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Effects of single and combined toxic exposures on the gut microbiome: current knowledge and future directions

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Abstract

Human populations are chronically exposed to mixtures of toxic chemicals. Predicting the health effects of these mixtures require a large amount of information on the mode of action of their components. Xenobiotic metabolism by bacteria inhabiting the gastrointestinal tract has a major influence on human health. Our review aims to explore the literature for studies looking to characterize the different modes of action and outcomes of major chemical pollutants, and some components of cosmetics and food additives, on gut microbial communities in order to facilitate an estimation of their potential mixture effects.

We identified good evidence that exposure to heavy metals, pesticides, nanoparticles, polycyclic aromatic hydrocarbons, dioxins, furans, polychlorinated biphenyls, and non-caloric artificial sweeteners affect the gut microbiome and which is associated with the development of metabolic, malignant, inflammatory, or immune diseases.

Answering the question ‘Who is there?’ is not sufficient to define the mode of action of a toxicant in predictive modeling of mixture effects. Therefore, we recommend that new studies focus to simulate real-life exposure to diverse chemicals (toxicants, cosmetic/food additives), including as mixtures, and which combine metagenomics, metatranscriptomics and metabolomic analytical methods achieving in that way a comprehensive evaluation of effects on human health.

Abbreviations


Keywords

Gut microbiota, chemical mixtures, environmental pollutants, toxicity, host health
Highlights:

• Heavy metals, pesticides, nanoparticles, polycyclic aromatic hydrocarbons, dioxins, furans, polychlorinated biphenyls and non-caloric artificial sweeteners affect the gut microbiome

• Metabolic, malignant, inflammatory and immune diseases are associated with the gut microbiome

• More studies are needed to simulate real-life exposure scenarios to diverse substances, including as mixtures
1. Introduction

The human gut, apart from its major role as regulator of host immunity and digestion of dietary nutrients, also accommodates a vast quantity of microorganisms (bacteria, viruses, fungi and protozoa) collectively known as the gut microbiota. The human intestinal microbiome is a dynamic community of great diversity, consisting of 500-1000 varieties of bacterial species, which constitute approximately 1800 different genera (Clemente et al., 2012; Qin et al., 2010). The overall genome of gut microbiota holds more than 3.3 million genes, 150 times higher than the human genome (Qin et al., 2010). Thus, the gut microbiome is currently considered as a discrete “organ” within the human body, which inevitably interacts with and determines our physiology (Forsythe and Kunze, 2013). The majority of these bacterial species mainly belong to the *Firmicutes* and *Bacteroidetes* phyla, which are the most dominant (up to 90% depending on the individual), followed by *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia* (Young, 2012). Although the human gut is not well populated at birth, bacterial inoculation begins soon post-partum, then utterly changes and stabilizes near adolescence remaining relatively intact until adulthood (Clemente et al., 2012). Early vigorous microbial modulation occurs due to maturation of immunity and dietary complexity, in addition to several factors being either innate (genotype) or exterior (lifestyle, dietary habits, exposure to xenobiotics). With numerous different interactions, the gut microbiota finally differentiates into a unique formation for each individual host, resembling a “bacterial fingerprint” (Goodrich et al., 2014; Yatsunenko et al., 2012). Recently, the critical role of gut microbiota in maintaining health has been generally appreciated and studied.

The composition and function of the gut microbiome is influenced by host genetics, and some taxa are even heritable. Twin studies have revealed that a total of 20% of the variation in abundance for ~10% of taxa was explained by inherited genetic variants (Rothschild et al., 2018). The presence of bacteria from the hereditable *Christensenellaceae* family has been associated with low body mass index (BMI), and its causative role in weight gain has further been confirmed by faecal transplants of the *Christensenellaceae* family to germ-free mice (Goodrich et al., 2014). On the other hand, studies on laboratory animals have suggested that diet dominates over genetics in determining gut microbiome composition (Carmody et al., 2015). The human gut microbiome is also highly dynamic: it can fluctuate according to diet within a few hours (David et al., 2014). However, little is known about the different factors explaining variations in gut microbiome composition. Only 10%–15% of gut microbiome variation between individuals can be explained by current knowledge (Schmidt et al., 2018). We hypothesize in this review that environmental
exposures to mixtures of chemicals can affect the gut microbiome, and that these effects have been underestimated.

Human populations are exposed to mixtures of environmental pollutants, including heavy metals, pesticides, nanoparticles (e.g., as components of cosmetics), persistent organic pollutants (POPs), and non-caloric artificial sweeteners (NAS) (Chowdhury et al., 2016; Jurewicz et al., 2013). Since these chemicals are omnipresent contaminants, humans are vulnerable to their exposure from various routes. Exposure to pollutants can promote the development of various diseases such as obesity, type 2 diabetes, metabolic disorders, and cancer (Casals-Casas and Desvergne, 2011). These pollutants have long-term effects. They can act sequentially or simultaneously as a mixture. However, the interaction between mixtures of environmental pollutants and the gut microbiota and whether it could be a cause of disease is not well elucidated (Mesnage et al., 2018). Although reviews on gut microbiome metabolism of xenobiotics have been published (Claus et al., 2016; Koppel et al., 2017), the subject has not been extensively studied and no reviews have considered the potential effects of chemical mixtures. Hence, this review will summarize the available literature and studies to show how gut microbiota are affected or altered in response to mixtures of pollutants, as well as adverse effects on human physiology.

2. The impact of the gut microbiome on host health

The gut microbiome is a metabolic “organ” participating in numerous host functions critical to maintain homeostasis. Gut microbiota influence the formation and structure of the intestinal mucus layer, influencing intestinal wall integrity (Jakobsson et al., 2015). It also controls energy metabolism, fermentation of dietary fibers and carbohydrates, digestion of proteins and peptides, biosynthesis of amino acids and vitamins, and provides defense against various pathogens (Sommer and Backhed, 2013; Spanogiannopoulos et al., 2016). Gut microbiota take part in bile acid metabolism, due to their capability to convert primary bile acids (initially produced in the liver) into secondary bile acids. Therefore, alterations in the population of gut microbiota could also change bile acid and energy metabolic pathways.

Another major function of the gut microbiota is the production of short-chain fatty acids (SCFAs) such as butyrate, propionate and acetate. SCFAs, especially butyrate, mainly act as an energy source for enterocytes and can interface with gluconeogenesis, energy metabolism, neuronal function, and intestinal immunity (Koh et al., 2016). SCFAs also possess an immunomodulatory capability (Neish, 2009), mediating anti-inflammatory processes (Borges-Canha et al., 2015). These properties of SCFAs contribute to a reduction in the risk of colorectal cancer (Koliarakis et al., 2018). Host immunity is modulated by gut microbiota: bacterial metabolites can interfere with either the adaptive or the innate immune
systems (Honda and Littman, 2016), regulate the proliferation and production of T cells (Ubeda and Pamer, 2012), and form the myeloid landscape (Thaiss et al., 2016).

The gut-brain axis is a major aspect of the gut microbiome. Shifts in the population of gut microbiota can directly affect neuronal function (Dantzer, 2009). The gut microbiome possesses a bidirectional communicative potency with the brain via vagus nerve activation (Wang et al., 2002). Moreover, the gut microbiota can control tryptophan metabolism and thus the bioavailability and synthesis of serotonin (Desbonnet et al., 2008). The secretion of gut peptides from enteroendocrine cells, which constitutes a major hormonal signaling pathway between the gut and brain, is modulated by the gut microbiome (Schele et al., 2013).

Structural shifts in microbiota populations, known as gut dysbiosis, have been associated with the development of various systemic or gut disorders. We previously reviewed the role of gut dysbiosis as a key component in the pathogenesis of colorectal cancer (Koliarakis et al., 2018). These diseases can also be caused by an exposure to environmental pollutants. Gut dysbiosis induced by early-life exposure to such chemicals has led to an increased prevalence of asthma and food allergies due to abnormal immunity (Russell and Finlay, 2012). Furthermore, gut bacteria could influence diabetes and obesity by changing the absorption, metabolism, or excretion of these contaminants (Snedeker and Hay, 2012).

Indeed, it has been reported that the gut microbiota promote the biotransformation of chemicals and other xenobiotics performing a plethora of reactions, mainly reduction, lyase reactions, functional group transfer, hydrolysis, and enzymatic transformation (Koppel et al., 2017). Approximately, 1369 environmental pollutants have been found to be metabolized by the human gut microbiome (Gao et al., 2010). Such compounds interact with the gut bacteria through numerous ways. Poorly absorbed pollutants reach ileum or cecum, or partition across the intestinal epithelium and therefore directly metabolized by the gut microbiome. Gut microbiota also presents the ability to deconjugate chemicals that have already undergone hepatic conjugation with glucuronic acid, reducing their polarity and molecular size, thus enabling their reabsorption, either in their original form, or as new toxic molecules. Furthermore, environmental xenobiotics may perturb the composition of gut microbiota resulting in gut dysbiosis, or can interact with the metabolic capacity of the gut microbiota, affecting the production and toxicity of bacterial metabolites. The large arsenal of enzymes of the gut bacteria participating in the aforementioned processes includes sulfatases, nitroreductases, b-lyases, azoreductases, and b-glucoronidases (Claus et al., 2016).

3. Chemical mixtures and the prediction of their effects
The study of toxic effects of single compounds provides reliable insight on health effects experienced after acute or chronic exposure (in case of intoxication or occupational exposures). However, human populations are always exposed to mixtures of chemicals. This is particularly true for chronic exposures at low environmental concentrations. Bisphenols, phthalates, pesticides, dioxins, furans, PCBs, brominated flame retardants, perfluorinated compounds and heavy metals were found in the blood of all pregnant women who gave birth in France in 2011 (Dereumeaux et al., 2016). Predicting the effects of these mixtures from the effects of single compounds poses a major challenge.

Some studies have suggested that chemical mixtures have toxic effects at regulatory admissible levels, which are not predictable from the effects of single compounds (Docea et al., 2018; Lukowicz et al., 2018). Strategies to study chemical interactions in mixtures have been proposed by the scientific committees of the European Union in 2001 (SCHER et al., 2012). They suggested a tiered approach in which both exposure and hazard is considered to model the risk arising from an exposure to a mixture. Chemicals can be grouped based on structural similarities or on their mode of action. Ultimately, an index can be calculated from regulatory guidance values (such as the acceptable daily intake) to estimate the risk arising from an exposure. Several research programs (EDC-MixRisk, EuroMix, EUToxRisk, HBM4EU and SOLUTIONS) have subsequently been initiated to address this issue (Bopp et al., 2018).

The range of approaches proposed are very limited for certain endpoints, which have been poorly studied including effects on the gut microbiome. For these reasons, other authors have proposed strategies to simulate real-life exposures in laboratory animals and directly estimate toxic effects (Tsatsakis et al., 2019; Tsatsakis et al., 2017). The aim of our review is to scrutinize the available literature in an effort to identify the different mode of action and effects of major chemical pollutants, and some cosmetic/food additives in order to facilitate an estimation of their mixture effects.

4. Heavy metals

Currently, heavy metals are considered a major factor of modern environmental pollution with significant health consequences (Liu et al., 2016). Heavy metal exposure has been linked to various toxicity mechanisms, such as oxidative damage, DNA breakdown, aberrant immunity, and tumorigenesis (Bajaj et al., 2013; Yu et al., 2016). Metabolism of heavy metals is strongly dependent on their chemical form, thus affecting their tissue distribution and bioactivity.
4.1 Arsenic

Arsenic (As), is an omnipresent environmental toxic contaminant present in inorganic (iAs) or organic (oAs) forms (Bhattacharya et al., 2007). Human exposure is mainly through dietary uptake from contaminated seafood, including fish and shellfish, with high levels of As (> 10 mg/L) also being detected in some water sources in the USA (Kozul et al., 2009). Arsenic exposure has been correlated with lung, liver, kidney, and bladder tumors (Hughes et al., 2011), since As is known for its tumorigenic properties (Wang and Mulligan, 2008). Inorganic arsenic is primarily ingested in the form of arsenate (iAsV) (Alava et al., 2012), and it is then reduced to arsinite (iAsIII) by the cecal microflora (Rowland and Davies, 1981). Subsequently, numerous methylation processes by gut microbiota participate in iAs metabolism, leading to synthesis of dimethylarsinic acid (DMAsV), the main excreted form of iAs (D C Rubin et al., 2014). Many secondary methylated compounds are produced during this detoxification process such as monomethylarsonous acid (MMAsIII), monomethylarsonic acid (MMAsV), and monomethylmonothioarsonic acid (MMMTAsV) (D C Rubin et al., 2014; Diaz-Bone et al., 2009; Van de Wiele et al., 2010). Furthermore, human colonic microbiota is able to methylate oAs in contaminated soil, as demonstrated in vitro (Van de Wiele et al., 2010). The toxicological importance of microbiota-mediated metabolism of As is not clear and more research is needed.

Lu et al. investigated C57BL/6 mice gut microbiota after 10 ppm As treatment for 4 weeks (Lu et al., 2014a). Arsenic exposure resulted in altered intestinal microbiota composition compared to controls, with significantly decreased abundance in some Firmicute (Clostridiales, Catabacteriaceae, Clostridiaceae) and Tenericute (Erysipelotrichaceae) members. This caused changes in the metabolism of indole-containing (indolelactic acid), isoflavone (daidzein), and bile acid derivatives. The same As exposure scheme increased Bacteroidetes and Verrucomicrobia, and decreased Firmicute (Catabacteriaceae, Clostridiaceae, Ruminococcaceae, Lachnospiraceae) and Tenericute (Erysipelotrichaceae) populations in interleukin(IL)-10 knockout mice (Lu et al., 2014b). These gut microbiota alterations were associated with significantly decreased DMAsV, as well as increased MMAsV and iAsV, in urine, indicating an impaired detoxification of iAs by the gut microbiome. Thus, changes in gut microbiota profiles markedly affect As biometabolism. Guo et al. (Guo et al., 2014) exposed ICR mice to a 90-day treatment with As (3 mg/L), iron (Fe) (5 mg/L), or both in order to study their effects on gut microbiota. The abundance of the Firmicutes, Proteobacteria and Tenericutes phyla increased, whereas a decrease was noted in Bacteroidetes, after metal exposure. Verrucomicrobia increased either in Fe alone or in both metal groups, while Acidobacteria and Cyanobacteria were elevated in As group. Firmicutes and Lactobacillus spp. decreased in mice exposed to As or Fe alone, but increased when metals were combined. Exposure to As or Fe diminished Bacteroides and Barnesiella genera.
The above alterations in microbial populations suggest an antagonistic effect of metal co-exposure on microbiota. The changes in gut microbiota composition were also associated with metagenomic alterations concerning carbohydrate, inorganic ion, and amino acid metabolism and transport (Guo et al., 2014).

