COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Toxicological interactions between xenobiotics and the human microbiota – a scoping paper

Introduction

1. At the March 2019 meeting, in horizon scanning, the COT expressed a wish to review the data on the effect of xenobiotics on the gastrointestinal microbiota and the effects of the microbiota on ingested xenobiotics and how these factors may need to be taken into account, if and as necessary, in risk assessment

2. The term human microbiota refers to the population of microorganisms (bacteria, viruses, fungi, protozoa) living in internal compartments and on the surface of human beings. This paper aims to describe changes in the population and function of the gut microbiota caused by exposure to components of, and contaminants present in the diet and the effects of the gut microbiota in modulating the toxicity of those substances. The search terms used for this paper are found in Appendix 1 and a list of abbreviations in Appendix 2.

3. Unless stated otherwise, the general information on the gut microbiota in this introduction (paragraphs 1 – 23) is taken from reviews by Rowland et al (2018), Jandhyala et al (2015) and Hollister et al (2014).

4. The majority of the internal and external compartments of the human body are inhabited by microorganisms. By far the greatest number and variety of microorganisms is present in the digestive tract, predominantly in the caecum. The majority of these are bacteria that fall into the phyla of the Gram-negative Bacteroidetes and the Gram-positive Firmicutes. Other relatively abundant phyla are the Actinobacteria, the Proteobacteria and the Verrucomicrobia but the full range of species varies from site to site and individual to individual and depends upon diet (David et al, 2014) and locality.

5. Although estimates vary, the number of organisms in the gut appears to exceed that of human cells in the whole body. Estimates of microbe/human cell ratios have decreased in recent years from 10:1 to about 1.3:1 but this depends upon the definition of a cell, for example, whether erythrocytes and platelets can be considered as true cells. (Sender et al, 2016). The commonly cited figures are that 5,000 to 10,000 species, or perhaps many more may be represented, although an individual may harbour many fewer species than this. The gene set of the gut

microbiota – the microbiome – is estimated at about 3 million genes, or about 150 times that of the human genome. All of the above values are estimates and numbers will vary between individuals

6. Identification of the composition of the microbiota was originally problematic because many of the bacteria are obligate anaerobes and/ or have precise pH, temperature or nutrient requirements and could not easily be cultured. Since the Mid-1980s, techniques for identifying bacterial communities from phylum to species level have improved dramatically in speed and efficiency. Sequencing the ribosomal 16s RNA gene has picked out stable and variable regions that can be used as a "fingerprint" to identify unculturable bacteria. The second genomics technique in use is Whole Metagenome Shotgun (WMS) sequencing. These methods cannot discern active from dead or quiescent organisms. High throughput "next generation" sequencing developed in the early 2000s has reduced sequencing time from months or years to hours or days. Large multinational research projects such as the European Metagenomics of the Human Intestinal Tract (MetaHIT) and the American Human Microbiome Project (HMP) have produced data on the microbiome in relation to health and disease. Transcriptomics, proteomics and metabolomics have been used to identify the gene expression and functions performed by the microbiota. (Review by Hiergeist et al (2015)

7. Changes to the relative composition of the gut microbiota, possibly leading to the overgrowth of normally relatively minor taxa and thence to a possible change in the balance of functions, is known as dysbiosis, which may have downstream effects on the health of the digestive tract and the individual as a whole.

8. The composition of the gut microbiota of new-borns appears to be influenced by the method of birth. Babies born by vaginal delivery acquire gut bacterial populations similar to those in their mother's gut and vagina while babies born via Caesarean section (C-section) acquire predominantly skin surface bacteria. (Milari *et al*, 2017). The first stool of C-section neonates has been found to have fewer microbial genes associated with amino and nucleotide sugar metabolism and more related to fatty acid metabolism, amino acid degradation and xenobiotic metabolism, with reduced bacterial diversity compared with those born vaginally (Mueller *et al*, 2017). Children have a more diverse gut population than adults, probably because diet preferences and lifestyle are still being established and habitual diets tend to decrease microbiota diversity (Heiman and Greenway, 2016).

9. Changes in the ratio of Firmicutes to Bacteroidetes ratio are frequently used in the literature to indicate dysbiosis, possibly caused by an ingested substance. Ley et al (2005) found that in homozygous genetically obese (ob/ob) mice, the population of the Firmicutes (F) was significantly increased and that of the Bacteroidetes (B) significantly reduced ($p<0.05$) relative to both wild-type and heterozygous (ob/wt) mice. The authors could not discern whether this difference was a cause or adaptive consequence of obesity. Koliada et al (2017) also found that adult humans had a F/ B ratio that increased in a manner that was significantly associated with

individuals' body mass index (P<0.005). However, these authors also pointed out that an earlier study by Schwiertz *et al* (2010) found that a reduction in the *F/B* ratio was associated with overweight in otherwise healthy human volunteers. In addition, Mariat *et al* (2009) reported that the *F/B* ratio in humans changed significantly with age, such that its median value in infants (aged 3 weeks to 10 months) was found to be 0.4, in adults (25 to 45 years) 10.9 and in elderly people (70 to 90 years) 0.6, indicating natural age-related changes in the make-up of the microbiota.

Metabolites produced by gut microbiota

Short Chain Fatty Acids (SCFAs)

10. The metabolism of carbohydrates that are non-digestible by the host, such as inulin, by various bacterial genera in the gut leads to the production of short-chain fatty acids, primarily acetate, propionate and butyrate, with other lesser components, including branched-chain acids. Butyrate appears to be an important nutrient for the gut epithelium, maintaining its barrier function and thus preventing "leakage" of gut antigens and pro-inflammatory molecules into the general circulation. Butyrate has been found to be effective in reducing the symptoms of ulcerative colitis in humans (Scheppach et al (1992).

11. Kimura et al (2013) investigated the action of SCFAs at adipose tissue-expressed GPR43 G-protein-coupled receptors in wild type and *Gpr*43$^{-/-}$ mice. The knockout mice were obese and the wild type were lean. Activation of the receptor was found to decrease insulin sensitivity and fat accumulation in adipocytes from white, but not brown, adipose tissue, and increase insulin sensitivity in muscle and liver. Acetate was found to suppress insulin-induced glucose and fatty acid uptake in adipocytes from wild-type but not *Gpr*43$^{-/-}$ mice Acetate moreover promoted phosphorylation of PTEN, a known downstream effector of GPR43, which blocks the insulin receptor cascade by dephosphorylating PIP_3. Thus, acetate was found to suppress the effect of insulin in adipose cells without directly affecting insulin receptors. The increase in insulin sensitivity in other tissues was thought to result from GPR43 activity increasing glucose uptake.

12. Oleskin and Shenderov (2016) briefly reviewed observed effects of SCFAs on host neurotransmitter function. Propionate and butyrate appeared to regulate expression of the gene for tryptophan hydroxylase, the rate-limiting step in serotonin synthesis, and decrease the activity of histone deacetylases, which appears to improve various neurological conditions, such as Parkinson's disease, depression and schizophrenia. Such beneficial actions of SCFAs were concentration-dependent since high concentrations, especially of propionate, had been associated with the expression of autism-related genes.

13. Acetate has also been found to mediate intestinal IgA release via activation of GRP43 receptors. This effect was not mimicked by butyrate and was independent of T cells. Acetate did not promote IgA production by directly stimulating B cells but by

activating retinoic acid production of regulatory dendritic cells, which then induced B cells to produce IgA.

14. Morrison and Preston (2016) reviewed recent evidence for the influence of acetate, propionate and butyrate on gut integrity, glucose homeostasis, lipid metabolism, appetite regulation and immune function. They concluded that “The multifaceted roles of SCFA suggest that they may play an important role over the life-course in protecting the body against deteriorating metabolic control and inflammatory status associated with Western lifestyles. “

15. SCFAs thus appear to be multifunctional effectors linking the metabolism of the gut microbiota to host physiology.

Bile acids

16. The conjugated (glycine in humans, taurine in rats) steroidal metabolites of cholesterol that are produced by the liver and stored in the gall bladder are primary bile acids. Primary bile acids are released into the lumen of the duodenum via the bile duct and act as lipid emulsifiers, producing micelles around fats and promoting their uptake. Conjugated bile acids are not reabsorbed by the small intestine but are excreted in the faeces. The gut microbiota are capable of deconjugation, regenerating free steroids that can undergo enterohepatic circulation. The metabolites of the primary bile acids produced by the microbiota are termed secondary bile acids. Bile acids have hormonal actions throughout the body, particularly through the farnesoid X and GPBAR1 (also known as TGR5) receptors.

Others

17. The gut microbiota are capable of synthesising B and K group vitamins, including biotin, cobalamin, folates, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine. Antibiotic treatment affects plasma prothrombin levels in people on a low-vitamin K diet. The *Bacteroidetes, Fusobacteria* and Proteobacteria appear to mostly account for these pathways, with lesser involvement of the Firmicutes and the *Actinobacteria.*

18. Roager and Licht (2018) reviewed the effects of bacterial metabolites of the amino acid tryptophan, arising initially from protein degradation, on host health. Tryptophan undergoes metabolism by the microbiota by oxidation, decarboxylation, diacylation, and amino transfer. The direct metabolites are processed further into products that act as signalling molecules (indole and 5HT), aromatic hydrocarbon receptor ligands and effectors in inflammatory bowel disease.

19. Anaerobic choline metabolism by the microbiota produces trimethylamine (TMA), acetate and ethanol. Dysbiosis leading to aberrant choline metabolism has been proposed as potential contributing factor in non-alcoholic fatty liver disease, and increased TMA in circulation has been mooted as a risk factor for cardiovascular disease and colon cancer. The choline utilization (cut) gene cluster in sulphate

reducing bacteria is thought to be responsible for this pathway. This gene cluster in human gut bacteria encoding TMA-lyase (cutC) is widely distributed across different phyla, but the pathway may also have been acquired in some strains via horizontal gene transfer (Krishnan *et al,* 2015).

Prebiotics and probiotics

20. Prebiotics are foods or components in foods that are supposed to act as substrates for “beneficial” bacteria, those that maintain the healthy functioning of the gut epithelium and restrict the growth of pathogenic species. Examples are inulin and oligofructose. Effects on the host resulting from the consumption of these substances appear to be reduction in blood very-low-density lipoprotein (VLDL), triglycerides and total cholesterol, reduction in gut inflammation and possibly protection against colorectal cancer. (Markowiak and Slizewska 2017).

21. Probiotics are bacteria that are ingested with the intent of maintaining the balance of the microbial communities in the gut, maintain the integrity of the epithelium and prevent the overgrowth of pathogens. Lactobacillus spp or Bifidobacterium spp are often added to yogurts and drinks with this stated intent. Fijan (2014) reviewed the field of probiotics and discussed health claims related to their use and concluded that care should be taken by people with existing conditions such as leaky gut, compromised immune systems or critical illnesses, not least because their effects seem to be strain-specific. It is, however, possible for a suitable organism with resistance to stomach acid and bile to reach its intended target of the large intestine and exert some re-balancing effect on a microbial community in dysbiosis, even if this presence of that organism may itself be transient.

22. Additives to the diet that provide both a prebiotic nutrient and a probiotic bacterium are termed synbiotics, Markowiak and Slizewska (2017) list clinical trials with a range of bacteria and fructo-oligosaccharides. Outcomes showed improvements in cases of obesity, insulin resistance and type 2 diabetes.

23. However, the effects of interventions to elucidate the effects of changes in the balance of the gut microbiota on health have largely been performed on animals and the responses of humans and animals differ. Faecal transplants from obese to lean or from lean to obese mice have been shown to lead to the recipient developing the opposite phenotype, but this has not been proven to occur in humans. The prevalence of various taxonomic groups of bacteria in the human GI tract has been correlated with type-2 diabetes, obesity and other conditions but evidence of causality is stronger in some cases than others. Wortelboer et al (2019) reviewed current progress in the use of faecal transplants in the treatment of various conditions and disease states. Whereas recurrent or refractory Clostridium difficile infection is now recognised has being amenable to this treatment, the evidence for efficacy in inflammatory bowel disease, ulcerative colitis, irritable bowel syndrome, Crohn’s disease and other conditions is hampered by variable results and the need for further studies. If the baseline involvement of the taxa of the microbiota in human

pathology has not been established, then the effect of xenobiotics and their metabolites is even more uncertain. The following is a description of effects largely observed in experimental animals, noting, where considered, how the changes may show correlation with the human GI flora.

Effect of xenobiotics on the gut microbiota

Metals

24. Constante et al (2017) found that the iron concentration in the food of C57BL/6 mice affected the colon luminal iron concentration and the composition of the microbiota at the species level without significantly affecting the ratio of the major phyla. Within a range of species, the growth of some increased and others decreased as the iron in the diet increased from deficient (5 mg/kg), through adequate (50 mg/ kg) to excessive (500 mg/kg). The iron formulation (ferrous sulphate (FS), ferrous bisglycinate (FBG) or ferric EDTA (FEDTA)) also exerted species-level differential effects.

25. Using dextran sodium sulphate (DSS) to mimic the mucosal inflammation seen in human inflammatory bowel disease (IBD), the same workers found that FEDTA exacerbated the inflammation whereas FBG and FS were generally protective. Moreover, the presence of iron (as FS) was found to potentiate the anti-inflammatory effect given by the probiotic bacterium Escherichia coli Nissle 1917.

26. Mahalhal et al (2018) reformulated a standard mouse diet to contain half (100 mg/kg) or twice (400 mg/kg) the usual concentration of iron and fed these diets to female C57BL/6 mice that had DSS-induced colitis. The DSS-treated mice on 100 mg/kg iron lost more weight and had more severe colitis than those with higher doses of iron, although the inflammation in the high dose group was also greater than in those with the standard food. Increased iron consumption in DSS-treated mice resulted in a trend to reduced levels of Bacteroidetes and Firmicutes and increased Proteobacteria and Actinobacteria, although the changes between day 1 and day 10 of treatment were not significant.

27. Werner et al (2010) fed either an iron-free diet or a diet containing iron sulphate that led to an estimated dose of 28 mg Fe/kg bw/day for 11 weeks to C57BL/6 wild-type or heterozygous $TNF^{\Delta ARF/WT}$ mice. The latter mice show impaired tumour necrosis factor synthesis and develop ileitis closely resembling human Crohn's disease. Groups of WT and $TNF^{\Delta ARF/WT}$ mice not fed the iron diet were injected intraperitoneally (ip) with $Fe(NO_3)_2$ complexed with nitrilotriacetic acid (together FeNTA) at 90 μmol/kg bw to restore liver non-haem iron. The other mice received isotonic saline ip. Gut luminal iron but not systemic iron was found to induce ileitis in the $TNF^{\Delta ARF/WT}$ mice, indicating that iron injections rather than oral supplements should be used in Crohn's disease patients with anaemia. A range of bacterial genera changed in proportion in the gut, with the iron-free diet, but of

particular note was a decrease in the genus Desulfovibrio, which has been suggested it be involved in the aetiology of inflammatory bowel disease, whereas Bifidobacterium, thought to protect against inflammation, increased.

