult female ICR mice by gavage (Sun *et al.*, 2021). Of the two treatment groups, 0.2 and 0.002 µm/g/day (~1.5×10^5 and ~1.5×10^3 particles/day, respectively), only the high dose had some effect. The lower dose was higher than microplastic concentrations found in the environment. The α- and β- diversities[9] of the faecal microbiome, evaluated on day 15 of the treatment, were not affected. However, the abundance of Bacteroidetes increased and Firmicutes decreased. KEGG prediction showed an increase in genes related to microbial amino acid metabolism. Although the host didn't present histopathological alterations of the colon, the increase in the expression of immunological markers indicated a mild inflammatory response. There was also a reduction in the mucin density. No correlations were made between microbiota and host-related alterations.

It is known that microplastics can adsorb different compounds and microorganisms on their surface. Therefore, they can act as carriers of environmental pollutants (e.g. persistent organic pollutants [POP], heavy metals, antibiotics), microorganisms and resistance genes (Amelia *et al*., 2021; Stenger *et al*., 2021), raising concerns regarding co-exposure effects in animals and humans. Based on this, Deng *et al*. (2020) carried out a 30-day exposure study to evaluate the effects of MP alone or contaminated with phthalate esters (PAEs) in a mouse model (ICR). The study parameters, e.g. type and concentrations of particles and PAEs, were carefully selected to be environmentally relevant and meaningful for health risk assessment. For example, the MP size was similar to those found in human stools (Schwabl *et al*., 2019), and the concentration (0.2 g/L, ~1.5×10^5 particles/L) was equivalent to that detected in drinking and bottled water (1.0×10^{-2} - 1.0×10^8 particles/m^3) (Koelmans *et al*., 2019; Mason, Welch and Neratko, 2018). Moreover, the PAEs used in the study (dibutyl phthalate, diethyl phthalate, dimethyl phthalate, and di-[2-ethylhexyl] phthalate) are among the priority pollutants listed by the United States Environmental Protection Agency (EPA, 2014), and the experimental concentrations (5 µg/L, and 50 µg/L PAE) used in the MP preparation are environmentally relevant (3.4–44.0 µg/L) (Clara *et al*., 2010; Deng *et al*., 2018). Because the intestinal accumulation of di-(2-ethylhexyl) phthalate (DEHP) was higher compared to the other PAEs, the authors evaluated only the effects of DEHP bound to MP or alone. In general, the effects were more evident after co-exposure to DEHP-MPs than MPs or DEHP alone. The histological evaluation of the intestine, and the biochemical and transcriptomics analyses, revealed that DEHP-MPs exposure caused adverse effects, including damage of the intestinal mucosa, inflammation, metabolic alterations of the lipid and energy metabolism and oxidative response. The impact of virgin MPs was more limited, affecting the expression of genes related to lipid metabolism and oxidative response. DEHP-MPs and, to a lesser extent, virgin MPs disrupted the intestinal microbiota. The co-exposure to DEHP-MPs did not change the richness of the faecal microbiota but altered the β-diversity and its composition (increased relative abundance of

[9] "In the study of the human microbiota, alpha diversity is used to describe the compositional complexity of a single sample, whereas beta diversity is used to describe taxonomical differences between samples" (Finotello, Mastrorilli and Di Camillo, 2016, p. 680).

Paraprevotellaceae and Lachnospiraceae families). Both DEHP-MP and virgin MP resulted in an increased abundance of Actinobacteria, *Lactobacillus*, *Adlercreutzia*, *Butyricimonas* and *Parabacteroides*. Although the influence of the microbiota on the host homeostasis has been described, the authors briefly mentioned the potential role of the altered genera in regulating the energy metabolism and immune response of the host. Authors gave three possible explanations for the stronger effects observed in the DEHP-MPs group compared to virgin MPs or DEHP alone: (1) higher intestinal accumulation of DEHP when carried by MPs, (2) MPs change the distribution, accumulation and release (slower) of the phthalate in tissues, and (3) MPs and DEHP may have common toxicity mechanisms.

The exposure of larval zebrafish to 20 mg/L polyethylene microplastic beads (10 to 45 µm) in tank water for 4 and 10 days (starting 5 days after postfertilization) resulted in limited effects (Kurchaba *et al*., 2020). The range of particle size used in this study mimics a mixed exposure of the MP found in the environment, and the dose is based on previous studies. Although the exposure did not appear to affect the overall metabolism and inflammatory response of the fish, there was an elevated oxidative stress response occurring in parallel to microbiome dysbiosis. The authors speculated that oxidative stress could promote the growth of bacteria, like those belonging to the phylum Bacteroidetes, whose relative abundance was increased at both testing points. Authors suggest that the co-occurrence of oxidative stress and dysbiosis may increase the host's susceptibility to diseases.

One additional study with blue mussels (*Mytilus edulis*) considered the weathering[10] effect that plastic particles undergo in the environment (Li *et al*., 2020b). The authors used a 1:1 mixture of two sizes (4 to 6 µm and 20 to 25 µm) of high-density polyethylene, which were used as examples of pristine or weathered (prepared in the laboratory) microplastics. Mussels were exposed to the two types of particles at two concentrations, low (0.2 mg/L, ~1,170 MPs/mL) and high (20 mg/L, ~117,000 MPs/mL) for one, three and six weeks. The low concentration was based on reported MP concentrations from existing surveys (Paul-Pont *et al*., 2018), and the high dose was 100-fold greater than the lower. After the exposure period, the mussels were monitored for two and eight additional days, as some countries require the depuration of bivalves for at least 48 hours before they are released to the market (Lee, Lovatelli and Ababouch, 2008). The research study investigated MP effects on the microbiota only. The exposure led to microbial alterations, with more significant effects at higher concentrations and with weathered particles. For example, the treatment with weathered particles increased the relative abundance of *Flavobacteriales* while *Oceanospirillales* decreased. The latter remained high even eight days post-exposure. Due to the differences found among the treatment groups for the different exposure times, the authors proposed unique microbial biomarkers for weathered MP (Chlamydiae, Rubritaleaceae, Verrucomicrobiales) and virgin MPs (Psychromonadaceae, Xanthomonadales,

[10] "In seawater, aging and weathering processes are almost inevitable for microplastics. These processes change physiochemical properties of microplastics including surface area, oxygen groups, crystallinity, and absorption/ leachate chemicals" (Li *et al*., 2020b, p.9).

Flavobacteriales). Some of the bacteria identified with abundant operational taxonomic units (OTUs)[11] were potentially pathogenic, some of which remained elevated even eight days post-exposure, potentially increasing the food safety risk.

OTHER PLASTICS: POLYAMIDE OR NYLON, POLYVINYL CHLORIDE

Studies reviewed in this section did not investigate any associations between MP-disturbed microbiome and health outcomes. In contrast to the rest of the articles analysed in this current review, both studies used soil organisms as research models.