Dheer et al. exposed C57BL/6 mice to chronic As treatment, consisting of 0, 10, or 250 ppb iAsIII, for 2, 5, or 10 weeks, and examined the effects on colonic microbiota composition and host metabolic capability (Dheer et al., 2015). The results demonstrated an increase in Bacteroidetes, which was mainly attributed to changes in the Ruminococcaceae family. On the other hand, Firmicutes decreased, mainly due to alterations in the Porphyromonadaceae and Lachnospiraceae families. Interestingly, these effects were dose and time-dependent.

Disturbance in the microbial biofilm adjacent to the colonic mucosa was responsible for the changes in microbiota composition. Elevated nitrate and nitrite levels in the colon, possibly correlating with upregulation of nitrite reductase in Bacteroides thetaiotaomicron, as well as increased toxic arginine derivatives, suggested a stimulation of nitrogen and amino acid host metabolism as a result of As exposure. Chi et al. (Chi et al., 2016), using the same As exposure scheme as Lu et al. (Lu et al., 2014a) in C57BL/6 mice, observed a greater β-diversity in gut microbiota of female mice versus male mice. Dorea spp. presented a diminished and increased abundance in female and male mice. Akkermansia spp. abundance was increased in female but remained unchanged in male animals. These alterations were associated with cell transport systems and metal resistance (such as zinc and mercury) in female, and sulfate, nitrogen, and carbon metabolism in male mice. Overall, it indicates that a sex-driven pattern underlies structural and functional alterations in gut microbiota as a result of As exposure. A more recent study by the same authors (Chi et al., 2017) further elucidated As-related effects. For this purpose, C57BL/6 mice were administered with iAsIII via gavage (100 ppb) for 13 weeks. As-treated mice presented decreased richness and diversity in the gut microbiome. A significantly lower number of Firmicutes (Coprococcus, Dorea, Lactococcus, Oscillospira, Ruminococcus) and a higher abundance of Akkermansia, Anaerostipes, and Bifidobacterium, was detected. Indeed, As-exposure enhanced genes involved in vitamin production (B6, B12, K2, folate, thiamin), lipopolysaccharide (LPS) synthesis, metal resistance (Cadmium, Zinc, and Cobalt), oxidative stress, and DNA repair mechanisms. The data obtained suggested a correlation between increased vitamin synthesis and elevated Bifidobacterium members, being the major source of vitamins in the gut (LeBlanc et al., 2013). On the other hand, there were perturbations in carbohydrate and pyruvate metabolism and a decreased SCFA biosynthesis. The reduced numbers of Firmicutes, such as Coprococcus, can be linked to a disturbance in SCFA production (Nicholson et al., 2012).

4.2 Lead
Lead (Pb) is another heavy metal, involved in large, major instances of environmental pollution, such as the Flint Water Crisis in Michigan in April 2014 (Heard-Garris et al., 2017; Mortada et al., 2015; Nelson, 2016). Pb is an omnipresent pollutant in soil, water, and air, but also in numerous consumer products such as old paint cans and dietary products. The use of tetraethyl-Pb as an additive in gasoline was also a source of exposure to this heavy metal for several decades although this practice has largely been discontinued. Consumption of marine products are also sources of exposure to Pb, with 0.3 mg/kg as the maximum Pb concentration allowed in fish in EU (Garcia-Barrera et al., 2012). A concentration of 5 μg/dl has been considered as the minimum hazardous blood level for Pb by the Center for Disease Control and Prevention (USA) (Betts, 2012), although lower concentrations may induce toxic effects (Gilbert and Weiss, 2006). Pb exposure causes central nervous system disorders, liver toxicity, inflammation, obesity via disturbance of metabolism, and gut microbiome dysbiosis (Breton et al., 2016; Breton et al., 2013a; S. Zhang et al., 2015).

Wu et al. (J. Wu et al., 2016) exposed the offspring of A^vy^-strain non-agouti (a/a) mice to Pb during gestation as well as lactation periods, through maternal drinking water (32 ppm) for a total of 40 weeks. Significant shifts were observed in the Firmicutes and Bacteroidetes phyla, with the former being more abundant than the latter. Maternal Pb exposure was associated with elevated populations of Barnesiella, Clostridium cluster XIVb, and Desulfovibrioaceae, while members of Enterorhabdus, Caulobacterales, and Lactococcus decreased. These alterations were also detected at the genus level, with diminished Akkermansia spp. and enriched Desulfovibrio spp. abundance in exposed mice. Since Akkermansia members maintain the homeostasis of the intestinal mucus layer, whereas Desulfovibrio members are trimethylamine-producing bacteria, such changes could influence the development of obesity, cardiovascular diseases, inflammatory conditions, and even colorectal cancer (Bae et al., 2014; Cani and Everard, 2014; Lukovac et al., 2014). In addition, sex-dependent alterations in body weights were correlated with the above shifts in gut microbiota composition. In another study by Gao et al. (Gao et al., 2017), C57BL/6 mice were treated with Pb chloride (10 ppm equivalent to 2 mg/kg BW per day) and fecal samples were obtained for analysis pre-, and at 4 and 13 weeks post-treatment. Gut microbiota phylogenetic diversity was inhibited in treated mice compared to controls. Specifically, Clostridiales, Blautia spp., Ruminococcus spp., Ruminococcaceae, Lachnospiraceae, and Oscillospira spp. populations diminished following Pb exposure. Moreover, Pb reduced synthesis of bile acids, vitamin E, and cholesterol, enhanced nitric oxide generation, energy deprivation, oxidative stress induction, and activation of defensive microbial mechanisms.

In a recent study (Xia et al., 2018), oral gavage of Pb (0.01, 0.03, or 0.1 mg/L) was administered to ICR mice for 15 weeks. Quantitative PCR and 16S rRNA gene amplicon sequencing analysis of cecal content and feces showed that Pb treatment perturbed the structure, abundance, and diversity of the microbiome. Bacteroidetes and Proteobacteria
increased, while *Firmicutes* decreased in response to Pb exposure. The known opportunistic pathogens *Parabacteroides* were also elevated. Alterations in SCFA-producing bacteria, including *Bacteroides*, *Oscillospira*, and *Ruminococcus* were also noted. Additionally, elevated levels of triglyceride (TG) and pyruvate in the liver, as well as changes in numerous intestinal metabolites (including glutamate, isobutyrate, alanine, and glycine among others), suggest a Pb-induced hepatic and gut metabolic disorder (Wang et al., 2016; Zmora et al., 2017).

### 4.3 Cadmium

Cadmium (Cd) is also a ubiquitous toxic metal with a great variety of sources, including numerous industrial products such as batteries, paint, electroplating, fertilizers and plastics (Alghasham et al., 2013). The daily intake of Cd in populations of developing countries such as China and Bangladesh is over the permitted level set by the European Food Safety Authority (2.5 μg/kg bw/day) (Al-Rmalli et al., 2012; Yuan et al., 2014; Zhang et al., 2014). Cadmium-related toxicity has been associated with cardiovascular diseases, hepatotoxicity, renal dysfunction, oxidative stress, osteoporosis, aberrant immunity, and tumorigenesis (Bajaj et al., 2013; Yuanxiang Jin et al., 2016; Ke et al., 2015; Liu et al., 2015; Solenkova et al., 2014).

In a study by Fazeli et al. (Fazeli et al., 2011), BALB/c mice received Cd chloride treatment (23 – 50 mg/kg) for a 45-day period. A sharp reduction of all bacterial species was detected in the fluid contents and biopsy samples from all intestinal regions after exposure. A greater Cd-mediated toxic profile was observed in the small intestine than in the colon and rectum, indicating a regional preference of Cd toxicity in the gut. Additionally, Gram(+) bacteria (*Bacillus cereus*, *Enterococcus* spp.) were more sensitive to Cd-related effects than Gram(-) bacteria (*Escherichiacoli*, *Klebsiella* spp.), possibly due a varied capability for metal ion uptake (Morozzi et al., 1986).

In another study by Liu et al. (Liu et al., 2014), BALB/c mice were administered with Cd chloride via gavage (20 and 100 mg/kg) for 3 weeks. The overall growth rate and abundance of gut microbiota greatly decreased after exposure. Specifically, the *Firmicutes*: *Bacteroidetes* ratio significantly decreased and the abundance of *Bifidobacteria* and *Lactobacilli* was suppressed. Moreover, disturbance of the gut barrier, elevated levels of tumor necrosis factor alpha (TNF-α), and reduced levels of SCFAs were observed in the colon, possibly reflecting the suppression of commensal bacteria as a result of Cd-mediated gut dysbiosis. Zhang et al. (S. Zhang et al., 2015) exposed mice to 10mg/L of Cd chloride for 10 weeks in order to assess the effects of subchronic low-dose Cd on gut microbiota. Microbial abundance assessed by quantitative PCR and 16S rRNA gene amplicon sequencing analysis in the cecum and feces, revealed that Cd exposure elevated *Bacteroidetes* members
(Bacteroidaceae, Paraprevotellaceae) and decreased Firmicutes (Lachnospiraceae, Ruminococcaceae, Clostridiaceae, Streptococcaceae) and γ-Proteobacteria populations. *Bifidobacterium longum* was also significantly decreased. These bacterial alterations were linked to an increased LPS production, which could be linked to the development of chronic liver disease such as cirrhosis (Giannelli et al., 2014; Rai et al., 2015; Xu et al., 2012).

In a more recent study, C57BL/6J mice were administered with a low-dose of Cd (100 nM) in early life, in order to assess the long-term effects on gut microbiota and metabolism in adulthood (Ba et al., 2017). Early Cd treatment enhanced adiposity (12 – 16 weeks) and induced hepatic lipid metabolism dysfunction with increased levels of serum TG, free fatty acids, and hepatic TG, especially in male compared to female mice. Cd-mediated gut microbiota alterations at 8 weeks, reflected by a decreased abundance of Firmicutes, in contrast to Bacteroidetes, and lower numbers of *Bifidobacterium* and *Prevotella*. This indicated that male gut microbiome were mostly sensitive to early Cd exposure, a fact that might be linked to fat accumulation and metabolic disorders in latter stages of life (Cox et al., 2014; Rodriguez et al., 2015). These sex-specific observations were also confirmed by fecal microflora transplant in germ-free mice.

### 4.4 Heavy metal mixtures

The exposure to heavy metals is ubiquitous and populations are more exposed to mixtures than single compounds. Consequently, testing of heavy metal mixtures constitutes a more relevant strategy for study designs trying to reflect real life exposure scenarios. Although no study has tested possible combined effects of heavy metals on the gut microbiome, a number of have focused on comparing the effects of different metal exposures on the gut microbiota within the same investigation. Studies have suggested that the toxicity of heavy metal mixtures on other systems can be explained using the concentration addition and independent addition models (X. Wu et al., 2016). Breton et al. (Breton et al., 2013b) treated BALB/c mice with Cd chloride (20 or 100 ppm) or Pb chloride (100 or 500 ppm) for 8 weeks. At the family level, the abundance of *Lachnospiraceae* decreased, while bacteria belonging to *Lactobacillaceae* and *Erysipelotrichaceae* (especially *Turicibacter* spp.) were increased, compared to controls. Interestingly, *Lachnospiraceae* reduction has been associated with gut inflammation (Lepage et al., 2011), and high numbers of *Turicibacter* have been indicative of an anti-inflammatory response (Presley et al., 2010). Thus, heavy metal exposure might promote bidirectional adaptive responses by gut microbiota in its susceptibility to inflammation (Breton et al., 2016).

Other heavy metals are less studied. However, in a recent study, C57BL/6 mice were orally administered with either Cd (100 mg/L), or Pb (1.83 g/L), or Copper (Cu) (1 g/L), or Aluminum...
(Al) (1.8 g/L) for 8 weeks (Zhai et al., 2017). Exposure to any metal significantly disturbed the abundance of Tenericutes, Actinobacteria, Proteobacteria, and Verrucomicrobia (Zhai et al., 2017). Cd-treated mice displayed elevated populations of Alistipes and Odoribacter and lower numbers of Mollicutes and Ruminococcaceae, whereas Pb exposure reduced the members of Anaerotruncus, Lachnoclostridium, Lachnospiraceae, Oscillibacter, Rikenellaceae, Ruminiclostridium, and Ruminococcaceae. Cu and Al have also been reported to cause toxic phenomena in humans and animals, although their impact on gut microbiota has received limited interest in contrast to Cd or Pb (Gaetke and Chow, 2003; Ward et al., 2001). Cu treatment resulted in a notable reduction in gut bacterial diversity compared to other metals. Cu perturbed the abundance of Alistipes, Allobaculum, Bacteroides, Mollicutes, Rikenellaceae, Ruminococcaceae, and Turicibacter (Zhai et al., 2017). Al treatment reduced the abundance of Anaerotruncus and elevated that of Odoribacter. In another recent study (Zhang et al., 2017), high levels of dietary Cu (120 and 240 mg/kg) significantly affected the diversity and composition of gut bacterial community in Sprague-Dawley rats, a fact that was associated with increased Cu accumulation in the rat ileum and colon. Moreover, alterations in Firmicutes genera including Ruminococcaceae, Defluviitaleaceae, Ruminococcaceae, Peptococcus, and mainly Anaerotruncus, were positively correlated with increased serum TNF-α, indicating that Cu-mediated changes in gut microbiota could promote an inflammatory response. In addition, it has been reported (Lerner et al., 2006) that Al exposure suppressed bacterial growth, induced microbiota dysbiosis, promoted bacterial translocation in mesenteric lymph nodes, impaired junctions in colonic epithelium, and stimulated inflammatory cytokine secretion, presenting a strong association with IBD. Interestingly, the abundance of Akkermansia was greatly reduced after long-term exposure to any metal (Zhai et al., 2017). All these findings suggest that heavy metal-induced changes in gut microbiota could contribute to the pathogenesis of various gut disorders, mainly inflammatory intestinal diseases and colorectal cancer (Jones-Hall et al., 2015; Png et al., 2010; Wang et al., 2012; Wu et al., 2013).

Combined effects of heavy metals with other compounds should also be explored. Ethanol has the ability to potentiate the toxicity of Cd on regional brain biogenic amine levels in rats. This suggests that industrial workers consuming alcohol could be more susceptible to Cd-induced toxicity (Flora and Tandon, 1987). Exposure to Cd could also enhance the accumulation of Pb (Mejia et al., 1997). A large number of studies describe mixture effects arising from the combination of heavy metals with pesticides such as dimethoate (with As, Cd, Pb, and Mercury) or CPF (with Nickel) (Institoris et al., 2001; Singh et al., 2017). Moreover, heavy metals metals including As, Pb, Chromium, Nickel and Cobalt have been found in commercial pesticide formulations, which indicate that co-exposure to these compounds is a likely scenario (Defarge et al., 2018).