28. Guo et al (2014) studied the effects if iron and arsenic on the microbiota and the expression of antibiotic resistance genes (ARG) in male ICR mice. Mice were exposed to water or aqueous solutions of arsenic (3 mg/l), iron (5 mg/l) or both at the same concentrations for 90 days, presumably in drinking water, although details were not given. After the dosing period, the animals were euthanased, their gut treated for histopathology and faecal samples taken for DNA profiling and to assess changes in the microbiota. Both arsenic and iron treatment resulted in a. increase in Firmicutes mostly at the expense of Bacteroidetes and Actinobacteria. There was a slight increase in carbohydrate metabolism in the bacterial community that may have been related to the increase in Firmicutes. Exposure to the metals slightly increased the abundance of ARG and changed their proportions, increasing those against tetracyclines predominantly. The metals alone and in combination had positive or negative effects depending on the gene in question.

29. Dheer et al 2015) investigated the effects of arsenic (sodium arsenite at 0, 10 or 250 ppb) in drinking water for 2,5 or 10 weeks on the structure and composition of the colonic microbiome in C57BL/6 mice. Transmission electron microscopy of the colonic mucosal layer showed an initially ordered stratification with small coccoid species near the epithelial layer, over laid by larger cocci and progressively more heterogeneous layers of cocci, rods and filamentous bacteria that extended into the lumen. After 5 weeks the structure showed signs of breakdown, with an apparent shift from stationary to growth phase and abundant spores. After 10 weeks the biofilm structure was no longer seen. Changes in community composition were seen on 16S rRNA analysis, but these were only marked at the 250 ppb dose. Arsenic caused an increase in the Bacteroidetes and a reduction in the Firmicutes at the phylum level, in particular, at the class level, an increase in Bacteroidia *and a* decrease in Clostridia, with minor classes growing and receding to various degrees. The mRNA expression of arsenic resistance genes arsA and arsB was progressively inhibited over time and that of pentahaem nitrite reductase increased, suggesting arsenic-specific gene expression changes. Concomitantly, liver nitrite and plasma arginine levels increased, and the authors suggested that these changes may lead to an increase in NO synthesis, with possible negative cardiac consequences.

30. Chi et al (2016) observed sex-related differences in the growth pattern and function of the microbiome of male and female mice and different effects on these parameters induced by arsenic. In females, up-regulated genes included those for mercury and for zinc resistance and trans-membrane transporters such as the glutathione-regulated K^+- efflux system. In males, for example, hexose phosphate uptake was down-regulated and denitrification up-regulated. The authors suggested that their work provided strong evidence that the sex of the host played a role in microbiome responses to toxicants, but that further work was required to clarify the relationship.

31. The same authors (Chi et al, 2017) studied the sex-specific effects of manganese on the gut microbiome of C57BL/6 mice. Several different mechanisms by which Mn^{2+} ions could disrupt the normal structure and function of the microbiota were proposed to account for observed effects: changes to quorum-sensing mechanisms that affect population density, motility and virulence; circumventing the homeostatic mechanisms in the gut that limit the concentration Mn^{2+} ions and enrich the population in some species; induction of oxidative stress that could influence growth and survival and change the homeostasis of other metals, particularly iron. Sex differences were seen in the expression of metal transport, DNA repair, antibiotic resistance and in the production of neurotransmitters and their precursors that could potentially influence the activity of the gut-brain axis and thence the host nervous system.

32. Gaulke et al (2018) tested the effects of arsenic in combination with marginal zinc deficiency on the microbiome of C57BL/6 mice. Arsenic exposure and marginal zinc deficiency individually affected the balance of the bacterial genera that the group tested, and in combination, reduced levels of zinc appeared to sensitise the microbiome to the effects of arsenic. There was also a relationship between zinc deficiency, arsenic and DNA damage and oxidative stress in the microbiome.

33. Dong *et al* (2017) investigated the effects of arsenic in drinking water on the intestinal microbiota of Bangladeshi children. High arsenic concentrations (218.8 ± 166.1 μg/l) correlated with a relative enrichment of bacteria in the Proteobacteria phylum ($p<0.03$) without statistically significant effects on the Bacteroidetes, Firmicutes or Actinobacteria. Of 322 genes that showed increased expression in the presence of arsenic, 78% (258) were found to be in antibiotic resistant bacteria. The E. coli genes associated with arsenic resistance that were seen in Bangladeshi children were not seen in children in a European cohort, where drinking water As levels are lower, suggesting that the Bangladeshi bacteria had adapted to the metal.

34. Liu et al (2014) examined the effects of 0, 20 and 100 mg/kg bodyweight cadmium on the gut biota of male Balb/c mice over 3 weeks of dosing. In a dose-related manner, cadmium appeared to reduce the overall bacterial growth rate, reduce the Firmicutes / Bacteroidetes ratio, reduce the gut population of Lactobacilli and Bifidobacteria. It also caused thinning of the intestinal mucus layer, increased the level of TNFα in the colon, indicating an inflammatory response, and reduced the production of short-chain fatty acids. The effects were most obvious after 3 weeks of dosing.

35. Ba et al (2017) looked at the effect of early-life exposure to cadmium (100 nM in drinking water) on gut microbiota and fat accumulation in C57BL/6 mice. Female mice were exposed to cadmium before and during pregnancy and weaning and their pups thereafter examined for the effects of the metal. In the male Cd treated group fat mass increased and microbial diversity decreased, and plasma levels of

triglycerides, total cholesterol, free fatty acids, leptin and HDL were significantly raised ($p<0.05$). In the same group, liver triglycerides increased, and fat deposits were observed. In the microbiota, the *Bacteroidetes* were increased at the expense of the Firmicutes. The authors performed a faecal transplant from male Cd-treated and control mice to male non-Cd-treated recipient mice and the fat mass of the Cd-faeces recipients increased significantly after 16 days.

36. Gao et al (2017) found that in female C57BL/6 mice treated for 13 weeks with 10 ppm lead (as lead chloride) in drinking water, the growth of microbiota followed different trajectories, ie population diversity was reduced, along with the expression of bacterial genes. Levels of vitamin E and bile acids produced by the microbiota were reduced by lead treatment and gene expression and metabolites related to nitrogen metabolism were variously increased or decreased, for example, urea decreased and the gene for copper-containing nitrite reductase (catalysing nitrite to nitric oxide) increased. Lead also negatively affected the genes involved in gluconeogenesis, activated oxidative stress-related genes and increased the activity of the gene for the phosphate ABC transporter, which could cause metal precipitation in the gut.

37. With similar methodology to that of Ba et al (2017), above, Wu et al (2016) exposed dams before and during pregnancy and weaning to lead (III) acetate at 32 ppm in drinking water and then analysed the effect on the microbiota of the offspring. Offspring body weights of the male lead-exposed mice were 11% greater than in the non-lead-exposed male mice but no such effect was seen between treatments in the females. No differences were seen in bacterial richness between the lead-dosed offspring and a control group not exposed to lead in the same manner but significantly fewer culturable aerobes ($p< 0.005$) and significantly more culturable anaerobes ($p<0.05$) were found in the faeces of the lead-treated group compared with controls. At phylum level, Bacteroidetes were reduced ($p<0.05$) and Firmicutes were increased ($p<0.005$). The authors speculated that this change in composition would change the metabolic pattern of the microbiota and, in combination with host physiology, account for the purported obesogenic effect of exposure to lead in humans.

38. Xia et al (2018) provided male ICR mice with drinking water containing lead at 0.01, 0.03 and 0.1 mg/l for 15 weeks. In the caecum, the Firmicutes were reduced by all levels of lead whereas a fall in Bacteroidetes was only significant at 0.1 mg/l ($p< 0.05$). The Proteobacteria and Actinobacteria were largely unaffected. In the faeces, the Bacteroidetes were increased at all time points by 0.1 mg/l, with a gradual upward trend whereas the Firmicutes followed a general downward trend at all doses. At the genus level, compositional changes took place in several genera, for example, the Desulfovibrio generally increased while the Ruminococci generally decreased. There were also indications that SCFA metabolism was perturbed. Concomitantly, plasma triglycerides and total cholesterol levels were raised ($p<0.05$) by 0.1 mg/l Pb and in the liver triglycerides were raised by 0.1 mg/l and pyruvate by 0.03 and 0.1 mg/l.

39. Breton et al (2013) exposed germ-free and specific-pathogen-free (SPF) C57BL/6 mice to 5, 20 and 100 ppm cadmium and lead (as the chlorides) in drinking water for 8 weeks. Faecal content in all of the mice increased in metal content with dose but the absolute values were greater in the germ-free animals than in the SPF ones. Levels of Cd and Pb at necropsy were also higher in blood, liver, kidney and spleen in germ-free compared with SPF animals. The authors recognised that anatomical and physiological differences in the germ-free mice compared with the SPF animals (expression of higher levels of metallothionein and changes in the microvilli and caecum wall) could account for these differences but the presence of the gut biota could also account for the reduced uptake.

40. Fazeli et al (2011) found that gavage dosing of male Balb/c mice with aqueous cadmium chloride at 23, 30, 37, 44 and 50 mg/kg bw for 45 consecutive days reduced the titre of a range of culturable anaerobic and aerobic bacteria in the small intestine, large intestine and rectum. Gram negative bacteria seemed more resistant to cadmium, with small numbers of E. coli and Klebsiella spp surviving at the highest doses that obliterated Lactobacillus, Clostridia, Pseudomonas, Enterococcus and Proteus in the large intestine. In contrast, Breton et al (2013) found that Cd in drinking water at 20 or 100 ppm for 8 weeks did not affect bacterial diversity as measured by ribosomal 16S RNA analysis in the caecum and faeces of female Balc/c mice. The relative proportions of different families and genera, however, were markedly affected by Cd exposure. The Breton group suggested that the Fazeli group's results may have been affected by relying on cultured bacteria rather than more sensitive molecular biological techniques.

41. Both marginal (1.5 ppm) and supplemented (20 ppm) copper in the diet of male weanling Sprague Dawley rats increased the gut Firmicutes/Bacteroidetes ratio but different families and genera within the Firmicutes phylum were responsible for the change in ratio of the phyla in each treatment. Some of the increased genera and families had previously been correlated with changes in liver metabolism leading to non-alcoholic fatty liver disease and non-alcoholic hepatic steatohepatitis (Song et al, 2017). Both low and high copper in the presence of fructose were associated with gut microbiota dysbiosis characterised by depletion of the genus Akkermansia, which is thought to be critical in maintaining gut barrier function.

42. Food-grade titanium dioxide was administered orally in drinking water to C57BL/6JAusb mice by Pinget et al (2019) for 3 weeks at 0, 2, 10 and 50 mg/kg bw/day. 16S rRNA gene sequencing from faecal samples revealed that TiO_2 had limited influence on bacterial diversity. although some genera were elevated (eg Parabacterioides, Lactobacilli and Allobaculum) but others (eg Aldercreutzia and unclassified Clostridiaceae) were decreased. TiO_2 at 50 mg/kg bw/day appeared to decrease acetate production (important for host-bacterial interaction) but increased levels of trimethylamine, a product of choline metabolism that has been associated with atherosclerosis. In tests with E. coli and E. faecalis, TiO_2 significantly increased

biofilm production ($p< 0.01$ and $p< 0.05$ respectively). Biofilm has been associated with colitis, reduced wound healing. and colorectal cancer.

43. Mao et al (2019) gavage dosed pregnant Sprague Dawley rats with TiO_2 nanoparticles (primary diameter 21nm, average hydrodynamic diameter 199.5 nm) at 5 mg/kg bw/day from day 5 to day 18 of pregnancy. Maternal blood glucose concentration rose in the TiO_2 NP treated animals which, if replicated in humans may leave pregnant women more at risk of gestational diabetes.

44. Silver nanoparticles (AgNP), like TiO_2 nanoparticles, are widely used in the food and other industries and human beings are thus exposed to them on a daily basis. They are used in food and food packaging materials for the antimicrobial action of the silver ions they release. Van den Brule *et al* (2016) found that oral exposure to silver nanoparticles (55 $\pm$ 2.7 nm in diameter) in a dose-related manner (0, 48, 480 and 4800 ppb in food pellets for 28 days) increased the Firmicutes/Bacteroidetes ratio in the ileum of mice. Within this, the balance of genera was changed beginning at the lowest dose tested. Newly generated Ag NP had a greater effect than aged particles, suggesting that changes in their structure takes place as they age. The authors suggested that sulphidation might be the process that reduces silver ion release.

45. Williams et al (2016) gavage dosed male and female Sprague Dawley rats twice-daily for 13 weeks with AgNP (10, 75 or 100 nm diameter at 9, 18 and 36 mg/kg bw/day), or silver acetate (AgOAC) at 100, 200 and 400 mg/kg bw. Post-mortem ileum scrapings were taken and plated on general and Lactobacillus-selective media and DNA was amplified by PCR to identify bacterial groups. The expression of host immune-response-related genes was also analysed. The AgNP showed antibacterial activity that decreased with increasing particle size. The positive control AgOAC was antibacterial at 100 mg/kg bw and increasingly toxic to the rats with dose, causing severe gastritis. The Bacteroidetes and the Firmicutes were both reduced in absolute terms at the lower doses of the 75 and 110 nm particles in male rats and the Firmicutes were reduced relative to the *Bacteroidetes* in both sexes by the high dose of 110 nm particles. The lower doses of the smaller nanoparticles, but not of the positive controls decreased the activity of genes associated with T-cell activity (such as FOXP3 and *GPR43*), the mucin gene MUC2 and genes related to microbial recognition in the gut (TLR2, TLR4 and NOD2).

46. In contrast, Wilding et al (2016) found very little effect on the microbiota in C57BL/6 mice dosed for 28 days with 10 mg/kg bw/day of 110 nm AgNP compared with a water control. The authors speculated that the lack of effect could have been a result of their relatively low dose of nanoparticles compared with other studies but also that other studies using culture methods to assess the antimicrobial affects may have been seeing the fragility of the bacteria under the conditions used rather than effects of the treatment per se.

47. Yin et al (2019) reported the production of silver nanoparticles in an *in vitro* model of the human gut microbiota (SHIME: the Simulator of the Human Intestinal Microbial Ecosystem). Faecal samples were collected from two healthy volunteers and incubated in a solution containing 1 mM silver nitrate at 37^{o}C for 48 hours. Spherical silver nanoparticles were observed under transmission electron microscopy on the surface and within microbial cells. The authors expressed concern that nanoparticles could be produce by gut microbiota, with unknown consequences for microbial and host health.