Horton *et al*. (2020) investigated the co-exposure effects of 1 percent nylon particles (10 g/kg; size < 50 µm, mean 13 to 19 µm) and six concentrations of a mixture of polybrominated diphenyl ethers (PBDE) – 3 000, 1 500, 750, 375, 188 and 94 ng/g – in pond snails (*Lymnaea stagnalis*). The MP concentrations used in this study were higher than concentrations found in aquatic environments. However, those of PBDE were equivalent to concentrations found in freshwater sediments. Results from this study indicated that MPs do not seem to affect the microbiota. After a four-day exposure, the diversity of the snail's gut microbiome was not significantly altered in any of the exposed groups (PBDE and/or microplastics, alone or combined). The relative abundance of Enterobacteriaceae increased, and Flavobacteriaceae decreased only at the highest PBDE concentration in the absence of microplastics. In addition, some microbial orders were present only in the PBDE treatments, specifically sulfate-reducing bacteria (Desulfobacterales and Syntrophobacterales). MP did not influence the uptake of PBDEs, and only BDE 47 from the PBDE mixture was reduced in the snail in the presence of MP.

Microplastics have also been used to evaluate the capacity of certain insect larvae to biodegrade plastics, a process that seems dependent on the gut microbiome. Larvae of mealworm beetles (*Tenebrio molitor*) were fed with polyvinyl chloride (PVC) microplastic powder (70 to 150 µm) without plasticizers for 16 days to evaluate their ability to biodegrade the plastic. The results indicated that the gut microbiome is involved in the depolymerization processes, which was confirmed by inhibiting microbial growth with gentamicin. The PVC MP exposure resulted in significant microbial community shifts, mainly affecting the abundance (increase) of four families: Streptococcaceae (mostly *Lactococcus*), Spiroplasmataceae (mostly *Spiroplasma*), Enterobacteriaceae and Clostridiaceae. About 80 percent of the larvae survived more than five weeks but did not complete their life cycle, with survival rates of 39 percent in three months.

[11] "In microbiology, a phylotype is an environmental DNA sequence or group of sequences sharing more than an arbitrarily chosen level of similarity of a particular gene marker. The most widely used phylogenetic marker is the small subunit ribosomal RNA gene. Two prokaryotic sequences are generally considered as belonging to the same phylotype when they are more than 97–98%. In prokaryotic microbiology, phylotypes, often referred to as Operational Taxonomic Units (OTUs), are a proxy for species" (Moreira and López-García, 2011, p. 1254). Given recent developments, some authors have called for an update to the 97 percent identity threshold (Edgar, 2018).

CHAPTER 4
DISCUSSION

The study of microplastics and nanoplastics and their impact on the environment and the health of living organisms is in its infancy. Critical knowledge gaps prevent risk assessors from carrying out comprehensive assessments to evaluate the health risk of these particles. Some studies are designed to understand the dynamics of microplastics in living organisms and their effects on the gut microbiome and the host's health. However, it is essential to understand and acknowledge the limitations of such studies, which are partly related to the lack of resemblance to real exposure scenarios, e.g. dose and physico-chemical properties of the test particles. In this discussion, we will address those gaps and limitations. We will use the studies described above as examples to provide information on the scientific status quo and to identify research needs to evaluate the impact of microplastics on the microbiome and host.

The manuscripts included in this review address the exposure of the microbiome to microplastics and nanoplastics in three different contexts. The first one is food safety and health, where studies evaluate the MP impact in mammalian models, which could be translated to potential health consequences in humans. The second context is environmentally relevant. Here, research focuses on aquatic animals, non-mammal vertebrates and invertebrates as microplastic contamination has been described more often in aquatic ecosystems. The last context is also environmentally important as it aims at the biological elimination of microplastics. In this case, research has looked into the capacity of the insect gut microbiome to biodegrade (e.g. depolymerization) microplastics.

MICROPLASTICS – POLYMER TYPE AND SIZE

Polystyrene (PS) is the most frequent type of plastic used in the reviewed microplastic studies (Auguste *et al.*, 2020; Gu *et al.*, 2020a; Gu *et al.*, 2020b; Huang *et al.*, 2020; Jin *et al.*, 2019; Jin *et al.*, 2018; Liu *et al.*, 2019; Lu *et al.*, 2018b; Luo *et al.*, 2019; Qiao *et al.*, 2019a; Qiao *et al.*, 2019b; Wan *et al.*, 2019). Although the authors did not give their reasons for selecting this plastic, it is likely related to the availability and versatility of this material. While PS is commonly used in research studies, it is not among the topmost abundant polymers produced. Between 2002 and 2014, the polymer production share in the European Union, the United States of America, China and India was (in decreasing order): high and low-density polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), and polyvinylchloride (PVC), and the primary waste generation was led by PE, PP, PET and PS (Geyer, Jambeck and Law, 2017). PE~PP > PS > PVC > PET are among the polymers most

frequently found by researchers in fresh- and drinking waters (Koelmans *et al*., 2019). PE (Deng *et al*., 2020; Kurchaba *et al*., 2020; Li *et al*., 2020a; Li *et al*., 2020b; Sun *et al*., 2021), and to a lesser extent PVC (Horton *et al*., 2020) and nylon (Peng *et al*., 2020) have also been used for research in connection to microbiome and host exposed to MP.

The effects of MP and NP in living organisms depend not only on their polymer composition but also on a combination of other particle-related factors. When plastics are released into the environment, they undergo ageing or weathering over time. This process can transform the plastic particles physically (e.g. mechanical abrasion or fragmentation into smaller fragments) or chemically (e.g. photo-oxidation, release or adsorption of chemicals). Such modifications can change the size, shape, and physico-chemical properties of microplastics, therefore altering how they interact with other substances and microorganisms in the environment (Duan *et al*., 2021).

The fate of MPs in the gut depends considerably on their size and is also influenced by surface characteristics, i.e. degree of ionization and hydrophobicity, and shape, as will be discussed later. The physical effects of microparticles include physical abrasion and irritation of the intestinal tissue (Paul *et al*., 2020), aggregation and accumulation in the gut lumen of the host. To reach the surface of epithelial cells, MPs have to cross the mucus layer, which protects the intestinal wall. EFSA has reported that microplastics < 150 µm may translocate across the gut epithelium, but the absorption may only be limited to ≤ 0.3 percent (EFSA, 2016). It is possible that intestinal cells uptake smaller particles (about 5 µm or less), and even smaller particles (less than 1.5 µm or more likely those in the nano-scale) could even be distributed systemically and accumulate in organs (EFSA, 2016; Paul *et al*., 2020).

Most of the particles used in the research studies were in the micro-scale ranging from 0.5 to 200 µm, with the majority between 5 to 50 µm. Only three studies evaluated nanoparticles between 50 and 100 nm (Auguste *et al*., 2020; Gu *et al*., 2020a; Gu *et al*., 2020b). Several studies were conducted with a single size, some compared the effects of two or three specific sizes, and some evaluated a mixture following a distribution within a size range. In some cases, the effects in the microbiota or the host differed depending on the particle size. In general, most of the evaluated particles led to changes in the microbiota diversity and composition. For example, exposure to both 0.5 and 50 µm PS beads led to gut dysbiosis in mice, although it was more apparent at the larger bead size (Lu *et al*., 2018b). Using the same particle sizes, Jin *et al*. (2018) also observed gut dysbiosis in zebrafish but the 0.5 µm particles induced gut inflammation. The type of alteration in the host can be size specific. For example, exposure to 5 and 50 µm PS beads led to different metabolomic profiles in zebrafish (Wan *et al*., 2019). Gu *et al*. (2020b) observed distinct size-specific effects caused by nano (100 nm) and microplastics (5 and 200 µm) in the function of intestinal immune cells and the gut microbiota of zebrafish.