5. Pesticides
Pesticides are chemical mixtures used for pest control in agricultural and domestic settings (Mostafalou and Abdollahi, 2017). A great variety of active ingredients is available such as organochlorides, organophosphates, and carbamates. Pesticides can be further classified as herbicides, insecticides, fungicides, or rodenticides depending on the target organism (Gilden et al., 2010). Their widespread use constitutes a major environmental issue because their residues accumulate in air, water, soil, and the food chain, with the latter being the main route of human exposure (Jin et al., 2015). Pesticide exposure has been linked to the development of cancer, neurodegenerative diseases, asthma, infertility, birth defects, autism, diabetes, and obesity (Mostafalou and Abdollahi, 2017). The antimicrobial capability of some pesticides implies that they may interfere with gut microbiota, and alter its metabolic function.

5.1 Organochlorine pesticides

Organochlorine pesticides constitute major environmental pollutants routinely detected in humans although the use of most has been banned in the majority of developed countries since the 1970s (Maisano et al., 2016; Vincetti et al., 2017; Michalakis et al., 2014). One of the most well-known organochlorine pesticides is the insecticide dichlorodiphenyl-trichloroethane (DDT) (Salihovic et al., 2016). Exposure to DDT has been linked to various health issues including hepatic, breast, and testicular tumors (Mrema et al., 2013), reproductive disorders (Freire et al., 2011), immunotoxicity (Mrema et al., 2013), neurotoxicity (Saeedi Saravi and Dehpour, 2016), and metabolic disease (Casals-Casas and Desvergne, 2011). DDT can be metabolized to dichlorodiphenyl-dichlorophenylethane (DDD) by rat gut microbiota (Mendel and Walton, 1966). There is also evidence suggesting metabolism of DDT to DDD by members of human gut microbiota (e.g. Eubacterium limosum) (Yim et al., 2008). Both DDT and DDD display endocrine disrupting activity presenting estrogenic and antiandrogenic properties in various tissues (Kim et al., 2014). Thus, it is not yet clear whether this metabolism contributes to detoxification or bioactivation of these compounds. Dichlorodiphenyl-dichloroethylene (DDE) is another major by-product of DDT metabolism through dehydrohalogenation. In a recent study of gut microbiota and bile acid metabolism, C57BL/6 mice were treated by oral gavage of DDE (1 mg/kg bw/day) for an 8-week period (Liu et al., 2017). The relative abundance of Firmicutes and Proteobacteria increased, whereas that of Bacteroidetes, Verrucomicrobia, and Actinobacteria was reduced. In addition, DDE altered bile acid composition and hydrophobicity, as determined by an increased cholic acid:β-muricholic acid ratio and elevated levels of the major secondary bile acids, such as deoxycholic acid and lithocholic acid. Furthermore, DDE exposure led to suppression of linked ileal absorption of bile acids, and stimulation of gene expression regarding hepatic synthesis of bile acids. Since gut microbiota are major determinants of bile acid metabolism
(Sayin et al., 2013), these microflora alterations were linked to the DDE-mediated changes in bile acid metabolism, possibly due to enhanced bacterial bile salt hydrolase activity.

5.2 Glyphosate

Glyphosate (GLP) is the stated active component of Roundup® (RU), the most broadly used GLP-based herbicide (GBH) worldwide (Benbrook, 2016). Agricultural use of GBH has massively increased in recent years through the introduction of GLP-resistant genetically modified (GM) crops (e.g., soy, maize, canola, sugar beet, cotton) and in pre-harvest desiccation processes in some crops especially cereals such as oats and wheat (Benbrook, 2016; Myers et al., 2016). This has led to a greatly enhanced dietary exposure to GLP and its metabolites (Toledo et al., 2014). It is believed that GLP primarily exerts its herbicidal activity through inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme of the shikimate pathway responsible for the biosynthesis of aromatic amino acids in plants (Boocock and Coggins, 1983). Since this pathway is absent in vertebrates, it is generally considered that GLP does not induce unfavorable health issues in higher animals and humans. However, studies have reported GLP- or GBH-associated adverse effects in mammals through various mechanisms (Mesnage and Antoniou, 2017a; Mesnage et al., 2015; Tarazona et al., 2017). This also includes the possibility of either GLP or GBH to induce microbiome dysbiosis since the shikimate pathway is present in some gut microbiota species (Moco et al., 2012). Indeed, GLP has been patented as an antibiotic with its EPSPS inhibitory capability being cited as the mechanism of action (US patent number US7771736B2). The first evidence that GLP could cause gut microbiome dysbiosis came from in vitro studies simulating the digestive system of agricultural poultry (Shehata et al., 2013) and bovine (Ackermann et al., 2015) species. Shehata and colleagues (Shehata et al., 2013) cultured pathogenic and beneficial bacteria representative of the poultry microbiome in the presence of a RU GLP-based herbicide formulation at increasing GLP equivalent concentrations (0.075, 0.15, 0.30, 0.60, 1.20, 2.40, 5.0 mg/ml). It was generally found that pathogenic bacteria (C. botulinum type A and B, C. perfringens, Salmonella Gallinarum, Salmonella Typhimurium, Salmonella Enteritidis, Escherichia coli) were highly resistant to RU (MIC value 5 mg/ml GLP). In addition, L. casei, L. buchneri, L. harbinensis, Staphylococcus aureus and Staphylococcus lentus were moderately resistant to RU (MIC value 0.60 and 0.30 mg/ml GLP respectively). Contrastingly, with the exception of Lactobacillus spp., all tested beneficial bacteria including E. faecalis, E. faecium and B. badius, B. cereus and B. adolescentis were highly
sensitive to RU with MIC values of 0.15, 0.15, 0.30 and 0.075 mg/ml GLP respectively (Shehata et al., 2013). In a follow-up investigation by the same group, Ackermann and colleagues used DAISYII-incubators filled with rumen fluid of a 4-year-old non-lactating Holstein–Friesian cow and cultured in the absence or presence of GLP at a final concentration of 0, 1, 10, and 100 μg/ml(Ackermann et al., 2015). Similarly to what was observed in cultures of poultry microbiota (Shehata et al., 2013), GLP had an inhibitory effect on select groups of ruminal bacterial species, but increased the population of pathogenic species. In addition, Botulinum neurotoxin (BoNT) was produced during incubation at high doses of GLP. The authors concluded that GLP causes dysbiosis, which favors the production of BoNT in the rumen (Ackermann et al., 2015). Another study employing the Rumen Simulation Technique (RUSITEC) in vitro culture system, investigated the effects of a GLP-based herbicide formulation at GLP equivalent concentrations of either 0.42 mg or 2.92 mg per litre (Riede et al., 2016). Following 5 days of culture, only subtle changes were observed such as a small increase in SCFA production and a reduction in NH3-N. In addition, minor changes in the composition of ruminal bacteria were found to take place but no shift towards a greater abundance of pathogenic Clostridia species. However, Clostridium sporogenes counts declined consistently. Based on these findings the authors concluded that there were no adverse effects on ruminal metabolism or bacterial composition in the presence of either the low or high dose of GLP-based herbicide formulation, including no stimulation of Clostridia growth (Riede et al., 2016). Thus, whether dysbiosis can arise in the rumen of bovine farm animals from GLP or GLP-based herbicide exposure at what is typically present in contaminated feed, remains to be resolved.

Honey bees can be affected by the exposure to GLP via an effect on their gut microbiome (Motta et al., 2018). In a study investigating susceptibility to infection by opportunistic pathogens, it was found that honey bees exposed to GLP were more susceptible to the pathogen Serratia marcescens. This was due to an effect of GLP on the abundance of bee gut bacteria that reduce pathogen susceptibility. The different bacteria inhabiting the gut of honey bees were not similarly sensitive to 5mg/L GLP. All strains of the core bee gut species, such as Snodgrassella alvi, possess an EPSPS of class I and were sensitive to GLP. This shift in gut microbiome composition can favor the colonization of honey bee gut microbiome by pathogens.

Of greater relevance to human health, a number of studies in laboratory rodents have also been conducted. Lozano et al. exposed male and female Sprague-Dawley rats
via drinking water to three different doses of RU (50 ng/L, 400 mg/L, 2.25 g/L GLP equivalent concentration) for a 2-year period (Lozano et al., 2018). At the phylum level, the relative abundance of Bacteroidetes increased compared to Firmicutes. Further analysis indicated a female-specific enrichment of Bacteroidales S24-7 and reduction of Lactobacillaceae after RU treatment. These in vivo findings could be explained via in vitro selective growth inhibition of Bifidobacteria, Clostridia, and Enterococci anaerobes as a direct response to RU. Cultivatable gut bacteria were also demonstrated to have differing sensitivities to RU with the additional detection of an RU-resistant Escherichia coli strain, a fact that was attributed to the lack of the EPSPS gene. Interestingly, the overall alterations in gut microbiota in RU-exposed rats essentially overlap with bacterial alterations observed in alcoholic fatty liver disease, obesity, and systemic inflammation (Serino et al., 2012; Yan et al., 2011). In another study, Swiss mice were administered via oral gavage with RU (250 or 500 mg/kg/day GLP equivalent dose) in order to assess the impact of RU on gut microbiota and neurobehavioral functions (Aitbali et al., 2018). Subchronic (6 week) and chronic (12 week) RU exposure induced significant changes in gut microbiota concerning bacterial abundance and diversity, as presented by reduced numbers of Firmicutes, Bacteroidetes, Lactobacillus, and Corynebacterium. A recent pilot study conducted by the Ramazzini Institute (Mao et al., 2018), exposed Sprague-Dawley rats from gestational day-6 to either GLP or RU at the US regulatory permitted chronic reference dose of 1.75 mg/kg bw/per day, and then orally after weaning to post-natal day 28 (PND 28) for a total of 13 weeks (PND 125). Microbiome profiling in fecal samples revealed notable modifications regarding microbiota composition in GLP- and RU-exposed rat pups especially at a pre-pubertal age (PND 31), including proliferation of Bacteroidetes (Prevotella) and reduction of Firmicutes (Lactobacillus) compared to controls. These results are consistent with previous studies which have reported an increased sensitivity of early life gut microbiota to various factors that could affect maturation of the microbiome with the possibility of this leading to disease (Jakobsson et al., 2010; Rodriguez et al., 2015). Another study also compared the effects of GLP and a GLP based herbicide on gut microbiota in male Sprague-Dawley rats (Nielsen et al., 2018). Animals were administered with either 2.5 or 25 mg/kg bw/day GLP or commercial herbicide formulation at 25 mg/kg bw/day GLP equivalent dose by oral gavage over a 2-week period. The study found little effect on the microbiota community from exposure to either GLP alone or the commercial herbicide formulation. This observation seemed to correlate with the availability of sufficient quantities of aromatic amino acids in the feed, which abrogated the need for bacteria to produce these essential nutrients via the shikimate
pathway. However, the authors concur that the situation may be different in humans (Nielsen et al., 2018). In addition, the short (2-week) duration of the study may also be a crucial limiting factor, as it does not reflect real world chronic population exposures. Thus, further longer term investigations comparing GLP and GLP-based herbicide effects on the gut microbiome are needed to ascertain whether they can give rise to dysbiosis with negative health consequences.

5.3 Insecticides

The organophosphate chlorpyrifos (CPF) is widely employed for pest control on fruit and vegetable crops as well as vineyards (Joly et al., 2013). Human CPF exposure occurs through ingestion of contaminated food and water. CPF is mainly metabolized by hepatic and gut CYP enzymes (Bolles et al., 1999; Joly et al., 2013; Poet et al., 2003). Numerous studies suggest that CPF could cause neurotoxicity via inhibition of acetylcholine esterase, impairment of gut permeability, and intervene in hormone signaling, and lipid and glucose metabolic pathways (Joly Condette et al., 2014; Reygner et al., 2016; Slotkin et al., 2015; Zafiropoulos et al., 2014; Petrakis et al., 2017). Joly et al. (Joly et al., 2013) experimented with CPF exposure in the in vitro Simulator of the Human Intestinal Microbial Ecosystem (SHIME) system at 1 mg/day for 30 days, and in vivo in Wistar rats (in utero 1 mg/kg/day until birth, and then the same dose until 60 days). CPF exposure increased abundance of Bacteroides spp. and Enterococcus spp. and reduced that of Bifidobacterium spp. and Lactobacillus spp., with these changes being especially notable in ileum and colon samples. In a more recent study by the same authors (Joly Condette et al., 2015), perinatal CPF exposure (1 or 5 mg/kg/day) induced similar alterations in the gut microbial community in rats. CPF was positively linked to perturbed intestinal development, marked by histologically notable structural changes in intestinal villi, disturbance of mucosal barrier function (mucin-2 or MUC2), enhanced splenic and hepatic microbial translocation, and upregulation of innate immune system genes (toll-like receptor or TLR2 and TLR4). In another study, Zhao et al. (Zhao et al., 2016) treated mice with oral CPF gavage (1 mg/kg bw) for 30 days, which resulted in significantly decreased Firmicutes and increased Bacteroidetes in gut bacterial populations. In addition, gut microbiome alterations were greatly correlated with changes in the urine metabolome. Perturbations of Bacteroidaceae, Bifidobacteriaceae, Enterobacteriaceae, Erysipelotrichaceae, Halobacteroidaceae, Lactobacillaceae, and Sphingobacteriaceae abundances showed a positive correlation with various products of energy, amino acid, phenyl, and bile acid metabolism. Lactobacillaceae were also positively associated with derivatives of SCFA metabolism, such as hexanoate and valerate. Elevated serum levels of LPS and diamine oxidase (DAO), in addition to histopathologically-detected colonic insults, reflected a colonic inflammatory response and irregular intestinal permeability as a result of the CPF exposure. To further investigate the effects of CPF on the gut microbiome, Fang et al. (Fang et al., 2018) exposed Wistar rats to either a daily normal fat
(NF) or high fat (HF) diet enriched with CPF (0.3 or 3 mg/kg bw) for a 9-week period. CPF treatment increased the abundance of various bacterial genera in NF- (Aerococcus, Anaeroplasma, Bacteroides, Brevundimonas) and HF-fed rats (Acinetobacter, Clostridium, Pseudomonas), which have been considered as opportunistic pathogens related to obesity and diabetes (Rasmussen, 2016; Woting et al., 2014). Alterations in SCFA-producing bacteria (e.g., Allobaculum, Roseburia, Clostridium and Bacteroides) were also detected in NF- and HF-fed rats similar to microbial changes in obesity and diabetes (Qin et al., 2012). Additionally, higher numbers of Allobaculum, Candidatus, and Sutterella correlated with neurotoxicity, β-cell dysfunction and pancreatic injury (Huang et al., 2017; Yu et al., 2017). All these findings indicated that CPF exposure could alter gut microbiota in a diet-specific manner.