48. Cattò et al (2019) studied the interactions between non-lethal concentrations of citrate-capped AgNP (1 μg/ml final concentration), human intestinal microbiota and a probiotic organism (Bacillus subtilis, *BS,* 10^7 cells/ml) in an in vitro batch incubation model. After 24 hours incubation, all conditions (control, AgNP, BS alone and AgNP-BS) led to a depletion of the Bacteroidetes with an increase in the Firmicutes and Proteobacteria. The other treatments all slightly raised the Bacteroidetes level, with treatment with BS alone having the greatest effect. AgNP and AgNP-BS treatment led to a 56% increase in the level of the Megasphoem genus, which has been associated with antibiotic resistance and stress response. At the species level, falls were noted in the titres of Faecalbacterium prausnitzii and Clostridium coccides/ Eubacterium rectales, a condition seen in patients with intestinal inflammation and ulcerative colitis. No treatment affected SCFA production, but AgNP-BS markedly counteracted functional changes induced by AgNP alone treatment, particularly in the microbiota's capacity for xenobiotic degradation and metabolism.

49. The effects of gold nanoclusters (AuNC) on the gut microbiota have also been investigated. Wang et al (2019) synthesised red-light-emitting glutathione-capped AuNCs and gavage dosed them at 1.7 mg/kg/day to female SPF BALB/c mice for 7 days. Faeces were collected at intervals up to the final dosing day and analysed by 16 S rRNA gene sequencing. Treatment with the AuNCs caused fluctuations in the titre of the microbiota at the phylum level but overall had no significant effect at this level up to 3 days. By day 7 the Proteobacteria had increased significantly. Some genera were affected in the AuNC treated group compared with the controls (Roseburia were depleted and Staphyllococcus, Ureoplasma and Methylobacterium were more abundant, $p<0.05$). These phylum and genus changes appear to be associated with a decrease in butyrate production and an increase in gut inflammation.

50. The examples above illustrate that a range of metals, including those added deliberately to the diet, may have effects on the consumer, if not directly then by affecting the composition and metabolism of the gut microbiota. Dose and dosage form may influence the effect of metals on the microbiota.

Pesticides

51. Liu et al (2017) gavage-dosed adult male C57BL/6 mice with p, p'-DDE (the major metabolite of DDT) and β-HCH (the major metabolite of HCH) daily at 1 and 10 mg/kg bw respectively for 8 weeks. Bacterial taxa were analysed by 16S rRNA gene sequencing and changes in bile acid profiles were followed by UPLC-MS analysis of gallbladder contents. RT-PCR was used to determine the mRNA expression of bile acid metabolic genes. Both p, p'-DDE and β-HCH reduced the relative abundance of the Actinobacteria ($p<0.001$ for p, p'-DDE) and the Candidatus Saccharibacteria without affecting the relative abundance of the Bacterioidetes, Firmicutes, Verrucomicrobia or Proteobacteria. Little change was seen at class or order level, but various genera were increased or decreased by both treatments. Both treatments stimulated the genes of bile acid production in the liver and stimulated hepatic bile acid transport. Treatment decreased the expression of ileal bile acid transport protein mRNA, but β-HCH increased the level of bile acid binding protein, suggesting that enterohepatic circulation of bile acids was reduced. The authors suggested that such changes could lead to disorders of cholesterol and triglyceride metabolism as well as glucose and energy homeostasis.

52. Zhang et al (2017) investigated the effect of the organochlorine insecticide endosulfan (0.5 and 3.5 mg/kg bw in corn oil daily by gavage for 2 weeks) on the metabolism of male ICR mice and their microbiome. As well as various positive and negative effects on mouse amino acid metabolism, serum hippurate levels fell with endosulfan treatment. This was associated with gut dysbiosis by the rationale that hippurate is a product of the reaction between glycine and benzoate, the latter arising from the gut microbiota. A further indicator was perturbation of choline metabolism, where increased levels of the choline metabolites dimethylalanine and trimethylamine N-oxide.

53. Nasuti et al (2016) investigated the effects of treating young (post-natal day (PND) 6 to 21) male and female Wistar rats orally with either corn oil or technical grade permethrin (75:25 trans:cis) at 34 mg/kg bw. 16S rDNA analysis was used to determine bacterial concentrations in faeces (log CFU/g) up to PND 141. Various Bacteroidetes species and Lactobacillus spp were increased by permethrin at PND21 ($p < 0.05$) but the effect waned over time and by PND141 the Bacteroidetes spp were significantly reduced ($p < 0.05$) relative to the control. SCFAs were variously affected: acetate was reduced at PND21, propionate was increased at PND 51 and butyrate was unaffected at all time points. Representative species of bacteria from culture collections or the human GI tract were also cultured and treated *in vitro* to determine a Minimum Inhibitory Concentration (MIC) for permethrin. Bacterial species Blautia producta and Bifidobacterium sp B1 were more sensitive to permethrin (MIC 0.4 μg/ml) than potentially pathogenic species such as Staphylococcus aureus and E. coli (MIC 3.2 and 1.6 μg/ml respectively).

54. In addition to its anticholinesterase activity, chlorpyrifos is suspected by some researchers of affecting insulin sensitivity and, through that mechanism, acting as an obesogen. Liang et al (2019) linked chlorpyrifos to insulin resistance via dysbiosis, leading to an increase in lipopolysaccharide that caused low-grade inflammation in

the gut and hence leakiness of the epithelial barrier. Mice fed a high fat diet exhibited all the signs of an increase of pro-inflammatory cytokines and the addition of chlorpyrifos did not significantly affect this. However, chlorpyrifos treatment of mice on a normal diet had symptoms similar to those on the high fat diet.

55. Fang et al (2018) gavage-dosed adult male Wistar rats for 9 weeks with a low and a high dose of chlorpyrifos (0.3 and 3 mg/kg bw respectively). Low dose chlorpyrifos plus a normal fat diet led to an increase in some genera of bacteria and a decrease in others to a greater extent than did the high dose. Both high and low dose chlorpyrifos affected the microbiota in similarly in the high-fat-fed rats. Changes in glucose and lipid metabolic hormones were seen with chlorpyrifos treatment. Although no toxicity relating to anticholinesterase activity was observed, the activity of AChE was reduced by chlorpyrifos treatment and the authors speculated that increased ACh in the gut may impact its immune function and lead to overgrowth of some bacterial genera that have been associated with changes in glucose and lipid metabolism.

56. Reygner, Lichtenburger, et al (2016) gavage dosed pregnant Wistar rats from gestational day 0 (GD0) to litter Day 21 (LD21) and their male pups from litter day 21 (LD21) to LD60 with 2 doses of chlorpyrifos (CPF 1and 3.5 mg/kg bw) in rapeseed oil, with and without inulin in their drinking water (approximately 3.7 mg/g bw/day). No cholinergic toxicity was seen in the dams with CPF and their body weight was unaffected by any treatment. CPF reduced the titre of Firmicutes and other SCFA-producing bacteria and inulin to some extent reversed this trend.

57. Reygner, Condette et al (2016) used the SHIME model to study the direct effects of below-threshold effects of below-threshold chlorpyrifos (1 mg/day) on the composition, diversity and metabolic functions of the human gut microbiota. Changes in the measured parameters were observed in the different compartments of the model but how they were observed depended upon the method used: PCR found no significant change in overall titre over 30 days whereas culturing found a significant increase in both aerobes and anaerobes. Cultured Bacteroides spp and Clostridia spp increased but Bifidobacterium spp decreased in the colon reactor. Of the SCFAs, changes were temporary increases or decreases in different compartments at different times. The authors suggested that although the changes measured were modest, they might have an impact that might affect health in very young infants.

58. Gao et al (2017) tested the effect of 4 mg/l diazinon in drinking water on the gut microbiome of male specific-pathogen-free C57BL/6 mice. Marked sex-specific changes in the relative titres of gut microbiota were observed at the genus level with some species markedly decreased in males but the majority increased, and the majority being decreased in females relative to controls. In conjunction with these changes, gene expression was also perturbed, especially those on the serotonin metabolic pathway.

59. In a similar experiment to that in Paragraph 57 (above) Gao et al (2018) investigated the effects of a chronic sub-neurotoxic dose of the organophosphate malathion (13 weeks' exposure in drinking water at 2 mg/l) on the gut microbiome of male specific-pathogen-free C57BL/6 mice. Different genera of bacteria were positively or negatively affected by malathion exposure, with genes for quorum sensing and those for flagellar proteins upregulated as well as others related to pathogenicity and virulence.

60. Gao et al (2019) dosed specific pathogen-free male C57BL/6 mice with aldicarb at 2 ppm (mg/l) for 13 weeks. At the end of the exposure period, 17 genera, as identified by 16S rRNA gene sequencing differed in proportion between the control and treatment groups, Genes for virulence, adsorption and bacteriocins were increased by aldicarb. Aldicarb disturbed the profiles of diglycerides, triglycerides and phosphatidylcholines in the liver, faeces and brain. Aldicarb also increased the expression of oxidative stress-related genes in the gut, and induced protein degradation and DNA damage.

61. The herbicide glyphosate is widely used in agriculture and is present in bread and other plant-based foods. This herbicide inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase in the aromatic amino acid synthetic pathway of plants and microorganisms. Since it is present in food, it is reasonable to expect some interaction with the gut microbiota. Nielsen *et al* (2018) tested glyphosate. (2.5 and 25 mg/kg bw/day) and its formulation Glyfonova™ (Cheminova A.S) (25 mg/kg bw/day) on male Sprague Dawley rats for 7 days by oral gavage and in vitro against E.coli. Growth of E. coli was inhibited in a dose-dependent manner but inclusion of aromatic amino acids in the culture medium alleviated this effect. Little effect was observed on the already diverse bacterial populations on the ileum, caecum and colon by glyphosate treatment and this was regarded as being due to the presence of already-sufficient amounts of aromatic amino acids in these compartments. The authors suggested that changes in the gut biota may, however, be more obvious in malnourished individuals.

62. Lozano et al (2018) found that female Sprague Dawley rats, but not males, treated with glyphosate in drinking water (50ng/l, 0.1 g/l and 2.5g/l) exhibited gut biota changes, with Bacteroidetes family S24-7 increasing and Lactobacilliaceae decreasing in 8 out of 9 treated animals. In vitro, Bifidobacteria, Clostridia and Enterococci were sensitive to glyphosate at 400 ppm, Lactobacilli above 5000 ppm and coliforms not sensitive at any of the concentrations used. The authors point out that resistance mechanisms against glyphosate exist in bacteria (for example Staub et al ,2012).

63. Aitbali et al (2018) used behavioural tests to assess the psychological state of male Swiss mice after acute, subchronic and chronic gavage dosing (once, six weeks or 12 weeks daily dosing) with glyphosate (250 or 500 mg/kg bw/day), formulated as Roundup™ (Monsanto). The mice were dosed, subjected to tests to assess locomotor behaviour, anxiety, depression-like behaviour and chronic stress

before being culled and their gut contents sampled. Subchronic and chronic treatment led to significant ($p< 0.01$, $p< 0.001$) dose dependent increases in anxiety and depression-like behaviour, whereas acute treatment did not. Concomitantly, total gut bacterial count was significantly reduced ($p<0.001$), and taxonomic diversity was altered, with Firmicutes, Corynebacterium, Bacteroidetes spp and Lactobacillus spp being depleted. Several possibilities for the linkage between the bacterial and behavioural effects were suggested: reduction in butyrate producing bacteria leading to leaky epithelium and entry of intestinal antigens that could affect the brain; Mn chelation, leading to depletion of Lactobacilli, which have been correlated with central nervous system health; reductions in aromatic amino acid levels and hence in the neurotransmitters produced from them, particularly serotonin.

64. Mao et al (2018) compared the effect of glyphosate alone and as formulated in Roundup™ at a human-equivalent dose on the gut microbiota of Sprague Dawley rats. Dams received glyphosate or Roundup™ in drinking water at a calculated glyphosate dose of 1.75 mg/kg bw/day from gestation day 0 until the end of weaning. The pups were then dosed in drinking water at the same rate until postnatal day (PND) 125. 16S rRNA gene sequencing revealed significant changes in the balance of the microbiota in the glyphosate- and Roundup™-treated dams and pups compared with a water control and also between glyphosate and Roundup™, suggesting an influence of the co-formulants in the commercial product. In the pups, changes were greatest prior to PND 31, a period corresponding to human puberty.

65. Two papers by Jun et al (2016 and 2018) reported the effect of the broad-spectrum fungicide imazalil on the gut microbiota and intestinal health of mice. In the first paper, male ICR mice were gavage dosed with 25, 50 or 100 mg imazalil / kg bw for 4 weeks. The most consistent finding was a fall in the relative abundance of the Bacteroidetes at all imazilil doses and in the Firmicutes and Actinobacteria at the highest dose and later time points. 100 mg/kg bw imazalil for 28 days coincided with an increase in inflammatory immune cell infiltration of the mucosal layer of the colon, indicating that treatment may have led to an inflammatory response in the gut. In the second paper, C57BL/6 mice were gavage dosed with 0.1, 0.5 or 2.5 mg imazalil/kg bw/day for 2,5 or 15 weeks. Caecal and faecal dysbiosis was observed as increases on Firmicutes, α-Proteobacteria and γ-Proteobacteria. The changes took place within the first 2 weeks of treatment and were stable by week 15. These changes were accompanied by reductions in mucous secretion and ionic transport in the gut. The dysbiosis caused by imazilil (2.5 mg/kg bw for 15 weeks ameliorated after 45 days of recovery.

66. Wu et al (2018a) treated male ICR mice with the fungicide propamocarb (PM) in drinking water for 4 weeks a 0, 3, 30 and 300 mg/l. No significant changes were seen in body weight or liver to body weight ratio, but changes were seen in the microbiota composition. In the faeces, the Bacteroidetes α-Proteobacteria *and* γ-Proteobacteria were reduced in a dose-dependent manner at each weekly time-point, the Firmicutes increased at 3 mg/l and then fell. In the caecum the pattern was different, with falls in Actinobacteria and β-Proteobacteria but little change in the

other phyla at 300 mg/l PM. SCFAs (propionate and isobutyrate) were increased, as were free choline, ethanolamine and trimethylamine. The latter compound is known to be oxidised to its N-oxide in the liver and its presence is possibly linked to atherosclerosis.