In nature, MP and NP differ not only in composition, size and concentration, but also in shapes. Some are produced for industrial use (primary microplastics: e.g. beads

in cosmetics, fibres in textiles, foams in packaging), and some are reshaped by weathering in the environment (secondary microplastics, e.g. fragments) (Toussaint *et al.*, 2019). After reviewing over 50 studies, Koelmans *et al.* (2019) reported that the most frequent particle shapes found in freshwater were fragments (35 percent) > fibres (25 percent) > films > foam > pellets > spheres. Most of the studies we have reported used spheric particles (beads). Qiao *et al.* (2019a) prepared fibres and fragments in the laboratory from commercial PS beads and compared the effects of the three particle shapes in zebrafish. They observed that particle accumulation and the consequent effects in the host and the gut microbiota were shape-dependent. Fibre was the particle shape leading to more severe outcomes in all parameters evaluated, i.e. higher accumulation, microbiota dysbiosis, intestinal damage and inflammation, and altered metabolism. Similarly, Li *et al.* (2020b) prepared a mix of weathered PE particles in the laboratory to evaluate their effects in blue mussels and also observed that higher concentrations and weathered particles had a higher impact on the gut microbiota than lower concentrations and virgin (non-weathered) particles.

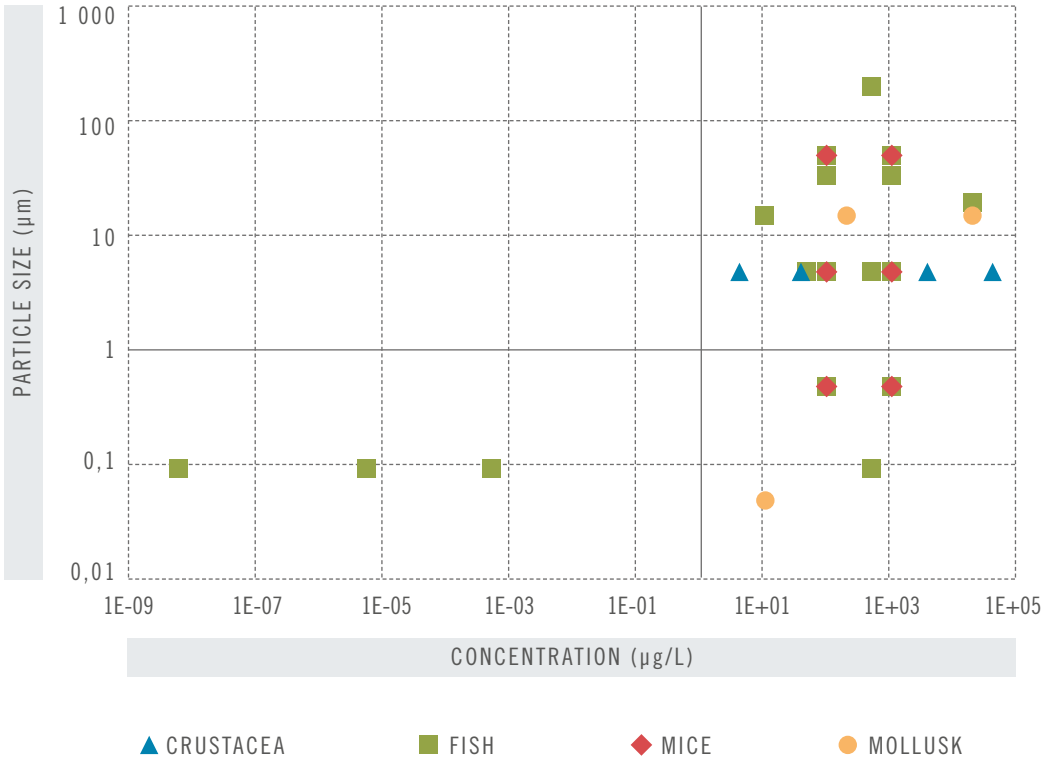
MICROPLASTICS – CONCENTRATION

Like other exposure studies, MP concentration is an essential component of the experimental design. The MP dose can be expressed differently, such as mass/kg or L (mg/L, for example) or the number of particles per kg or L. For a fixed concentration mass/kg or L, the particle count is inversely proportional to the particle size. While most studies in this review provide the concentration in mg or µg per kg or L, some indicate only the number of particles per unit of volume or weight or report both. Many manuscripts clarify the reasoning for dose selection, which is usually based on high-end concentrations of MP found in environmental surveys (such as oceans or rivers) or concentrations reported in drinking water. However, in some cases, scientists select doses based on previous research studies. Except for one study using 100 nm nanopolystyrene beads with as low as 10 particles per L (5.5×10^{-12} mg/L), the doses used in the rest of studies ranged from as low as 6 µg/L to 40 mg/L, although the majority were in the range between 100 and 1 000 µg/L. Some of the effects in the microbiota seem to be dose-dependent, being more evident at higher MS particle concentrations (Jin *et al.*, 2018; Li *et al.*, 2020b; Wan *et al.*, 2019). Effects in the host, e.g. intestinal damage (Qiao *et al.*, 2019b) and gut inflammation (Wan *et al.*, 2019), seem more apparent at higher doses. At the nano level, two different studies using 100 nm PS in two fish species, and at different concentrations, resulted in opposing results: increased microbiota diversity in zebrafish exposed to 1.1×10^{11} particles/L (Gu *et al.*, 2020b), but no change was observed at any dose in yellow croaker for this parameter, even at the highest dose of 1.8×10^{13} particles/L (Gu *et al.*, 2020a). However, in the latter study, changes in the microbiota structure were observed even at the lowest dose tested (10 particles/L).

Most of the studies did not report estimations of MP particles intake. Only two studies conducted in mice provided daily exposure to polyethylene MP, 6, 60 and 600 µg/day (Li *et al.*, 2020a) and 100 mg/kg/day (about 5.25×10^4 particles/day)

(Deng *et al.*, 2020). Particle intake is easier to control when given via gavage, in the feed or drinking water of caged animals than in water tanks. In the absence of intake estimations, providing accurate exposures and relating exposure to health risk outcomes is challenging.

FIGURE 4. **RELATION OF EXPERIMENTAL PS AND PE PARTICLE SIZE/CONCENTRATION USED IN THE STUDIES INCLUDED IN THIS REVIEW**



The mice studies treated with polyethylene microplastics have been excluded from this chart as the concentration unit could not be converted to (µg/L). Details of the studies can be found in Annex I – Findings

Source: Authors' own elaboration.

MICROPLASTICS – SURFACE PROPERTIES AND ADSORPTION OF CHEMICALS

Another point to consider when evaluating particles is the surface properties and the ability of the particles to bind chemicals and release them into the environment. Auguste *et al.* (2020) exposed Mediterranean mussels to amino-modified nanopolystyrene particles and observed changes in the immune parameters and the hemolymph microbiota composition. However, it would have been useful to compare these results with virgin or other types of surface modifications to assess the relative impact of the different particle modifications in the host and the microbiome.