Permethrin (PERM) is a frequently used pyrethroid (Barr et al., 2010). Although PERM presents a greater safety profile than other insecticides (Casida and Durkin, 2013), its exposure is not considered benign. To investigate its toxicity on gut microbiota, Nasuti et al. (Nasuti et al., 2016) administered PERM (34 mg/4 mL/kg bw) to Wistar rats by oral gavage, from PND 6 to PND 21 for a period of 4 months. Alterations were observed in fecal microbiota of PERM-exposed rats compared to controls, with increased populations of Enterobacteriaceae and Lactobacillus and reduced abundance of Bacteroidetes, including Bacteroides, Prevotella, and Porphyromonas. Decreased acetic acid levels after PERM treatment indicated impaired SCFA production, possibly reflecting the changes in bacterial composition. Additional in vitro experiments revealed a greater PERM-induced antimicrobial effect on commensal bacteria such as Bifidobacterium and Lactobacillusparacasei, allowing gut colonization by opportunistic pathogens such as Staphylococcusausaureus and Escherichia coli. These microbiota compositional alterations may also be associated with PERM-mediated neurotoxicity (Nasuti et al., 2014). In a recent study (Alhasson et al., 2017), C57BL/6J mice established with Gulf War Illness (GWI) were exposed to a chemical mixture including PERM (200 mg/kg) for 13 days, in order to assess the neurological and intestinal toxicity of PERM. The results revealed gut dysbiosis with significantly reduced abundance of Bacteroidetes in contrast to Firmicutes and Tenericutes, which were more abundant than in control animals. At the genus level, several bacterial species were enriched in GWI mice such as Allobaculum, Coprococcus, Turicibacter, Dorea, and Ruminococcus. Intestinal permeability also increased, as revealed by a reduction in Occludin protein levels and an increase in Claudin-2 protein levels. The resultant endotoxemia promoted TLR4 stimulation in the brain and gut, causing the inflammatory release of IL-1β and monocyte chemoattractant protein-1 (MCP-1) in the frontal cortex as well as the small intestine.

5.4 Fungicides
Imazalil (IMZ) is a broad-spectrum fungicide, mainly used to prevent post-harvest loss from fungal infestations in vegetable and fruit crops (Sepulveda et al., 2015). Human exposure mainly occurs through ingestion of such contaminated food products (Masia et al., 2015; Ruiz-Rodríguez et al., 2015). Studies in animal model systems have reported IMZ-induced endocrine disruption and neurotoxicity (Y. Jin et al., 2016; Tanaka, 1995). Jin et al. (C. Jin et al., 2016) administered ICR mice with an IMZ-enriched diet (25, 50, and 100 mg/kg bw) for 28 days. The overall diversity and richness of gut microbiota decreased in the highest dose IMZ-exposed mice, as revealed by 16S rRNA gene amplicon sequencing of cecal and fecal samples. Further taxonomic analyses revealed higher numbers of Firmicutes, Actinobacteria, Acidobacteria, and Chlorobacteria, and lower numbers of Bacteroidetes, Proteobacteria, and Cyanobacteria after IMZ treatment. In particular, a reduced abundance of commensal bacteria, including Lactobacillus and Bifidobacterium, and increased pathogenic bacteria, such as Desulfovibrio, were associated with enhanced inflammatory infiltration, increased fecal levels of lipocalin-2 (Icn-2), and upregulation of IL-1β, IL-22, TNF-α, and interferon (IFN)-γ in the colon, in accordance with previous studies (Huang et al., 2015; Petersson et al., 2011). Thus, high-dose IMZ-mediated gut dysbiosis induced colonic inflammation. In another recent study (Jin et al., 2018a), C57BL/6 mice were treated with IMZ (0.1, 0.5, and 2.5 mg/kg bw) for 2, 5, or 15 weeks, in order to evaluate the effects of this fungicide on gut microbiota composition and intestinal barrier function. Microbiome analysis by 16S rRNA gene amplicon sequencing in cecal and fecal samples revealed high-dose dependent alterations in bacterial richness at 15 weeks, with a reduced abundance of Bacteroidetes, Lactobacillus, Parabacteroides, and Prevotella, and an elevated proportion of Clostridiales, Helicobacteraceae, and Oscillospira in IMZ-exposed mice. It was also proposed that these alterations could enhance bacterial invasion of the intestinal mucus layer, decrease mucus synthesis, and impair ion transport through downregulation of genes related to the cystic fibrosis transmembrane conductance regulator (CFTR), since gut microbiota are strongly associated with intestinal barrier function (Bhatia et al., 2015; Zmora et al., 2017). Thus, IMZ-induced gut dysbiosis could result in intestinal barrier dysfunction.

Epoxiconazole (EPO) is a broad-spectrum azole-classified fungicide, which is utilized for the prevention of damage caused by common wheat pests such as rust (Puccinia triticina) and leaf blotch (Septoria tritici) (Paveley et al., 2001; Zarn et al., 2003). As a result, EPO-contaminated wheat products are the main route for human exposure. EPO presents fetotoxic effects in rats after gestational or in utero exposure (50 mg/kg), which were associated with elevated testosterone and decreased estradiol levels in maternal serum (Taxvig et al., 2007; Taxvig et al., 2008). Xu et al. (Xu et al., 2014) were the first to evaluate the effects of EPO on gut microbiota. In this study, Sprague-Dawley rats were fed a diet containing EPO (0, 4, and 100 mg/kg bw/day) for a 90-day period. The abundance of Firmicutes reduced, whereas fecal populations of Bacteroidetes and Proteobacteria were enriched following EPO exposure. Moreover, concerning bacterial families Lachnospiraceae, Enterobacteriaceae, and
Bacteroidaceae numbers were increased while Lactobacilaceae decreased in EPO-treated rats.

Carbendazim (CBZ) is a broad-spectrum carbamate-classified fungicide, which has been associated to endocrine disruption, reproductive toxicity and hepatic oxidative stress (Jiang et al., 2014; Selmanoglu et al., 2001; Yu et al., 2009). In a study by Jin et al. (Jin et al., 2015), CBZ was orally administered (100 and 500 mg/kg bw) to ICR mice for 28 days. The diversity of cecal microflora significantly decreased following CBZ exposure. Taxonomic analysis in cecal samples detected enrichment of Deferribacteraceae, Desulfovibrionaceae, Lachnospiraceae, and Ruminococcaceae, as well as a reduction in the abundance of Bacteroidaceae, Christensenellaceae, Paraprevotellaceae, Porphyromonadaceae, Prevotellaceae and Rikenellaceae. Elevated levels of TG and lipid accumulation in the liver, in addition to upregulation of genes related to hepatic and adipose TG synthesis and lipogenesis, indicated a hepatic lipid metabolism disorder in CBZ-treated mice. Meanwhile, elevated serum levels of IL-1β and IL-6 pointed towards a CBZ-induced inflammatory response. All these findings in combination with low hepatic and intestinal CBZ bioaccumulation, suggested that a CBZ-mediated interaction with gut microbiota might contribute to disturbed lipid metabolism and inflammatory stimulation, underlying a functional interplay between the liver and gut (Cammarota et al., 2014). A similar recent study (Jin et al., 2018b), proposed a possible mechanism, which connects CBZ-induced gut dybiosis and lipid metabolism disorder. According to this investigation, CBZ ingestion perturbs the gut microbiota, leading to a downregulation of fasting induced adipose factor (Fiat) and alterations in SCFA production, thus increasing intestinal absorption of dietary TG and lipids. In parallel, CBZ promotes deeper bacterial invasion in the colonic mucus layer, which results in increased mucus secretion, reduced IL-17α defense secretion, and thus in aberrant colonic immunity with subsequent inflammation. These changes caused hyperlipidemia and increased serum TNF-α, inducing decreased hepatic lipid synthesis and enhanced storage in adipose tissue in addition to systemic inflammation.

5.5 Pesticide mixtures

In real world scenarios, populations are exposed to mixtures rather than individual pesticides, which play a major role in potential health issues concerning the gut system due to antimicrobial activities that they sometimes cause. Exposure to these chemical mixtures in daily life routines could have serious effects on the intestine since it is the first organ system to be exposed to such residues. Thus, the gastrointestinal tract maybe the most susceptible vital organ of consumers exposed
to a combination of multiple pesticide residues through food and water intake either alone or as a mixture. Although several single chemicals have been associated with bacterial metabolism, studies investigating mixtures of pesticides are rare.

Studies with insects, especially honey bees, have been undertaken and have found an association between gut microbiota and pesticide exposure applied within agricultural fields. One of the latest surveys identified multiple pesticide residues, both beekeeper and grower applied, in honey bee hives (Mullin et al., 2010). The microbial community associated with the gut has been shown to influence the growth and health of insects (Dillon and Dillon, 2004; Martinson et al., 2012). Medications applied in the hive may alter the structure and function of the microbiome and thus affect gut function and overall health of the bee colony (Alaux et al., 2010; van Engelsdorp et al., 2008). Four pesticides were studied to investigate the potential synergism of frequently used compounds at realistic exposure levels to bee larvae. Regarding the mode of action for chlorothalonil, the tendency towards antagonism of brood toxicity at the lower dietary chlorothalonil fluvalinate mixture concentration, may be associated with alternative peripheral mechanisms such as gut microbial detoxification that may be overwhelmed at higher doses where more internal neurotoxic effects of the pyrethroid can prevail (Zhu et al., 2014).

An investigation of the metabolic effects resulting from a chronic dietary exposure to a low-dose pesticide cocktail in mice, studied a mixture of six pesticides commonly used in France, namely boscalid, captan, CPF, thiofanate, thiacloprid and ziram (Lukowicz et al., 2018). The pesticide mixture was administered to wild-type and constitutive androstane receptor-deficient (CAR<sup>-/-</sup>) male and female mice for 52 weeks via a standard chow at doses exposing animals to the tolerable daily intake (TDI) of each pesticide. It was found that wild-type exposed female mice exhibited fasting hyperglycemia, a higher reduced glutathione:oxidized glutathione liver ratio and perturbations of gut microbiota-related urinary metabolites compared to control wild-type animals. Significant differences were observed in the concentration of gut microbiota related metabolites in the urine of wild-type exposed female mice as well as lower levels of metabolites from the methylamine pathway (trimethylamine is derived from dietary choline fermentation by commensal bacteria and metabolized in to trimethylamine oxide in the liver), higher levels of 3-indoxylsulfate (a metabolite derived from the conversion of dietary tryptophan to indole by enteric bacteria and further conversion of indole in the liver and phenyl derivatives p-cresol glucuronide and phenylacetylglycine), which were also lower in the urine of pesticide-treated wild-type females. These perturbations were observed only after 48 weeks of pesticide
exposure and after metabolic perturbations, such as hyperglycemia were established, which could imply that perturbations in the gut microbiota are a consequence of the pesticide-induced metabolic disorders and not the cause.

The results of the above studies regarding pesticide exposure and gut microbiota are summarized in Table 1.

Table 1. Summary of various experimental studies reporting the impact of pesticides and mixtures on gut microbiota and their effects on host health