67. Wu et al (2018b) followed up on the earlier paper, finding that PM (1, 3 or 10 mg/l) in drinking water reduced the faecal expression of genes involved with bile acid synthesis and transport. Even at these lower doses (compared with the earlier paper) trimethylamine levels were increased by PM.

68. Xu et al (2014) found that the broad-spectrum fungicide epoxiconazole (4 or 100 mg/kg bw/day) altered the composition of the gut microbiota in female Sprague Dawley rats at the phylum level, increasing the Bacteroidetes and the Proteobacteria at the expense of the Firmicutes. The high dose markedly increased liver weight ($p < 0.001$), and reduced serum total bilirubin and cholinesterase, suggesting liver damage, although ALT and AST were unaffected. At the family level, both Lachnospiraceae, which are associated with protective butyrate production and Enterobacteriaceae, which are associated with inflammation, were increased by high-dose epoxiconazole.

69. Tu et al (2019) dosed SPF C57BL/6 mice with 1 ppm (mg/l) of the widely-used herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in drinking water for 13 weeks. 2,4-D treatment changed the richness and composition of the murine gut microbiota, enriching Bacteroidetes, Chlorobi, Chloroflexi, Spirochetes and Thermotogae but not Acidobacteria, which were enriched in the control group. Genes involved in the metabolism of amino acids were differentially changed in abundance by treatment, and the genes involved in urea metabolism were increased., indicating a disturbance in nitrogen metabolism. Of particular note was a reduction in the plasma concentration of acylcarnitines, suggesting perturbation of fatty acid metabolism and perhaps increased risk of neurological disorders. Acylcarnitine level were correlated with a reduction in the microbial species Xylanimonas cellulosilytica,

70. Schneeberger et al (2018) treated hookworm-positive adolescents aged 15 to 18 years from Cote d'Ivoire with four anthelminthic drug regimes (tribendimidine +ivermectin. tribendimidine + oxantel pamoate and albendazole + oxantel pamoate for three weeks. On treatment arm 2, the titre of *Bacteroidetes* in stool samples was increased at 24 hours but had reduced to baseline by the end of treatment. Increases were noted for biotin metabolism, folate synthesis and N-glycan biosynthesis were concurrently increased.

Antibiotics

71. Antibiotics alter the structure of the human gut microbiota. At least 42 genera have been found to be sensitive to the effects of a range of 68 different antibiotics. However, the effects of antibiotics are difficult to ascertain using the commonly used 16S rRNA genetic analysis since this technique detects all bacteria in a population,

including those that are dead, dormant and quiescent. Bacteria in the gut that are susceptible to antibiotics are replaced by others that fulfil the same functions but are resistant to treatment, but this can also lead to an imbalance of function causing detrimental effects on the host such as greater risk of obesity and/or type 2 diabetes. (Review by Ferrer et al (2017).

72. The growth of antibiotic resistant bacteria in the gut appears to be affected differently by oral and iv injection, depending upon the antibiotic in question. Zhang et al (2013) inoculated male C57BL/6 mice with either Enterococcus spp expressing the tetracycline resistance gene Tet^r or E. coli strains expressing the ampicillin resistance gene Amp^r and then exposed the mice to the corresponding antibiotic either orally or by tail vein injection. Gut antibiotic resistance increased with treatment but for ampicillin, which is largely excreted in the urine, the effect was smaller for iv than for oral administration. Tetracycline, however, is excreted by both the kidney and the GI tract, so the microbiota were exposed by both routes of treatment.

73. Leclercq et al (2016) studied the effect of in utero and post-natal exposure to penicillin V on the gut microbiota and behavioural parameters in mice. Pregnant dams received low doses of antibiotic (31 mg/kg bw/ day) for a week before giving birth and up until the end of weaning (PND21). A second group received the same dose of penicillin with the addition of 10^9 cfu probiotic Lactobacillus rhamnosus JB-1 per day. Control animals received water. Gut microbiota were identified and behavioural tests were applied to the animals. In the pups receiving antibiotic alone, by PND21 the Actinobacteria were markedly increased at the expense of the Bacteroidetes, the Firmicutes remaining largely unchanged. After 21 more days of recovery, the Actinobacteria were much decreased, although still at a greater level than control and the Bacteroidetes, the Firmicutes had both increased in proportion. A similar pattern was seen in the presence of the probiotic although the changes were not so great. In the behavioural tests, antibiotic did not affect locomotor activity but decreased anxiety in males and increased aggression, whereas the probiotic decreased anxiety in females. The Lactobacillus appeared to prevent antibiotic-induced decrease in sociability in males and females. A significant ($p<0.01$) increase in the expression of frontal cortical but not hippocampal arginine vasopressin receptor B1, which has been associated with aggressive behaviour, was observed in both males and females treated with penicillin. Inflammatory cytokines were increased by antibiotic treatment in both sexes but was partially ameliorated by the probiotic in females.

74. Han et al (2016) gavage dosed male and female Wistar rats with 4-epi-oxytetracycline (4-EOTC), a major oxytetracycline metabolite at 0.5, 5 and 50 mg/kg bw for 15 days. Faecal samples were taken every 4 days After 15 days of treatment the level of Firmicutes was only slightly increased in both genders. The Bacteroidetes dropped from about 25% to 18 % in males and from about 34% to 11% in females at the high dose. The Actinomycetes also increased dramatically in response to the medium and high-level treatment, in particular Bifidobacteria. The

families Lactobacilliaceae (associated with good gut health) and Helicobacteriaceae (associated with poor gut health) were both relatively depleted. The expression of the tetracycline resistance genes tetQ and tetO increased in a dose dependent manner. This study showed that antibiotic metabolites may have tangible effects on the gut microbiota, the consequences of which have yet to be determined.

75. Zhan et al (2018) considered the effect of antibiotics on the gut microbiota and the possibility that the resulting changes in microbial balance may increase the bioavailability of other xenobiotics, in this case triazine herbicides. Male Sprague Dawley rats were gavage dosed with a mixture of ampicillin, neomycin, gentamicin, metronidazole (at 1.75 mg/day each) and vancomycin (0.875 mg/day) for 14 days. On the third day, 2 or 20 mg/kg bw each of atrazine, simazine, ametryn, terbuthylazine and metribuzin mixed. Antibiotic treatment increased the bioavailability of all the triazines without affecting microbial or hepatic triazine metabolism. The area under the curve of blood triazine concentration was significantly higher in microbiota deficient rats than in normal microbiota rats. The authors concluded that use and abuse of antibiotics may increase the toxicological risks of exposure to xenobiotics.

76. Cho et al (2012) Investigated the effect of sub-therapeutic doses of antibiotics on the growth of juvenile mice and impact on their gut microbiota. The study was to investigate the mechanism by which low doses of antibiotics can act as growth promoters, as is practiced in farm animals. Weanling C57BL6 mice were exposed through drinking water to penicillin, vancomycin, penicillin-plus-vancomycin or chlortetracycline for 7 weeks. Percentage fat weight increased in all antibiotic groups without lean weight change and bone mineral density also increased. The blood level of the intestinal-derived hormone glucose-dependent insulinotropic polypeptide increased with the antibiotics and there was a trend towards hyperglycaemia. The composition of the microbiota taxa altered, with a bloom in the Lachnospiriaceae family and the Firmicutes/ Bacteroidetes ratio increased. Butyrate synthesis gene (butyrylCoA transferase) copy number decreased at 3 weeks but had increased, along with measured SCFAs by 6 weeks, providing the energy for increased adiposity.

77. Nagano et al (2019) treated 2 groups of female BALB/c mice with antibiotics. One group (CM) received 0.02% ciprofloxacin and 0.1% metronidazole and the second group received 0.05% vancomycin in drinking water for 3 weeks. The Bacteroidetes and Actinobacteria were depleted and the Proteobacteria increased. The F/B ratio therefore increased. At the family level, the Streptococcaceae increased in the CM treated mice and the Lactobacillaceae and Enterobacteraceae increased in the vancomycin treated mice. The authors commented that different antibiotics appear to affect the gut microbiota differently, possibly leading to different disease susceptibilities.

<u>Miscellaneous</u>

78. Zhang et al (2018) exposed 4 species of common gut bacteria (E. coli, Bacteroides fragilis, Clostridium sporogenes and Streptococcus gallolyticus) to a cocktail of 29 xenobiotics (plasticisers, colorants, flame retardants and personal care products) at a final concentration of 1mM/compound. Compounds included bisphenol A, rhodamine B, triphenylphosphate and triclosan. The cocktail was added to growth media to give an exposure concentration of 10 or 100 nM for each compound, to mimic the range of human exposures. Minimal effects were seen on bacterial growth and morphology over 48 hours. Multiple changes in metabolite profiles were observed at the 100 nM level, affecting sulphur-containing amino acids, putative neurotransmitters, intermediates of energy metabolism and oxidative stress products. Possible mechanisms of action within the cocktail of compounds were recognised as diverse, including acting as surrogate electron acceptors, upregulating microbial GABA production and increasing angiogenesis. Responses differed between species and between species sharing the same growth habit, eg anaerobiosis. In addition, the authors pointed out that the involvement of secondary metabolites had not been addressed and would require a more comprehensive study.

79. Montassier et al (2017) investigated the effect of chemotherapy with a cocktail of agents (bis-chloroethylnitrourea, etopside, aracytine and melphalan) on the gut microbiota of 28 patients with non-Hodgkin's lymphoma. There was a reduction in the abundance of the Firmicutes ($p=0.0002$) and the Actinobacteria ($p=0.002$) and increases in the Proteobacteria ($p=0.0002$) after 7 days' treatment. Functional shifts were noted, with reductions in amino acid, nucleotide and energy metabolism but increased inflammation-related signal transduction and glycan metabolism. A decrease in butyrate-producing bacteria suggested that the epithelial mucus layer may have been reduced and was suggested as a possible linkage between the known condition of chemotherapy-induced Gi mucocitis and dysbiosis.

80. Banarjee et al (2016) observed that subcutaneous implantation of slow-release pellets containing morphine, leading to a serum concentration of the drug of ~ 1μM, reduced the titre of Bacteroidetes in C57BL/6 mice. Within the Firmicutes, morphine treatment elevated the families Enterococcaceae, Staphyllococcaceae, Bacillaceae, Streptococcaceae and Erysipelotrichaceae. Gut barrier homeostasis was negatively affected and bacteria (mainly Gram positive) were found to translocate through the epithelium in the morphine treated animals. Toll-like receptor 2 knock-out and μ-opioid receptor knock-out mice showed little or no dysbiosis or epithelial dysfunction when treated with morphine. Chronic morphine also reduced the bile acid deconjugating activity in the gut and thus reduced bile acid enterohepatic circulation. The authors felt that further studies would be needed to clarify the temporal relationships between morphine exposure and its effects on the microbiota and the host.

81. Hill-Burns et al (2017) observed changes in the gut microbiota of Parkinson's Disease (PD) patients that appeared to be brought about by the disease itself and the type of drug used in its treatment. Stool samples from 197 PD and 130 control

patients were analysed. PD patients were also analysed for medication-induced changes. Disease duration was correlated with an increased abundance of Ruminococcaceae ($p = 0.0005$). Earlier reports had found that PD was associated with increased abundance of Akkermansia, Lactobacillus and Bifidobacterium, with reduced levels of Lachnospiraceae. This study found that catechol-O-methyltransferase (COMT) and anticholinergic drugs reduced Bifidobacterium levels and increased the Lachnospiraceae. PD-induced depletion in the latter family is correlated with a decrease in SCFA production, with its adverse effects on host physiology. The authors speculated that the initial lesion in PD may be in the gut, which then has downstream effects in the CNS.

The effect of food components on the gut microbiota.

82. Roca-Saavedra et al (2018) reviewed the effect of minor food components on the gut microbiota and vice versa. For example, polyphenols found in a variety of fruit, vegetables and beverages both alter community composition via their antimicrobial actions and are metabolised to products with increased bioavailability. Other plant-derived compounds, such as flavonols, tannins and resveratrol, promote some taxa and inhibit the growth of others and are metabolised in ways that may affect their reported effects on host health,

83. Maier et al (2017) fed human adults (26 women and 13 men) with reduced insulin sensitivity on high or low carbohydrate diets and supplemented groups of them with high-resistance starch (HRS, high amylose cornstarch) or low-resistance starch (LRS, high amylopectin cornstarch) in a cross-over design study with 2 weeks on each diet with a 2-week washout period between diets. Faecal and blood samples were taken before and after each diet-consumption period. The gut microbiota were affected most by the low carbohydrate diet with high-resistance starch. The high resistance starch appeared to improve meal-to-meal regulation of blood glucose but also led to higher plasma levels of trimethylamine-N-oxide, which has been linked to increased risk of cardiovascular disease. In addition, HRS was found to increase the Firmicutes/ Bacteroidetes ratio and increased the numbers of butyrate- and propionate-producing genera. Genes related to lipid metabolism were variously up- or down-regulated by the HRS diet.

84. Viennois et al (2017) exposed male C57BL6 mice to either carboxymethylcellulose (CMC) or polysorbate-80 (P80) at 1% w/v in drinking water and looked at the effects of the emulsifiers on DSS-induced colitis-associated cancer and the gut microbiota. Alterations were observed to be induced by the emulsifiers in the bacterial community at phylum, class and order levels, with a decrease in the Firmicutes and increase in Bacteroidetes, leading to more pro-inflammatory conditions. Genes for the virulence-associated bacterial products lipopolysaccharide (LPS) , and flagellin, Toll-like4 and Toll-like 5 receptors were increased without an increase in bacterial load, suggesting altered species composition. Concomitant with increased inflammation, tumour burden increased.

85. Shinohara et al (2010) observed that apple pectin consumption was associated with an improved intestinal environment because isolates of “beneficial” bacteria such as Bifidibacteria and Lactobacillus from faecal samples from healthy human individuals were capable of metabolising this carbon source, whereas other, potentially harmful species such as Escherichia coli and Clostridium perfringens were not. Sahasrabudhe et al (2018) also observed that lemon pectins with various levels of methyl esterification ameliorated doxorubicin-induced ileitis in mice via activation of Toll-like receptor 2-1 but this effect did not appear to be mediated via microbial SCFA production. The authors concluded that the microbiota may not always be involved in the effects of xenobiotics.

86. Costantini et al (2017) review the effects of consuming omega-3-fatty acids on the structure and function of the gut microbiota. Results of the few (9) clinical studies reported were variable with the major phyla unaffected, increasing or decreasing in different studies. In some cases, potentially pathogenic genera were reduced in comparison to beneficial ones. Oils differed in being from plants in some cases and fish in others. In animals, omega-3-fatty acid deprivation resulted in anxiety and depression-like behaviour and increased activity in the hypothalamic-pituitary-adrenal (HPA) axis and gut inflammation along with Firmicutes/ Bacteroidetes imbalance, and all of these conditions were improved by supplementation. The authors concluded from their review that omega-3-fatty acids were beneficial for gut microbiota, leading to greater epithelial integrity and function of the immune function and thence of the gut-brain axis.