Manufactured plastics can contain additives (e.g. fillers, plasticizers, colourants, stabilizers, processing aids). These additives could be potentially released into the environment. Moreover, microplastics can bind organic materials and chemicals present in the environment (Fackelmann and Sommer, 2019; Jacob *et al*., 2020; Lu *et al*., 2019). For example, plastics of hydrophobic nature can adsorb hydrophobic chemicals or persistent organic pollutants from the environment (e.g. polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and dichlorodiphenyltrichloroethane). The interactions between environmental contaminants and MP and their transfer from the environment to living organisms are complex and challenging to understand, as many factors (microorganisms, sediments, pH, etc.) will influence these processes (Lusher, Hollman and Mendoza-Hill, 2017). MPs and NPs have been reported to modulate the toxicity of different types of contaminants like mercury (Barboza *et al*., 2018), cadmium (Banaee *et al*., 2019; Lu *et al*., 2018a; Miranda, Vieira and Guilhermino, 2019), and polychlorinated biphenyls (PCBs) (Rainieri *et al*., 2018).

Two studies evaluated the effects of the co-exposure to MP and environmental pollutants. The study by Deng *et al*. (2020) observed the capacity of PE MPs to adsorb, transport and release different phthalate esters in a mouse model. The co-exposure di-(2-ethylhexyl) phthalate (DEHP)-MP resulted in more diverse and more severe effects than DEHP or virgin MPs alone. The effects included increased intestinal permeability and inflammation, induction of the immune response, oxidative stress and disturbed lipid and energy metabolisms. The co-exposure also altered the gut microbiome composition. Snails (*Lymnaea stagnalis*) were also co-exposed to nylon MP and a mixture of PBDEs (Horton *et al*., 2020). In the presence of MP, the uptake of one of the PBDEs (BDE 47) was reduced. Moreover, the microbiome did not seem to be affected significantly in this study. Other studies have also shown that the presence of MP and NP can lower the toxicity of contaminants like polycyclic aromatic hydrocarbons (PAHs) in fish embryos and larvae (Trevisan *et al*., 2019).

Laboratory experiments can replicate certain real-life scenarios. However, reproducing the dynamics of environmental microplastic–pollutant interactions is challenging. Although the effects of combined exposure remain unclear, tolerable daily intake (TDI) values for some plastic additives and contaminants have been established, making an individual assessment of these chemicals at those levels in the gut microbiome and the host meaningful. However, the combined exposure of microplastics and pollutants is necessary for a more realistic picture for risk assessment purposes.

Microplastics can also be colonized by microbial communities creating "plastispheres", which may include pathogenic species like *Vibrio parahaemolyticus*, biofilm-producing bacteria and harmful algae (Amaral-Zettler, Zettler and Mincer, 2020; FAO, 2020; Wright *et al*., 2020). Furthermore, microbial communities on microplastics have the potential to enhance the horizontal transfer of antibiotic resistance genes (Arias-Andres *et al.*, 2018). Currently, there is insufficient data on the occurrence of pathogens on microplastics, and therefore they are not included in risk profiling (Lusher, Hollman and Mendoza-Hill, 2017). None of the studies included in this review has evaluated any of these microbiological aspects.

MODELS

The limited number of research studies evaluating the impact of microplastics and nanoplastics on the microbiome of living organisms indicates that this is an aspect that is just starting to gain attention, and it is likely to expand in the near future. We found only six studies conducted in mammals, specifically mice (mostly ICR, but also C57BL/6 and CD1) (Deng *et al*., 2020; Jin *et al*., 2019; Li *et al*., 2020a; Lu *et al*., 2018b; Sun *et al*., 2021). Research in mammals is relevant as they provide toxicity data typically used to assess the health risk in humans. Moreover, mammal microbiomes are more similar to that of humans than non-mammal microbiomes. All mice studies were conducted in adult males, except two: one on adult females (Sun *et al*., 2021) and the second exposed dams (F0) to MP during pregnancy and lactation (Luo *et al*., 2019). The authors did not explain the preference of males over females. Future research should evaluate if microplastic exposure leads to gender-specific effects on the gut microbiota and the animal model. Also, more studies are needed to address early exposure to MPs and NPs. The first days of life are critical for establishing the gut microbiome (Arrieta *et al*., 2014) and the impact of dietary components as primary modulators of the microbial population (Clarke *et al*., 2019).

Due to numerous reports showing microplastic contamination in aquatic environments and the growing concern about their potential effects on living organisms, most studies selected aquatic organisms as experimental models. A recent report indicates that about 55 percent of the species known to ingest microplastics in aquatic habitats are of commercial importance (Lusher, Hollman and Mendoza-Hill, 2017). Therefore, the exposure of animals to microplastics not only has relevance from the environmental perspective. It is also important from the food safety point of view as humans can end up consuming animals that have accumulated microplastics. The studies we have reviewed are mostly conducted in zebrafish (*Danio rerio*) (Jin *et al*., 2018; Kurchaba *et al*., 2020; Qiao *et al*., 2019a; Qiao *et al*., 2019b; Wan *et al*., 2019), which is a non-commercial fish that is very common in aquaria, like guppies (*Poecilia reticulata*) (Huang *et al*., 2020). However, other studies used commercial species such as the large yellow croaker (*Larimichthys crocea*), a popular fish in China grown in offshore aquaculture (Gu *et al*., 2020a), the crustacean Chinese mitten crab (*Eriocheir sinensis*), commonly found in the highly polluted Yangtze estuary (Liu *et al*., 2019), and the bivalve molluscs blue mussel (*Mytilus edulis*) and Mediterranean mussel (*Mytilus galloprovincialis*) (Auguste *et al*., 2020; Li *et al*., 2020b). This type of mollusc is of particular interest because they are consumed whole, and since they are filter feeders, they can concentrate contaminants at a higher rate than other aquatic organisms (Lee, Lovatelli and Ababouch, 2008). To minimize exposure in humans, many countries require that bivalves be first cleaned in pools before they are sold on the market. Another mollusc, the freshwater snail *Lymnaea stagnalis* was also used to evaluate the effect of co-exposure to nylon MP and PBDEs on the model's health and gut microbiome (Horton *et al*., 2020). This type of invertebrate seems to be an appropriate model for evaluating organic pollutants as they can bioaccumulate these types of compounds (Amorim *et al*., 2019). The microbiome of non-commercial and non-mammal species, such as fish

and molluscs, could be used as a toxicological endpoint for microplastic exposure (or co-exposure to microplastics and environmental pollutants). This makes sense only when it has been demonstrated that the microbiome alteration is characterized and is a more sensitive endpoint than existing ones.

Although less related to the previous studies, it is interesting to consider using the beetle *Tenebrio molitor* Linnaeus larvae, commonly known as mealworms, for its capacity to biodegrade PVC (Peng *et al.*, 2020). This is an environmentally relevant area of research that is gaining interest as several studies report the ability of different insects to consume and potentially degrade various types of plastics with the direct participation of the gut microbiome (Lear *et al.*, 2021). It contributes to the evidence reported for other microbiomes, such as the soil microbiome (Zrimec *et al.*, 2020).

EXPOSURE TIMES

The exposure periods used in the different experiments are more similar in the five mice studies (30 and 42 days) than in those involving fish and crabs (7 to 42 days). The two exposure studies on molluscs had a duration of four days. Given the continuous presence of microplastics in the environment, it may be more appropriate to design studies resembling chronic exposures and monitor the impact of microplastics over extended periods. The challenge here is to determine a suitable time length and sampling frequency.

IMPACTS ON THE HOST AND THE MICROBIOTA

Most studies have limited the evaluation of the effects of microplastics to the gastrointestinal tract, including the gut microbiota. The parameters evaluated in the intestinal tract include the determination of MP accumulation in the gut, alterations of the mucosal structure, changes in the mucus layer and gut permeability, inflammatory and immune response as well as oxidative stress. All studies find some degree of alteration in the host following MP exposure. In some cases, alterations of biochemical markers and gene expression are observed in the absence of, or subtle, intestinal histopathological changes (Gu *et al.*, 2020b; Huang *et al.*, 2020; Sun *et al.*, 2021). In addition to the physical abrasion of MP, the accumulation of plastic in the gut can also induce satiety in the organism and decrease food consumption (Wright, Thompson and Galloway, 2013). This may result in a reduction in body weight and alterations in energy metabolism, which are some of the effects reported in several studies (Deng *et al.*, 2020; Huang *et al.*, 2020; Lu *et al.*, 2018b; Qiao *et al.*, 2019a; Wan *et al.*, 2019). A limited number of studies have investigated the impact of MP beyond the gastrointestinal tract and focused on evaluating parameters related to liver function and metabolism. The only mice study evaluating intergenerational effects of MP exposure (F0 only) resulted in metabolic disorders in F0 (Luo *et al.*, 2019). The authors associated these alterations with microbiota dysbiosis and dysfunction of the intestinal barrier. Although F1 and F2 didn't present significant microbiota alterations, they suggested an increased risk for developing metabolic disorders.

In general, and based on a limited number of studies, the severity of effects seems to be directly proportional to the MP concentration and dependent on the particle shape. The one study covering the broadest range of particle sizes – nano and micro sizes (100 nm to 200 µm) – resulted in size-specific alterations of intestinal cells (Gu *et al.*, 2020b).

Regarding the microbiota, Huang *et al.* (2020) suggested that MPs could act as stressors and induce an inflammatory response in the host, which could select for certain microorganisms and lead to microbial dysbiosis. The assessment of the gut microbiota was mainly limited to evaluating alterations in diversity and composition by sequencing the regions V3 and V4 of the 16S rRNA gene. The functional evaluation of the microbiome was basically limited to the predictive assessment of KEGG pathways (Gu *et al.*, 2020a; Gu *et al.*, 2020b; Jin *et al.*, 2019; Luo *et al.*, 2019; Sun *et al.*, 2021). The effects of nanoplastics and microplastics observed in the diversity and composition of microbiotas across the different studies are very heterogeneous. For this reason, it is challenging to compare results and determine commonalities, except for the observed proportional dose-effect relationship, which is not unexpected. A plausible explanation for result heterogeneity is the low number of studies and differences in experimental design (animal model; polymer type, microplastic size, shape and concentration; exposure times). This highlights some of the critical gaps in microbiome and microplastic research: the need for standard methodologies and the availability of suitable reference microplastic particles. Moreover, at this point, it remains questionable whether MP effects caused by one type of polymer can be extrapolated to other classes, and if the effects observed *in vivo* are translatable to the human context.

From these studies, it is not possible to evaluate whether long-term MP exposure could lead to the development of chronic disorders, and whether a potentially MP-altered microbiome would contribute to it. Moreover, additional research is needed to evaluate if – and to which extent – the gut microbiome can biodegrade microplastics, and if the resulting degradation products could further impact the host.

RISK ASSESSMENT

There are currently many open fronts in microplastic research due to the relative novelty of the topic, the lack of definitions and the limitations of current analytical methodologies to detect, identify and characterize these particles. In addition, the multiple physical-chemical characteristics of microplastics (composition, size, shapes, surface properties) as found in nature make it challenging to characterize them as hazards.

The global production, consumption and disposal of plastics have become an issue of concern. Exposure to microplastics and nanoplastics is particularly concerning for several reasons. In addition to their potential to accumulate in living organisms across the food web, microplastics can contain additives as part of their composition that can be released into the environment. Moreover, they can adsorb environmental pollutants or be colonized by microorganisms, including pathogens. As polymers

alone or combined with environmental pollutants and pathogens, microplastics can reach living organisms, potentially leading to adverse effects and gut microbiome alterations. However, it is very challenging to estimate the accurate exposure to MP (or co-exposure to MP and environmental contaminants) and assess health risks.

Microplastics have been found in many food products, including fish, seafood, sea salt, meat, milk, fruits, vegetables, drinking water and beverages (EFSA, 2016; Hirt and Body-Malapel, 2020; Paul *et al.*, 2020). However, there is limited data on oral exposure to microplastics. Still, several attempts have been made to estimate microplastic uptake with very heterogeneous results. An early study estimated the annual dietary exposure to MP among those European consumers who eat the most mussels to be about 1.1×10^4 microplastic particles (Toussaint *et al.*, 2019; Van Cauwenberghe and Janssen, 2014). More recent data shows that American adults and children consume $7.4 \times 10^4 – 1.1 \times 10^5$ MP particles annually (Cox *et al.*, 2019). The maximum annual uptake of MP in adults has been estimated to be 4.6×10^5 particles from tap water and 3.5×10^6 from bottled water (Danopoulos, Twiddy and Rotchell, 2020). The estimation from marine products is as high as 66×10^3, 28×10^3 and 36×10^3 particles/day from fish, crustaceans, and molluscs, respectively (Hantoro *et al.*, 2019). A study evaluated MP in fruit and vegetables and estimated a daily intake from apples of 4.62×10^5 MP particles for adults and 1.41×10^6 for children (Oliveri Conti *et al.*, 2020). Several explanations have been provided to explain the variability of such figures. Paul *et al.* (2020) criticized the study from Cox *et al.* (2019) mentioned above because it did not consider variability due to consumers' consumption behaviour and particle size, which did not include nanoplastics. Recently, a team from the Joint Research Center (JRC) from the European Commission evaluated the average consumption of food products and the global exposure to microplastics (Toussaint *et al.*, 2019). They concluded that it is currently impossible to calculate the total annual exposure to microplastics per capita or compare such exposure to other sources of contamination. The reasons given are the high variability of available contamination levels – influenced by the absence of harmonized definitions and methodologies, lack of reference materials – and the need to retrieve regional consumption data. In an earlier report, EFSA also highlighted the lack of reliable MP occurrence data in food, standardized analytical methodologies, as well as toxicological data from humans and animals (EFSA, 2016).

The few studies included in this review showed effects of microplastics on the microbiota of aquatic and soil organisms, and rodent models. However, they did not provide scientific evidence about the biological relevance of such microbial alterations. Instead, several of the authors speculated about the potential contribution of microbial disturbances to metabolic disorders. With the available information, it is not possible to decipher whether the gut microbiome enhances the impact of microplastics on the host or if microbial dysbiosis is the consequence of the host's response to microplastics, as suggested by Huang *et al.* (2020). Establishing causality and the underlying mechanisms of the host–gut–microbiome interactions using standardized methodologies would contribute to assessing better the impact and risk posed by microplastics.

In summary, using microplastics–microbiome research data for risk assessments and regulatory decisions requires further discussions by a group of multidisciplinary experts. The considerations for discussion could include:

> identification of microplastic exposure conditions causing biologically relevant alterations of the microbiome;

> co-exposure to MP and environmental contaminants;

> identification and characterization of microbiome alterations biologically relevant for the host;

> distinction between transient or permanent microbiome changes;

> relationship between microplastic-induced microbiome changes, gender and age, with especial focus on the long-term implication of early exposure to microplastics (e.g. development of metabolic disorders);

> determination of causality between the microbiome and host alterations. Scientific evidence characterizing the mechanisms involved in the microbiome-host interactions;

> discussions about microbiome endpoints and the suitability of microbiome data for inclusion in risk assessment;

> translatability of *in vivo* and *in vitro* microbiome-related data into the human context.

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CHAPTER 5
RESEARCH GAPS AND OPPORTUNITIES

In addition to the need for definitions of microplastics and microbiome, more research is needed to better understand the effects of microplastic exposure on living organisms and gut microbiome–host interactions. The number of *in vivo* research studies conducted to date, especially those aimed at evaluating the impact of MP on human health, is very limited. There is a need to expand on this area using standardized *in vivo* models.

The design of newer studies should consider:

- ages and genders.
- more rigorous and appropriate use of statistical methods and modelling approaches (Xia and Sun, 2017);
- more realistic exposure context:
  - most commonly used plastics, size distributions and shapes found in the environment, such as fragmented polyethylene particles;
  - different surface properties;
  - long-term MP exposure periods; and
  - co-exposure to MP and environmental contaminants (chemical or biological).
- dose-response relationships to help establish health-based values; and
- other members of the microbiome, e.g. fungi, virus, archaea.

There is a substantial amount of scientific information needed to elucidate microplastics–microbiome–host interactions and their relevance for health. For example, additional research efforts are needed to:

- Determine the effects and risks of smaller microplastics and nanoplastics not only at the intestinal level but also systemically.
- Evaluate whether the microbiome enhances, diminishes or contribute to the effects of microplastics in the host.
- Evaluate the microbiome function, almost neglected in microplastic–microbiome studies.

> Determine causality and the mechanistic evidence that confirm if gut dysbiosis is (1) a direct effect of microplastics on the microbial population, (2) an indirect outcome resulting from the host response to microplastics or (3) a combination of both.

> Evaluate the potential long-term health effects of microplastic–microbiome interactions, e.g. the development of non-communicable diseases.

> Evaluate the capacity of the gut microbiome to biodegrade microplastics and determine the effects and risks of resulting products.

In order to produce quality data and enable reproducibility and inter-study comparability – essential elements of robust risk assessments – it is necessary to:

> Harmonize experimental protocols (e.g. sampling, handling, storage and processing microbiota samples).

> Define and establish appropriate quality controls to minimize the risk of cross-contamination of microplastics in experimental settings.

> Standardize particles or fit-for-purpose reference materials that are industrially produced and mimic natural degradation processes (Brachner *et al*., 2020). This would include different polymers, specific sizes or more realistic size distributions, and shapes, different surface properties, and particles with defined amounts of chemicals. As previously noted, most of the studies used commercial beads with characteristics that do not resemble the properties of naturally occurring microplastics. They are likely to behave differently in biological systems, providing an inaccurate toxicological picture.

> Validate and standardize analytical methodologies. This would include analytical methods used in microbiome research, including conventional techniques and multi-omic approaches.

Other areas needing additional research are those aiming to:

> Investigate the fate of microplastics and nanoplastics under food processing conditions (such as heating or microwaving).

> Characterize the physico-chemical properties of the particles under gastrointestinal conditions.

> Evaluate the bioavailability and their interactions with other food components.

In addition to data quality, more emphasis and guidance should be paid to interpreting, reporting and communicating microbiome-related data. Consideration should not only be paid to statistically significant results. Negative (e.g. no alterations) outcomes are also relevant and are commonly underreported.

As described above, there are numerous gaps and limitations in our understanding of microplastic prevalence and health risk. To tackle the many gaps and needs discussed above, it is necessary to establish a prioritization strategy to guide future research.

CHAPTER 6
CONCLUSION

The number of available studies evaluating the impact of microplastics on the gut microbiota is very limited. The research discussed in this review shows that microplastics induce alterations in the gut microbiota of all animal models used. However, the characteristics of such microbial disturbances vary from study to study. This is partly explained by the heterogeneity of experimental designs (animal models; MP type, sizes, shapes and doses), lack of standardized analytical methods and MP reference materials, making inter-study comparisons and drawing conclusions very challenging. The microbiota of aquatic animals was evaluated as an endpoint parallel to the analysis of other parameters in the host. The effects of MP on the host were limited to the intestinal tract in most cases. Alterations observed after MP exposure included altered intestinal structure and function, gut inflammation and increased oxidative stress. In addition, MPs were able to induce alterations in lipid and energy metabolism in rodents. Although based on very limited number of studies, the effects are dose-, size- and shape-dependent. While some authors speculated on the potential role of the altered microbiome in the development of metabolic alterations, they did not support this possibility with scientific evidence. In addition, the biological relevance of the microbial alterations described in those studies is not clear.

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ANNEX I
FINDINGS

TABLE AI.1 **SUMMARY ARTICLES REPORTING THE IMPACT OF POLYSTYRENE ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

PARTICLE SIZE	PARTICLE SHAPE	DOSE	MODEL AND METHOD	ANIMALS PER TREATMENT GROUP (N)	PERIOD	IMPACT ON GUT MICROBIOTA	HEALTH OUTCOMES	REFERENCES
0.5 and 50 µm	Sphere	100 and 1 000 µg/L (about 1.456×10^{10} particles/L for 0.5 µm and 1.456×10^{4} particles/L for 50 µm) in drinking water	> Mice ICR (male) > qPCR phyla-specific primers > 16S (V3-V4) rRNA gene sequencing	n = 8	5 weeks	qPCR (caecal and faecal content) - Dose-dependent: ↓ Firmicutes and α-Proteobacteria, Actinobacteria (no change in faeces) 16S rRNA gene (caecal content) – High dose: Phylum level: ↓ Firmicutes, Bacteroidetes, Verrucomicrobia ↑ Actinobacteria Genus level: ↓ *Oscillospira*, *Anaerostipes* ↑ *Parabacteroides*, *Prevotella*, *Dehalobacterium*, *Ruminococcus*, *Bilophila*, *Bifidobacterium*, *Adlercreutzia*, *Plesiomonas*, *Halomonas*, *Acinetobacter*	> Decreased mucus secretion in the colon Particle accumulation in the colon > Hepatic lipid metabolism disorder (The authors speculate about the potential role of gut dysbiosis on the observed metabolic alterations in the host)	(Lu *et al.*, 2018b)
5 µm	Sphere	100 µg/L (1.456×10^{6} particles/L) 1 000 µg/L (1.456×10^{7} particles/L) in drinking water	> Mice ICR (male) > qPCR phyla-specific primers > 16S (V3-V4) rRNA gene sequencing	n = 8	6 weeks	qPCR (caecal content): ↓ 100 um: Firmicutes and β-Proteobacteria; 1 000 um: α-Proteobacteria, γ-Proteobacteria 16S rRNA gene (caecal content): ↓ Phyla: Actinobacteria Genus level: ↓ *Prevotella*, *Dehalobacterium*, *Turicibacter*, *Bifidobacterium*, *Phascolarctobacterium*, *Lachnospira*, *Haemophilus*, *Adlercreutzia*, *Megamonas*, *Blautia*, *Dialister*, *Veillonella* ↑ *Coprococcus*, *Anaeroplasma*	Intestinal barrier dysfunction (particle accumulation, decreased mucus secretion and expression ion transport-related proteins) and metabolic disorders (Arg and Tyr metabolism, bile acids) (The authors speculated about the potential role of gut dysbiosis on the observed metabolic alterations in the host)	(Jin *et al.*, 2019)
5 µm	Sphere	Dams: 100 and 1 000 µg/L MPs in drinking water	> Mice ICR (dams during gestation and lactation; and pups F1) > 16S (V3-V4) rRNA gene sequencing > Transcirptomics (liver)	Dams = 6 Pups F1 = 5	Dams: 6 weeks Pups F1: PND 42 PND 280 Pups F2: PND 42	Dams: ↑ Actinobacteria and Epsilonbacteraeota (high dose) ↑ *Oscillibacter*, *Helicobacter*, Ruminococcaceae_ UCG-003, *Anaerotruncus*, *Ruminiclostridium*_9, Lachnospiraceae_UCG-010, *Corynebacterium*_1, *Bacteroides*, and Ruminococcaceae_UCG-009 ↓ uncultured_bacterium_f_Muribaculaceae, unclasified_f_Peptostreptococcaceae, *Turicibacter*, and *Bifidobacterium* Pups F1 and F2: > Gut microbiota did not change significantly	> F0 lipid metabolic disorder and intestinal barrier dysfunction > F1 and F2: Risk of metabolic disorder after early exposure (The authors speculated about the potential role of gut dysbiosis on the observed metabolic alterations in the host)	(Luo *et al.*, 2019)

continues

PARTICLE SIZE	PARTICLE SHAPE	DOSE	MODEL AND METHOD	ANIMALS PER TREATMENT GROUP (N)	PERIOD	IMPACT ON GUT MICROBIOTA	HEALTH OUTCOMES	REFERENCES
0.5 and 50 µm	Sphere	100 and 1 000 µg/L (about 1.456×10^{10} particles/L for 0.5 µm and 1.456×10^{4} particles/L for 50 µm) in tank water	> Zebrafish adult male (AB strain) > 16S (V3-V4) rRNA gene sequencing	n= 4/5	14 days	↓ Phylum Bacteroidetes and β-Proteobacteria ↑ Phylum Firmicutes	> Dysfunction of gut barrier > Gut Inflammation	(Jin *et al.*, 2018)
5 and 50 µm	Sphere	100 and 1 000 µg/L in water In tank water	> Zebrafish larvae (AB strain) > 16S (V3-V4) rRNA Gene Sequencing	n= 5	7 days	All doses: ↓ γ-Proteobacteria 1 000 µg/L 5- and 50-µm: ↓ Bacteroidetes; ↑ Firmicutes	> Alteration of energy metabolism > Oxidative stress > Potential neurotoxicity	(Wan *et al.*, 2019)
Beads: 15 µm Fragments: 4-40 µm (peak at 15 µm) Fibres: diameter 15 µm, length: 20 to 100 µm	Spheres, fragments, fibres	10 µg/L microplastics: Beads and fragments: ~5 400 particles/L fibres: ~680 particles/ L	> Zebrafish adult (AB strain) > 16S (V3-V4) rRNA gene sequencing,	n= 16 (triplicates)	21 days	Shape dependent gut dysbiosis (fibres > fragments > beads): ↓ Actinobacteria, *Pseudomonas* and *Aeromonas*, ↑ Proteobacteria, *Gordonia*	Shape dependent effects (fibres > fragments > beads): Intestinal disturbances: mucosal damage, increased permeability, inflammation and metabolism disruption.	(Qiao *et al.*, 2019a)
5-µm	Sphere	50 µg/L (7.3×10^{2} particles/L) 500 µg/L (7.3×10^{3} particles/L)	> Zebrafish (*Danio rerio*) adult > 16S (V4) rRNA gene sequencing	n = 205 per group	21 days	Changes in α-diversity. Highest MP concentration: ↓ Proteobacteria ↑ Fusobacteria	Intestinal inflammation, oxidative stress and disruption of lipid metabolism.	(Qiao *et al.*, 2019b)
100 nm 5 µm 200 µm	Sphere	500 µg/L (1.1×10^{11} particles/L for 100 nm MP; 9.1×10^{5} particles/L for 5 µm MPs; 14 particles/L for 200 µm MPs)	> Zebrafish (*Danio rerio*) adult female and male > Metagenomics	n = 110 per group n = 9 to study gut microbiota	21 days	Increased the abundance of pathogenic bacteria: 100 nm MP induced more diverse changes ↑ *Aeromonas*, *Actinobacillus* and *Mycoplasma* 5 µm MPs: ↑ *Staphylococcus* 200 µm MPs: ↑ *Vibrio*, *Acinetobacter*, *Porphyromonas*, *Haemophilus*, *Neisseria* and *Lactococcus*	All particle sizes: dysfunction of intestinal immune cells Nanoparticles: higher effects on intestinal secretory cells	(Gu *et al.*, 2020b)
100 nm	Sphere	L: 10 items/L (5.50×10^{-12} mg/L), M: 10^{4} items/L (5.50×10^{-9} mg/L), H: 10^{6} items/L (5.50×10^{-7} mg/L)	> Large yellow croaker (*Larimichthys crocea)* juvenile > 16S (V3-V4) rRNA gene sequencing	n= 200 per treatment (triplicates) n = 3 to study gut microbiota	14 days	↑ Bacteroidetes (L + M), Firmicutes (L), *Lactobacillus* (L), *Parabacteroides* (L+M) and *Alistipes* (M) ↓ Proteobacteria (L+M)	Reduced intestinal immune and digestive activity Poor growth performance Total mortality increases proportional to nanoplastic concentration	(Gu *et al.*, 2020a)
32 to 40 µm	Sphere	100 and 1 000 µg/L	> Juvenile guppy (*Poecilia reticulata*) > 16S (V3-V4) rRNA gene sequencing	n = 20 per treatment (triplicates) n = 5 to study gut microbiota	28 days	↑ Proteobacteria (high dose), Rhizobiaceae ↓ Planctomycetes, Microbacteriaceae	Altered digestive activity and stimulation of the immune response	(Huang *et al.*, 2020)

continues

PARTICLE SIZE	PARTICLE SHAPE	DOSE	MODEL AND METHOD	ANIMALS PER TREATMENT GROUP (N)	PERIOD	IMPACT ON GUT MICROBIOTA	HEALTH OUTCOMES	REFERENCES
5 µm	Sphere	0, 0.04, 0.4, 4 and 40 mg/L (microbiota evaluated at 40 mg/L only)	> Chinese mitten crab (*Eriocheir sinensis*) juvenile females > 16S (V3-V4) rRNA gene sequencing	n = 6 per treatment (triplicates)	7, 14 and 21 days (microbiota studied only after 21 day exposure)	↓ Nitrospirae, Firmicutes, Bacteroidetes, *Dysgonomonas*, *Acinetobacter* ↑ Cyanobacteria, Chloroflexi, Fusobacteria, and Proteobacteria, *Pseudomonas*, *Rhodococcus*	Disturbed immune and enzymatic activity Altered gene expression of immunity factors	(Liu *et al.*, 2019)
50 nm amino-modified nanopoly-styrene	Sphere	10 µg/L	> Mussels (*Mytilus galloprovincialis* Lam.) > 16S (V4) rRNA gene sequencing	n = 5-6 (x5 replicates)	4 days	↓ *Mycoplasma*, *Shewanella*, *Tenacibaculum*, *Pseudoalteromonas* ↑ Arcobacter-like, *Psychrobium*, *Vibrio*	Alteration of innate immune response	(Auguste *et al.*, 2020)

Source: Authors' own elaboration.

TABLE AI.2 **SUMMARY ARTICLES REPORTING THE IMPACT OF POLYETHYLENE ON THE GUT MICROBIOME AND ITS EFFECTS ON THE HOST'S HEALTH**

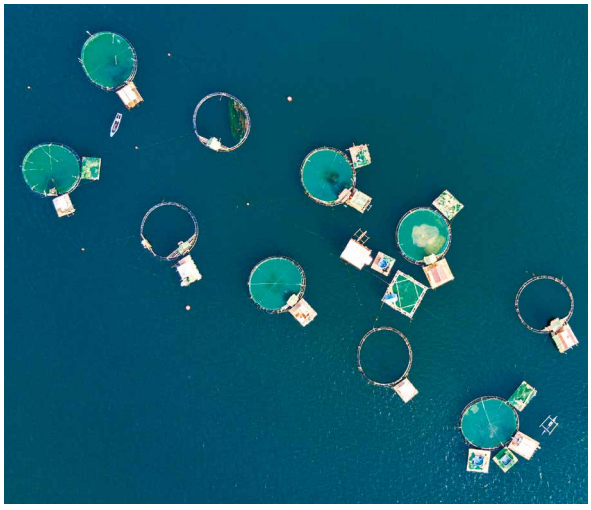
PARTICLE SIZE	PARTICLE SHAPE	DOSE	MODEL AND METHOD	ANIMALS PER TREATMENT GROUP (N)	PERIOD	IMPACT ON GUT MICROBIOTA	HEALTH OUTCOMES	REF.
10 to 150 µm	Spheres	6 (L), 60 (M), and 600 (H) µg/day in feed	> Mice C57BL/6 (male) > 16S (V4) rRNA gene sequencing	n = 20	5 weeks	↓ Bacteroidetes (M,H); *Parabacteroides* (L,M,H) ↑ Firmicutes (M,H), Melainabacteria (L,M,H); *Staphylococcus* (L,M,H), *Lactobacillus* (M) *Dubosiella* (M), *Blautia* (H), *Desulfovibrio* (H)	Intestinal inflammation	(Li *et al.*, 2020a)
45 to 53 µm	Spheres	100 mg/kg/day (~ 5.25×10^4 particles/day) Virgin and PAE contaminated (DBP, DEP, DMP and DEHP) By oral gavage	> Mice CD-1 adult 16S (V3-V4) rRNA gene sequencing	n = 6 per treatment	30 days	DEHP-contaminated MP and virgin MP: ↑ Actinobacteria, *Lactobacillus*, *Adlercreutzia*, *Butyricimonas*, *Parabacteroides* DEHP-contaminated MP: ↑ Paraprevotellaceae, Lachnospiraceae	Intestinal inflammation and metabolic disorders	(Deng *et al.*, 2020)
1 to 10 µm	Spheres	0.002 and 0.2 µg/g/day (~1.5 x 10^5 and 1.5 10^3 particles/g, approx.) In corn oil – oral gavage > microbiota structure tested only in high dose	> Mice ICR female adults > 16S (V3-V4) rRNA gene sequencing (faeces)	n = 12 per treatment	30 days	Highest dose: No changes in diversity ↓ Firmicutes ↑ Bacteroidetes	No histological and immunopathological changes in colon High dose: decreased mucin density, reduced mucus secretion	(Sun *et al.*, 2021)
10 to 45 µm	Spheres	20 mg/L	> Larval zebrafish (*Danio rerio*) > 16S (V4) rRNA gene sequencing	n = 20 per treatment (6 replicates)	4 and 10 days	↑ Bacteroidetes	Oxidative stress	(Kurchaba *et al.*, 2020)
Equal parts mix: 4 to 6 µm and 20 to 25 µm of high-density polyethylene	Sphere, weathered beads	0.2 mg/L (~1,170 MPs/mL) 20 mg/L (~117 000 MPs/mL)	> Blue mussels (*Mytilus edulis*) > 16S (V3-V4) rRNA gene sequencing	n = 40 per tank and 4 per microbiota sampling	1, 3 and 6 weeks, 2 and 8 day post-exposure	Weathered particles: ↑ Flavobacteriales, Chlamydiales ↓ Oceanospirillales	-	(Li *et al.*, 2020b)

MP: microplastic; PAE: phthalate esters; DBP: dibutyl phthalate, DEP: diethyl phthalate, DMP: dimethyl phthalate, DEHP: di-(2-ethylhexyl) phthalate
Source: Authors' own elaboration.

TABLE AI.3 **SUMMARY ARTICLES REPORTING THE IMPACT OF MISCELLANEOUS PLASTICS (NYLON AND PVC) ON THE GUT MICROBIOME AND THEIR EFFECTS ON THE HOST'S HEALTH**

PARTICLE SIZE	PARTICLE SHAPE	DOSE	MODEL AND METHOD	ANIMALS PER TREATMENT GROUP (N)	PERIOD	IMPACT ON GUT MICROBIOTA	HEALTH OUTCOMES	REFERENCES
Nylon Heterogeneous fragments < 50 µm (mean size 13–19 µm)	Not mentioned (powder form)	1% ~ 10 g/kg Mix polybrominated diphenyl ethers (PBDE: 47, 99, 100, 153 and PBB-153) at 3 000, 1 500, 750, 375, 188, 94 ng/g	> Snail (*Lymnaea stagnalis*) Adult > 16S (V3-V4) rRNA gene sequencing	n = 6	4 days	Diversity and composition of the snail microbiome was not significantly altered in the presence of MP. Highest concentration of PBDE: ↑ Enterobacteriaceae ↓ Flavobacteriaceae	-	(Horton *et al.*, 2020)
PVC 70 to 150 µm	Not mentioned (powder form)	Larvae fed only with PVC MP	> Mealworm beetle (*Tenebrio molitor*) larvae > 16S (V3-V4) rRNA gene sequencing	n= 60	16 days	↑ Streptococcaceae (mostly *Lactococcus*), Spiroplasmataceae (mostly *Spiroplasma*), Enterobacteriaceae, Clostridiaceae	-	(Peng *et al.*, 2020)

Source: Authors' own elaboration.



THE IMPACT OF MICROPLASTICS ON THE GUT MICROBIOME AND 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 microplastics on the gut microbiome and potential health implications. The main aspects analysed are (1) the effects of microplastics on the composition, diversity and function of gut microbiome using *in vivo* or *in vitro* models; (2) health implications resulting from the microplastic–microbiome interactions and underlying mechanisms; (3) the establishment of causality; and (4) influence of the gut microbiome on microplastic biodegradation. 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–microplastic interactions. The outcomes will provide information which can be used to improve food safety policies.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO)

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