<table>
<thead>
<tr>
<th>Pollutant</th>
<th>Dosing regimen</th>
<th>Animal model/Methods of microbiota analysis</th>
<th>Impact on gut microbiota</th>
<th>Outcomes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDE</td>
<td>1 mg/kg bw per day via oral gavage for 8 weeks</td>
<td>- C57BL/6 mice - 16S rRNA gene sequencing</td>
<td>(-): Bacteroidetes, Verrucomicrobia, Actinobacteria (+): Firmicutes, Proteobacteria</td>
<td>Alterations in hepatic and enteric bile acid metabolism and profiles through enhanced activity of bacterial salt hydrolase</td>
<td>(Liu et al., 2017)</td>
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<tr>
<td>GLP (Roundup)</td>
<td>0.075, 0.15, 0.3, 0.6, 1.2, 2.4, or 5 mg/ml in vitro for 5 days</td>
<td>- Bacterial strains of poultry microbiota - MALDI-TOF mass spectrometry</td>
<td>- Highly resistant: (MIC: 5 mg/ml) C. botulinum type A and B, C. perfringens, Salmonella Gallinarum, Salmonella Typhimurium, Salmonella Enteritidis and E. coli - Moderately resistant: (MIC: 0.6 and 0.3 mg/ml) L. casei, L. buchneri, L. harbinensis, Staphylococcus aureus and Staphylococcus lentus - Highly sensitive: (MIC: 0.3, 0.15 and 0.075 mg/ml) E. faecalis, E. faecium and B. radius, B. cereus and B. adolescentis</td>
<td>Higher resistance of pathogenic bacteria than beneficial bacteria to GLP</td>
<td>(Shehata et al., 2013)</td>
</tr>
<tr>
<td>Treatment</td>
<td>Concentration</td>
<td>Duration</td>
<td>Method</td>
<td>Findings</td>
<td>Reference</td>
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<tr>
<td>GLP</td>
<td>0, 1, 10, and 100 μg/ml in vitro for 48h</td>
<td>- Bovine rumen fluid cultures</td>
<td>(-): Most members of ruminal microbiota NS: <em>C. botulinum</em> type B</td>
<td>Dysbiosis with enhanced production of Botulinum neurotoxin</td>
<td>(Ackermann et al., 2015)</td>
</tr>
<tr>
<td>GLP (Plantaclean)</td>
<td>0.42 or 2.92 mg/L in vitro for 5 days</td>
<td>- RUSITEC culture system</td>
<td>None significant</td>
<td>No adverse effects on ruminal metabolism or bacterial composition</td>
<td>(Riede et al., 2016)</td>
</tr>
<tr>
<td>GLP</td>
<td>5 or 10 mg/L for 5 days</td>
<td>- Adult worker bees</td>
<td>(-): <em>Snodgrassella alvi</em>, <em>Bifidobacterium</em>, and <em>Lactobacillus</em></td>
<td>Increased susceptibility to pathogens (<em>Serratia marcescens</em>)</td>
<td>(Motta et al., 2018)</td>
</tr>
<tr>
<td>GLP (Roundup)</td>
<td>50 ng/L, 400 mg/L or 2.25 g/L in drinking water for 2 years</td>
<td>- Sprague-Dawley male and female rats</td>
<td>- Both genders (-): <em>Firmicutes</em> (+): <em>Bacteroidetes</em> - Female rats (-): <em>Lactobacillaceae</em> (+): <em>BacteroidalesS24-7</em></td>
<td>Overlap with bacterial alterations observed in alcoholic fatty liver disease, obesity, and systemic inflammation</td>
<td>(Lozano et al., 2018)</td>
</tr>
<tr>
<td>GLP (Roundup)</td>
<td>250 or 500 mg/kg per day for 6 or 12 weeks</td>
<td>- Swiss male mice</td>
<td>(-): <em>Firmicutes</em>, <em>Bacteroidetes</em>, <em>Lactobacillus</em> and <em>Corynebacterium</em></td>
<td>Neurobehavioral dysfunction</td>
<td>(Aitbali et al., 2018)</td>
</tr>
<tr>
<td>GLP or Roundup</td>
<td>1.75 mg/kg bw per day since gestation, and through drinking water after weaning for 13 weeks</td>
<td>- Sprague-Dawley rats</td>
<td>(-): <em>Firmicutes</em> (-): <em>Lactobacillus</em> (+): <em>Bacteroidetes</em> (Prevotella)</td>
<td>Early life exposure shapes gut microbiota maturation</td>
<td>(Mao et al., 2018)</td>
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<tr>
<td>GLP or Glyfonova</td>
<td>2.5 or 25 mg/kg bw per day via oral gavage for 2 weeks</td>
<td>- Sprague-Dawley rats</td>
<td>None significant</td>
<td>Sufficient dietary levels of aromatic amino acids reduce the antimicrobial effect of GLP</td>
<td>(Nielsen et al., 2018)</td>
</tr>
<tr>
<td>CPF</td>
<td>- 1 mg/day in vitro for 30 days - 1 mg/kg per day since gestation, and through oral gavage after weaning until 60 days of age - SHIME and Wistar rats - Biomérieux strips</td>
<td>(-): <em>Bifidobacterium</em> spp. and <em>Lactobacillus</em> spp. (+): <em>Bacteroides</em> spp. and <em>Enterococcus</em> spp.</td>
<td>- Intestinal barrier dysfunction - Bacterial translocation - Stimulation of innate immune system (Joly et al., 2013; Joly Condette et al., 2015)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPF</td>
<td>1 mg/kg bw per day via oral gavage for 30 days</td>
<td>Mus musculus male mice - 16S rRNA gene sequencing</td>
<td>(-): <em>Firmicutes</em> (Bacteroidaceae, <em>Clostridiaceae</em>) and <em>Bifidobacteriaceae</em> (+): <em>Bacteroidetes</em> (Erysipelotrichaceae, <em>Sphingobacteriaceae</em>, Halobacteriaceae, <em>Lactobacillaceae</em>) and <em>Enterobacteriaceae</em> - Colonic inflammation - Irregular intestinal permeability (Zhao et al., 2016)</td>
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<tr>
<td>CPF</td>
<td>0.3 or 3 mg/kg bw per day in NF or HF diet for 9 weeks</td>
<td>Wistar male rats - 16S rRNA gene sequencing</td>
<td>- NF diet group (+): <em>Anaeroplasma</em>, <em>Bacteroides</em>, <em>Aerococcus</em>, <em>Allobaculum</em>, <em>Roseburia</em>, <em>Anaeroplasma</em>, <em>Candidatus Saccharimonas</em> and <em>Sutterella</em> - HF diet group (+): <em>Acinetobacter</em>, <em>Pseudomonas</em>, <em>Clostridium</em>, and <em>Candidatus Arthromitus</em> - Increased risk of obesity and diabetes - Neurotoxicity, β-cell dysfunction and pancreatic injury (Fang et al., 2018)</td>
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<tr>
<td>PERM</td>
<td>34 mg/4 ml/kg bw per day via oral gavage for 4 months</td>
<td>Wistar male and female rats - qPCR</td>
<td>(-): <em>Bacteroidetes</em> (Bacteroides, <em>Prevotella</em>, <em>Porphyromonas</em>) (+): <em>Enterobacteriaceae</em> and <em>Lactobacillus</em> - Impaired SCFA production - Neurotoxicity (Nasuti et al., 2016)</td>
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<tr>
<td>PERM</td>
<td>200 mg/kg orally for 13 days</td>
<td>C57BL/6J male mice - MoBio PowerMag Microbiome kit</td>
<td>(-): <em>Bacteroidetes</em> (+): <em>Firmicutes</em>, <em>Tenericutes</em>, <em>Allobaculum</em>, <em>Coprococcus</em>, <em>Tunicibacter</em>, <em>Dorea</em> and <em>Ruminococcus</em> - Intestinal and neuronal inflammation (Alhasson et al., 2017)</td>
<td></td>
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<tr>
<td>IMZ</td>
<td>25, 50, or 100 mg/kg bw per day in diet for 28 days</td>
<td>- ICR male mice - 16S rRNA gene sequencing</td>
<td>- Bacteroidetes, Proteobacteria, Cyanobacteria, Lactobacillus and Bifidobacterium (+): Firmicutes, Actinobacteria, Acidobacteria, Chlorobacteria and Desulfovibrio</td>
<td>Colonic inflammation (C. Jin et al., 2016)</td>
<td></td>
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<tr>
<td>IMZ</td>
<td>0.1, 0.5, or 2.5 mg/kg bw per day in drinking water for 2, 5, or 15 weeks</td>
<td>- C57BL/6 male mice - 16S rRNA gene sequencing</td>
<td>- Bacteroidetes, Lactobacillus, Parabacteroides and Prevotella (+): Clostridiales, Helicobacteraceae and Oscillibacteriaceae</td>
<td>Intestinal barrier dysfunction (Jin et al., 2018a)</td>
<td></td>
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<tr>
<td>EPO</td>
<td>0, 4, or 100 mg/kg per day in diet for 90 days</td>
<td>- Sprague-Dawley female rats - 16S rRNA gene sequencing</td>
<td>- Firmicutes and Lactobacillaceae (+): Bacteroidetes, Proteobacteria, Lachnospiraceae, Enterobacteriaceae and Bacteroidaceae</td>
<td>Hepatotoxicity (Xu et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>CBZ</td>
<td>100 or 500 mg/kg bw per day in diet for 28 days</td>
<td>- ICR male mice - 16S rRNA gene sequencing</td>
<td>- Bacteroidaceae, Christensenellaceae, Paraprevotellaceae, Porphyromonadaceae, Prevotellaceae and Rikenellaceae (+): Deferribacteraceae, Desulfovibrioaceae, Lachnospiraceae and Ruminococcaceae</td>
<td>- Hepatic lipid metabolism disorder (Jin et al., 2015)</td>
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6. Nanoparticles

Nanoparticles (NPs) of extremely small diameter (≤ 100 nm) are broadly used in many different industrial and medical sectors (Bergin and Witzmann, 2013). Various NP-based manufactured products include cosmetics (UV protection), toothpaste (biofilm inhibition), electronic components, and scratch-free paints (Mahmoudi et al., 2011). The ability of NPs to

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cross biological barriers also make them valuable in the medical field, allowing for precise hyper-selective accumulation of chemotherapeutic agents at tumor sites (Blanco et al., 2015; Wilhelm et al., 2016). Moreover, NPs are widely used in the food industry as enhancers of many physicochemical food properties, improving mechanical endurance of food containers, and aiding in the detection of any food-related contaminant, thus extending shelf-life and quality of dietary products (Eleftheriadou et al., 2017; Frohlich and Roblegg, 2012; Sotiriou and Pratsinis, 2010). Human exposure to NPs can also be achieved via inhalation, transdermal absorption, and oral intake routes. The extensive and intentional presence of various NPs, such as silver (Ag), zinc oxide (ZnO), titanium dioxide (TiO₂), and silicon dioxide (SiO₂) in food products, significantly increases the potential of NP ingestion (Bouwmeester et al., 2014; Peters et al., 2014). As a result, the gut and its resident microbiota may be greatly exposed to NPs. In addition, NPs demonstrate antibacterial properties possibly through NP-derived ions and oxidative stress induction (Fröhlich and Fröhlich, 2016; Hadrup and Lam, 2014; Sharifi et al., 2012).

Among available NPs, AgNPs are the most frequently used and well-studied, being the dominant component for almost half of NP-based consumer products worldwide (Vance et al., 2015). The mean dietary exposure level of AgNPs is estimated at 70-90 μg/day, being presented mostly as a food additive (E174) on beef (Kahru and Ivask, 2013; Wijnhoven et al., 2009). Following oral consumption of AgNPs, ileal absorption and accumulation into Peyer’s patches occurs. Although AgNPs are nearly harmless with respect to eukaryotic cells, they present a strong bactericidal capability (Prabhu and Poulose, 2012; Zarei et al., 2014). In one of the first studies that evaluated the effect of AgNP exposure on gut microbiota, Hadrup et al. (Hadrup et al., 2012) treated Wistar rats with different doses of AgNP (2.25, 4.5 or 9 mg/kg bw) daily for 28 days. Quantitative PCR analysis of cecal samples revealed perturbations of Firmicutes and Bacteroidetes phyla following AgNP treatment. In addition, AgNP exposure showed favorable toxicity with no adverse biochemical or pathological effects, findings consistent with previous reports (Y.S. Kim et al., 2010; Loeschner et al., 2011). A study conducted by Das et al. (Das et al., 2014) was the first to assess the impact of AgNPs on an in vitro replication system of human fecal microbiota. For this purpose, a synthetic anaerobic bacterial community, defined as a microbial ecosystem therapeutic-1 (MET-1) culture established from stools having being collected from a healthy human donor, was exposed to varying concentrations of AgNPs (25, 100, and 200 mg/L) for a 48-hour period. A significant decrease in culture-derived bacterial gas production, alterations of fatty acid methyl ester profiles, indicated a shift towards a more pathogenic bacterial profile. These findings were further corroborated through PCR-denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene amplicon sequencing analysis, which revealed notable perturbations in bacterial culture composition following AgNP treatment. There was a reduction in the abundance of Bacteroidesovatus, Eubacteriumrectale, Roseburiafaecalis, Rosuburiaintestinalis and Ruminococcustorques, which are considered as immunoprotective and anti-inflammatory
species (Segain et al., 2000; Wexler, 2007). In contrast, species that have been linked to IBD such as Escherichiacoli and Raoutella spp. (Roediger, 2008) were enriched. The authors concluded that AgNP ingestion could promote deleterious consequences on human gut microbiota.

Williams et al. (Williams et al., 2015), investigated for the first time the in vivo effects of AgNPs on the gut microbiome in rodents. Discrete AgNP concentrations (9, 18, and 36 mg/kg bw/day) were prepared and administered orally to Sprague-Dawley rats for a total of 13 weeks. PCR analysis of ileal samples revealed evidence of AgNP related dose- and size-dependent alterations in the ileo-mucosal bacterial community. More specifically, at the phylum level, AgNP exposure led to reduction in Firmicutes and enrichment in Bacteroidetes. These changes were mainly attributed to lower proportions of commensal bacteria Bifidobacterium and Lactobacillus, and an enhanced abundance of opportunistic Gram(-) pathogens Bacteroides and Enterobacteriaceae. In addition, it was observed that low-dose AgNP exposure elicited the downregulation of ileo-mucosal immunomodulatory genes, such as MUC3, TLR2, TLR4, GPR43, and forkhead box P3 (FOXP3). A more tolerable immunity mediated by T-reg cells may be responsible for the TLR2 and FOXP3 associated profiles (J.H. Wang et al., 2002). The authors proposed a possible mechanism of intestinal immune modulation as a result of AgNP ingestion. Prolonged interaction between gut microbiota and AgNPs enables preferential binding to the large numbers of charged groups in the cell wall of Gram(+) bacteria with subsequent deleterious effects (Li et al., 2013). In parallel, enhanced Gram(-) interplay with various epithelial and secreted molecules (TLR2, TLR4, MUC2, MUC3), and trans-mucosal transportation by immune cells (dendritic cells and macrophages), could stimulate an intestinal immune response and thus release of inflammatory cytokines, such as IL-10 and transforming growth factor beta (TGF-β). Hence, oral exposure to AgNP, especially of small size (10 nm) may induce an intestinal immune response correlated to gut microbiota perturbations. However, an earlier study (Wilding et al., 2016) using varying sizes of AgNPs (20 and 110 nm), coated with either polyvinylpyrrolidone (PVP) or citrate, and prepared for oral administration (10 mg/kg bw) in C57BL/6NCrl mice on a daily basis for 28 days, reported no considerable AgNP related perturbations regarding composition, diversity, and structure of cecal microbiota after 16S rRNA gene sequencing. This was possibly due to differences in sampling methods. In another study, van den Brule et al. (van den Brule et al., 2016) tested if a human relevant dosage of AgNPs could affect the composition of gut microbiota in C57BL/6 mice orally treated with pellets enriched with AgNPs (0, 46, 460 and 4600 ppb) for 28 days. Although AgNP treatment resulted in no-apparent toxicity regarding animal physiology and body weight, analysis of fecal DNA samples indicated dose-dependent changes in the colonic microbiota. An increased abundance of Firmicutes and a reduction of Bacteroidetes phyla were detected in AgNP treated mice owing to altered populations of bacterial families Lachnospiraceae and Bacteroidales S24-7. This study signaled that ingestion of aged AgNPs is less toxic to the gut microbiota than freshly prepared
nanomaterials, which may be due to sulfidation and confined release of ions (Axson et al., 2015; Walczak et al., 2013). More recently, Javurek et al. (Javurek et al., 2017) exposed Sprague-Dawley rats during 12 weeks to either cubic or spherical AgNPs (3.6 mg/kg bw). The gut bacterial community was altered in a shape-specific manner, with cube-shaped AgNPs reducing the relative abundance of Bacteroides uniformis, Christensenellaceae, Clostridium spp. and Coprococcus eutactus, whilst populations of Aggregatibacter pneumotropica, Corynebacterium spp., Dehalobacterium spp., Oscillospira spp. and Peptococcaceae diminished in response to sphere-shaped AgNPs. These perturbations in gut microbiota were correlated with anxiety and exploratory behavioral patterns in AgNP exposed rats as revealed by elevated plus maze (EPM) testing.