87. Patrone et al (2018) fed female C57BL/6 N mice with standard mouse diet or with isoproteic diet containing soy (highly polyunsaturated) or coconut oil (highly saturated). The mice were weighed daily and culled at 2 or 8 weeks. Weight gain was similar with both high-fat diets, but the coconut-oil-fed mice showed a higher blood cholesterol level after 8 weeks. Coconut oil also produced a greater relative abundance of some genera, for example Allobaculum and Anaerofustis and depletion of Akkermansia compared with soy oil. The saturated fat diet also reduced the microbiota metabolism of fatty acids, amino acids, terpenoids and polyketones and xenobiotic metabolism in general. These changes were, however, only correlative and further study was suggested to understand the mechanisms involved.

Sweeteners

88. Bian et al (2017) gavage dosed male and female CD-1 mice with acesulfame-K for 4 weeks at a dose of 37.5 mg/kg bw. Male but not female mice gained weight significantly ($p<0.01$) over the dosing period. Concomitantly, male mice exhibited significant increases in some genera of their microbiota and females had decreases in others. Genes associated with carbohydrate absorption and metabolism were decreased in females and increased in males. In male mice the microbiome genes associated with inflammation and virulence, such as LPS and flagellar proteins were upregulated. Organic acid and bile acids were affected differently in male and female

mice, suggesting changes in microbiota-host cell cross-talk. However, the authors recognised that this study had limitations (sample size, lack of consideration of other factors such as food intake, and body composition) that confounded the extrapolation to humans.

89. Wang et al (2018) studied the bacteriostatic effects of 4 non-nutritive sweeteners (sucralose, saccharin, acesulfame-K and rebaudioside A (from Stevia)) on E. coli in vitro and on the microbiota, food intake and body weight of mice. Acesulfame-K and saccharin (0.25% w/v) exerted a bacteriostatic effect on two E, coli strains in liquid culture, as measured by $OD_{600,}$ whereas sucralose did not. Rebaudioside (2.5% w/v) inhibited the growth of E. coli strain HB101 on agar, but not that of strain K-12. In vivo, a high fat diet in mice increased food (and hence calorie) intake and reduced water intake and sucralose had no effect on this but high fat also reduced faecal output and sucralose partially reversed this effect ($p<0.05$). On normal diet, mice fed sucralose showed a significant increase in the Firmicutes ($p<0.05$) and a trend to reduced Bacteroidetes ($p = 0.117$), without changes in the other major phyla. At the genus level, sucralose significantly increased the abundance of the Bifidobacteria but not the Clostridia. The authors concluded that non-nutritive sweeteners exert a variety of effects on the microbiota with consequences for the host that should be followed up.

90. Rodriguez-Palacios et al (2018) studied the effect of the sweetener Splenda (1% sucralose, 99% maltodextrin w/w) on the microbiota of a mouse model of Cohn's disease-like ileitis. Supplementation of mouse diet with Splenda led to microbiota dysbiosis characterised by over growth of 5 classes within the phylum Proteobacteria. without any significant effect on the Firmicutes or Bacteroidetes. Tissue myeloperoxidase activity, which is linked to gut inflammation was also increased in Splenda-treated mice.

91. Biam et al (2017) dosed male C57BL6 mice for 6 months with sucralose in drinking water at 0.1 mg/ml (calculated to give a dose equivalent to the human ADI of 5 mg/kg bw/day) and investigated its effects on the balance and metabolism of the gut microbiota. The growth of a range of different bacterial genera was altered, in some cases increased and in others decreased. Genes for LPS synthesis and flagellar components and fimbriae were up-regulated by sucralose. The faecal metabolome was also affected, with changes in the concentrations of quorum-sensing molecules, amino acids and their derivatives and bile acids. In the host animals, there was increased hepatic gene expression for pro-inflammatory enzymes, such as matrix metalloproteinase 2. The authors recognised that the dose of sucralose used was high compared with most human doses and that their genetic and metabolite profiling needed verification by further work.

92. Uebanso et al (2017) looked at the effect of low (40 mg/kg bw/day) or medium (200 mg/kg bw/day) doses of xylitol on the gut microbiota and lipid metabolism of male C57BL/6 mice in combination with a high fat diet. The Bacteroidetes were reduced in both the control and high-fat fed mice by the medium level of xylitol, The

high fat, medium dose regimen also increased the Firmicutes phylum and the Prevotella genus within the Bacteroidetes. High fat diet induced hypertriglyceridemia and hypercholesterolemia, but xylitol had no effect on this. Nor did xylitol affect glucose tolerance, despite previous correlation of abundance of Prevotella in glucose intolerance and insulin resistance in humans and mice. Effects on recipient gut microbiota of faecal transplant from high fat plus medium xylitol mice to high fat diet mice were minor and transient, apart from a small, albeit significant ($p<0.05$) increase in total plasma cholesterol.

93. Lobach et al (2019) reviewed the area of low/ no-calorie sweeteners on the gut microbiota. Papers on acesulfame-K, aspartame, cyclamate, saccharin, neotame, sucralose and rebaudioside A were discussed. Changes in the balance of the microbiota are noted in some studies but not in others and different studies highlight different bacterial genera. Most studies used doses higher than the ADI and could not therefore be equated with the dose of these compounds as generally used by humans. The review reported that metabolic studies in mice, rats, and humans have shown that sucralose is largely unabsorbed by the gut but is not a substrate for gut microbiota. No change in the metabolic profile was seen after 1 year exposure, indicating no microbial metabolic adaptation, even with very high doses. Neither saccharin nor acesulfame K underwent gastrointestinal metabolism, but, in contrast to sucralose, both of these latter sweeteners were rapidly absorbed and excreted unchanged in the urine. steviol glycosides passed unabsorbed through the upper portion of the gastrointestinal tract but in the colon the sugar moieties attached to the steviol backbone were removed by the gut microbiota, primarily of the Bacteroidaceae family. Steviol was not a substrate for the intestinal microbiota and was absorbed from the colon intact. Following absorption, it was conjugated with glucuronic acid, and primarily excreted in humans as steviol glucuronide via the urine. The authors concluded that considering the extensive safety databases that have evolved over the years for these structurally unrelated sweeteners, there was little in the papers on the microbiome to suggest that they raised safety concerns at their currently applied levels.

Ethanol

94. Mutlu et al (2009) gavage-dosed male Sprague Dawley rats twice daily with 2 – 3 ml of ethanol, increasing over 2 weeks to a maintenance dose of 8 mg/kg for a total of 10 weeks of dosing. Control rats received an isocaloric dose of dextrose. Some mice had supplemented diets including prebiotic oats or the probiotic bacterium Lactobacillus rhamnosus Gorbach-Goldin (GG). After 10 weeks of dosing, significant changes ($p<0.05$) were seen in the diversity, richness and evenness of the colonic population of the ethanol dosed rats compared with the dextrose controls. Addition of oats or the probiotic Lactobacillus, however, maintained all the measured parameters at control levels but the authors recognised that the pre- and pro-biotic groups were small and further work would be required to confirm their findings.

95. Xie et al (2013) investigated changes in the metabolic products of the gut microbiota of male Sprague Dawley rats that were fed a liquid diet of protein, fat and carbohydrate, or a similar diet where some of the carbohydrate content was replaced with a calorific equivalent of ethanol. Intestinal metabolites were measured in the stomach and distal portions of the gut by LC-TOFMS. The control rats had a signature distribution of amino acids, fatty acids and steroids and their derivatives. The distribution changed with ethanol treatment such that branched-chain amino acids were generally increased and short-chain fatty acids, particularly acetate, were increased in the stomach and colon. The general trend of the other metabolites was reduction in all compartments with ethanol treatment. Since many of these metabolites were thought to affect host immune response and health, the authors suggested that alcohol consumption could have effects on the host via the microbiota.

96. Bull-Otterson et al (2013) treated male C57BL/6 mice with a liquid diet with ethanol or isocaloric maltodextrin for 6 weeks. 16S rRNA sequencing revealed that over time the ethanol-treated group's microbial balance changed, with slightly reduced Firmicutes, markedly reduced Bacteroidetes and over growth of the Actinobacteria and Proteobacteria. These changes were concurrent with an increase in lipopolysaccharide production, and hepatic steatosis and injury. Faecal pH also rose, providing conditions for other minor genera to grow. Treatment with Lactobacillus rhamnosus GGat 1x 109 cfu/mouse /day over the dosing period ameliorated the dysbiosis, LPS production, hepatic TNFα-expression and to some extent plasma ALT activity. The authors speculated that the protective effect of the L. rhamnosus may have been partially due to its ability to produce SCFAs, thus lowering faecal pH, inhibiting the growth of potentially pathogenic species and maintaining intestinal barrier function.

97. Lowe et al (2017) and Lowe et al (2018) treated female C57BL/6 mice with a liquid diet with or without calorie-matched 5% ethanol for 10 days, followed by a bolus dose of ethanol or sugar, and followed the inflammatory response in the liver and central nervous system. Some of the mice were also treated with a cocktail of antibiotics that reduced the gut bacterial titre. With ethanol, no changes in bacterial diversity were observed but there was an increase in the Actinobacteria and a decrease in the Verrucomicrobia, the latter almost entirely due to a decrease in the genus Akkermansia, which is known to be decreased in mouse models of obesity and type-2 diabetes. Ethanol treatment led to an inflammatory response as seen by neutrophil infiltration of the gut, and, through increased leakiness, inflammatory cytokine release in the brain.

Mycotoxins

98. Liew et al (2019) investigated the effect of aflatoxin B1 (AFB1) on the gut microbiota of male Sprague Dawley rats and the effect of co-treatment with the probiotic bacterium Lactobacillus casei Shirota (Lcs). Rats were gavage dosed with either phosphate-buffered saline (PBS) or 25 μg AFB1/ kg bw for 5 days a week for 4

weeks. A third group were dosed for 5 days with 10^9 cfu/ day and then treated with AFB1 at the same rate as the AFB1 only group. Faecal samples were taken using metabolism cages after the end of the dosing period. At the phylum level, AFB1 Had minimal effect (a slight reduction in Bacteroidetes with minor increases in Firmicutes and Proteobacteria, whereas there were marked changed at the genus level, only partially remediated by the probiotic. However, the diversity of the three groups showed that the AFB1 – treated bacterial communities clustered together distinctly from the control and probiotic groups. Alloprevatella spp were noted to be much more abundant in the AFB1 only group, but not in the control or AFB1 – plus – probiotic group. This genus has been associated with succinic acid production, which had been linked to inflammation and malignant transformation of cells. The authors recommended further studies on Lcs with regard to AFB1 toxicosis.

99. Wang et al (2015) found a dose-response relationship between aflatoxin B1 dose (5, 25 and 75 μg/ kg bw) and gut microbiota diversity in male F344 rats. The rats were gavage dosed 5 days per week for 4 weeks with AFB1 or DMSO control and then faeces were collected in metabolism cages for analysis. The genetic diversity in the AFB1-treated groups was lower than that in the control in the order con> 5 >25 = 75. No major shifts were seen at the phylum level but species within the Firmicutes were either unchanged or increased in abundance, except for the tested lactic acid bacteria, which were reduced, and some within the Bacteroidetes were unchanged or slightly reduced in a dose-dependent manner. The authors suggested that the variable inter-species response to AFB1 may be due to variations in microbiota content and function.

100. Saint-Cyr et al (2013) Assessed the effects of No-Observed-Adverse Effect-Level doses of deoxynivalenol (DON) on human gut flora transplanted into germ-free male Sprague Dawley rats. After allowing the faecal transplants 2 weeks to stabilise, rats were administered DON at 100 μg/kg bw by gavage for 4 weeks. Faecal samples were collected weekly up to day 27 and then on day 37 and frozen until analysis. DON exposure increased the titre of the Bacteroides/Prevotella group of organisms during dosing ($p< 0.01$), but this declined to control levels before the end of the experiment. The Bifidobacteria, Clostridium leptum group and the Lactobacillus/Leuconostoc/Pedococcus group were unaffected by DON but E.coli was significantly reduced ($p< 0.01$) and this reduction persisted until the end of the experiment. The authors suggested that since DON at its toxicological NOAEL had effects on the gut microbiota with unknown physiological effects the approach of establishing a microbiological NOAEL for veterinary drugs should be considered for mycotoxins

Environmental Pollutants

101. The environment contains a rich variety of chemical entities that may enter the digestive tract of an animal and have toxicological consequences either directly or by affecting the composition and/ or functioning of commensal organisms. Inhaled PM2.5 and PM10 particles from natural (forest fires, volcanoes) and man-made (vehicle exhausts and smoking) may be delivered to the digestive tract from the

lungs by the action of the mucocilliary escalator system in the trachea. Direct effects on the growth of gut microbiota or metabolic disturbances may then ensue from the presence of metals and organic components, such as PAHs, leading to the development of inflammation-associated conditions (Salim et al 2013).

102. Claus et al (2016) reviewed the involvement of the gut microbiota in the toxicity of environmental chemicals. For example, the microbiota are capable of oxidising the widespread environmental pollutant benzo[a]pyrene (BaP) to its DNA-reactive 7-hydroxy derivative and deconjugate the hepatic product of phase 2 metabolism of BaP back to the parent compound. PCBs can be methylsulphonated by gut bacteria to products that are implicated in lung dysfunction, azo dyes such as Sudan 1 can be cleaved into potentially carcinogenic aromatic amines and melamine can be oxidatively deaminated to cyanuric acid, which may cause nephrotoxicity. This is in addition to the development of dysbiosis induced by the toxicity of the ingested chemical.

103. Snedeker and Hay (2012) reviewed evidence for the contribution of gut microbiota and environmental chemicals in the development of obesity and diabetes. They pointed out the associations that have been reported between gut dysbiosis in obese and diabetic individual and the possible correlation between exposure to various pollutants, such as heavy metals, insecticides, and putative oestrogenic chemicals and suggested that the interaction was a subject to which resources should be applied.

104. Ribière et al (2016) gavage dosed male C57BL/6 mice for 28 consecutive days with BaP at 50 mg/kg bw, a dose known to induce genotoxic and carcinogenic effects. Daily faeces samples were taken. The mice were culled and samples of their gut were taken for DNA analysis of the mucosal bacteria and for histology. The ileum and colon of the BaP-treated mice showed a moderately increased histology score for inflammation as well as epithelial erosion when compared with saline or sunflower seed oil(vehicle) – treated animals. The Bacteroides phylum increased in the faeces and the Verrucomicrobia phylum decreased. Genera considered beneficial, such as Lactobacillus and Akkermansia, decreased in the BaP animals' faeces. In the mucosa, different taxa increased and decreased, depending upon location in the intestine. The balance changed across the taxa and with time of exposure to BaP: potentially protective species first bloomed and then declined as exposure continued and the population became more pro-inflammatory. The authors speculated that BaP exposure could trigger or exacerbate conditions such as IBD or colorectal cancer.