The impact of various NPs other than those based on Ag on gut microbiota, has also been evaluated. TiO$_2$NPs have been broadly used in numerous products including cosmetics, sunscreens and toothpastes, which could be accidentally ingested (Kaida et al., 2004). More importantly, such NPs have been utilized as additives (colorant E171) in various food products especially in the confectionery field (pastries, candies, chocolate, chewing gums, toppings) with their estimated daily adult intake being 0.45 mg/kg (Peters et al., 2014; Weir et al., 2012). TiO$_2$NPs exhibit mild toxicity and are able to cross the intestinal epithelium through transcytosis (Koeneman et al., 2010). Cerium oxide (CeO$_2$)NPs are commonly used in many consumer products such as cosmetics, sunscreens, personal care, paints, and coatings (Auffan et al., 2009). Research has demonstrated that CeO$_2$NPs can be bactericidal particularly to some strains such as Escherichia coli, and also may impair eukaryotic cell membrane function and structure (I.S. Kim et al., 2010; Zeyons et al., 2009). Similarly to CeO$_2$NPs, ZnONPs exposure can occur through similar routes (Keller et al., 2013). In addition, ZnONPs may be ingested after consumption of nutritional supplements (vitamins) or after dissociation from food packaging (Rincker et al., 2005). Studies show that ZnONPs can cause dose-dependent and species-specific bactericidal effects (Yamamoto, 2001), as well as perturbing proliferation, viability, and membrane penetration of eukaryotic cells (I.S. Kim et al., 2010). However, the in vivo toxicity of ZnONPs has not yet been fully clarified (Aillon et al., 2009). Taylor et al. (Taylor et al., 2015) investigated the effects of exposure to these metal oxide NPs at environmentally similar doses (ZnONPs, CeO$_2$NPs, and TiO$_2$NPs, at 0.01 μg/L, 0.01 μg/L, and 3 mg/L respectively), for five days in vitro in a model colon reactor containing the gut bacterial community extracted from a healthy human donor. Although shifts in the microbial composition were not assessed, the study indicated important phenotypic alterations of colonic microbiota regarding the production of SCFAs, sugar capacity of extracellular polymeric substance (EPS), cell hydrophobicity, and electrophoretic mobility in response to NPs, thus displaying impaired microbiota stability. The above changes were more discrete for TiO$_2$NPs, compared to the other NPs tested. Moreover, reduced production of butyrate was associated with CeO$_2$NP exposure.
Silica dioxide (SiO$_2$) NPs are another category of NPs mainly used as food additives (E551) as an anti-caking factor in powder products (spices, flour, sugar, coffee, dried milk, table salt), with daily intake being estimated at 1.8 mg/kg (Dekkers et al., 2011). In an in vivo study conducted by Lecloux et al. (Lecloux, 2015), C57BL/6 mice were fed a diet enriched with SiO$_2$ NPs or AgNPs at human relevant doses (5, 50 or 500 ppm and 46, 460 or 4600 ppb respectively) for 28 days. Even though NP-exposure had no deleterious consequences on animal physiology and histology, the richness of gut microbiota was significantly decreased. More specifically, AgNP exposure led to increased abundance of Firmicutes together with a reduction in Bacteroidetes, while a decreased abundance in Actinobacteria was observed in SiO$_2$ NP-exposed mice in a dose-dependent manner. However, since these findings have only been reported in abstract form, they require further investigation (Pietroiusti et al., 2016). Recently, the effects of AgNPs, SiO$_2$ NPs, and TiO$_2$ NPs on colitis development and gut microbiota were evaluated. In this study (Chen et al., 2017), dietary relevant doses of these NPs (2.5 mg/kg bw/day) were administered to CD-1 mice for a 7-day period. Exposure to AgNPs resulted in histological alterations and an inflammatory response related to colitis, in addition to changes in microbiota abundance including a significant reduction of Firmicutes (Lactobacillus) and elevation of Bacteroidetes (Alistipes, Bacteroides, and Prevotella) levels.

On the other hand, regarding SiO$_2$ NPs and TiO$_2$ NPs, stimulation of the colonic secretion of IL-1β, IL-6, and TNF-α was the only toxic outcome observed. SiO$_2$ NP treatment induced changes in Firmicutes and Bacteroidetes contrary to that observed in AgNPs, with enhancement of Alistipes, Lactobacillus, Oscillibacter and Prevotella genera, whereas Bacteroides were reduced. TiO$_2$ NP ingestion resulted in no notable shift in gut microbiota. The latter findings are in agreement with an in vitro study of TiO$_2$ NP exposure in human relevant doses using a model intestinal microbiota community, which caused no significant changes in bacterial populations (Dudefoi et al., 2017).

Another type of NP are carbon nanotubes (CNTs). CNTs could be either single-walled (SWCNTs) or multi-walled (MWCNTs) in structure. The wide usage of CNTs in many fields such as agriculture, environment (water filters), biomedicine (as drug carriers) and industry have raised great concerns about their possible effect on human health (Cote et al., 2007; De Volder et al., 2013). The characteristics of CNTs enable their exposure through many routes including inhalation and transdermal absorption, or ingestion, either directly, or after mucociliary clearance. The antibacterial properties of CNTs have been demonstrated (Kang et al., 2008). An in vitro study by Chen et al. (Chen et al., 2013) reported that SWCNTs or MWCNTs possess a broad-spectrum bactericidal potency against human gut Gram(+) or Gram(-) bacteria, pathogenic or non-pathogenic, both rod- or sphere-shaped (Lactobacillus acidophilus, Bifidobacterium adolescentis, Escherichia coli, Enterococcus faecalis and Staphylococcus aureus) via various mechanisms including binding-mediated microbial wall lysis and cell membrane impairment. However, these antimicrobial effects were more prominent in SMCNTs than MWCNTs. Hence, selective CNT usage might be useful against
opportunistic pathogens and antibiotic-resistant bacteria. A more recent in vivo study by the same authors (Chen et al., 2018) experimented on CD-1 mice treated by oral gavage of SWCNTs at varying doses (0.05, 0.5, and 2.5 mg/kg bw/ day) for 7 days. The highest dose of SWCNTs administered resulted in intestinal permeability, caused histological lesions and stimulated the production of IL-1β, IL-6 and TNF-α. These alterations were correlated with shifts in gut microbiota, with a decrease in *Firmicutes* and increase in *Bacteroidetes* populations, and increased abundance of *Alistipes, Bacteroidales S24-7* and *Lachnospiraceae bacterium A4*, which are considered as pro-inflammatory genera (Ormerod et al., 2016; Saulnier et al., 2011; Ye et al., 2008).

### 7. Persistent organic pollutants: polycyclic aromatic hydrocarbons, dioxins, furans and polychlorinated biphenyls.

Persistent organic pollutants (POPs) are highly toxic, semi-volatile, synthetic chemical compounds that are extremely resistant to biodegradation and therefore remain in the environment for extended time periods, thus accumulating in the food chain and consequently in living organisms (White and Birnbaum, 2009). POPs have been associated with global health issues such as diabetes, obesity, autoimmunity, reproductive disorders, cancer, and developmental defects (Lee et al., 2014; Vassilopoulou et al., 2017). The toxicity of POPs is partly mediated through binding with the aryl hydrocarbon receptor (AhR), which results in various biological responses (Jin et al., 2014).

Polychlorinated biphenyls (PCBs) are a large group of chemicals having been broadly utilized in the manufacture of many products such as lubricants, cooling liquids, transformers, hydraulic fluid and capacitors due to their unique electrochemical properties. PCBs are linked to cancer (especially breast cancer), aberrant immunity, reproductive impairment, neurological defects and metabolic disturbances (adipose inflammation, disrupted insulin and glucose tolerance) (Baker et al., 2015; Buck Louis, 2014; Mesnage et al., 2018; Park et al., 2010). Improper disposal of PCBs from hydraulic or electrical systems into the environment raises the risk of human exposure through inhalation, transdermal absorption, as well as via water or food consumption. PCBs are initially metabolized in the liver by CYP enzymes, resulting to the production of arene oxide intermediates. In mammals, the next main metabolic pathway is hydroxylation of the intermediates generating biphenylols, which are then excreted (Grimm et al., 2015). However, PCB metabolism can also occur through the mercapturic acid route. In this pathway, the arene oxide intermediates are conjugated to glutathione, which are then further severed to PCB-cysteine conjugates. Subsequently, PCB-thiols are formed after catalysis by bacterial cysteine S-conjugate β-lyase, which are then methylated in the gut to PCB-methyl sulfides, and finally transformed into PCB-methyl sulfone derivatives (MeSO2-PCB) following intestinal absorption and hepatic oxidation (Grimm et al., 2015). Hence, these
reflect the involvement of the gut microbiome in PCB metabolism (Bakke et al., 1982). Furthermore, removal of chlorine atoms and cleavage of the phenyl rings in PCBs has been found to be at least mediated by the gut bacteria *Clostridium perfrigens* and *Clostridium beijerinckii* (De et al., 2006). MeSO₂-PCBs accumulate in lung, liver, kidney and adipose tissue in rodents and humans, where it exerts toxicological effects (Brandt et al., 1982; Shigematsu et al., 1978). Choi et al. (Choi et al., 2013) explored the interplay between the gut microbiota, PCB exposure and exercise in C57BL/6 mice. Half of the mice exercised for a 5-week period, whereas the other half were sedentary. The mice were then orally treated with a PCB mixture (PCB138, PCB153, PCB180) at environmentally relevant doses (150 μmol/kg in total) for 48 hours, and fecal samples were analyzed with 16S rRNA gene sequencing. The overall abundance of gut microbiota was significantly reduced in PCB-exposed mice, with a notable deleterious effect on *Proteobacteria*. However, these changes were more prominent in sedentary mice, while the exercise level was positively correlated with higher bacterial diversity and attenuation of antibacterial changes. Moreover, members of *Erysipelotrichaceae* were decreased in exercised mice exposed to PCBs. Interestingly, studies have indicated an elevated abundance of *Proteobacteria* and *Erysipelotrichaceae* in association with IBD and colorectal cancer respectively (Chen et al., 2012; Nagalingam and Lynch, 2012). In this study it was hypothesized that the exercise-induced intestinal secretion of bile acids, might have caused selective antibacterial effects on gut microbiota (Choi et al., 2013). Nevertheless, the role of exercise in shaping the gut microbiota response to PCBs and other chemicals is yet unclear (Clarke et al., 2014).

As previously mentioned, POPs as well as PCBs, mostly bind to AhR to elicit their toxic properties, and studies suggest that AhR function is a major factor of gut homeostasis (Monteleone et al., 2013). Zhang et al. (L. Zhang et al., 2015) experimented on the impact of 2,3,7,8-tetrachlorodibenzofuran (TCDF), a specific PCB pollutant, on gut microbiota composition and host metabolome of C57BL/6J mice in relation to AhR activity. For this purpose, wild-type and AhR-knockout mice were dietarily exposed to a high-dose of TCDF (24μg/kg bw/day) over the course of 5 days. Exposure to TCDF significantly modified gut microbiota in wild-type mice, with a reduction in *Firmicutes*, *Clostridia* and *Oscillibacter* spp. and enrichment of *Bacteroidetes*, *Flavobacteria* and *Butyrivibrio* spp. in the cecum, in addition to a deleterious effect on the ileal populations of segmented filamentous bacteria (SFB). These bacterial alterations were correlated with changes in host gut metabolome including elevated synthesis of bile acids and SCFAs (propionate and butyrate), impaired hepatic metabolism of glucose and fat and enhanced intestinal inflammatory response. These metabolic disruptions were attributed to a TCDF-mediated downregulation of FXR and induction of bacterial fermentation. On the other hand, the gut microbiome and metabolic profile of AhR-knockout mice were not markedly affected after TCDF treatment, indicating a possible AhR-dependent pattern of TCDF toxicity that could contribute to other metabolic disorders and obesity (Myre and Imbeault, 2014).
In order to further evaluate the interaction between PCBs and obesity, a recent study conducted by Chi et al. (Chi et al., 2018) treated C57BL/6 mice with a mixture of PCBs (P77, P126, P153) at different doses (5 mg/kg of P77 or P153, and 50 μg/kg of P126) for 6 weeks, or combined with a HF diet for 20 weeks. PCBs and/or a HF diet greatly influenced gut microbiota elevating Firmicutes and reducing Bacteroidetes phyla, an alteration which has been positively correlated with obesity (Ley et al., 2005). Furthermore, PCB treatment led to increased body weight, higher adiposity and inflammation via upregulation of TNF-α, IL-6 and inducible nitric oxide synthase (iNOS). The co-enhancement of these changes with a HF diet suggests a possible vulnerability of obese individuals to PCB exposure, which could be mediated by increased TLR4 signaling (Kim et al., 2012). Recently, the role of gut microbiota as a modulator between PCB exposure and bile acid metabolism was further investigated. In this study (Cheng et al., 2018), C57BL/6 mice were orally administered with environmentally relevant doses of a Fox River Mixture of PCBs (6 or 30 mg/kg bw/day) for 72 hours. Low-dose PCB exposure markedly increased the members of Akkermansia muciniphila, Bacteroides, Bifidobacterium, Clostridium scidens and Enterococcus, which are known mediators of bile acid metabolism (Klaassen and Cui, 2015; Pierre et al., 2016). Indeed, elevated serum and intestinal levels of bile acids were detected in PCB-exposed mice, indicating a positive association with gut microbiota variations. Furthermore, these bacterial alterations induced the production of tauro-muricholic acids alpha and beta, which might contribute to the disruption of bile acid homeostasis through their antagonistic potency on FXR (Sayin et al., 2013).

Another types of POPs are dioxins, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which are also known to be modulators of AhR activity (Whitlock, 1990). TCDD and other dioxins are by-products of organic waste, wood, or fuel combustion, and the herbicide industry. They can contaminate seafood and dairy farm animals following water pollution. Thus, human exposure is mainly dietary (Yamashita et al., 2000). The primary effects of TCDD on human health are aberrant intestinal immunity, immune cell depletion and liver dysfunction (Fader et al., 2015). Lefever et al. (Lefever et al., 2016) indicated that CD-1 hyperglycemic mice injected with streptozotocin, and exposed orally to TCDD (6 μg/kg BW) for 26 weeks, showed substantial alterations in gut microbiota composition. At the phylum level, TCDD treatment enriched the numbers of Firmicutes and reduced that of Bacteroidetes, whereas at the family level the populations of Lactobacillaceae and Desulfovibrionaceae were notably elevated and Prevotellaceae were decreased in the fecal samples tested. These changes in the gut microbiome were negatively associated with liver toxicity, and positively correlated with diminished CD3+NK+ T-cells after chronic TCDD exposure, findings that are consistent with those of previous studies (De Minicis et al., 2014; Van Kaer et al., 2015). Since gut dysbiosis has been linked to increased bacterial species that harbor antimicrobial resistance genes (ARGs) (Larsen et al., 2010), Stedtfeld et al. (Stedtfeld et al., 2017b)
recently evaluated the impact of TCDD (0 to 30 \( \mu \)g/kg) on the gut microbiota of C57BL/6 mice for either 28 or 92 days. Quantitive PCR analysis of fecal samples revealed a significant increase in the abundance of \textit{Enterobacteriaceae} family members that typically harbored ARGs, including multidrug-resistant genes to various antibiotics (aminoglycoside, macrolide, and erythromycin), within 8 days of TCDD treatment. In order to further examine the interaction between TCDD, gut microbiota, and host immunity, C57BL/6 gnotobiotic mice were supplied via oral gavage of TCDD (30 mg/kg) for a 28-day period, following colonization with SFB and \textit{Bacteroides fragilis} (Stedtfeld et al., 2017a). TCDD exposure suppressed the expression of host ileal immune cell genes, such as TLR5 and genes associated with major histocompatibility complex class II (MHC-II), possibly favoring the enrichment of SFBs. Additionally, the populations of \textit{Bacteroides fragilis} were reduced in response to TCDD, upregulating polysaccharide A genes, including \textit{wcfQ}, as a sign of distress. Hence, the results indicate a possible modulating relationship between TCDD-mediated responses and gut microbiota.

Polycyclic aromatic hydrocarbons (PAHs) are POPs of major concern, as they are highly toxic and omnipresent environmental pollutants. Ingestion of contaminated vegetables and charcoal-grilled or smoked meat products is the main route of human exposure (Lee et al., 2016; Veyrand et al., 2013). PAH exposure may also occur through inhalation of air particles generated by tobacco smoke, household heating and engine exhaust by fuel combustion, which could also reach the gut through mucociliary clearance (Mutlu et al., 2011). PAHs also behave as AhR ligands, and their toxicity spectrum includes endocrine disrupting (estrogenic or anti-estrogenic) and tumorigenic properties (lung or bladder cancers) (Bosetti et al., 2007).

One of the most well-described compounds of the PAH group is benzo[a]pyrene (B[a]P), which has been characterized as a human carcinogen group 1 class of compound due to its genotoxic, mutagenic and tumorigenic activity observed in human and animal model studies (Hudson et al., 2013; White et al., 2016). After biometabolism by human gut microbiota, hydroxy-metabolites of B[a]P are produced (mostly 7-OH B[a]P), which possess strong estrogenic as indicated by in vitro studies (Van de Wiele et al., 2005), suggesting that the colonic microbiome is responsible for the bioactivation of PAHs. In addition, detoxification of B[a]P can be reversed by the human or rodent gut microbiota, which re-enables its toxicological potency (Renwick and Drasar, 1976). Once B[a]P enters the enterohepatic circulation, it is further converted by CYP enzymes in the liver and the gut to diol-epoxide derivatives (mainly B[a]P-7,8-dihydrodiol-9,10-epoxide), which are highly carcinogenic after forming DNA adducts (van Herwaarden et al., 2009). In a study by Ribiere et al. (Ribière et al., 2016), C57BL/6 mice were administered orally with a toxicologically relevant dose of B[a]P (50 mg/kg BW) for 28 days to examine its impact on the intestinal mucosa and its associated microbiota. B[a]P treatment markedly perturbed the abundance of the fecal and mucosal gut microbiome, increasing pro-inflammatory bacteria belonging to the families \textit{Alcaligenaceae}, \textit{Bacteroidaceae}, \textit{Erysipelotrichaceae}, \textit{Paraprevotellaceae}, \textit{Paratuberculosisaceae}, \textit{Ruminococcaceae}, \textit{Streptococcaceae}, \textit{Lachnospiraceae}, and \textit{Rikenellaceae}.
Porphyromonadaceae and Turicibacter. Contrastingly, populations of beneficial bacteria including Lactobacillaceae, Lachnospiraceae, Mucispirillum, Ruminococcaceae and Verrucomicrobiaceae were reduced. These alterations were correlated with histologically detected signs of ileal and colonic moderate inflammation, thus creating an intestinal susceptibility for the development of IBD and colorectal cancer (Chen et al., 2012; Morgan et al., 2012). Although recently no substantial shifts in the bacterial communities of human fecal microbiota were observed in vitro (Defois et al., 2017), acute B[a]P exposure greatly altered gut bacterial activity, regarding the volatolome (synthesis of volatile organic compounds) and the metatranscriptome, in a dose-dependent manner. More specifically, various pathways concerning co-factor, vitamin, aromatic and cell wall compound metabolism, in addition to DNA replication and repair mechanisms were stimulated, while pathways related to microbial chemotaxis, carbohydrate and glucose metabolism were suppressed. These changes imply that B[a]P can modify the gut microbial ecosystem.

The results of the above studies regarding POP exposure and effects on gut microbiota are summarized in Table 2.

Table 2. Summary of various experimental studies reporting the impact of persistent organic pollutants (POPs) and mixtures on gut microbiota and their effects on host health

<table>
<thead>
<tr>
<th>Pollutant</th>
<th>Dosing regimen</th>
<th>Animal model/method of microbiota analysis</th>
<th>Impact on gut microbiota</th>
<th>Outcomes</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>PCB mixture</td>
<td>150 µmol/kg via oral gavage, with or without exercise, for 48 hours</td>
<td>- C57BL/6 male mice</td>
<td>- Sedentary group: Proteobacteria, Exercise group: Erysipelotrichaceae</td>
<td>Attenuation of PCB-induced alterations through exercise</td>
<td>(Choi et al., 2013)</td>
</tr>
<tr>
<td>TCDF</td>
<td>24 µg/kg per day in dough pills for 5 days</td>
<td>- Wild-type C57BL/6 male mice, AhR+/-</td>
<td>- Wild type mice: Firmicutes, Clostridia, Oscillibacter spp. and SFB, (+): Bacteroidetes, Flavobacteria and Butyribiobio spp., (-): AhR+/- mice, None significant</td>
<td>Stimulation of bile acid and SCFA synthesis, - Hepatic lipid and glucose metabolism, - Intestinal inflammation</td>
<td>(L. Zhang et al., 2015)</td>
</tr>
<tr>
<td>PCB mixture (77, 126, 153)</td>
<td>6 mg/kg (P77 or P153) and 50 μg/kg (P126) via oral gavage per week for 6 weeks and then with HF diet for 14 weeks</td>
<td>- C57BL/6J female mice - 16S rRNA gene sequencing</td>
<td>(-): Bacteroidetes (+): Firmicutes</td>
<td>Increased body weight, adiposity and systemic inflammation (Chi et al., 2018)</td>
<td></td>
</tr>
<tr>
<td>Fox River PCB mixture</td>
<td>6 or 30 mg/kg per day via oral gavage for 3 days</td>
<td>- C57BL/6J female mice - 16S rRNA gene sequencing, qPCR</td>
<td>(+): Akkermansiamuciniphila, Bacteroides, Bifidobacterium, Clostridiumscidens, and Enterococcus</td>
<td>Alterations in bile acid metabolism (Cheng et al., 2018)</td>
<td></td>
</tr>
<tr>
<td>TCDD</td>
<td>6 μg/kg bw biweekly via oral gavage for 26 weeks</td>
<td>- CD-1 hyperglycemic male mice - 16S rRNA gene sequencing</td>
<td>(-): Bacteroidetes and Prevotelaceae (+): Firmicutes, Lactobacillaceae and Desulfovibrionaceae</td>
<td>Hepatic and immune toxicity (Lefever et al., 2016)</td>
<td></td>
</tr>
<tr>
<td>TCDD</td>
<td>0 to 30 μg/kg every 4 days via oral gavage for 28 or 92 days</td>
<td>- C57BL/6 female mice - qPCR</td>
<td>(+): Enterobacteriaceae</td>
<td>Enrichment of ARG-harboring bacterial groups (Stedtfeld et al., 2017b)</td>
<td></td>
</tr>
<tr>
<td>TCDD</td>
<td>30 mg/kg every 4 days via oral gavage for 28 days following colonization</td>
<td>- C57BL/6 germ-free female mice - 16S rRNA gene sequencing, qPCR</td>
<td>(-): Bacteroidesfragilis (+): SFB</td>
<td>Modulation of host immune and inflammatory response (Stedtfeld et al., 2017a)</td>
<td></td>
</tr>
<tr>
<td>B[a]P</td>
<td>50 mg/kg bw per day via oral gavage for 28 days</td>
<td>- C57BL/6 male mice - 16S rRNA gene sequencing</td>
<td>(-): Lactobacillaceae, Lachnospiraceae, Mucispirillum, Ruminococcaceae and Verrucomicrobiaceae (+): Alcaligenaceae, Bacteroidaceae, Erysipelotrichaceae, Paraprevotellaceae, Porphyromonadaceae and Turicibacter</td>
<td>Ileal and colonic inflammation (Ribière et al., 2016)</td>
<td></td>
</tr>
</tbody>
</table>
8. Non-caloric artificial sweeteners

Non-caloric artificial sweeteners (NAS) are food additives, which substitute the taste effects of sugar along with reduced dietary caloric intake (Gardner et al., 2012). Since obesity has become a worldwide health issue, NAS have been a favorable alternative providing a seemingly healthier lifestyle. It is reported that approximately 22% of obese individuals drink beverages enriched with NAS on a daily basis (Bleich et al., 2014), and consumption of NAS-associated products is very popular in countries such as the USA and Japan (Swithers, 2013). NAS usage is considered as beneficial especially for individuals suffering from obesity, diabetes, or glucose intolerance, controlling weight gain, glycemic load and caloric levels (Fitch and Keim, 2012; Gardner et al., 2012). However, several studies have reported associations between NAS and elevated risk of metabolic disorders (Pereira, 2014) such as hypertension (Kim and Je, 2016), obesity (Swithers, 2013), and type 2 diabetes (Imamura et al., 2015). Saccharin (Sac), aspartame (Asp), sucralose (Suc), and acesulfame-potassium (Ace-K) are the most well-known and widely used NAS with each having a distinct chemical structure and metabolism (Yang, 2010).

Saccharin (Sac), a naphthalene derivative, was the first NAS with a 400 times greater sweetening power than sugar and a maximum recommended dose of 5 mg/kg bw/day. It is slowly absorbed by the gut and excreted in the urine, and has been linked to disturbed glucose tolerance (Abou-Donia et al., 2008). Aspartame (Asp), a di-amino acid ester, has a 180-200 times greater sweetening power than sugar and a maximum recommended dose of 40 mg/kg bw/day. Asp is completely hydrolyzed in the gut into aspartic acid, methanol, and phenylalanine and then absorbed (Butchko et al., 2002). Asp consumption has been correlated with neurobehavioral disorders and deregulation of glucose and weight control in diabetes (Humphries et al., 2008) and has also been found to have carcinogenic properties (Soffritti et al., 2014). Sucralose (Suc), a chlorinated disaccharide, possesses a 600 times higher sweetening power than sugar, has a maximum recommended dose similar to Sac, and it is generally not absorbed being mainly excreted (65-95%) intact in feces (ADA, 2004). Suc
ingestion has been reported to cause gut microbiota disturbances and to interfere with the bioavailability of orally-administered medications (Abou-Donia et al., 2008). Most recently, Suc has been demonstrated to induce hematopoietic neoplasias in male mice (Soffritti et al., 2016). Lastly, acesulfame-potassium (Ace-K), an acidic cyclic sulphonamide derivative, possesses a 200 times higher sweetening power than sugar and has a maximum recommended dose of 15 mg/kg bw/day, and is mostly (95%) excreted intact in the urine (ADA, 2004). High doses of Ace-K have been linked to enhanced glucose uptake by enterocytes by stimulating translocation of the GLUT2 transporter (Zheng and Sarr, 2013).

Since the main route of human exposure to these NAS is via ingestion, there is a distinct possibility that these compounds might also influence the status of the gut microbiota including its biochemical activity.

In a study by Abou-Donia et al. (Abou-Donia et al., 2008), Sprague-Dawley rats were orally exposed to Splenda®, a commercial Suc-based sweetener (Suc 1.1% + maltodextran), in several doses (100, 300, 500, and 1000 mg/kg) for a total of 12 weeks. Fecal analyses revealed gut microbiota dysbiosis, with a significant reduction, even at the lowest dose, in the abundance of anaerobic bacteria, particularly those belonging to Bacteroides, Bifidobacterium and Lactobacillus, which are generally considered as beneficial species, whereas the numbers of Enterobacteriaceae were not affected in treated animals. Furthermore, Splenda® treatment led to an increased mean fecal pH, a finding that may be associated with a decrease in SCFA-producing bacteria (Wong et al., 2006). Such microbiota-mediated changes could result in irritable bowel syndrome, IBD, obesity, or colorectal cancer (Fooks and Gibson, 2002).

Suez et al. (Suez et al., 2014) have described that oral administration of various common NAS (Sac, Suc, or Asp) via drinking water to C57BL/6 mice for 11 weeks induced glucose intolerance in response to all three substances compared to controls. Since this impairment was more pronounced in Sac-exposed mice, the authors further assessed the effect of Sac alone at a human relevant dose (5 mg/kg bw/day). Fecal 16S rRNA gene amplicon sequencing analysis showed a Sac-mediated gut dysbiosis, with proliferation of Bacteroides and Clostridiales, and reduction of Firmicutes and Lactobacillus members. Interestingly, no disturbance in glucose tolerance was observed in either antibiotic-treated or germ-free mice. In contrast, transplantation of gut microbiota, from the feces of Sac-consuming mice or in vitro cultures exposed to Sac, to germ-free mice perturbed glucose homeostasis and altered their gut microbiome in a similar manner. Further analysis demonstrated enhanced gut fermentation pathways of glycans into SCFAs such as acetate or propionate in Sac-exposed mice (Markle et al., 2013; Turnbaugh et al., 2006). Thus, NAS, especially Sac, directly influences gut microbiota composition and function and may be causative in the disruption of glucose metabolism. In addition, the authors investigated the effects of NAS in a human cohort study of non-diabetic individuals (Suez et al., 2014) who mentioned frequent Sac
consumption as validated by a food questionnaire. Sac exposure was positively correlated with increased fasting serum glucose, glucose tolerance, glycated hemoglobin A1c, central obesity, and serum alanine aminotransferase. Significant correlations were also reported between Sac consumption and altered gut microbiota, characterized by elevated *Actinobacteria*, *Deltaproteobacteria* and *Enterobacteriaceae* phyla. Finally, the researchers also conducted a small intervention trial in seven healthy human volunteers (Suez et al., 2014), who reported no previous NAS intake, and who were treated with Sac at the maximum recommended intake dose (5 mg/kg bw) daily for 7 days. The majority of these individuals (4/7), presented glucose intolerance and thus termed as “responders”, while the rest (3/7) designated as “non-responders” since their glucose levels remained unaffected by Sac ingestion. The gut microbiota composition of “responders” was also notably changed as opposed to “non-responders”. Transplantation of fecal microbiota only from “responders” to germ-free mice induced glucose intolerance and altered their gut bacterial profiles, demonstrating an increase in the abundance of *Bacteroides fragilis* and *Weissella*, whereas that of *Candidatus Arthromitus* was reduced. These results suggest a more personalized response of human gut microbiota to NAS exposure, possibly depending on the compositional variations between individuals and the initial structure of the gut microbiome.