105. Defois et al (2017) investigated the effect of BaP on the microbiota in samples of human faeces in *vitro.* Samples from two donors were incubated with BaP in sunflower oil at 0.005, 0.05, and 0.5 mg/l. The composition of the microbiota was determined by 16S-rRNA gene sequencing and changes a range of volatile compounds produced by the bacteria (the "volatolome") caused by the BaP was assayed using solid-phase extraction coupled with GC-MS. No significant changes

were observed in the microbiota at the phylum level with only minor changes at the family level as a result of BaP treatment, but the baseline composition differed between the two donors. Seven volatile products were detected by the GC-MS system and tentatively identified from the literature of internal data, with a seventh of unknown identity. All detected compounds were significantly changed in level by at least 0.5 mg/l BaP with some (benzaldehyde and 2-methylphenol) showing significant increases and others (such as 2-hexylfuran and butylbutanoate) showing significant decreases. After 24 hours of incubation a wide range of metabolic pathway genes were upregulated by 0.5 mg/l BaP in both samples (21 in sample1, 31 in sample 2) but only 3 in each sample, different in each, were downregulated. The authors considered that the microbiota were adapting to the presence of the BaP and in doing so their change in metabolism could have down-stream effects on the gut and host health.

106. Defois et al (2018) studied the effect of a range of environmental pollutants (TCDD, PhIP, α and γ HBCDD, BaP, deltamethrin and a mixture of PAHs) on the gut microbiota from a human volunteer *in vitro*. The compounds were tested at 0.005, 0.90, 2.60, 5, 21 and 38 μg/l. Of the volatile microbial products assayed, 5, 2, 7 and 4 were significantly altered in concentration by deltamethrin, PhIP, TCDD and the PAhs, respectively. These included ketones, xylenes and phenols. BaP and PAH exposure in total up-regulated 613 genes and down-regulated 419 genes.

107. Fader et al (2017) examined the changes in the levels of the bacterial genes *bsh* and *baiCD* that are responsible for bile acid deconjugation and dihydroxylation, activities that are carried out by the gut microbiota, in response to exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Male C57BL/6 mice were gavage dosed every 4 days over a 28-day period with TCDD at 0, 0.01, 0.1, 0.3, 1, 3, 10 or 30 mg/kg bw. DNA was extracted from faecal samples and probed with various primer sets for the two genes. Results suggested that TCDD increased the relative abundance of species in the genera Lactobacillus, Clostridium, Streptococcus and Listeria. The authors suggested that disturbances in the structure of the microbiota may contribute to the pleiotropic effects of TCDD in its toxicity to the host.

108. Gnotobiotic (germ-free) female C57BL/6 mice were used by Stedtfeld et al (2017) to study interactions between TCDD and segmented filamentous bacteria - SFB - (Candidatus Savagella) in the AhR-induced regulation of regulatory T cells (T_{reg}) in the gut. The mice were colonised by either Bacteroides fragilis as a representative commensal organism alone or in combination with the SFB and treated with either TCDD at 30 mg/kg bw or sesame oil vehicle. Other mice were treated with TCDD or vehicle in the presence of the SFB alone. In general, genes related to T cell differentiation were downregulated in response to TCDD and upregulated in response to SFB whereas the B. fragilis exerted a lesser effect. The effect of SFB on the response of T_{reg} was also seen in the spleen, blood and mesenteric lymph nodes. The authors suggested that continued work on the immune regulatory effects of the gut bacteria may lead to treatments for intestinal pathogens and autoimmune diseases.

109. Zhang *et al* (2015) studied the effect of a persistent organic pollutant similar to TCDD, 2,3,7,8-tetrachloribenzofuran (TCDF) on microbiota-host relations via the Aryl Hydrocarbon Receptor (AhR). C57BL/6 mice (Ahr $^{-/-}$ and Ahr $^{+/+}$) were trained to eat dough pills containing TCDF to give a dose of 24 μg/kg bw/ day for 7 days, urine and faeces were collected daily and then the animals were culled and blood, liver, intestine, caecum and caecal contents were taken. TCDF reduced the Firmicutes ($p= 0.033$) and increased the Bacteroidetes ($p=0.035$) without affecting the Actinobacteria and decreased the Firmicutes/ Bacteroidetes ratio by about 40%. TCDF significantly reduced the levels of SFB in the ileum of the Ahr $^{+/+}$ mice but not in the Ahr $^{-/-}$ mice after 7 days. TCDF increased the caecal content of SCFAs but reduced the oligosaccharide content. In the faeces, TCDF increased the content of lysine, glutamine and alanine as well as propionate and butyrate. Bile acid metabolism was also disrupted by the TCDF,

110. The dioxin-like polychlorinated biphenyl126 (PCB 126) has been found to increase gut inflammation and significantly alter the caecal Firmicutes /Bacteroidetes ratio in mice, with significant overgrowth of the Verrucomicrobia (Petriello et al, 2018). Male *Ldtr*$^{-/-}$ mice (negative for low-density lipoprotein receptor and a mouse model for human diabetes and related pathologies) were gavage dosed with 1 μmol/kg bw of PCB 126 at weeks 2 and 4 of a 12-week study. Faeces were collected for analysis at 72 hours and 4 weeks after the initial dose and then at the end of the experiment, when intestinal and caecal samples were taken. PCB 126 reduced overall bacterial diversity and caused significant reductions ($p<0.05$) in a number of measured genera, including Bifidobacterium, Lactobacillus and Ruminococcus but an increase in Akkermansia (all $p<0.05$). Inflammatory cytokines were increased in the gut by PCB 126 treatment. Formic acid was increased, but acetate, propionate and butyrate were not affected. Plasma levels of insulin, c-peptide and glucose-dependent insulinotropic peptide were increased but glucagon-like peptide-1 was decreased. The authors concluded that PCB was pro-inflammatory and led to metabolic disruption but admitted that more work was needed to establish a causal link between these effects and dysbiosis.

111. Lai et al (2018) gavage dosed female CD-1 mice with perfluorooctane sulfonic acid (PFOS) at 0.3 or 3 mg/kg bw/day for 7 weeks. On day 50 the animals were given an oral glucose tolerance test before being culled and samples of blood, liver and caecum taken. PFOS did not affect overall gut bacterial diversity. Within the major phyla, some taxa of bacteria increased and others decreased. Of note was a low-PFOS-dose-induced increase in the genus Turicibacter, which had previously been associated with an increase in blood cholesterol. The genus Allobaculum, a SCFA-producing genus that had previously been correlated with insulin resistance and obesity, also increased. The authors recommended further investigations into mechanisms that linked PFOS exposure to gut microbial metabolism and downstream effects.

112. Li *et al* (2017) observed that the metabolism of polybrominated diphenyl ethers (PBDE), specifically BDE-47 (2,2,4,4-tetrabromodiphenyl ether) and BDE-99 (2,2,4,4,5-pentabromodiphenyl ether) by mouse liver was modified by the presence or absence of gut microbiota. Conventional C57BL/6 mice were gavage dosed daily for 4 days with BDE-47 or BDE-99 at 10 or 100 μmol/kg bw. Germ-free mice were gavage dosed similarly with the PBDEs but only at 100 μmol/kg bw. Germ-free mice treated with BDE-47 had higher liver levels of the 5-OH metabolite but lower levels of 4 other metabolites than did conventional mice. With BDE-99, 4 major metabolites were reduced in germ free mice. Merely the absence of gut biota altered the expression of Cyp genes in the liver (Cyp2c subfamily upregulated, Cyp3a downregulated) and different Cyps were affected by the PHDEs depending on microbiota status. Phase 2 metabolic genes (UDP-glucuronyl transferases, sulfotransferases, glutathione-S-transferases) and transmembrane carrier protein genes were generally upregulated by germ-free status and this was enhanced by the PBDEs, especially by BDE-99. The mechanisms behind these changes were not resolved.

113. Wang et al (2019) found that triphenylphosphate (TPHP), gavage dosed to pregnant female ICR mice at 1000 μg/kg bw changed the gut microbiota of their offspring by increasing the abundance of bacterial class Erysilelotrichia and Bacilli and decreasing the class Clostridia. At the genus level, the abundances of Allobaculum, Tunicibacter and Lactobacillus were increased. Changes were noted in the levels of microbial metabolites: bile acids, SCFAs, taurine, succinate and methanol were increased, and glycine, isoleucine and alanine were decreased. The authors suggested that these changes could lead to the development of metabolic syndromes.

Food contact materials

114. Groh et al (2017) reviewed the effect of food contact materials on gut health, including the gut microbiota. They recognised that a large number of potentially antimicrobial compounds that are used in food contact materials such as packaging or added directly to foods have so far been insufficiently studied for any statement on their effects to be made. The polymer chitosan is known to be antimicrobial (Gram+ > Gram -) and has been shown in pigs and humans to reduce the *Firmicutes* / *Bacteroidetes* ratio. Other compounds found to affect the composition of the microbiota in mammals, but without obvious antimicrobial properties, are diethylphthalate, methyl paraben, polysorbate-80 and carboxymethylcellulose. Many of these changes have been seen to be accompanied by gut inflammation.

115. Wang et al (2019) investigated the effect of the endocrine disrupting chemical mono-2-ethylhexylphthalate ester (MEHP) on cholesterol metabolism in male C57BL/6 mice, having found that female mice were less sensitive to its effects. MEHP was dissolved in olive oil and the mice were gavage dosed once a day for 4 weeks at 0.05 mg/kg bw/day. The phthalate increased the titre of the Firmicutes, reduced the Verrucmicrobia and led to a 1,5-fold increase in the Firmicutes/Bacteroidetes ratio. At the genus level, MEHP reduced Akkermansial and

Alloprevotella and increased genera that have been associated with cholesterol metabolism, such as Intestinimonas and Coprobacter. Concomitantly, increases were noted in host adipocytes, total, high-density and low-density serum cholesterol (but not triglycerides), AhR, PXR and transcription factors associated with adipogenesis, such as Srebp1 and C/EBPα. The authors suggested that alterations in the makeup of the microbiota may contribute to these effects.

116. Bisphenol A (BPA) was found to reduce the species diversity in the gut microbiota of male CD-1 mice dosed at120 mg/l in drinking water for10 weeks (Lai *et al*, 2016). The changes were similar to those seen with a high sucrose (2g/ kg bw) diet. BPA treated mice had an increased titre of the phylum Proteobacteria, as did those on a high fat diet, indicating dysbiosis. The family Helicobacteriaceae, which has been associated with the development of inflammatory bowel disease, was also markedly increased. The phylum Firmicutes was reduced by high fat diet and BPA, and this fall was in the class Clostridia, a condition also noted by the authors to be found I individuals suffering from type 2 diabetes. Thus, BPA was deemed to disrupt the microbiota in a manner potentially detrimental to health.

117. Javurek et al (2016) supplemented the diet of female California mice with BPA and, for comparison with a more potent oestrogen, with ethinyl oestradiol (EE), both at 50 mg/kg feed, prior to mating up until weaning of their offspring. The male parental mice were also put on the BPA diet from mating until offspring weaning. Faecal DNA was collected from parental and F1 generations. In the parental mice, treatment with BPA or EE induced sex-dependent changes in microbiota composition (some taxa increased, such as Bacteroides, Mollicutes, Prevotellaceae and Sutterella) in males and females Bifidobacterium increased in F1 females. Many of the taxonomic changes were those associated with up- or down-regulated metabolic pathways associated with IBD, metabolic diseases or colorectal cancer. Contrary to the currently held belief that the gut microbiota of vaginally delivered offspring mirrors that of the mother, the F1 generation in this study differed from that of both parents and treatment-induced changes in milk composition was given as a possibility for this,

118. Lu et al (2018) dosed ICR mice with polystyrene microplastic (MP) particles of 0.5 or 50 μm diameter at 100 or 1000 μg/l in drinking water for 5 weeks. Faecal samples were collected over the course of the experiment. Samples of blood, liver, colon and caecal content were taken at the end of the experiment. Q-PCR revealed that both particle sizes reduced the relative abundance in the caecum of the Firmicutes, Actinobacteria and α-Proteobacteria without significantly affecting the level of *Bacteroidetes*, although there was a slight downward trend..16S rRNA gene sequencing showed an increase in Proteobacteria and Actinobacteria at the high dose of 0.5 μm particles but only of Proteobacteria with the 50 μm particles, at the expense of the Firmicutes, not the Bacteroidetes. Gut genes associated with the secretion of mucin were down-regulated by both MP sizes and both dose and hepatic lipid metabolism was decreased at the high doses. The authors pointed out that the doses of particles used here were higher than those seen in the environment

and further work needed to be carried out, but indicated the potential health risks of their ingestion,

Effects of the microbiota on xenobiotics.

119. The metabolism of xenobiotics from various sources by the gut microbiota is a two-edged sword, like that performed by host enzymes, in that it may lead to products that protect the host from chemical damage or degrade relatively innocuous chemicals to active products. For example, sulphates and sulphur-containing amino acids in the diet may be reduced to hydrogen sulphide, which may lead to reduced functioning of colonic epithelial cells, inflammation and increased rates of colon cancer. Nitrate reduction results in the production of nitroso compounds, which are known DNA alkylating agents. Conversely, metabolism of plant-derived flavonoids and glucosinolates by various genera of gut bacteria have been related to reduced levels of colorectal cancer. (Hullar et al, 2014)

120. Li et al (2019) reviewed the methylation and demethylation of mercury by the gut microbiota of fish, terrestrial invertebrates and mammals. Papers from as early as 1975 showed that anaerobic incubation of inorganic mercury in closed off loops of rat intestine and in the rumen of red deer could lead to methylmercury (MeHg) production. Incubation of Hg Cl_2 with a human faecal suspension under anaerobic conditions also produced MeHg and some of the bacteria capable of this process were identified as Staphylococci, Streptococci and E. coli.

121. Methylmercury demethylation is a function of which the rat and marine fish gut biota are capable and one which confers protection against the neurotoxic effect of organic mercury in the diet. Guo et al (2018) tested the effect of methyl mercury on the microbiota in slurries prepared from the faeces of two healthy human individuals (designated A and B) and found that there were marked inter-individual differences in demethylation. A balanced diet, a diet rich in carbohydrates and a diet rich in protein were tested on the ability of faecal slurry to demethylate methylmercury and the effect was enhanced by increased protein, but only in individual A. After 48 hours of methyl mercury treatment, Proteobacteria growth had reduced the proportion of the major phyla in individual A except in the high carbohydrate group, where there was marked growth of the Actinobacteria. The pattern in individual B was markedly different, with MeHg-carbohydrate having minimal impact on the microbiota profile but Hg and the other diets increasing the proportions of other minor phyla. On genetic profiling, the gut biota from neither individual expressed the mer operon, the best characterised mercury-resistance mechanism in bacteria, so the actual demethylation mechanism remained unresolved.

122.

123. A number of reviews on the effects of the gut microbiota on the metabolism by, and thence influence in the pharmacological or toxicological effects of xenobiotics were discovered in literature searches:

124. Saad et al (2012) listed a number of microbial metabolic actions that modify the effects of ingested chemicals such as enhancing the conversion of HAA into more potent mutagens, hydrolysing glucosides to release aglycones, potentiating host drug metabolism, activation of prodrugs and increasing or reducing toxicity depending on the drug in question.

125. Li and Jia (2013) listed the metabolic actions of microbiota-expressed enzymes on a range of drugs. Activities included reduction, hydrolysis, diacylation, deamination, proteolysis, ring opening and group scission. These actions led to xenobiotic activation or inactivation, increased absorption and increased activity, leading to either the desired therapeutic effect, reduction of this effect or unwanted toxicity.

126. Kim (2015) covered a range of drugs and concentrates on mechanisms activation by microbial metabolism. Mechanisms covered are azo, nitro, sulphoxide, N-oxide, C=C, O-N and C-N reduction, deglycosylation, ring fission, desulphation, deamination, hydroxylation and dihydroxylation. Antibiotics potentiated some effects and antagonised others, although the mechanism behind this difference was not discussed.

127. Currò (2018) reviewed the action of the gut microbiota on various pharmaceuticals, with examples. These included the activation of prodrugs, such as aminosalicilates by Clostridium and Eubacterium in the distal gut where the released drugs then act as anti-inflammatories against colitis, and anthranoid laxatives that are glycosides activated predominantly by Bifidobacterium species. Digoxin was reduced and deactivated predominantly by the species Eggerthella lenta. Bacterial β-glucuronidases have been implicated in the GI tract toxicity associated with the chemotherapeutic agent irinotecan and non-steroidal anti-inflammatory drugs.

128. Jourova et al (2016) reviewed a wide range of enzymatic transformations that are known to take place in the gut, presumably by the microbiota but only a few have been characterised to the genus or species level. Those identified in this paper were: paracelamol O-sulphation and C-S cleavage of paracetamol-3-cysteine by Clostridium difficile, reduction of digoxin by Eggerthella lenta, hydrolysis of the antiviral sorivudine by Bacteroides spp, nitroreduction of nitrazepam leading to teratogenicity by Clostridium leptum and increased activity of the anthelmintic levanosole by thiazole ring opening caused by Bacteroides and Clostridium spp.

129. The metabolite p-cresol, derived from the protein amino acid tyrosine appears to compete for the same microbiotic O-sulphation pathway as paracetamol. The presence of a unique gut microbial community with different metabolic capacities

may thus explain the difference in paracetamol metabolism and potential toxicity in different individuals (Clayton et al, 2009)

130. Exposure of murine gut microbiota to the NSAID indomethacin appears to affect its own pharmacokinetics and pharmacodynamics by coincidentally altering the composition and hence available metabolic capacity. Liang *et al*, (2015) gavage dosed male C57BL6 mice with indomethacin at 10 mg/kg bw in PEG400 and culled the animals 6h later in an acute study. They dosed a second group of mice at 20 mg/ kg bw in food and culled these at 7 days for a chronic study. Blood, urine and faeces were collected for microbiota and drug metabolism analysis. Acute and chronic indomethacin treatment increased bacterial richness in the lumen of the middle and distal large intestine. Peptococcaceae increased in the lumen and Erysipelotrichaceae. Increased in the mucosa. After 5 days of treatment, the Firmicutes and Bacteroidetes were decreased, with a concomitant increase in the Proteobacteria. The bacterial de-glucuronidation of the drug after enterohepatic circulation was reduced in parallel with the microbiota changes, leading to reduced reabsorption and increased elimination and a shortened half-life.

131. Velmurugan et al (2017) analysed the blood biochemistry of people who were occupationally exposed to organophosphates and dosed BALB/c mice with monocrotophos (MCP) to assess effects on glucose tolerance related to metacolism by the gut microbiota. In the workers, eighteen percent of the people directly exposed to OPs had diabetes compared with 6% of those indirectly exposed and half of those with the condition in each group had no family history of the disease. Plasma AChE activity was unaffected in the pesticide workers. BALB/c mice were administered MCP orally in drinking water at 28 μg/kg bw (10x the theoretical maximum daily intake) for 180 days. The MCP treated mice showed increased blood glucose levels ($p<0.0001$) beginning after 60 days' treatment without changes in body weight or plasma AChE activity. Lipid peroxidation, indicating oxidative stress, was also increased ($p< 0.01$). Faecal transplants between MCP-fed and control animals suggested that the gut microbiota were responsible for the observed glucose intolerance. The OP was found to induce faecal expression of enzymes for glucose and nucleotide metabolism, phosphate transport and vitamin biosynthesis. Acetate produced by microbial metabolism of the OP was found to induce gluconeogenesis, and a trend for higher acetate levels was found in the faeces of the diabetic OP workers.

132. Chi et al (2019) studied the effect of disrupting the gut microbiota of female C57BL/6 mice with antibiotics on the toxicity of arsenic (250 ppb or 1 ppm) in drinking water. The concentrations of the arsenic metabolites monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) were measured in urine. The MMA/DMA ratio, a marker of As toxicity, was increased in the urine of the microbiota-disrupted mice compared with mice with a normal flora, indicating that As metabolism was affected by the gut microbiota. Faecal As levels were lower in the microbiota- disrupted mice, suggesting that the microbiota absorb As, allowing less to be absorbed by the host. Changes were noted in host metabolism: While As had no

effect on the expression of the major As methylating gene 3mt, 250 ppb reduced the expression of other genes associated with 1-carbon metabolism and 1 ppm reduced the liver level of S-adenosylmethionine. Moreover, 250 ppb altered the expression of p53 and other genes associated with hepatocellular carcinoma in microbiota-disrupted mice compared with control animals. The authors suggested that the microbiota thus affected the toxicity of As on the host.

133. Humblot et al (2007) investigated the possibility that bacterial β-glucuronidase could enhance the carcinogenesis of the food process genotoxic compound 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) by reversing host phase 2 glucuronidation when the conjugate re-enters the gut lumen in bile. Gnotobiotic (germ-free) male F344 rats were gavage dosed with 1 ml of an overnight culture of E. coli TG1 that either expressed or was deficient in β-glucuronidase. The rats then received 90 mg/kg bw IQ or corn oil and were culled 4 hours later. Comet assays on colonocytes and hepatocytes from the treated rats showed that the presence of β-glucuronidase led to a much longer tail length in the former cells than the latter, suggesting greater DNA damage. Thus, the authors suggested that the microbiota could play a role in the colonic carcinogenicity of food borne IQ.

134. Gratz et al (2018) assessed the microbial metabolism of the masked mycotoxin deoxynivalenol-3-glucoside (DONGlc). Such conjugates are formed as a result of plant phase II metabolism of DON and are stored in plant cell vacuoles as a response to fungal infection. In this study the Intestinal contents from healthy pigs fed a commercial diet lacking in antibiotics and probiotics were spiked with DON, DONGlc or the metabolite deepoxy-deoxynivalenol (DOM-1) and incubated anaerobically for up to 17 hours. LC-MS/MS analysis showed that the rate of DONGlc degradation and thus free DON release increased from the jejunum contents to the egested faeces, although the difference between caecum, colon and faeces was non-significant. No DOM-1 was produced in any compartment. Bacterial genera in each compartment were identified, but not those responsible for the DONGlc hydrolysis. Slow constant release of DON from DONGlc in the distal intestine was expected to have toxic consequences but these were not determined.

135. Beer et al (2019) examined the glycerol-dependant metabolism of heterocyclic aromatic amines (HAA) by the human faecal microbiota. HAAs are process contaminants of meat cooking, some of which are known experimental animal carcinogens and potential human carcinogens (IARC 2015). A range of HAAs were incubated with human faecal suspensions under strictly anaerobic conditions in the presence and absence of glycerol. Glycerol is known to be metabolised by various gut bacterial species, including *Lactobacillus*, to the reactive compound reuterin. Metabolites were analysed by RP-HPLC-TOF-MS/MS. A range of reuterin conjugation products were recovered. Reuterin conjugation blocks the exocytic amino group of HAAs and is thought to reduce their ability to bind DNA.

Statins

136. Individuals are known to differ in their hypolipidaemic response to treatment with statins. Kaddurah-Daouk et al (2011) studied the potential genetic and non-genetic differences between good-and poor-responders to simvastatin in relation to the enteric metabolome. Plasma samples were analysed from participants in a clinical trial involving 944 Caucasian and African-American men and women with total cholesterol levels of 160 – 400 mg/dl (4.2 – 10.4 mmol/l), who were treated with 40 mg simvastatin/day for 6 weeks. There was a strong relationship between response to the statin and a higher level of secondary, bacterially derived, bile acids. The level of coprestanol, the reduced metabolite of cholesterol, produced in the gut also correlated positively with good response to the statin. The authors suggest that such knowledge could lead to developing microbiota-altering dietary interventions that could improve patients' response to statins.

137. He et al (2017) also considered whether the gut microbiota may have a role in the individualistic response to simvastatin. To this end, male C57BL/6J mice were fed a high fat/cholesterol diet for 8 weeks before being gavage dosed simvastatin (20 mg/kg bw once daily) for 4 weeks, with or without antibiotic (imipenem: clistatin sodium, 100 mg/kg bw). The diet raised total, HDL and LDL cholesterol and triglycerides, the statin reduced the levels of all of these lipids but in combination with the antibiotic the levels rose again. Both the antibiotic and the antibiotic-plus-statin treatment groups' microbiota were different from the control and high fat groups' as well as being different from each other, suggesting that each treatment affected the balance of bacteria. Hepatic CYP7A1, CYP7B1 and FXR proteins, involved in bile acid and cholesterol homeostasis, were stimulated by the statin in high-fat-fed mice, and this effect was attenuated by antibiotic treatment, indicating the involvement of the gut microbiota in the actions of simvastatin,

138. The statin lovastatin is a prodrug that requires hydrolysis to its β-hydroxy metabolite to be activated to its HMG-CoA reductase-inhibitory form. The observation that a cell-free preparation of rat or human faeces, known as fecalase, caused lovastatin degradation prompted Yoo *et al* (2014) to investigate the involvement of the gut microbiota in the activation of this drug. Both human and rat fecalase preparations were found to metabolise lovastatin, but when the rat preparation was made from the faeces of animals that had been treated with ampicillin or a mixture of cefadroxil, oxytetracycline and erythromycin, levels of activity were less than half that of the control. In live rats, the activity of three measured microbial enzymes – β-D-glucuronidase, α-L-rhamnosidase and β-D-glucosidase was reduced to almost zero after 3 days of antibiotic treatment. Metabolism of the statin by the gut microbiota contributed almost as much as that by the liver and the authors suggested therefore that patients who co-administered. an antibiotic would have a reduced level of the activated metabolite and hence reduced effectiveness of lovastatin.

Probiotics

139. Wu et al (2017) proposed the term "gut remediation" for the action of the probiotic bacterium Lactobacillus plantarum TW1-1 in reducing the faeces and tissue

concentrations and effects of Cr(VI) in mice. Male and female Kunming mice were administered 1 mM potassium dichromate in drinking water for 7 weeks, with a gavage dose of 1 x 10^9 cfu of TW1-1 every second day. The probiotic reduced the level of tissue oxidative stress and inflammation induced by the Cr and appeared to protect the liver against changes seen on histological sections. Faeces from Cr-TW1-1 treated mice showed increased ability to reduce Cr(VI) to Cr(III), but this was not seen in mice treated with TW1-1 without Cr. The *Bacteroidetes* and the *Firmicutes* were decreased by Cr and the probiotic partially reversed this trend. The authors suggested that TW1-1 might be considered for treating heavy metal toxicity in humans.

140. Feng et al (2019) reviewed the gut remediation of a range of contaminants by probiotics. *Lactobacillus* spp have mostly been used and have been found to reduce oxidative stress caused by, or increase the excretion of, heavy metals (Cd, Hg, Pb, As), protect against the toxicity of pesticides (endosulfan, malathion, chlorpyriphos-plus-parathion) and restore microbial diversity after antibiotic (ampicillin, streptomycin, clindamycin) treatment.

141. Zhai et al (2019) studied the effect of a probiotic bacterium, Lactobacillus plantarum CCFM8661 on the excretion of lead in male C57BL/6 mice with a pre-existing excess body burden of the metal. Mice were allowed to drink water with a lead content of 1g lead carbonate/L for 8 weeks and were then gavage dosed with either skimmed milk or skimmed milk containing the probiotic for 4 weeks. Other groups received the lead and the probiotic followed by the bile acid chelator cholestyramine, GW4064 (an agonist of farnesoid X receptor) or a cocktail of antibiotics. The probiotic reduced the lead content of the blood ($p<0.001$), liver and kidneys ($p<0.01$). Cholestyramine increased faecal bile acid and lead concentrations and reduced blood lead. Antibiotic and GW4064 treatment raised blood Pb and lowered faecal Pb compared with the probiotic alone, indicating that the probiotic acted through the farnesoid-X receptor to affect lead enterohepatic circulation.

142. Theilmann et al (2017) investigated the ability of a known human gut bacterium, Lactobacillus acidophilus to metabolise dietary plant glucosides. Their premise was that the aglycone moiety of the glucosides would then be secreted by the bacterium and serve as a substrate for further metabolism by other microbial species into bioactive products, which may be beneficial or toxic to the host depending upon the molecule. L. acidophilus grew on amygdalin, salicin (from willow bark), vanillin 4-O-β-glucoside (from vanilla), polydatin (from grapes), esculin (from dandelion coffee) and frexin (from kiwi fruit). The metabolism of amygdalin, esculin and salicin were compared. Lactate increased as the glucosides were metabolised and aglycones were produced in the external supernatant, with esculin and salicin being preferred substrates.

143. Wang *et al* (2018) observed that the bacterial strain Bacillus cereus BC7 protected mice against liver damage caused by the mycotoxin zearalenone. The bacterium was isolated from mouldy animal feed and was found to be able to utilise

zearalenone as its sole carbon source, being able to degrade 90.4% of 2 mg/l zearalenone in 48 hours at 37^{o}C. Degradation also took place in simulated gastric fluid. Female BALB/c mice were gavage dosed with saline, zearalenone (10 mg/kg bw), BC7 (6.9 x 10^{7} cfu) or zearalenone-plus-BC7 once daily for 2 weeks after which the animals were euthanised and tissue, blood and faeces samples taken. Zearalenone markedly increased the titre of the Bacteroidetes at the expense of the Firmicutes and the co-administration of BC7 returned the balance almost to control levels although at genus level all of the treatments were markedly different from the control. Concomitant to this, BC7 ameliorated the liver damage caused by zearalenone in terms of histological appearance of the tissue, organ weight, and AST/ALT release. The authors suggested that BC7 could be used as a feed additive as a probiotic and a zearalenone removal agent.

144. Wang et al (2019) found that pre-treatment of male and female KM mice with either of two Lactobacillus species isolated from Tibetan yaks (L. *pseudomesenteroides* (LP) and L. johnsonii (LJ)) ameliorated the diarrhoea and morbidity caused by *E. coli* O124. The *E. coli* infection resulted in pathological changes to the spleen and damage to the duodenum and the probiotic treated animals showed recovery from these effects. At the phylum level, *E. coli* caused a large increase in the Firmicutes at the expense of the Bacteroidetes in the duodenum, but not in the ileum; in the colon the phylum Deferribacteres overgrew. Both probiotics ameliorated the effect in the duodenum and colon, made Fimicutes overgrowth worse in the jejunum and, in the ileum, LP reversed the effect but LJ did not. The authors concluded that overall, there were grounds for the use of these two species as probiotics to treat intestinal infections in animals.

Risk assessment

145. Considering the multitude of interactions between the gut microbiota and chemicals of various classes ingested with food, Licht and Bahl (2018) considered how this knowledge may affect the risk assessment processes commonly used in toxicology. In addition to the physiological differences between experimental animals and humans that lead to uncertainty in the extrapolation between species, the composition and metabolic capacity of the microbiota differs and overlaps in unknown ways. To mitigate this, the authors suggested that animals in toxicological studies could be modified by vendors in a number of ways:

to each have as wide a range of microbiota to cover the widest range of activities;

to have standardised microbiota or

each animal to have its microbiota analysed and correlation made between this and the findings of the experiment in that animal.

Careful consideration should also be given to co-caging, randomisation and the effects of coprophagy and environmental contact on individual animals.

146. Velmurugan (2018) proposed a toxicological risk assessment protocol for the gut microbiota. The questions to be resolved were the effects of a chemical on the structure and function of the microbial community, the former of which could be addressed by whole genomic DNA isolation and 16S rRNA gene sequencing to assess dysbiosis and the latter by mass spectrometry techniques. The author outlined a workflow diagram of the steps he proposed. The use of germ-free mice that could be inoculated with human-like bacterial populations and the *in vitro* SHIME system were highlighted. The substrate used in the SHIME system could then be transplanted into a suitable host animal to assess the effect of the changes observed *in* vitro in a whole animal. Single bacteria or whole communities could also be assessed by "gut on a chip" microfluidic technology.

Derivation of microbiological health-based guidance values

147. The International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guidance document GL36(R2) (2019) outlines recommendations for deriving a microbiological acceptable daily intake (ADI) for a veterinary medicine with suspected antimicrobial properties. The procedures for deriving ADIs, based on either disruption of the intestinal colonisation barrier or on overgrowth of drug resistant species, using *in vivo* or *in vitro* methods, are outlined. This guidance updates guidance GL36(R) (2004, implemented 2013).

148. The 85th JECFA recommended that "…studies be conducted according to internationally recognized standards using at least 10 strains of the relevant genera of intestinal bacteria sourced from faecal samples of healthy donors taking into consideration recent scientific knowledge from molecular and metagenomic studies on intestinal microbial community composition; and that in vitro or in vivo studies be conducted using a range of concentrations of the antimicrobial agent, from residue levels to therapeutic levels, and that these studies address the effects." (JECFA 2018).

https://apps.who.int/iris/bitstream/handle/10665/259895/9789241210171-eng.pdf;jsessionid=D2F57DBBF206C8B3EF596864AF35E376?sequence=1

149. Points to be considered before a microbiological HBGV for a particular xenobiotic (as applied by JECFA to veterinary drugs) would be considered necessary are:

1 Are residues of the drug and/or its metabolites microbiologically active against representatives of the human intestinal flora?
2 Do residues enter the human colon?
3 Do the residues entering the human colon remain microbiologically active?

150. In 2016, JECFA produced a guidance document on the derivation of a microbiological acute reference dose (ARfD). Disruption of the colonisation barrier is

relevant to acute exposure and therefore would be the basis of a microbiological ARfD.

https://www.who.int/foodsafety/chem/jecfa/Guidance_ARfD.pdf

151. The calculations for microbiological ADIs and ARfD are similar to one another in format. For the derivation relating to the use of in vitro models using defined bacterial strains of bacteria, the formula derived by JECFA is as follows:

$$HBGV = \frac{POD\ (MIC_{calc}\ or\ NOAEC) \times correction\ factors \times colon\ volume}{Fraction\ of\ oral\ dose\ available\ to\ microbiota \times body\ weight}$$

Where
HBCV = health-based guidance value (ADI or ARfD)

POD = Point of Departure = Minimum Inhibitory Concentration or No-Observed-Adverse-Effect-Concentration.

MIC_{calc} = calculated minimum inhibitory concentration. MIC_{calc} represents the lower 90% confidence limit for the mean MIC_{50} (the minimum inhibitory concentration for 50% of strains) for the 10 most relevant and sensitive human colonic bacterial genera. An intrinsically resistant bacterial genus should not be included.

Correction values (where appropriate) take into account considerations not used for the microbiological ADI that may be appropriate to the microbiological ARfD. For example, a factor of 3 to allow for temporal dilution during gastrointestinal transit and for dilution by consumption of additional meals. Others may take into account the inoculum effect on MIC determinations, pH effects on the MIC, and possibly other physico-chemical-specific factors of the growth conditions used in testing.

The fraction of an oral dose available for colonic microorganisms should be based on in vivo measurements for the drug administered orally. Alternatively, if sufficient data are available, the fraction of the dose available for colonic microorganisms can be calculated as 1 minus the fraction (of an oral dose) excreted in urine.

The value assumed for the volume of the colon has recently been increased from 220 ml to 500 ml.

Body weight = 60 kg.

152. JECFA (2018) state that "… data from in vitro studies (continuous culture flow chemostats) and in vivo models (human volunteers, animal models and human microbiota– associated animals) are evaluated by the Committee for both microbiological end-points. However, data from these studies can be problematic in determining a microbiological ADI and/or ARfD. This is due to the small sample size in the animal studies; insufficient data and low power of studies in human volunteers (because of small numbers of subjects); concentrations of antimicrobial agent

generally not being adequate to determine a chronic or acute dose with no effect; and the lack of validation of the in vitro and in vivo test models….and… Therefore, the Committee recommends that in vitro or in vivo studies be conducted using a range of concentrations of the antimicrobial agent, from residue levels to therapeutic levels. Such studies should address the predominant bacterial strains that inhabit the gastrointestinal tract when determining if levels of antimicrobial residues in animal-derived food after consumer ingestion can increase the population of antimicrobial-resistant intestinal bacteria in the gastrointestinal tract."

Conclusions

153. The composition of the microbial community of the gastrointestinal tract is complex, consisting of a relatively small number of major phyla, within which the taxonomic groups account for an enormous range of species. The number and range of species present depends upon the local conditions and thus position in the gut, the major repository being the large intestine, particularly the caecum.

154. The gut microbiota, by the interaction of their metabolites initially with the intestinal epithelium, have been associated with both gut- and systemic-disease states. Examples include Crohn's disease, diabetes, obesity and neurological disturbances. Enteric and systemic immune function also appears to be impacted by dysbiosis caused by ingested xenobiotics.

155. A wide range of substances can influence the species balance of the gut microbiota to the extent that almost anything ingested affects structure and function and by implication, host health.

156. The gut microbiota also appear to participate in the activation and deactivation of ingested substances, including effecting or affecting the action of some pharmaceuticals, and thus may lead to toxicity, or to intended, reduced or prolonged pharmacological action.

157. The presence of some bacterial taxa has been associated with metabolic defects in the host and others with good health and barrier function of the epithelium, but the mechanisms involved have yet to be fully elucidated.

158. The whole body of literature on the subject is vast. This paper has used relatively narrow search terms and thus has just scratched the surface of the microbiota and their interaction with ingested xenobiotics.

159. Given the current interest in personalised medicine, it is possible that attempts will be made to utilise an individual's gut microbiota to tailor treatments for gastrointestinal or systemic pathologies in which they or their metabolic capacity are involved. Achieving this would also allow a precise, personal risk assessment of the toxicity of an encountered xenobiotic. This, however, would require knowledge of the

organisms and the causal links involved, which in most cases is currently unavailable.

Questions for the Committee

1 In view of the wide range of toxicants that appear to be capable of affecting the balance of the gut microbiota and which may be present in food, would it be useful to consider extending the establishment of microbiological ADI values beyond veterinary drugs?

2 Does the Committee have any views on the potential for the use of selected probiotic species to mitigate the toxicity of xenobiotics in humans?

3 Can the Committee identify any areas for research that they would feel it useful to follow to increase the understanding of microbiota/ xenobiotic/toxicity relations?

4 Would the Committee be happy for a statement to be prepared from this paper as an overview of the current state of knowledge in this area?

5 Does the Committee have any further comments on this paper?

Secretariat

October 2019

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Appendix 1

Search terms in PubMed

The search terms used fot this paper were largely of the format:

Microbiome OR microbiota AND “X” and toxicity AND gut, where “X” was:

Heavy metal
Insecticide
Herbicide,
Xenobiotic,
Pyrethroid
Organophosphate
DDT
DEET
Glyphosate
Food contact materials,
Polyamines
Drug metabolism
Food additive
Antibiotics
Alcohol
Function
Composition
Coccidiostat
Fungicide
Aldrin
Dieldrin
Flame retardant
Dysbiosis
Sweeteners
Bisphenol A
Chlorpyrifos
Gold
Tin
Mercury
Antimony
Nickel
Silver
Titanium
PAHs
Emulsifiers
Probiotic
Prebiotic

Some references were found in the reference lists of papers acquired in the PubMed searches.

Metabolite

Appendix 2

Abbreviations

5HT	5-hydroxytryptamine, serotonin
ABC	ATP-Binding-Cassette trans0-membrane transport protein
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADI	Acceptable daily intake
AFB1	Aflatoxin B1
AgNP	Silver nanoparticles
AgOAC	Silver acetate
AHR	Aromatic hydrocarbon receptor
ALT	Alanine aminotransferase
AMR	Antimicrobial resistance
ARfD	Acute reference dose
ARG	Antimicrobial resistance gene
As	Arsenic
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AuNC	Gold nanoclusters
BC	*Bacillus cereus*
BDE	Brominated diphenylether
BPA	Bisphenol A
BS	*Bacillus subtilis*
CCAAT	A DNA transcription initiation site
Cd	Cadmium
C/ERPα	CCAAT/enhancer-binding protein alpha
cfu	Colony-forming units
CNS	Central nervous system
CPF	Chlorpyrifos

Cr	Chromium
CYP	Cytochrome P450
DDT	Dichlorodiphenyltrichloroethane
DMA	Dimethylarsinic acid
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOM-1	Deepoxydeoxynivalenol
DON	Deoxynivalenol
DONGlc	Deoxynivalenol-3-glucoside
EE	Ethinyl (o)estradiol
F1	First generation offspring
FAO	United Nations Food and Agriculture Organization
FBG	Ferrous bisglycinate
Fe	Iron
FeEDTA	Ferrous ethylenediaminetetraacetic acid
FOXP3	Forkhead immune-regulatory protein P3
FS	Ferrous sulphate
FXR	Farnesyl-X receptor
GC	Gas chromatography
GI	Gastrointestinal
GPR	Orphan G-protein-coupled receptor
HAA	Heterocyclic aromatic amines
HBCDD	Hexabromocyclododecane
HDL	High-density lipoproteins
Hg	Mercury
HMG-CoA	Hydroxymethylglutarate coenzyme A
HPA	Hypothalamic-pituitary-adrenal
HRS	High-resistant starch
IARC	International Agency for Research on Cancer
IBD	Inflammatory bowel disease

IgA	Immunoglobulin A
IQ	2-amino-3-methylimidazo[4,5-f]quinoline
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	Liquid chromatography
LDL	Low-density lipoproteins
LPS	Lipopolysaccharide
MCP	Monocrotophos
MeHg	Methyl mercury
MIC	Minimum inhibitory concentration
μM	Micromolar
μm	Micrometre
MMA	Monomethylarsonic acid
Mn	Manganese
MP	Microplastic
mRNA	Messenger RNA
MS	Mass spectrometry
nM	Nanomolar
NO	Nitric oxide
NOAEL	No-observed-adverse-effect-level
NOD	Interbacterial flavinoid signalling receptor
OP	Organophosphate
p,p-DDE	Dichlorodiphenylethylene
PAH	Polycyclic aromatic hydrocarbon
Pb	Lead
PBDE	Polybrominated diphenylether
PBS	Phosphate-buffered saline
PCB	Polychlorinated bisphenyl
PCR	Polymerase chain reaction
PD	Parkinson’s disease
PFOS	Perfluorooctane sulphonic acid

PhIP	2-amino-1-methyl-6-phenylimidazole[4,5-b]pyridine
PiP3	Phosphatidylinositol-3,4,5-trisphosphate
PM	Propamocarb
PM2.5, 10	Airborne particles of 2.5, 10 μm diameter
PND	Post-natal day
PTEN	Phosphatase and tensin homolog
PXR	Pregnane-X receptor
Q-PCR	Quantitative polymerase chain reaction (aka RT-PCR)
RP-HPLC	Reversed-phase high performance liquid chromatography
rRNA	Ribosomal riboneucleic acid
RT-PCR	Real-time polymerase chain reaction
S	Svedberg unit of centrifugal sedimentation time (10^{-13} seconds)
SCFA	Short-chain fatty acid
SPF	Specific-antigen-free
spp	Species (plural)
Srebp1	Sterol regulatory element-binding protein
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCDF	2,3,7,8-tetrachlorodibenzofuran
TiO_2	Titanium dioxide
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TOF	Time-of-flight
UDP	Uridine diphosphate
UPLC	Ultra Performance Liquid Chromatography
VLDL	Very-low-density lipoproteins
WHO	World Health Organization