Similar findings were reported in a study by Palmnas et al. (Palmnas et al., 2014), where Sprague-Dawley rats were administered with low doses (5-7 mg/kg bw/day) of Asp through drinking water, and fed either on a standard chow or a HF diet. Asp consumption led in increased fasting glucose and abnormal insulin resistance in both dietary rat groups despite favorable effects on body composition, due to reduced caloric uptake. The gut microbiota was also substantially altered, showing increased abundance of *Enterobacteriaceae* and *Clostridium leptum*. Furthermore, diet-specific Asp changes were observed with elevated numbers of *Roseburia* spp. In addition, Asp-treated rats showed higher serum propionate levels, implying an Asp-mediated gut dysbiosis favoring SCFA producing bacteria (Puertollano et al., 2014), which in turn could elicit hepatic gluconeogenesis (De Vadder et al., 2014). Asp conversion into its cyclic dipeptide 2,5-diketopiperazine (DKP), as well as methanol produced via Asp metabolism may be responsible for its antibacterial effects (Caldwell, 1989; Martins-Teixeira and Ivone, 2007).

The role of Ace-K, concerning gut microbiota composition and metabolism, was not thoroughly explored until a recent study by Bian et al. (Bian et al., 2017a) where CD-1 mice were exposed to Ace-K by gavage (37.5 mg/kg bw/day) for a 4-week period. Ace-K treatment markedly induced perturbations in the gut bacterial community and metabolic pathways in a sex-dependent manner. Specifically, male mice showed significant weight gain, enhancement of functional bacterial genes related to energy or carbohydrate metabolism, and increased abundance of *Firmicutes* and *Bacteroides*. In contrast, female mice showed a reduction in several bacterial metabolites (d-lactic acid, succinic acid, 2-oleoylglycerol) and a decrease in
Lactobacillus, Clostridium, Ruminococcaceae and Oxalobacteraceae. In addition, elevated expression of genes involved in an inflammatory response to LPS synthesis was observed in Ace-K-exposed mice. The impact of exposure to human relevant doses (5 mg/kg) of other NAS, such as Suc (Bian et al., 2017b) or Sac (Bian et al., 2017c) was further evaluated on the gut microbiome and host inflammatory response in C57BL/6 mice for 6 months. Suc and Sac treatment markedly affected the composition of gut microbiota. Ruminococcaceae (Ruminococcus), which have previously been associated with IBD (Willing et al., 2010) were increased, whilst Dehalobacteriaceae (Dehalobacterium), Lachnospiraceae (Anaerostipes and Ruminococcus) and Streptococcaceae (Streptococcus) that have been characterized as anti-inflammatory bacteria (Fernández et al., 2016) were decreased in Suc-exposed mice. On the other hand, Sac treatment increased the population of Corynebacteriaceae (Corynebacterium) that have been linked to chronic inflammation (Chamulitrat et al., 1995), and reduced Lachnospiraceae (Anaerostipes, Dorea and Ruminococcus), which are considered as protective species against inflammation (Fernández et al., 2016). Upregulation of pro-inflammatory hepatic pathways including iNOS, TNF-α, and matrix metalloproteinase-2 (MMP-2) was observed in both NAS groups. Hence, it was hypothesized that ingestion of either Suc or Sac disrupted the gut microbiota population towards a more inflammatory profile, which in turn enhanced various bacterial inflammatory pathways (LPS, bacterial toxins, flagella and fimbriae), stimulated the expression of bacterial pro-inflammatory metabolites (quinolic acid), and suppressed that of anti-inflammatory metabolites (kynurenic acid, ethanolamide derivatives), finally resulting in hepatic inflammation (Bian et al., 2017b; Bian et al., 2017c).

Gut microbiome dysbiosis caused by Suc has also been reported by Uebanso et al. (Uebanso et al., 2017). These authors found disruptions in the gut microbiome and host metabolism in response to Suc but not to Ace-K consumption at the maximum recommended dose (15 mg/kg bw/day) for 8 weeks. The relative abundance of Clostridium cluster XIVa was reduced in the fecal microflora of Suc-treated mice, a fact that was correlated with elevated ratio of secondary/primary bile acids, particularly cholic acid (Ridlon et al., 2006), and decreased luminal levels of butyrate which have been associated with aberrant gut immunity and colitis (Atarashi et al., 2011).

9. Discussion

The understanding of xenobiotic metabolism by bacteria inhabiting the gastrointestinal tract is necessary to estimate the potential mixture effects of environmental pollutants. Exposure to heavy metals, pesticides, NPs, PAHs, dioxins, furans, PCBs, and NAS affects the gut microbiome and associates with the development of various diseases through many mechanisms (Figure 1). Collective data from the above studies indicate that ingested pollutants and their mixtures interact with the gut microbiota altering its composition at the
expense of commensal bacteria. As a result, the altered bacterial metabolism impacts the intestinal epithelium, stimulating inflammatory cascades and impairing the gut barrier. The stimulation of immune response and the release of various cytokines progress towards systemic inflammation, furthering various gut or systemic pathological conditions (Ramezani and Raj, 2013; Thomas et al., 2017).

Figure 1. Interaction between toxicant mixtures and gut microbiota and the subsequent pathological effects. (1) Toxicant mixtures reach the gut through ingestion. (2) Exposure to such chemicals leads to various microbial shifts in gut microbiota, favoring the growth of pathogenic bacteria while causing deleterious effects on beneficial bacteria. (3) Bacterial metabolism is also altered leading to decreased production of beneficial SCFAs (e.g. butyrate), bile acid dysmetabolism, and increased synthesis of bacterial toxins and pathogenic metabolites. (4) These changes impair the epithelial cells through stimulation of TLR-mediated inflammatory pathways and elevated ROS production. Moreover TJ proteins are damaged, thus increasing gut permeability and infiltration of lamina propria by bacterial metabolites, pollutants, and pathogenic bacteria. (5) Consequently, host immunity is induced with activation of DCs and MPs, differentiation of T-cells, and secretion of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α). (6) Modulation of gut immune response and systemic inflammation could result in the development of several diseases, including type 2 diabetes, obesity, IBD, CRC, hepatic inflammation and metabolic disorder, and neurobehavioral dysfunction due to disruption of gut-brain axis.
Gut microbiota may be a key player in the toxicity of these contaminants and thus it should be considered as a biomarker for exposure to environmental chemicals. Although the results of the studies reviewed were not consistent across different investigations conducted, gut microbiome profile alterations resulting from the exposure to different environmental pollutants were generally comparable. A reduction in alpha diversity, as well as an elevation in the abundance of bacterial families, which are generally known as pathogens such as Enterobacteriaceae, is an effect encountered in many studies. This is most likely a marker of ill health rather than a mode of action indicating a direct effect of a given chemical on the gut microbiome, and thus a causative link with disease. The induction of aberrant immune response in the gut, which subsequently results in systemic inflammatory response, thus furthering the development and progression of various diseases, could be a possible mechanism underlying these interactions (Figure 2). Although the gut microbiome harbors a large range of enzymatic functions, which can be interfered with chemicals, few studies provide a direct mechanism linking microbiome profile alterations to an effect on bacterial metabolism. Most studies are performed with high doses causing acute toxic effects, which are not representative of real life exposure scenarios. This is in line with historical paradigms in toxicology but not necessarily the most suitable to study chemical mixture effects (Tsatsakis et al., 2018b). Future studies should focus on elucidating the detailed endogenous mechanisms of the interference between chemical exposure at human relevant doses and gut dysbiosis, as well as the long-term effects on host health. This can be achieved in laboratory animals (Tsatsakis et al., 2017), or in simulators of the human gut microbiome (Garcia-Villalba et al., 2017). Key parameters such as genes, environmental exposure, lifestyle, diet and the microbiota all play a role when interfering with each other upon their combined interactions, affecting the bacterial and host metabolome, hence alternative phenotypes appearing (Figure 3).
Figure 2. Induction of dysregulated immune response following gut exposure to toxicant mixtures. Changes in gut microbiota composition and metabolism, as well as impairment of gut epithelium, due to the interaction between gut microbiota and toxicant mixtures, lead to increased infiltration of the lamina propria by various bacterial and chemical components that inevitably interact with immune cells. MΦs are recruited from circulating differentiated monocytes through stimulation of CCR2 receptor, which are then activated (possibly through IFN-γ and TLR signaling), thus causing phagocytosis and secretion of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), ROS and NO. Furthermore, DCs are also activated via antigenic recognition and TLR stimulation, resulting in antigen presentation to naive Th0 cells. This fact combined with several secreted cytokines triggers the polarization of Th0 cells to Th1 (via IL-12 from MΦs and DCs), Th2 (via IL-4 mainly from Bas and NKTs) and Th17 (via IL-1, IL-6 and TGF-β from DCs, and IL-23 from MΦs and DCs) cells. Subsequently, numerous pro-inflammatory cytokines are also produced from these cells after their differentiation. Moreover, the secretion of IL-1 and IL-6 from reactive gut ECs further fuels the activation of MΦs, DCs and T cells. All above phenomena create a condition of aberrant gut immunity, thus inducing systemic inflammatory response which constitutes a causative factor for many diseases.

Figure 3. Genetics, exposure to various environmental chemicals, lifestyle, nutritional habits and the composition of microbiota (especially in the gut), greatly impact the host and the bacterial metabolome through their combined effects. Under such conditions, the bidirectional interaction between these metabolomes form a metabolic fingerprint, that inevitably determines the host phenotypic status.

The study of the gut microbiome should also be included in the battery of tests performed by companies when they apply for market approval of their chemical products. This can be included either in the long-term tests performed to evaluate chronic toxicity or as a separate assay, which can be performed in rodents or in simulators of the human gut microbiome. The gut microbiome should also be included in the development of physiologically based pharmacokinetic (PBPK) model systems, which would include the potential for the gut microbiome to metabolize xenobiotics.

The study of pesticide effects on the gut microbiome should also take into account the fact that substances are not single chemicals, but mixtures made of an active ingredient, which is mixed with other compounds (collectively known as co-formulants or adjuvants), which are added to increase the stability and penetration of the active principle (Mesnage and Antoniou, 2017b). Only active ingredients are tested in the battery of regulatory tests and co-formulants are considered as inert diluents although they can be more toxic than the active ingredient itself (Mesnage et al., 2013). Moreover, farmers are never using pure active ingredients. There is also confusion in the scientific community when authors report that they tested a pesticide (e.g. GLP) although they tested a commercial formulation containing other toxicants.
(e.g. RU). This creates reproducibility issues because pesticide formulations containing a
similar active ingredient can have very different profiles (Mesnage et al., 2015; Mesnage et
al., 2019). It is thus important to emphasize that the products, which are tested in studies of
chemical mixtures, and which are considered as single ingredients, are themselves
sometimes mixtures. Studies have shown that GLP has different toxic effects on the gut
microbiome in comparison to its commercial formulations (Mao et al., 2018; Nielsen et al.,
2018).

We found that a search of the available literature revealed that alterations of gut microbiome
caused by these xenobiotics were poorly reproducible, possibly due to differences in
analytical methods, animal models, dosage, and duration of exposure.

Furthermore, it is important to note that in the majority of the studies whose findings we have
summarized in this review investigated the composition of the gut microbiome by 16S rRNA
gene amplicon sequencing, which is not capable of revealing a true comprehensive picture of
both the quality and quantity of gut microbiota. Thus, future work should employ a shotgun
metagenomics approach, which allows the identification of whole genomes and thus
circumvents the problem of the choice of appropriate PCR primers. As a result, metagenomics allows a higher resolution compared to 16S rRNA gene amplicon analysis and
covers a larger range of microorganisms (bacteria, fungi, viruses and small eukaryotes).
However, a gut metagenomics is still very limited with respect to providing information of the
biological, functional activity of the gut microorganisms that are detected. Biological
pathway analysis is possible with current metagenomics analytical pipelines but it remains
limited due to the quality of database annotations and the completeness of the pathways.
Thus, adding a metatranscriptomics (Lavelle and Sokol, 2018) and metabolomics (Zierer et
al., 2018) analysis to directly gain insight into biochemical activity of the gut microbiome is
increasingly seen as an important tool to understand the function of the gut microbiome. Co-
analysis of the gut metagenome, metatranscriptome and metabolome is even more essential
to perform when one considers that is possible for predominant bacterial species in the gut to
be less transcriptionally active, and thus possibly less contributive to gut microbiome
biochemical status, than lower abundant organisms (Schirmer et al., 2018). Thus, a significant
change in gut microbiome activity stemming from exposure to a toxicant will be missed by
simply looking at the compositional profile of various microorganisms that are present.

Strategies implemented to study mixture effects mostly rely on the knowledge of the
chemicals’ modes of action. Overall, few studies were conducted with a level of detail
sufficient to understand the mode of action on microbial communities. The lack of knowledge
of the metabolism of xenobiotics by the gut microbiome does not allow an accurate prediction
of effects of chemical mixtures. We therefore recommend that new studies be undertaken,
which simulate real-life human exposures in laboratory animals and assess the effects of
long-term exposures to chemical mixtures (Hernandez and Tsatsakis, 2017). The responsible pathways of xenobiotic bioconversion should also be further examined. In addition, the impact of early exposure to chemical mixtures must also be evaluated, as the shape of gut microbiota determines future susceptibility to different disorders at later stages of life. However, in designing future studies to address these gaps in our knowledge, it is important that an experimental design based on a chronic, low-dose exposure to a specific pesticide or other chemical mixture, truly mimics a 'real-life human exposure scenario', otherwise this can raise questions regarding the correct interpretation of results and their human health implications due to study limitations. This also challenges the validity of regulatory safety limits (Tsatsakis et al., 2018a). Some studies (Docea et al., 2018; Hernandez et al., 2013; Tsatsakis et al., 2016; Tsatsakis et al., 2017) have incorporated experimental designs to address these issues and similar methods should be applied also when focusing on effects on the gut microbiome.

10. Conclusions

In this review, we have summarized the various studies underlying the complex bidirectional interaction between gut microbiota and environmental pollutants including heavy metals, pesticides, NPs, PAHs, dioxins, furans and PCBs, and non-caloric artificial sweeteners. Exposure to these chemicals can clearly induce gut dysbiosis and affect the metabolic functions of the microbiome of the host in numerous pathways. Such alterations can possibly lead to several metabolic, malignant, inflammatory, or immune diseases. It is important to note that many alterations in gut microbiome composition can be secondary consequences of toxic effects on internal body organs and systems, and thus they cannot be considered as a mode of action indicative of a direct effect of a given chemical on the gut microbiota. However, gut dysbiosis that results from internal toxicity, may further aggravate the disease condition. We also conclude that variations in bacterial abundance profiles cannot be used to define the mode of action of a chemical in predictive modeling of toxicant mixture effects. We recommend that future studies be undertaken, which simulate real-life human exposures and which evaluate the effects of long-term exposures to toxicant mixtures and which employ a multiomic (metagenomics, metatranscriptomics, metabolomics) analytical approach.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.
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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: