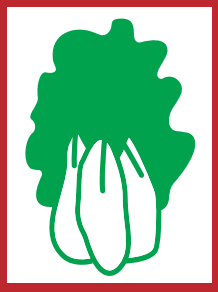
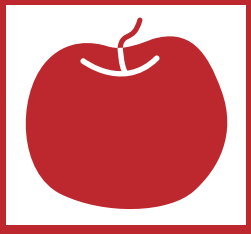
EFFECTS OF XENOBIOTICS ON GUT MICROBIOTA AND RELATED BILE ACID METABOLISM

WEIJIA ZHENG





Propositions

1. The in vitro batch fermentation model is an effective tool to elucidate host microbiota-metabolism interactions. (this thesis)

2. The gut microbiota and its metabolism need to be considered in future pesticide safety evaluations. (this thesis)

3. Chemical biology-derived probes will play a pivotal role in drug discovery.

4. Combined “omics” approaches provide a holistic view for toxicity studies.

5. To live is to balance.

6. Freedom is to say no.

Propositions belonging to the thesis, entitled
‘Effects of xenobiotics on gut microbiota and related bile acid metabolism’.

Weijia Zheng
Wageningen, 2 May 2023

Effects of xenobiotics on gut microbiota and related bile acid metabolism

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Effects of xenobiotics on gut microbiota and related bile acid metabolism

Weijia Zheng

Thesis

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CHAPTER 1

General Introduction

1.1 Background and aim of the thesis

The gut microbiome consists of trillions of microorganisms residing in the intestine and has a mutualistic relationship with its host (Backhed et al., 2005). The gut microbiome plays an important role in maintaining host health, enhancing the immune system and regulating host metabolism thereby defining a host-microbe metabolic interaction (Turnbaugh et al., 2006; Tremaroli and Backhed, 2012; Nicholson et al. 2012). It has been reported, for example, that germ-free (GF) mice have reduced adiposity, a phenotype that can be reversed by colonization with a normal gut microbiota (Bäckhed et al., 2004). It is generally believed that gut microbiota is a critical factor contributing to host metabolism and health, by producing numbers of bioactive compounds that act as a signal to the host by activating cognate receptors in various cells (Holmes et al., 2012). Such signalling metabolites may include, for example, bile acids.

Bile acids which are synthesized from cholesterol in the liver, excreted via the biliary tract into the gastrointestinal tract, and further modified by gut microbes, contribute to the host metabolic phenotype and hence to disease risk (Gérard, 2013). Metabolomics has been demonstrated to be a valuable tool in studies on metabolic patterns and related mechanisms of toxicity (Robertson et al., 2011). Metabolic patterns, such as also bile acid profiles, are known to be sensitive to external environmental stimuli, and relative subtle changes in the metabolome can be detected in response to exposure to xenobiotics, like antibiotics and pesticides (Behr et al., 2019; Li et al., 2019).

Antibiotic-induced alterations of gut microbiota and bile acid profiles in in vivo animal models have been previously reported (Behr et al., 2018; Behr et al., 2019; Kang et al., 2019; Theriot et al., 2016). For example, in the studies reported by Behr et al. (Behr et al., 2018; Behr et al., 2019), antibiotics from five different classes including lincosamides, glycopeptides, macrolides, fluoroquinolones, and aminoglycosides were reported to be able to significantly alter the rat gut microbial community and the related bile acid profiles. In addition, the toxicity of

pesticides towards non-target organisms like gut microbiota has also gained raising attention in recent years (Defois et al., 2018). Some studies on gut microbiota alterations and host outcomes induced by exposure to pesticides such as organochlorine pesticides, have been recently published (Defois et al., 2018). As another example, in work evaluating the impact of carbamates on gut microbiota, an altered abundance of *Firmicutes* as well as changes in lipid profiles were reported in aldicarb treated C57BL/6J mice (Gao et al., 2018). In other studies, effects of the pyrethroids bifenthrin and permethrin on gut microbiota causing dysbiosis have been reported (Li et al., 2021; Nasuti et al., 2016). However, studies on effects of antibiotics or pesticides on the gut microbiome and metabolic profiles were mostly carried out in in vivo animal models, and there has been no study reporting on an in vitro fermentation model able to mimic and reproduce the in vivo alteration of intestinal bacteria and related bile acid profiles upon exposure of the microbiota to xenobiotics.

Therefore, the aim of the present thesis was to develop such an in vitro model for studying the potential effects of test compounds on the transformation of bile acids by fecal microbiota; subsequently, lincosamide antibiotics were used for the evaluation of this model by the comparison of results obtained in the newly developed in vitro model and reported previously in a 28 day in vivo study (Behr et al., 2019). This in vitro model system consisted of anaerobic fecal incubations and was optimized to enable detection of (altered) bile acids profiles by LC-MS/MS, while bacterial profiles were determined by 16S rRNA gene sequencing analysis. As a first proof of principle the in vitro model system was applied to study the variation of gut microbiota induced by the lincosamides, and to characterize the dynamic interactions between gut microbiota and the bile acid pool. Subsequently, the impact of pesticides including organophosphates, carbamates, and pyrethroids was assessed using the newly defined in vitro model. Further validation of the in vitro fermentation model was achieved by comparison of the effects of the pyrethroid cyhalothrin on gut microbiota and bile acid profiles in the

in vitro model system to effects on these endpoints induced by this pesticide in an in vivo mouse study.

1.2 Gut microbiota

Coelomate animals have an internal body cavity involving the gut which is coevolved with a diverse range of symbiotic gut bacteria and other microorganisms collectively known as the gut microbiota (Gordon, 2012). A mutually beneficial relationship between the host and the resident gut microorganisms contributes to the production of metabolites and the evolutionary fitness of the host (Hosokawa et al., 2006). Topographical and temporal variation influence and condition the diversity and composition of the microbial communities within and between individuals of the host species. Particular bacterial species have been linked to particular growth or maturation phases of the host (Gordon, 2012; Dominguez-Bello et al., 2010). In humans, the primary individual microbiota first appear at birth (Ravel et al., 2011; Torrazza and Neu, 2011), and the subsequent shaping of the microbial landscape is driven by a series of complex and dynamic interactions including diet, life-style, disease, and antibiotic use throughout life. As an example, the gut microbiota influences the development of the host immune system, which in turn influences the gut microbial composition. This crosstalk begins at birth, and is transmitted by a vast array of signalling pathways including multiple classes of molecules. These immune-mediated signalling processes coupled with other direct chemical interactions between gut microbes and host, act on organs such as gut, liver, muscle, and brain. Together these complex interactions comprise a series of host-microbe metabolic axes (Nicholson et al., 2012).

The host-microbe metabolic axis comprises an interactive chemical communication between specific host cellular pathways and a series of microbial species and their activities and metabolites in multiple directions. Within these metabolic axes, a variety of bacterial genomes can sequentially direct metabolic reactions, carrying out combinatorial metabolism of substrates, such as the

production of bile acids, choline, and short-chain fatty acids that are essential for host health (Nicholson and Wilson, 2003). The production of these metabolites by gut microbes contributes to the host metabolic phenotype potentially influencing host health. The composition of the core gut microbiota is considered to be essentially stable throughout adulthood. However, some gut components are dynamic and biologically and metabolically flexible, and could respond to many perturbations through the alteration of microbial composition hence influencing health or disease risk (Clemente et al., 2012). The incidence of gut dysbiosis in western populations has been reported to be continuously rising during the past 60 years, which has been associated with a variety of factors, exemplified by changes in diet, environmental stresses, exposure to xenobiotics (such as pesticides and antibiotics), or life-style-related diseases (such as diabetes and obesity)(Giambò et al., 2021; Liu et al., 2020).

Hence, increasing attention is focused in recent years on the development of new therapeutic tools for determining and manipulating the gut microbial composition to benefit host health. A better understanding on how variations of the gut microbial community and the subsequent metabolic consequences mediated by specific bacterial species, will contribute to disease risk as well as health sustainability, will point the way to new therapeutic interventions and disease prevention strategies (Holmes et al., 2012). In the present project, the focus is on the effects of antibiotics as well as pesticides on gut microbiota mediated modulation of bile acid profiles.

1.3 Bile acids

Bile acids play a significant role as biological detergents in the intestine, aiding the breakdown and adsorption of lipids (Begley et al., 2005). In general, they are synthesized in the liver from cholesterol, stored in the gallbladder of human and released into the small intestine following consumption of food. Notably, a gallbladder is present in mice (Mus musculus) but not in rats (Rattus norvegicus), respectively (Mann et al., 1920; Martins and Neuhaus, 2007). In animals like rats,

bile acids are excreted from the liver directly into the small intestine via the bile duct (Yang et al., 2017). In liver hepatocytes, bile acids are synthesized by a series of enzymatic reactions predominantly via two routes involving the classic and alternative pathway. The classic pathway mediates the synthesis of the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) from cholesterol through cytochrome P450 enzymes (CYPs), as shown in **Figure 1.1**. The bile acid synthesis from cholesterol is first performed by the liver-specific 7α-hydroxylase (CYP7A1), and continued by CYP8B1 together with CYP27A1 to generate CA or CDCA (de Aguiar Vallim et al., 2013). In addition to the classic pathway, less than 10% of the bile acids in the pool are produced via the alternative pathway, which is initiated by CYP27A1 and via CYP7B1 yields CDCA in human (Chiang, 2009; Li and Chiang, 2014), while instead of CDCA mice produce muricholic acids (MCAs), predominantly beta-MCA (βMCA) as primary bile acids (Sayin et al., 2013)(**Figure 1.1**).

Prior to secretion into bile of human, CA and CDCA are first conjugated in the liver to either a taurine or glycine moiety to generate T/G-CA and T/G-CDCA (Falany et al., 1994), while in mice βMCA is conjugated with taurine to primarily generate tauro-βMCA (TβMCA), and then these conjugated bile acids are stored and eventually released into the intestines (**Figure 1.1**). Following release into the duodenum after meals, the conjugated bile acids are subject to chemical modifications by the microbiota (**Figure 1.1**). A first step in this bacterial modification is deconjugation of these conjugated bile acids followed by the generation of secondary bile acids. The deconjugation of glycine- or taurine-conjugated bile acids is a prime and key step in the bacterial modulation of bile acids, and is carried out by the action of microbial bile salt hydrolase (BSH)(Jones et al., 2008). BSH cleaves the amide bond to release the amino acid group and liberate unconjugated bile acid moieties, modifying the bile acids to more lipophilic compounds. The majority of the deconjugated bile acids is reabsorbed and circulates back to the liver. Part of the deconjugated primary bile acids

(approximately 5%) that escapes this enterohepatic recycling enter the colon and are further metabolized by the colonic microbiota. Some bacterial species exemplified by *Eubacterium* and *Clostridium XIVa* clusters possess the ability to 7α-dehydroxylate primary unconjugated bile acids to generate secondary bile acids (Ridlon et al., 2013).

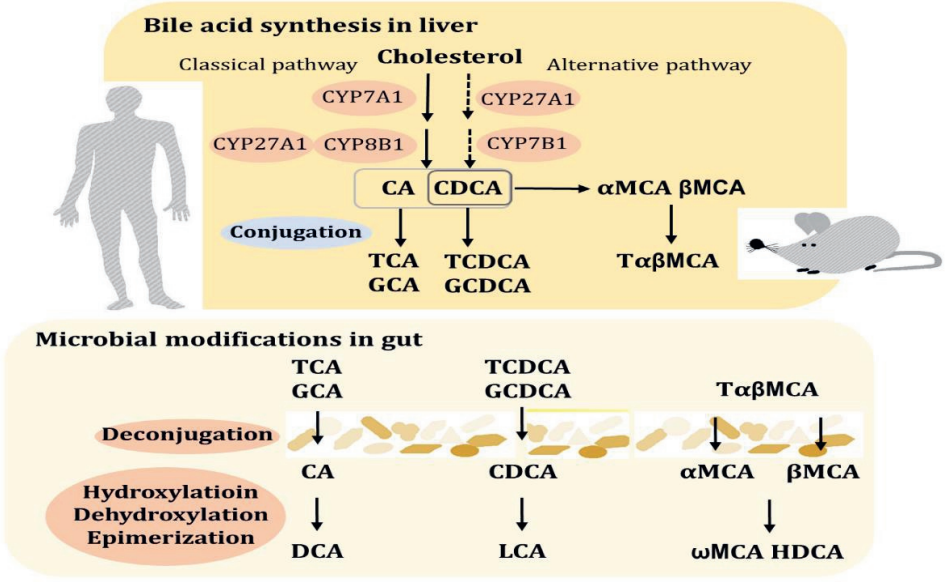

Figure 1.1 Bile acid synthesis and metabolism in human and mice.

As shown in **Figure 1.2**, in human the majority of the non-reabsorbed CA and CDCA are converted to deoxycholic acid (DCA) and lithocholic acid (LCA) respectively, and further to ursodeoxycholic acid (UDCA)(Ridlon et al., 2006). In mouse intestine, after the deconjugation of TβMCA, βMCA is primarily converted to the secondary bile acid omega-MCA (ωMCA) by 6β-epimerization, catalyzed by the mouse gut microbiota (Eyssen et al., 1983). These secondary bile acids are important signalling molecules that are potent activators of the bile acid receptors (Ridlon et al., 2014), for example, DCA has been known to activate a number of cell-signalling pathways (Hylemon et al., 2009). Since the human liver is incapable of 7α-hydroxylation, secondary bile acids return to the liver via reabsorption and the portal vein, and external stimuli could cause high levels of the secondary bile

acids such as DCA accumulating in human feces and serum, an observation which is widely reported to be associated with colon cancer and some cholesterol gallstone diseases (Backhed et al., 2005).

Overall, the bile acid profile composed of conjugated, primary, and secondary bile acids as it circulates between gut, liver and the periphery can regulate several host processes, and play a crucial role in health.

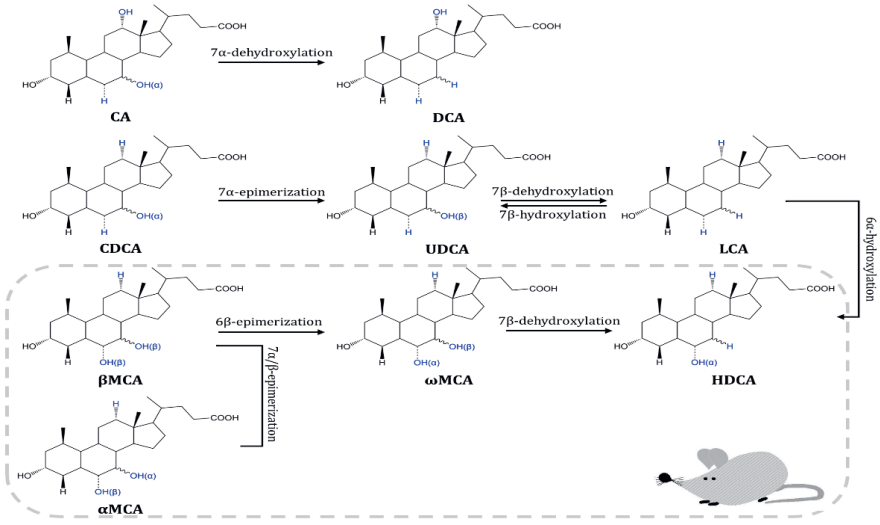

Figure 1.2 Primary and secondary bile acid transitions by the intestinal microbiota.

1.4 Antibiotics

Antibiotics are analogues of microbial or fungal metabolites that at low concentrations inhibit the growth of other microorganisms. They are low-molecular-weight substances with a defined chemical structure having a relative mass of at most a few thousand, and involve not only natural products of microorganisms but also the semisynthetic antibiotics which are obtained by chemical modification of the microbial metabolites (Lancini et al., 1995). The large variety of antibiotics is produced by an array of widely diverse microorganisms, and the taxonomic distribution of the strains involved is not uniform. More than 50% of antibiotics are produced by the bacterial order *Actinomycetales* and in particular the genera *Streptomyces*. *Eubacteria* such as the sporogenic *bacilli* are

known to have the capacity of producing particular classes of antibiotics (Lancini et al., 1995). In addition, two fungal genera, including *Aspergillus* and *Penicillium*, also produce a relatively high number of antibiotics (Teixeira et al., 2012). In contrast to the great variety of chemical structures and producing strains, the biological reactions involved in the synthesis of antibiotics can be grouped into a few fundamental biosynthetic pathways, and small changes in these pathways can give rise to diverse substances (Lancini et al., 1995).

Antibiotics have the ability to form a complex with molecules that are essential for the growth of bacterial cells by binding with molecules of various nature, thus inhibiting their function (Zimmer et al., 1990). Commonly, these molecules are proteins with enzymatic activity involved in the functioning of DNA or ribosomal RNA (Dethlefsen et al., 2008; Garcia et al., 2011). Hence to induce inhibition, several weak bonds must form between functional groups of the antibiotic and those of the target molecules, together providing sufficient strength and formatting a stable complex. This reveals that chemical structures of antibiotics determine their biological activity, identify the functional target groups and how they interfere with molecular activity (Lancini et al., 1995). Thus, antibiotics can be classified on the basis of their chemical structures and the resulting mode of action. Based on this classification, the most common antibiotic groups are: β-lactams, sulfonamides, monobactams, carbapenems, aminoglycosides, glycopeptides, lincosamides, macrolides, polypeptides, tetracyclines, quinolones, and fluoroquinolone (Chu et al., 1996).

During the last decades, concerns emerged on the use of antibiotics not only because of the clinical role of antibiotics but also because of unintentional residues in the environment and the potential of developing resistance (Aminov, 2009). The wide use of antibiotics has led to their ubiquitous occurrence in most natural and artificial systems. Soil, sediment, sludge, groundwater, tap water, plants, and aquatic animals have been reported for contamination with antibiotics (Halling-Sørensen et al., 1998; Kümmerer, 2009; Heberer, 2002). Thus, the antibiotic

residues in fish meat, drinking water, or fruits and vegetables which are irrigated by the contaminated water could enter human bodies by various food products. For example, in previous work on screening of antibiotic residues in fresh milk in Nepal, it was reported that 81% of milk samples were positive for amoxicillin (68–802 μg/kg), 41% for sulfadimethoxine (31–69 μg/kg), 27% for penicillin G (13–353 μg/kg), and 12% for ampicillin (0.5–92 μg/kg), and most of the residual antibiotics were found to exceed the maximum residue limits (MRLs)(Khanal et al., 2018). Recently, it has been reported that antibiotic treatment has tremendous impact on the overall taxonomic composition of the gut microbiota appearing fast and being surprisingly long-lasting. Within days after administration of an antibiotic, profound compositional effects on luminal and mucosal bacteria were noted resulting in decreased taxonomic richness (Dethlefsen et al., 2008; Neulinger et al., 2015) together with a significant upregulation of resistance genes, which may even last for years (Jernberg et al., 2007; Jakobsson et al., 2010). In the past five years, some studies reported that the treatment of mice or rats with broad-spectrum antibiotics such as cefoperazone, clindamycin, and vancomycin, streptomycin, roxithromycin, or lincomycin could induce a changed gut microbial community and resulting changes in fecal bile acid profiles (Behr et al., 2019; Theriot et al., 2016; Zhang et al., 2014). Notably, in the work of Behr et al. (Behr et al., 2019) it was shown that the treatment of rats with lincosamides induced the most significant alterations in host bile acid metabolism compared to other antibiotics tested including roxithromycin, sparfloxacin, streptomycin, and vancomycin. Hence, the two lincosamides were selected as the representative antibiotics for the studies in the present thesis.

1.5 Pesticides

Since the discovery of DDT in 1939 (Mellanby, 1992), numerous pesticides have been developed and extensively applied worldwide. They are substances or mixtures of substances which are intended for repelling or lessening the damage of any pest (Eldridge, 2008). The pest could be insects, plant pathogens, weeds,

mollusks, and microbes which are detrimental for humans because they destroy food or property and carry or spread diseases. The well-known pesticides involve insecticides, herbicides, fungicides and rodenticides; in addition to these common used pesticides, growth regulators, plant defoliants, and surface disinfectants are also frequently applied (Yadav et al., 2015). Pesticides are widely used primarily in the health sector for protection of humans against vectors of diseases like mosquitoes, and on agricultural crops for protection against pest damage. Pesticides are potentially toxic to non-target organisms including humans, probably posing risks to public health when exposure exceeds established health based guidance values like an ARfD (acute reference dose) or ADI (acceptable daily intake).

Pesticides differ in their physical and chemical properties, and they are normally studied under the respective groups according to their characters. Currently, there are three methods for classification of pesticides based on their mode of entry, their function (organisms they kill) or their chemical structures (Drum, 1980). The classification according to the chemical structure and nature of active ingredients is the most commonly used, since it gives a clear clue on the efficacy, and physical and chemical properties of the pesticides. Meanwhile, the information on chemical and physical characteristics is not only useful in evaluating the mode and rates for application, but also for defining precautions for protection, and for setting up the screening and supervision methods. Based on chemical structure, the synthetic organic pesticides are classified into four dominant groups including organochlorines, organophosphorus pesticides, carbamates and pyrethroids (Buchel, 1983)(**Figure 1.3**).

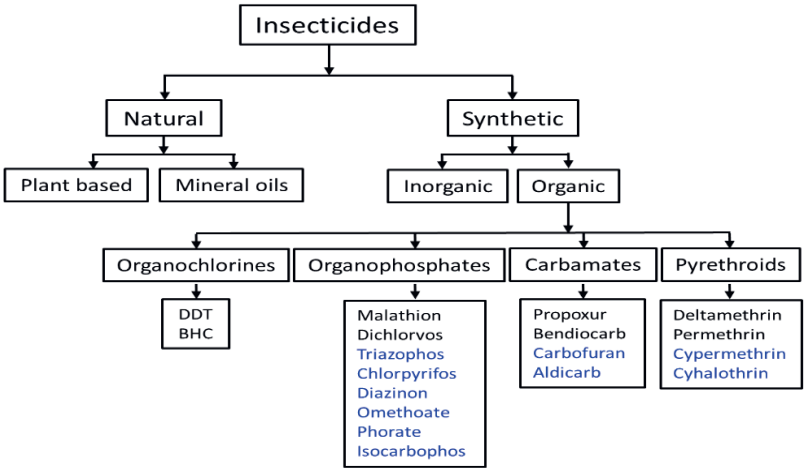

Figure 1.3 Classification of pesticides (pesticides studied in this thesis are highlighted in blue).

Organochlorine (OC) pesticides are organic compounds containing five or more chlorine atoms. They are the first group of pesticides ever synthesized and extensively used as insecticides. They exert their toxicity via binding to the nerve membrane and by interfering with the transmission of nerve impulses via disturbing the sodium or potassium ion balance across nerve membrane, resulting in convulsions, paralysis, and eventually death (Kaushik and Kaushik, 2007). Representative examples of OC pesticides include DDT, lindane, endosulfan, aldrin, and chlordane (Kaushik and Kaushik, 2007).

Organophosphate (OP) pesticides are another category of broad spectrum pesticides with multiple functions, including contact, stomach, and nerve poisons. In addition, OP pesticides are biodegradable, which results in lower environmental pollution and slower development of pest resistance (Martin, 1968). OPs act as cholinesterase inhibitors leading to a prolonged presence of the neurotransmitter acetylcholine in a synapse, an effect that is toxic to vertebrates and invertebrates. Due to the inhibition of acetylcholinesterase (AChE) induced by OPs, nervous impulses ultimately fail to move across the synapse which can result in a rapid twitching of voluntary muscles followed by paralysis and death.

Extensively used OP insecticides are triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, and phorate (Liu et al., 2019; Sapbamrer and Hongsibsong, 2014)(**Figure 1.4A**).

Carbamates (CBMs) are structurally similar to OP pesticides, however, OPs and CBMs are derived from phosphoric acid and carbamic acid, respectively. The working principle of CBMs and OPs is similar since both mostly act by altering the activity of AChE thereby affecting the transmission of nerve signals leading to death (Drum, 1980). CBMs are also easily degraded in the natural environment, and the CBMs carbaryl, carbofuran, aldicarb, and propoxur are commonly used (Koc et al., 2008); among them, carbofuran and aldicarb were selected to be studied in the present study (**Figure 1.4B**).

Pyrethroid (PYR) pesticides are synthesized and mimic the structure of natural pyrethrins; relatively, they have longer residual effects than pyrethrins. It has been found that the pyrethroids primarily act on the sodium and chloride channels which drive the transport of ions through cell membranes (Bradberry et al., 2005), thereby reducing the threshold for the action potential of nerve and muscle cells, leading to repeated stimulation (Sichilongo, 2004). Previously, their carcinogenic, neurotoxic and immunosuppressive properties and potential for reproductive toxicity in mammals have been reported (WHO, 2005). Of all PYRs cypermethrin and cyhalothrin were reported to be the most extensively used, therefore, they were selected for the studies in the present thesis for their effects on intestinal microbiota and related bile acid profiles (Liu et al., 2007)(**Figure 1.4C**).

Some previous studies reported that environmental pollutants including especially pesticides could affect the gut microbiota causing an altered composition and diversity (Lee et al., 2005). Notably, an altered gut microbial community and altered lipid profiles were found to be highly associated with exposure to insecticides including nitenpyram (Yan etal., 2020) and aldicarb (Gao et al., 2018). Additionally, changes in hepatic and gut microbiota-mediated bile acid metabolism have been reported in mice after treatment with the OCs

dichlorodiph, enyldichloroethylene and β-hexachlo rocyclohexane (Liu et al., 2017). As a result, the pesticide-induced toxicity towards non-target organisms of the gut bacterial community and the related effects on metabolism are recently gaining increasing attention (Shim et al., 2009). Hence in the current project, pesticides involving OPs, CBMs, and PYRs were studied for their impact on gut microbial and bile acid profiles.

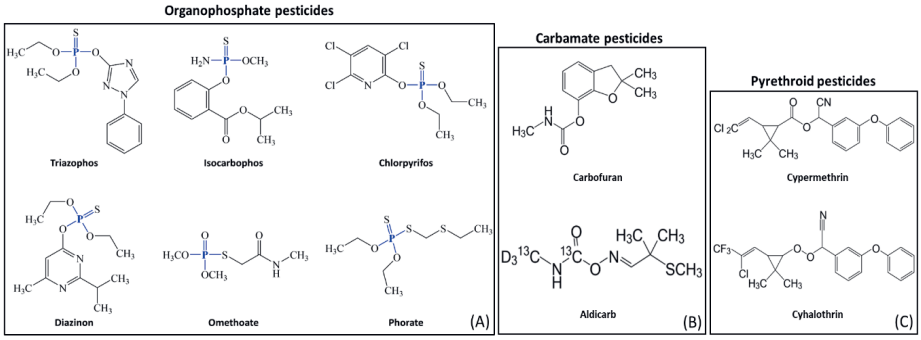

Figure 1.4 Chemical structures of the pesticides studied in the present thesis for their effects on intestinal microbiota and related bile acid profiles, including (A) six OPs, including triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, and phorate; (B) two CBMs, including carbofuran and aldicarb; (C) two PYRs, including cypermethrin and cyhalothrin.

1.6 In vitro models to study the microbiome

The host-microbe interactions consisting of activities from the gut microbiota, intestinal epithelial cells, neuronal, and immune cells are critical for the maintenance of host homeostasis. The gut microbiota could be influenced by environmental, nutritional, and cognitive stress. To date, studies only reported a few factors which can induce microbial dysbiosis such as pesticide exposure (Giambò et al., 2021) or obesity (Liu et al., 2020), and the bidirectional relationship between host and microbiome pertaining to health and disease is still not well understood. Hence in the past decade, research in this area is rapidly expanding, with especially in vivo models being widely utilized for studying effects

of environmental stimuli on the gut microbiota. In addition to the in vivo models, in modern life sciences in vitro models are increasingly employed and have also been shown to be of use for studying some specific bacterial species or pathways, shortening the experimental time, and following the principles of the 3Rs (Replacement, Reduction and Refinement).

Particularly in studies on the microbial community, in vitro fermentation models were extensively applied including the continuous Simulator of the Human Intestinal Microbial Ecosystem (SHIME) and batch fermentation models (Pérez-Burillo et al., 2021). The SHIME is a complex and large system which is composed of five stages, two to mimic gastrointestinal digestion and three to mimic colonic fermentation; meanwhile, all five stages are operated in a way to mimic different portions of the entire gastrointestinal tract incorporating stomach, small intestine and different colon regions by controlling pH and nutrients (Van de Wiele et al., 2015). The obvious drawback of the SHIME is that the experiment takes over one month involving 1–2 weeks for the stabilization of the microbial communities, and another 1–2 weeks for experimental running after adding the studied target component (Agans et al., 2018); additionally, only one sample can be studied at a time. In contrast, batch fermentation models enable many samples to be studied at once; meanwhile, these batch cultures are convenient in that they are only carried out in small test tubes and are running within a short period of time of usually 24–48 hours (Ludwig et al., 2013; Coles et al., 2005). Although batch fermentation models can be less physiologically relevant and may have the problem of bacterial waste accumulation, they are useful tools for initial screening of the studied compounds and showing effects of the targeted chemicals on the gut microbial community and their metabolites; particularly, the physiological relevance can be improved by optimizing the culture conditions. Thus, in the present thesis, a 24 h batch fermentation in vitro system was optimized for studying the effects on the gut microbiota and related bile acid metabolism and utilized in the current project.

1.7 Outline of this thesis

Gut microbiota is considered a cornerstone of maintaining the health status of its host since it not only facilitates harvesting of nutrients and energy from ingested food, but also produces numerous metabolites that regulate host metabolism. One such class of metabolites, the bile acids, are synthesized from cholesterol in the liver and further metabolized by the gut microbiota into secondary bile acids. These bile acids can regulate diverse metabolic pathways and also in return modify gut microbial composition directly or indirectly. In addition, the gut microbial and bile acid profiles can be altered by acute stress factors such as antibiotics and pesticides. In this project, an in vitro fecal fermentation model that enabled detection of effects on gut microbiota and related bile acid homeostasis was first established; subsequently, this developed in vitro model system was utilized for the evaluation of the effects of selected pesticides on gut microbiota and the related bile acid profiles. In a final study, an in vivo study on the impact of one selected pesticide was carried out to evaluate the altered gut bacterial community and bile acid profiles induced by this pesticide and the developed in vitro model.

Background information and an outline of the thesis are provided in the present chapter, **Chapter 1**.

In a previous rat in vivo study (Behr et al., 2019), Behr et al. reported the alterations of bile acid profiles in rat plasma and fecal samples induced by various antibiotics. Results obtained showed that the antibiotic group of lincosamides had a large impact on host bile acid profiles especially on the fecal bile acids, an effect likely mediated via effects of the antibiotics on the gut microbiota and their bile acid metabolism. In **Chapter 2** of the thesis an in vitro batch fermentation model was established and optimized for the study of the effect of antibiotics and other chemicals on the gut microbiota and related bile acid profiles. To this end a method for quantification of bile acid profiles by LC-MS/MS was first developed, and the process of fecal sample preparation and fermentation using anaerobic

incubations was optimized. Lincosamides were utilized for evaluating this in vitro model by comparison of effects induced by lincomycin and clindamycin to results on the effects of these lincosamides on bile acid profiles reported in the 28-day in vivo rat study.

Subsequently, this developed model was applied for exploring the impact of pesticides on gut microbiota and related bile acid profiles. Studies on gut microbiota toxicity of pesticides just started to be a topic of interest in recent years, and very few studies have reported on pesticide-induced gut microbial disorder so far. Hence, after a literature search on the presence of pesticide residues in agricultural products of China, ultimately ten pesticides, including organophosphates, carbamates, and pyrethroids, were selected and studied in **Chapter 3** and **Chapter 4**. In **Chapter 3**, six organophosphates including triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, and phorate were investigated whereas in **Chapter 4** two carbamates carbofuran and aldicarb, as well as two pyrethroids cypermethrin and cyhalothrin, were evaluated. LC-MS/MS and 16S rRNA sequencing were utilized for determining the bile acid and bacterial profiles respectively. The impact of the various pesticides on gut microbiota and related bile acid profiles was characterized by principal component analysis (PCA). The possible relationships between significantly altered bile acid levels and changes in gut microbiota at the family level were characterized by pearson correlation analysis. Among all tested pesticides in **Chapter 3** and **4**, the one that manifested the most substantial effects on gut microbiota and related bile acid profiles was selected for the final in vivo study. Hence, the toxic isomer of cyhalothrin, lambda-cyhalothrin, was selected for the in vivo study.

Chapter 5 describes this in vivo study for assessing the impact of lambda-cyhalothrin on the gut microbial community and bile acid profile. C57BL/6 mice were used for this 28-day oral dose toxicity study, and two dose levels of 1 and 2 mg/kg bw per day were employed. Plasma samples were taken at day 7, 14, an 28 whereas fecal samples were taken at the 28th day from lambda-cyhalothrin treated

mice and controls, and all acquired samples were processed and measured for their bile acid pools. In addition, the collected fecal samples were analyzed for their bacterial profiles by 16S rRNA sequencing, and clinical signs of all studied mice were noted as well. Results obtained were analyzed for the impact of lambda-cyhalothrin on mouse bacterial and bile acid profiles, as well as the correlation between the two profiles.

Chapter 6 presents a detailed discussion of the results obtained including a comparison between results obtained in the in vitro and in vivo models, the interactions between gut microbiota and bile acid profiles, the impact of chemical compounds on the gut microbial community, the advantages and disadvantages of the in vitro vs. in vivo models, and the potential health effects induced by altered bile acid profiles.

Finally, **Chapter 7** provides a concluding summary of the results obtained in this thesis.

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2

CHAPTER 2

A simple in vitro fermentation model to detect alterations in microbiota dependent bile acid metabolism

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Abstract

A previous in vivo study showed that lincosamide antibiotics (lincomycin and clindamycin) could induce changes in the gut bacterial community, leading to significant changes in fecal bile acid profiles. Herein, our aim is to develop an animal alternative in vitro model for studying gut microbiota-dependent bile acid profiles induced by xenobiotics. The effects of lincosamides were evaluated using this model, and results obtained were verified by comparing with those of the previous in vivo study. Fecal sample processing and bile acid incubation conditions were developed and optimized using feces collected from Wistar rats, and prepared samples were incubated for 24 h with or without lincosamides. Upon treatment of the fecal gut microbiota with lincosamides primary and secondary bile acids showed obviously increased and decreased levels respectively. Moreover, the changes in bile acid profile could be linked to a reduced richness of family *Erysipelotrichaceae* and *Bacteroidacea*. The consistent consequences of in vivo and in vitro provides a proof of principle for further application on elucidating effects of other xenobiotics on the gut bacterial community and related bile acid metabolism, thereby contributing to the 3Rs (replacement, reduction and refinement) in animal testing.

Keywords: metabolomics; fecal bile acid; gut microbiota; in vitro model system, lincosamides

2.1 Introduction

It is increasingly appreciated that the gut microbiota contributes to health and disease (Wahlström et al., 2016). It has been reported that the reduced adiposity of germ-free (GF) mice can be reversed by colonization using a normal gut microbiota (Bäckhed et al., 2004); as a result, the gut microbiota has emerged as a critical factor contributing to host health and metabolism. However, the profile of intestinal bacteria could be altered by a variety of factors, especially antibiotics, such as lincosamides (Sung and Lee, 2008). Representatives of lincosamide antibiotics, such as lincomycin and clindamycin, which are normally applied for the treatment of protozoal diseases according to their antibacterial properties (Spížek and Řezanka, 2004).

It is generally believed that the gut microbiota contributes to host metabolism by converting various bioactive compounds, such as bile acids, that may signal to the host by activating cognate receptors in sensitive cells (Holmes et al., 2012). It has been reported that the gut microbiota is involved in the biotransformation of bile acid through deconjugation, dehydroxylation, and reconjugation of these molecules (Hirano et al., 1982). Moreover, bile acids may also display antimicrobial activity that can damage bacterial cell membranes, leading to inhibition of bacterial overgrowth and protection of the liver and intestine against inflammation (Kurdi et al., 2006; Torres-Fuentes et al., 2017). Thus, the altered composition of intestinal microbiota caused by oral administration of antibiotics could further result in the disorder of bile acid profiles and other unknown health effects.

However, bile acid metabolism is complex, since bile acids are initially modified by liver enzymes and upon secretion into the intestines subsequently modulated by the microbiota, which in return regulate the size and composition of the bile acid pool (Forman et al., 1995; Seol et al., 1995). Some studies have reported on antibiotic-induced alterations of gut microbiota and bile acid production in in vivo animal models (Behr et al., 2019; Kang et al., 2019; Theriot et al., 2016). Previously, characterization of bile acid pools was mostly achieved from samples of animal in vivo work, and thus far, only a few studies have developed in vitro batch models to enable quantification of the dynamic intestinal bile acid pool (Martin et al., 2018). In particular, there has been no study reporting on an in vitro fermentation model able to mimic and reproduce the alteration

of intestinal bile acid profiles observed upon administration of antibiotics or other drugs in vivo.

The aim of the present study was to develop and evaluate such an in vitro model for studying the potential effects of test compounds on the processing of conjugated bile acids by the rat intestinal microbiota. To this end, a model consisting of anaerobic fecal incubations was characterized in which (altered) bile acid profiles could be quantified using LC–MS/MS, and bacterial profiles were determined by 16S rRNA gene sequencing analysis. The study design for sample processing and data acquisition of the current study is presented in **Figure 2.1**. As a first proof of principle, the in vitro model system was applied to study the variation in gut microbiota and bile acid profiles induced by lincosamides to characterize the dynamic interactions between gut microbiota and the bile acid pool and to enable evaluation of the in vitro model by comparison to available in vivo data on the effect of these antibiotics on bile acid profiles in rats orally exposed to lincomycin and clindamycin (Behr et al., 2019).

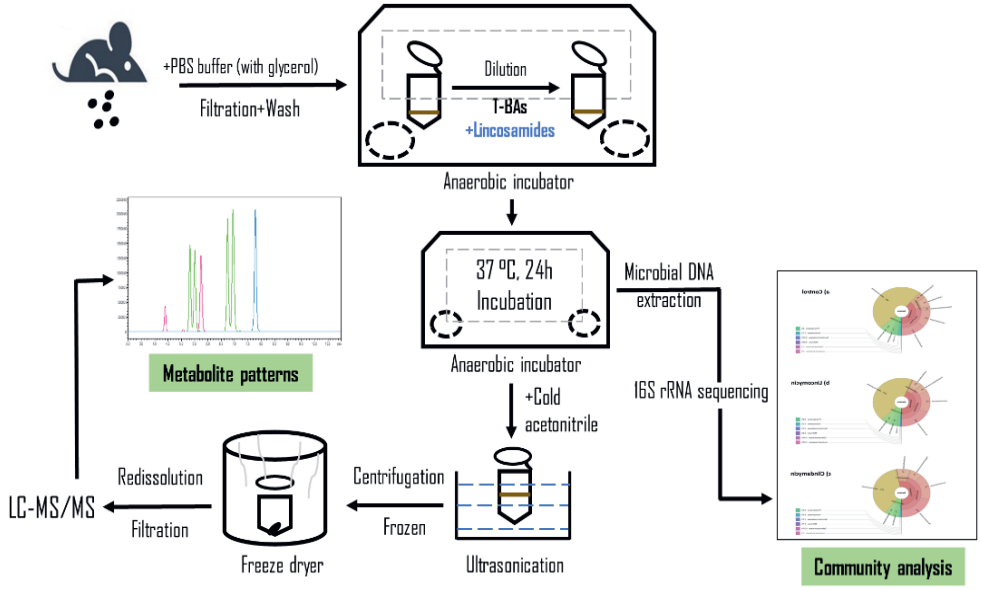


Figure 2.1 Scheme presentation and study design for bile acid profiling in control and antibiotic-treated rat feces and the corresponding community analysis is shown. Fecal samples for bile acid profiling were taken at 0, 6, 12, and 24 h, while samples for 16S rRNA analysis were taken only at 24 h of anaerobic incubation.

2.2 Material and methods

2.2.1 Chemicals and solvents

The test compounds lincomycin hydrochloride and clindamycin hydrochloride were procured from Sigma Aldrich (Zwijndrecht, The Netherlands). Bile acids were supplied by Merck KGaA (Darmstadt, Germany) or Cambridge Isotope Laboratories (Tewksbury, USA). Methanol and acetonitrile (ACN) were acquired from Biosolve BV (Valkenswaard, The Netherlands), and formic acid was ordered from VWR CHEMICA (Amsterdam, The Netherlands). Phosphate-buffered saline (PBS) was secured from Gibco (Paisley, UK), and para-Pak SpinCon tubes were supplied by Fisher Scientific (New Hampshire, USA). A solution of mixed taurine conjugated bile acids (T-BAs) containing 500 µM taurocholic acid (TCA), 100 µM tauroursodeoxycholic acid (TUDCA), and 100 µM tauro-β-muricholate (TβMCA) was prepared in PBS. The mixture of labelled bile acids ([2,2,4,4-d4] cholic acid and [2,2,4,4-d4] lithocholic acid) obtained from Merck KGaA was dissolved at a final concentration of 100 µM (for each) in methanol as an internal standard for LC–MS/MS analysis.

2.2.2 Fecal sample preparation

Fresh fecal samples from Wistar rats (20 males and 20 females) were kindly provided by BASF (Ludwigshafen, Germany). Feces from individual animals were obtained by physical stimulation of the abdomen to trigger defecation, after which the fecal samples were weighed and immediately transferred into an anaerobic solution of 10% (*v/v*) glycerol in PBS and diluted to a final fecal concentration of 20% (*w/v*). Pooled samples were manually stirred by a sterile glass wand and flushed with N_2 gas. Subsequently, samples were filtered using a woven sterile medical gauze dressing (provided by HeltiQ, Wolvega, The Netherlands) under anaerobic conditions (85% N_2, 10% CO_2 and 5% H_2, in a Bactron EZ anaerobic chamber)(Sheldon, Cornelius, USA), and aliquoted samples of the resulting fecal slurry were stored at -80 °C until further use.

2.2.3 Anaerobic incubations and extraction of bile acids

Conditions for the anaerobic incubation of rat feces and subsequent bile acid analysis were optimized to achieve an effective extraction and adequate recovery of the bile acids and to ensure sufficient activities of the gut microbiota. To remove residual endogenous fecal bile acids, filtered fecal samples containing 20% feces (*v/v*) were washed twice using

equal volumes of anaerobic PBS ($V_{fecal\ sample}$: V_{PBS} =1: 1) by vortex mixing for 1 min and centrifugation at 2,000×g for 5 min at 4 °C under anaerobic conditions. Supernatants were removed, and PBS was supplied to obtain the same sample volume as before washing. Subsequently, the samples were incubated with an externally added bile acid mixture. The 200 µL incubation system was composed of 120 µL washed fecal sample (final concentration 1.5 g feces/mL), 20 µL mixed solution of T-BAs providing a final concentration of 50 µM TCA, 10 µM TUDCA and 10 µM TβMCA, 20 µL concentrated stock solutions of lincomycin (final concentration: 16.32 mM) or clindamycin (final concentration: 10.41 mM) in anaerobic PBS buffer or 20 µL PBS for the control without antibiotic. The final concentrations of lincosamides to be tested in the developed in vitro model were based on the dose levels used in a literature reported in vivo study in rats (Behr et al., 2019) and determined as follows:

$$\text{Exposure concentration in vitro (mM)} = \frac{\text{dose level in vivo (mg/kg bw)} * \text{body weight (kg)}}{\text{molecular weight (mg/mmol)} * \text{rat gut volume (mL)}} \times 1000\ \text{(mL/L)}$$

The dose levels used in the reported in vivo study amounted to 300 and 200 mg/kg body weight for lincomycin and clindamycin, respectively (Behr et al., 2019), and a rat body weight of 0.25 kg as well as a gut volume of 11.3 mL were applied (Davies and Morris, 1993), as summarized in **Table 2.1**.

Table 2.1 Calculation of the exposure concentrations of lincosamides used in the fecal incubations

Antibiotics	Dose level in vivo (mg/kg/bw) (Behr et al., 2018)	Total exposure amount (mg)	Exposure to gut* (mg/mL)	Molecular Weight	Exposure concentration (mM)
Lincomycin	300	75	6.64	406.54	16.32
Clindamycin	200	50	4.42	424.98	10.41

* A gut volume of 11.3 mL was applied (Davies and Morris, 1993).

Samples were prepared and incubated in an anaerobic chamber (Sheldon, Cornelius, USA) with an atmosphere of 85% N_2, 10% CO_2 and 5% H_2 at 37 °C and terminated at 0 h, 6 h, 12 h and 24 h by adding 200 µL ice-cold acetonitrile to terminate the reaction. Samples were then removed from the anaerobic incubator and placed on ice for 10 min followed by 5 min ultrasonication to rupture the cell membranes of microbes and collect bile acids. Subsequently, centrifugation at 21,500 ×g for 15 min at 4 °C was applied to precipitate microorganisms, particles, and proteins. The upper layers were collected and stored at -

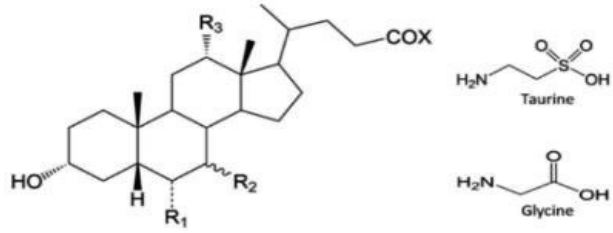
80 °C overnight followed by 8-hour freeze drying. The powder obtained was redissolved in 100 µL of nano-pure water and methanol (1/1, *v/v*) and vortexed for 2 min followed by centrifugation at 21,500 ×g for 5 min. The supernatant was removed and loaded onto a 96-well filter plate (PALL AcroPrep, PTFE 0.2 µm). To 90 µL of filtered extract, 10 µL internal standard was added. The obtained solutions were shaken briefly and subsequently transferred to vials for further analysis by LC–MS/MS.

2.2.4 Measurement of bile acids by LC–MS/MS

LC–MS/MS detection and quantification of bile acids was performed using a Shimadzu Nexera XR LC-20CE SR UPLC system coupled with a Shimadzu LCMS-8050 mass spectrometer (Kyoto, Japan) with an electrospray ionization (ESI) interface. Electrospray ionization (ESI) in turbo negative ion mode was performed with the triple quadrupole tandem mass spectrometric (MS/MS) system, and multiple reaction monitoring was utilized for data acquisition. Individual standard solutions (1 µg/mL) of each of the bile acids were employed to optimize the precursor ion, product ion, declustering potential and collision energy by direct injection into the mass spectrometer. The chemical structures, as well as parent and fragment masses of 24 major unconjugated bile acids and their glycine and taurine conjugates, are shown in **Table 2.2**. Samples were loaded, and the target bile acids were separated on a Kinetex 100A C18 column (2.1*50 mm, 1.7 mm) provided by Phenomenex (Torrance, USA). The mobile phases consisted of 0.01% formic acid in distilled water (solvent A), a mixture of methanol and acetonitrile (v/v=1/1)(solvent B), and acetonitrile containing 0.1% formic acid (solvent C). The column temperature was 40 °C, the flow rate was 0.5 mL/min, and the injection volume was 2 µL. The total run time was 16 minutes with the following gradient profile: 95% A, 0% B and 5% C (0-2 min), slowly changed to 30% A, 70% B, and 0% C from 2 to 7.5 min, rapidly reversed to 2% A, 98% B, and 0% C in 0.1 min, then kept at 2% A, 98% B, and 0% C from 7.6 to 10 min, then changed to 70% A, 30% B, and 0% C in 0.5 min; in the end, slowly returned to the initial conditions of 95% A, 0% B and 5% C from 10.5 to 13 min, then maintained at these conditions until 16 min for equilibration.

The Postrun and Browser Analysis function from LabSolutions software (Shimadzu, Kyoto, Japan) was employed to obtain the peak areas of the satisfactory extracted ion chromatogram (EIC) for each target.

Table 2.2 MRM data acquisition parameters of the LC–MS/MS procedure for unconjugated bile acids and conjugated bile acids. Unconjugated bile acids involve primary bile acids (CA, CDCA, αMCA and βMCA) and secondary bile acids (DCA, LCA, UDCA, HDCA, and ωMCA). Conjugated bile acids include tauro-bile acids (X=taurine) and glycol-bile acids (X=glycine).



Analytes	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision energy (eV)	R1	R2	R3	X
Cholic acid (CA)	407.3	407.3	25	-H	-OH(α)	-OH	-OH
Taurocholic acid (TCA)	514.4	514.4	30	-H	-OH(α)	-OH	-taurine
Glycocholic acid (GCA)	464.3	74.0	43	-H	-OH(α)	-OH	-glycine
Chenodeoxycholic acid (CDCA)	391.3	391.3	15	-H	-OH(α)	-H	-OH
Taurochenodeoxycholic acid (TCDCA)	498.4	498.4	55	-H	-OH(α)	-H	-taurine
Glycochenodeoxycholic acid (GCDCA)	448.3	74.0	43	-H	-OH(α)	-H	-glycine
Deoxycholic acid (DCA)	391.3	391.3	15	-H	-H	-OH	-OH
Taurodeoxycholic acid (TDCA)	498.4	498.4	55	-H	-H	-OH	-taurine
Glycodeoxycholic acid (GDCA)	448.3	74.0	43	-H	-H	-OH	-glycine
Lithocholic acid (LCA)	375.3	375.3	30	-H	-H	-H	-OH
Taurolithocholic acid (TLCA)	482.3	482.3	54	-H	-H	-H	-taurine
Glycolithocholic acid (GLCA)	432.3	74	55	-H	-H	-H	-glycine
Ursodeoxycholic acid (UDCA)	391.3	391.3	15	-H	-OH(β)	-H	-OH
Tauroursodeoxycholic acid (TUDCA)	498.4	498.4	55	-H	-OH(β)	-H	-taurine
Glycoursodeoxycholic acid (GUDCA)	448.3	74.0	43	-H	-OH(β)	-H	-glycine
Hyodeoxycholic acid (HDCA)	391.3	391.3	15	-OH(α)	-H	-H	-OH
Taurohyodeoxycholic acid (THDCA)	498.4	498.4	55	-OH(α)	-H	-H	-taurine
Glycohyodeoxycholic acid (GHDCA)	448.3	74.0	43	-OH(α)	-H	-H	-glycine
α-Muricholate (αMCA)	407.3	407.3	25	-OH(β)	-OH(α)	-H	-OH
Tauro-α-muricholate (TαMCA)	514.4	514.4	30	-OH(β)	-OH(α)	-H	-taurine
β-Muricholate (βMCA)	407.3	407.3	25	-OH(β)	-OH(β)	-H	-OH
Tauro-β-muricholate (TβMCA)	514.4	514.4	30	-OH(β)	-OH(β)	-H	-taurine
ω-Muricholate (ωMCA)	407.3	407.3	25	-OH(α)	-OH(β)	-H	-OH
Tauro-ω-muricholate (TωMCA)	514.4	514.4	30	-OH(α)	-OH(β)	-H	-taurine

2.2.5 Microbial Taxonomic Profiling and Total Bacterial Load

A total of 240 µL diluted and filtered fecal slurry containing 20% feces (v/v)(as described in Section 2.2.2) and 30 µL PBS mixed with 30 µL stock solution of lincomycin (final concentration: 16.32 mM) or clindamycin (final concentration: 10.41 mM) was incubated

under anaerobic conditions for 24 h at 37 °C, and stored at -80 °C overnight (3 aliquots per group). Subsequently, DNA was isolated from the fecal slurries using a bead-beating procedure coupled with the customized MaxWell 16 Tissue LEV Total RNA Purification Kit (XAS1220; Promega Biotech AB, Stockholm, Sweden). DNA isolates underwent triplicate polymerase chain reaction (PCR) reactions of the 16S rRNA gene V4 region (515-F; 806-R), and PCR products acquired were purified, pooled, and sequenced (Illumina NovaSeq 6000, paired-end, 70 bp; Eurofins Genomics Europe Sequencing GmbH, Konstanz, Germany).

To quantify the total bacterial load in each individual fecal slurry, quantitative PCR (qPCR) was carried out. PCR products were generated by amplification using 16S V3–V4 primers (F-NXT-Bakt-341F: 5′-CCTACGGGNGGCWGCAG-3′ and R-NXT-Bakt-805R: 5′-GACTACHVGGGTATCTAATCC-3′). During index PCR, barcodes for multiplexed sequencing were introduced using overhang tags. A sequencing library was prepared from the barcoded PCR products and sequenced on the Illumina MiSeq next-generation sequencing system (Illumina Inc.). Signals were processed to *.fastq-files, and the resulting 2 × 250 bp reads were demultiplexed. Microbiota identification was performed by clustering the sequences at a 97% identity threshold defining operational taxonomic units (OTUs), according to the taxonomy of the SILVA 132 16S rRNA sequence database.

2.2.6 Data Analysis

Metabolic profile data acquisition and processing were carried out using the Lab solutions software of the LC-TQ-MS system. Graphics were presented by using GraphPad Prism 8.2 (San Diego, USA), and chemical structures were drawn by using ChemDraw 18.0 (PerkinElmer, Waltham, USA). The results are shown as the mean ± standard deviation (SD). 16S rRNA analysis data were analyzed with R version 3.6.1 and QIIME 2 view. Statistical significance was determined by a one-way ANOVA with a Dunnett/Bonferroni correction for multiple tests and results were considered significant when $p < 0.05$.

2.3 Results and discussion

2.3.1 Bile acid measurement by LC–MS/MS

Figure 2.2 A-C show the chromatograms of unconjugated bile acids, glycine conjugated bile acids and taurine conjugated bile acids, respectively. Good separation with an identified retention time for each bile acid and high sensitivity with limits of

quantification ⩽ 0.05 μM for all targets were obtained after the development and optimization of the LC–MS/MS detection method.

To obtain a higher intensity of signals for all these bile acids, a pseudo-MRM method, which applies the same product ion as the parent ion under an optimized collision energy, was employed. Since all these unconjugated bile acids have no 12-hydroxyl group that is fragmented merely via dehydration/dehydrogenation, no discriminative fragments were exhibited regardless of sites and epimerization of the hydroxyl groups on the skeleton (Lan et al., 2016). Thus, fragments such as *m/z* 407.3 > 407.3 for CA and *m/z* 391.3 > 391.3 for CDCA were used for detection of the unconjugated isomers and stereoisomers (Yin et al., 2017). Upon testing both ESI+ and ESI-, the negative ionization mode was selected since it provided more stable and stronger signals. Table 2 summarizes the MRM data acquisition parameters of the LC–MS/MS procedure for unconjugated bile acids and conjugated bile acids.

To optimize the chromatographic separation of the bile acids, various mobile phases with various counterions in the pH range of 3∼10 were evaluated, since due to their different pKa values, different effects of acidity on the retention of bile acids were observed. The unconjugated bile acids and glycine conjugates usually have pKa values in the range of 4∼6, while the pKa values of taurine conjugates are less than 2.0 (Carey et al., 1972). Therefore, decreasing the pH of the mobile phases markedly increased the retention of glycine-conjugated bile acids and unconjugated bile acids, which improved their chromatographic separation with minimal influence on taurine bile acids. Meanwhile, the ionization efficiency and therefore the signal intensity in ESI- mode are also strongly dependent on mobile phase constituents and pH (Alnouti et al., 2008). Thus, based on our findings, the mobile phases separately adding 0.01% and 0.1% formic acid were finally selected in a gradient of methanol, acetonitrile, and distilled water.

In addition, the hydrophilicity of these bile acids based on their nucleus and side chain structures is another important factor influencing chromatographic separation. For example, tri-hydroxy bile acids (CA, MCA) elute earlier than di-hydroxy bile acids (CDCA and DCA), which in turn elute earlier than mono-hydroxy bile acids (LCA). This elution behaviour may be attributed to the orientation of the hydroxyl substitutions and their ability to form intramolecular H-bonds (Ikegawa et al., 1996). According to this, a

reversed-phase C18 column was tested and selected to obtain the optimized separation for all the targets.

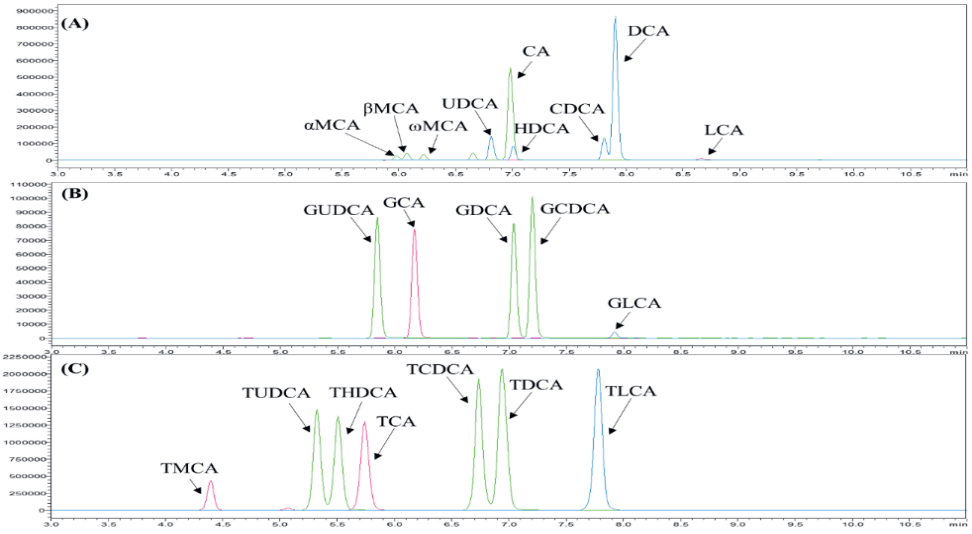


Figure 2.2 UPLC chromatography graphs of (A) unconjugated bile acids (blue: 391.3>391.3; green: 407.3>407.3), (B) glycine conjugated bile acids (blue: 432.3>74.0; green: 448.3>74.0; pink: 464.3>74.0) and (C) taurine conjugated bile acids (blue: 482.3>482.3; green: 498.4>498.4; pink: 514.4>514.4) in a mixed standard solution with final concentration of 1 µM standard for each. The time range displayed is from 3 to 11 min.

2.3.2 Development and optimization of the in vitro anaerobic fecal incubation model

An anaerobic fecal batch culture model in PBS was developed to study bacterial metabolism. **Figure 2.3** shows the bile acid levels in incubations with freshly isolated (no treatment), PBS-washed, and T-BA supplemented fecal samples at t=0, is the latter also reflecting the ultimate optimized protocol applied for subsequent studies on the effects of the selected antibiotics (**Figure 2.3C**). In samples with freshly isolated rat feces, both secondary bile acids (ωMCA, HDCA, DCA and LCA) and primary bile acids (βMCA, αMCA, and CA) were readily detected, as shown in **Figure 2.3A**. Glycine-conjugated bile acids (G-BAs) were not detected, which is in line with a previous study reporting a low proportion of G-BAs (<1%) in rat liver (Lin et al., 2019). Levels of T-BAs were present below the detection limits, while these are known to be readily excreted from the liver into the intestines. This implies that the bile acid profile in freshly isolated fecal samples does not reflect the bile acid profile in the intestine before metabolism by the gut microbiota as

such. To define a bile acid content of the fecal incubations more in line with what would be expected in the intestines, fecal samples were washed and supplemented with a mixture of T-BAs. After washing the freshly isolated rat fecal samples with PBS, the amount of the detected bile acids was significantly reduced, leaving ωMCA, HDCA, βMCA and DCA at levels amounting to 8.58%, 12.67%, 9.74%, and 25.84% of the levels detected in the freshly isolated fecal samples (Fig. 3). Subsequently, to obtain the final incubations, TCA, TβMCA, and TUDCA were added to the fecal incubations at levels amounting to final target concentrations of 50 μM TCA, 10 μM TUDCA, and 10 μM TβMCA to better simulate the bile acid composition of the gut environment. The levels and total amount of TCA, TβMCA, and TUDCA were chosen based on a previous study (Lin et al., 2019), which reported a composition of 70.82% TCA, 13.47% TβMCA, and 11.08% TUDCA present in rat liver and thus expected to be transferred to the intestinal lumen. Thus, a rough ratio of 5:1:1 for TCA, TβMCA, and TUDCA was added to imitate the in vivo gut levels of these bile acids. Furthermore, as a result of the apparently extremely fast deconjugation of these T-BAs, at t=0, the incubations contained the conjugated bile acids TCA, TUDCA, TβMCA, and TαMCA at concentrations somewhat lower than the added amounts while also substantial levels of the corresponding deconjugated primary bile acids were detectable (**Figure 2.3** and **Table S2.1**). For example, upon supplementation of the washed samples with TCA, CA instantly increased as well accompanied by an instant reduction of the TCA levels from 50 mM (added) to approximately 30 mM (detected), pointing at apparently swift deconjugation of TCA. In this way, fecal incubations with a reasonable combination of conjugated, primary and secondary bile acids could be obtained, reflecting intestinal levels better than the residual bile acid levels in the fecal samples.

It is also of importance to note that some bile acids, such as DCA, LCA, CDCA and their derivatives, have strong hydrophobicity (Májer et al., 2014), and high concentrations could alter bacterial membrane permeability, leading to disturbed gut microbiota (Islam et al., 2011; Ramírez-Pérez et. al, 2018). Thus, the modification of the initial bile acid pool at the beginning of the incubation was also used to avoid such a negative effect on bacteria. A washing step using anaerobic PBS (equal volume as the filtered fecal sample) followed by centrifugation at 2,000×g for 5 min was applied. Following these optimization steps, the total amount of T-BAs and unconjugated bile acids present in the incubations at t=0 were comparable at the start of the incubations (**Table S2.1**). The supplemented sample

thus obtained was used for further studies on the effects of antibiotics on gut microbiota and the resulting effects on bile acid composition and metabolism.

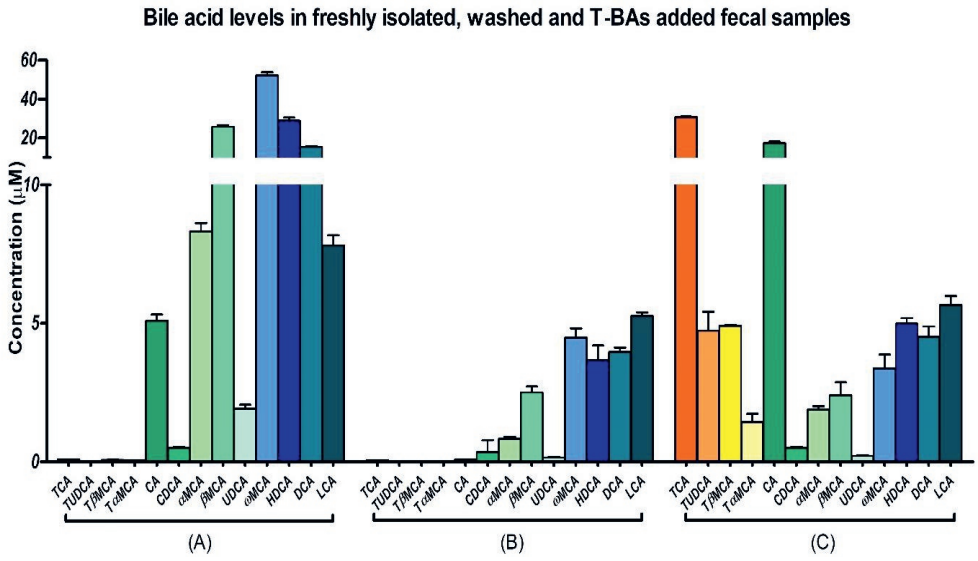


Figure 2.3 Bile acid profiles at t=0 h in incubations with 20% rat feces (v/v) from (A) freshly isolated rat fecal samples, (B) freshly isolated rat fecal samples washed twice using anaerobic PBS, and (C) washed fecal samples with the addition of a mixed T-BA solution of TCA, TUDCA, and TβMCA at final concentrations of 50 µM, 10 µM, and 10 µM Data presented are the mean ± SD from 3 independent experiments.

In addition to the modification and optimization of the initial bile acid profile in the in vitro fecal incubations, the time of incubation was also optimized. This is because the variation in bacteria upon prolonged incubation times could affect the reproducibility and linearity of the results. In a previous study on conjugated bile acid processing by the human gut microbiota, an incubation period of 24 h was applied, which is relevant for intestinal transit times (Martin et al., 2018); finally, samples taken at 0, 6, 12, and 24 h were selected for final bile acid analysis. Additionally, ice-cold acetonitrile was utilized for not only stopping the reactions but also as the solvent to extract the bile acids efficiently from the fecal incubations.

2.3.3 Alteration of bile acids

After optimization of the conditions for the anaerobic fecal incubations and the subsequent sample preparation protocol, incubations in the absence and presence of the selected antibiotics lincomycin and clindamycin were performed. **Figure 2.4A** presents

the changes in bile acid composition during 24 h anaerobic incubations in the absence (control) or presence of lincomycin and clindamycin. From these data, it follows that during the 24 h lincosamide treatment of the fecal samples, resulted in significant changes in the bile acid profile. **Figure 2.4B** shows the principal component analysis (PCA) according to the bile acid profiles in the antibiotic-treated and control samples taken at 24 h of incubations, and the clustered controls with an obvious separation with lincomycin and clindamycin treated groups reveals the effects of lincosamides on gut-mediated bile acids metabolism.

In line with the optimized conditions (**Figure 2.3C**) at the start of the incubations, especially the primary bile acids CA, αMCA, and βMCA (except for CDCA), and the secondary bile acids DCA, LCA, ωMCA, and HDCA were detected along with a minor amount of UDCA. In the samples treated with either lincomycin or clindamycin, effects on bile acid pools were readily observed compared to the control incubation, with changes being most pronounced upon 24 h of incubation. During the 24 h incubation, the levels of most bile acids changed to a significantly larger extent in the presence of the antibiotics than in the control, in most cases resulting in lower concentrations, while for CA, the opposite was observed, resulting in higher levels of CA at the end of the 24 h incubation. The CA levels in the control were as low as 0.3±0.04 μM at the end of the 24 h incubation, while 50% of the original amount of CA was still detected in the lincomycin- and clindamycin-treated samples. These higher residual levels of CA in both antibiotic-treated samples were accompanied by a comparable reduction in the level of its secondary bile acid DCA, indicating an effect of the antibiotics on the bacteria responsible for this conversion. Similar effects were observed for the formation of other secondary bile acids, including UDCA, LCA, and HDCA. In particular, the production of DCA, LCA and HDCA in the antibiotic-treated samples was only half of that in the control group. The effects of the antibiotics on αMCA and βMCA were less pronounced and not significant at the end of the 24 h incubation. In line with the rapid deconjugation observed when optimizing the incubation protocols, the taurine-conjugated bile acids TCA, TUDCA, TαMCA, and TβMCA, which were present at the start of the incubations at levels of approximately 42 mM in total (**Table S2.1**), were almost completely deconjugated at the first time point in all incubations.

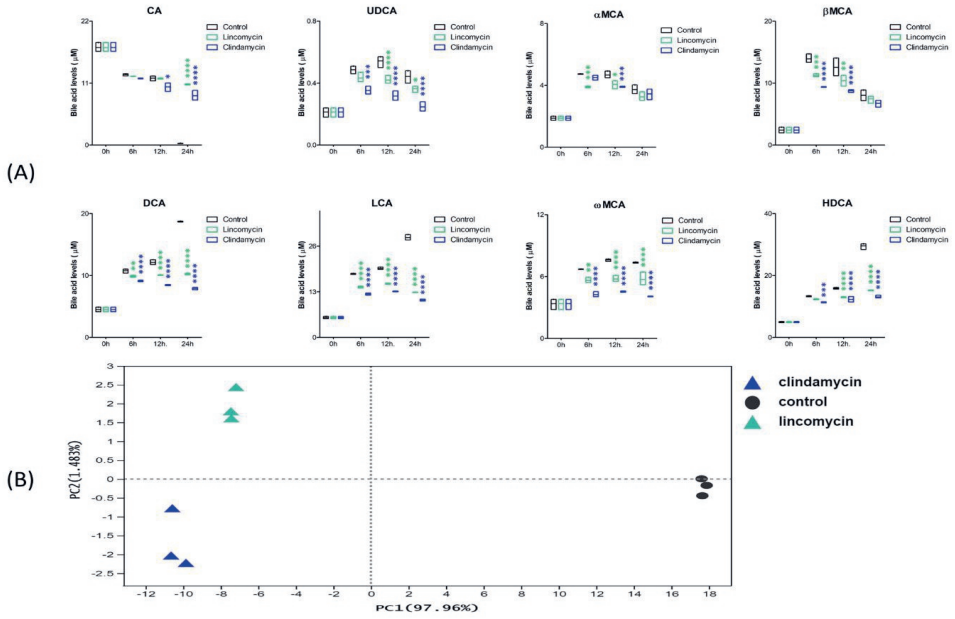


Figure 2.4 Time-dependent changes in bile acid concentrations in anaerobic fecal incubations at 0, 6, 12, and 24 h in control (black), lincomycin-treated (green), and clindamycin-treated (blue) fecal samples are shown in (A)(n=3 per group). The bile acids of CA, αMCA, βMCA, UDCA, DCA, LCA, ωMCA, and HDCA were detected and quantified in control and treated samples taken from the anaerobic fecal incubations at the time points indicated (****p <0.0001, *** 0.0001≤ p <0.001, ** 0.001≤ p <0.01, * 0.01≤ p <0.05 indicates a difference of the antibiotic treated samples from the control samples without antibiotic at the corresponding time point). The principal coordinate analysis (PCoA) based on the bile acid profiles of control and treated fecal samples taken at 24 h is shown in (B).

2.3.4 Evaluation of the in vitro model for studying alterations in microbiota-dependent bile acid metabolism by comparison to in vivo data

To evaluate the use of the in vitro incubations for elucidating effects on microbiota-mediated bile acid metabolism, **Figure 2.5** presents a comparison of the changes observed in the bile acid profiles induced by lincomycin and clindamycin in the newly defined in vitro model and data reported in a previous in vivo study (Behr et al., 2019). The overview thus obtained reveals that for most of the primary and secondary bile acids studied, including CA, βMCA, DCA, LCA, ωMCA, and HDCA, the effects observed in this in vitro model match the in vivo data, with increased levels of CA and decreased or unmodified levels for the other bile acids upon antibiotic treatment. For the remaining

two bile acids, UDCA and αMCA, the in vivo data in males and female rats did not match, and the female data are in line with what was observed in the in vitro model using mixed gender fecal samples. Taken together, it is concluded that upon the treatment of the gut microbiota with antibiotics, similar perturbations of gut microbiota-mediated bile acid metabolism were found in the in vitro model and the in vivo situation for both primary and secondary bile acids. In addition, **Figure 2.6** shows the bioconversion pathways of added TCA, TβMCA, and TUDCA to further explain the alteration of the bile acid profile.

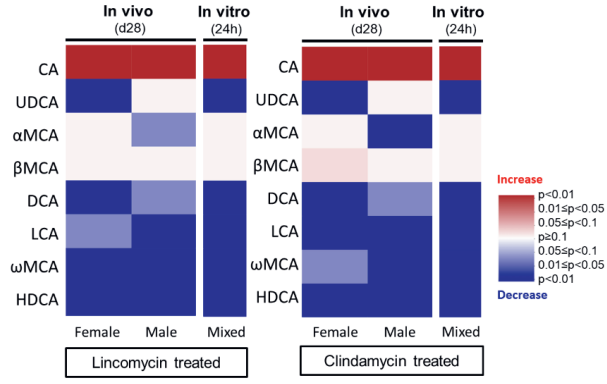


Figure 2.5 Comparison of the effect of lincomycin and clindamycin on bile acid concentrations in the in vitro anaerobic fecal incubations (mixed gender) at 24 h with the in vivo effects detected in feces of male and female rats exposed to lincosamides vs. feces of control rats on day 28 (Behr et al., 2019). Red boxes indicate a significant increase, blue boxes indicate a significant decrease, and the intensity of the color corresponds to the significance of the change. P values are presented in light and dark colors.

The results of both the in vivo (Behr et al., 2019) and the present in vitro study reveal the inhibition of CA decomposition upon antibiotic treatment, and these findings have also been reported in other in vivo studies (Shimizu et al., 2011; Theriot et al., 2016). This increase in levels of CA and the accompanying decrease in DCA as the primary product of CA upon treatment of the microbiota with antibiotics, observed in both the current in vitro and the literature reported in vivo study (Behr et al., 2019), indicate a reduction in the 7α-dehydroxylation activity of the intestinal microorganisms (shown in **Figure 2.6**). In addition to the formation of the major CA metabolite DCA, **Figure 2.6** also shows that a small amount of CA can be converted to the secondary bile acid LCA via oxidation and dehydroxylation. The accumulated CA and decreased production of LCA revealed a slower oxidation/dehydroxylation induced by antibiotic administration as well. Notably, LCA

and UDCA can be converted to each other by 7β-hydroxylation and 7β-dehydroxylation, respectively (Wahlström et al, 2016; Tonin and Arends, 2018); therefore, the reduction in LCA could be accompanied by a reduction in UDCA.

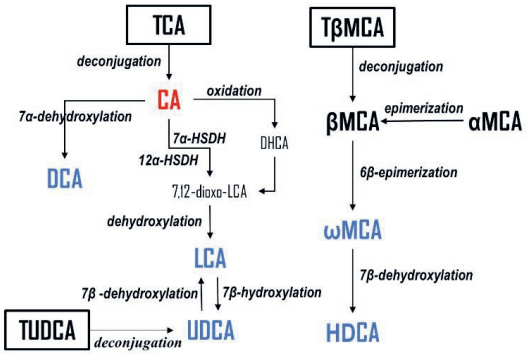


Figure 2.6 Pathways of fecal T-BA transformation. T-BAs (in black boxes), including TCA, TβMBA, and TUDCA, transform to CA, βMCA, and UDCA by deconjugation; then, the primary bile acids CA and βMCA (some of which can be produced from αMCA) are further converted to various secondary bile acids, including DCA, LCA, UDCA, ωMCA, and HDCA, through 7α/β-dehydroxylation, 7β-hydroxylation, epimerization, and oxidation (Zhang and Klaassen, 2010; Wahlström et al, 2016). In addition, on the pathway of CA transforming to LCA, the intermediate 7,12-dioxo-LCA can be generated via a special oxidation by 7α-hydroxysteroid dehydrogenase (7α-HSDH) and 12α-hydroxysteroid dehydrogenase (12α-HSDH) produced by gut microbiota (Tonin and Arends, 2018). Bile acids showing a significant increase or decrease in the present study are colored red and blue, respectively.

On the other hand, the added TβMCA rapidly deconjugated to βMCA, followed by conversion to ωMCA and further transformation to HDCA (**Figure 2.6**). Additionally, it was observed (**Figure 2.5**) that the production of ωMCA and HDCA was significantly reduced upon treatment with the antibiotics, while an alteration of βMCA was not obvious. Therefore, the slower production of ωMCA could be linked to weakened 6β-epimerization, leading to a reduced level of HDCA. Meanwhile, the βMCA levels may be dependent on a dynamic equilibrium between weakened 6β-epimerization and stronger epimerization of αMCA, the latter in line with the decreased level of αMCA in antibiotic-treated male rats (Wahlström et al, 2016), providing support for this speculation. In addition, sex differences were found in the in vivo study with respect to the levels of UDCA, αMCA, and βMCA (only in the clindamycin-treated group), as reported in some previous studies

where obvious differences were found between male and female mice in metagenomic analysis involving bile acid metabolism (Sheng et al., 2017; Xie et al., 2017).

2.3.5 Microbial Taxonomic

Figure 2.7A and **B** presents the results of the 16S rRNA analysis of the gut microbiota composition of the fecal samples from control rats incubated in vitro either without (control) or with lincomycin or clindamycin, showing the relative microbial profile for the phylum and dominant families. The figures reveal that the altered abundance of bacterial species is relatively small but consistent. At the phylum level, the in vitro fecal microbial community upon 24 h of in vitro incubation for the control sample was composed mainly of *Firmicutes* and *Verrucomicrobia*, followed by *Bacteroidetes* and *Proteobacteria*. In lincomycin- or clindamycin-treated samples, *Proteobacteria* abundance was obviously reduced compared to that in the control group, whereas the relative abundance of *Verrucomicrobia* and *Bacteroidetes* showed a slight increase and decrease, respectively. At the family level (**Figure 2.7B**), *Akkermansiaceae*, *Ruminococcaceae*, and *Lachnospiraceae* dominated in rat fecal microbial communities, and no obvious alterations of the three bacterial species were shown in the antibiotic-treated samples compared to controls. In converse, the relative abundance of *Erysipelotrichaceae* significantly reduced in both antibiotic-treated samples ($0.0001 < p < 0.001$), and the richness of *Bacteroidaceae* also markedly reduced in lincomycin ($0.01 \leq p < 0.05$) and clindamycin ($0.0001 < p < 0.001$) groups compared to control. In addition to these changes, the family of *Prevotellaceae* and *Lactobacillaceae* also presented a significant alteration but differently in the antibiotic-treated groups. Relative abundance of *Prevotellaceae* showed the obviously decrease only in the clindamycin-treated fecal samples ($0.001 \leq p < 0.01$), while the richness of *Lactobacillaceae* only significantly reduced in the lincomycin-treated group ($0.01 \leq p < 0.05$). Although the discrepancy of changes was shown between the bacterial profiles in different antibiotic-treated samples (**Figure 2.7B**), effects of the antibiotics on the rat gut microbiome were obviously revealed and further described by the principal coordinate analysis (PCoA) of the Bray–Curtis distance matrix based on the obtained bacterial profiles (OTUs) in **Figure 2.7C**, showing the clustered controls with a significant separation from the antibiotic-treated fecal samples. Moreover, the alpha diversity of the samples with or without in vitro antibiotic treatment presented by the Chao and Shannon indexes was shown in **Figure 2.7D**, revealing the

biodiversity and the relative abundance of species. The total amount as well as biodiversity of the bacterial species presented a slight increase and decrease respectively in the lincomycin and clindamycin treated groups, with no significance shown.

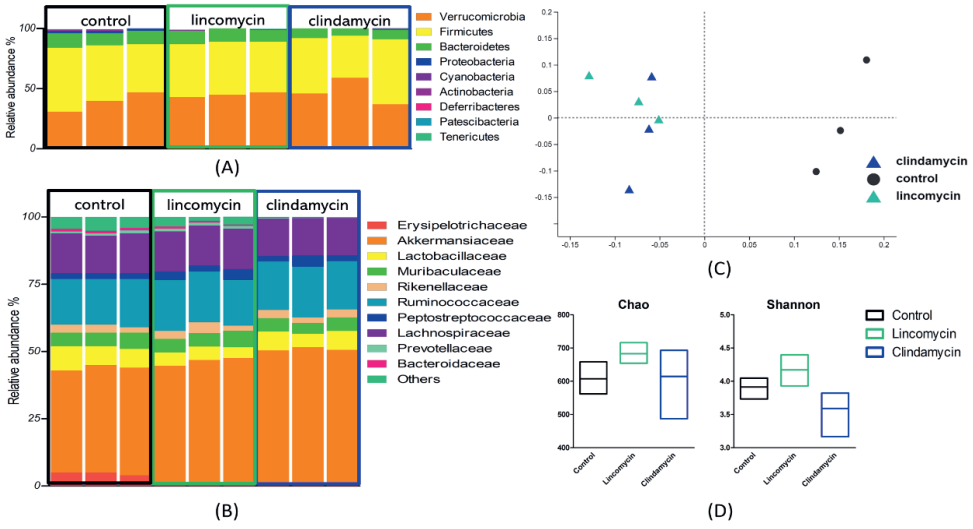


Figure 2.7 Relative abundance of gut microbes in control and lincomycin- or clindamycin-treated pooled fecal samples (n=3) from Wistar rats at the (A) phylum and (B) dominant family levels are described and all the samples were taken after a 24 h incubation. Based on the data of OTUs obtained from these samples, the principal coordinate analysis (PCoA) of the Bray–Curtis distance matrix and the alpha diversity measured by the Chao and Shannon index are shown in (C) and (D) respectively, plotted for control (black), lincomycin-treated (green), and clindamycin-treated (blue) fecal samples.

The studies published previously which focused on the gut microbial community induced by the exposure of antibiotics achieved the same results as those observed in the present study. For example, a previous mouse in vivo study has reported the changes in gut microbial communities induced by gentamicin, and *Erysipelotrichaceae* was also found to be mutated further leading to changes in short-chain fatty acids and primary bile acids (Zhao et al., 2013). In another work, the decreased abundance of *Erysipelotrichaceae* affected by antibiotics in human gut microbiota was reported by Yun (Yun et al., 2017). In addition to the altered richness of *Erysipelotrichaceae*, the reduced abundance of *Lactobacillaceae* and *Bacteroides* was also found to be in line with a review on the modification of gut microbiota by antibiotics (Ianiro et al., 2016). Notably, *Lactobacillaceae* is one of the beneficial bacterial species, the abundance of which could

significantly decrease by the treatment of antibiotics particularly lincomycin (Tang et al., 2021), and the decrease was shown in the current study as well.

2.3.6 Correlation of the changes in the microbiota with those in bile acid metabolism

The correlation of the effects of the antibiotics on the microbiota with their effects on the bile acid profiles may shed some light on the microbiota-related changes in bile acid profiles upon treatment with lincomycin or clindamycin, which is presented by the pearson correlation coefficients as shown in **Figure 2.8**. *Erysipelotrichaceae* as well as *Akkermansiaceae* were shown highly relative to the bile acid profiles especially secondary bile acids involving DCA, LCA, ωMCA, and HDCA. In particular, *Erysipelotrichaceae* which showed a positive correlation with the levels of fecal secondary bile acids significantly reduced on the relative abundance in antibiotic-treated samples, leading to the decreased proportions of DCA, LCA, ωMCA, and HDCA (**Figure 2.4**) after the 24 h incubation, whereas *Akkermansiaceae* presented the negative relation to the production of fecal secondary bile acids. Meanwhile, *Bacteroidaceae* also showed a significant positive correlation with the fecal secondary bile acids, and the reduced richness of *Bacteroidaceae* could result in the decreased production of secondary bile acids, especially in clindamycin-treated samples. In addition, *Prevotellaceae* and *Lactobacillaceae* were also shown to be highly relative to the secondary bile acids in the lincomycin- and clindamycin-treated groups respectively.

Together with the bile acid transformation pathways shown in **Figure 2.6** and the previously reported studies, the correlation between the bacterial species and the conversion of individual bile acids could be more clear. For example, the accumulation of CA and the reduction in DCA may result from a reduction in 7α-dehydroxylation activity, which may be attributed to the loss of microorganisms belonging to the phylum *Firmicutes* known to contain 7α-dehydroxylation activity (Gérard, 2013; Jia et al., 2018), such as the reduced abundance of *Lactobacillaceae* and *Erysipelotrichaceae* observed in the current study. Meanwhile, as it is known that epimerization and oxidation of bile acids are also carried out by gut microbes (Guzior et al., 2021), the reduced relative abundance of microbes that possess epimerization and oxidation capabilities would result in decreased production of some secondary bile acids, such as ωMCA and LCA. As observed in this study, the reduced production of ωMCA and LCA upon antibiotic administration could be suspected to be associated with the weakened epimerization of βMCA and the weakened

oxidation of CA (converting to 7,12-dioxo-LCA), respectively, and further related to the bacterial species of *Bacteroidaceae*, which has also been reported by Jia (Jia et. al., 2018). In addition to dehydroxylation, oxidation, and epimerization, deconjugation is another traditional distinct pathway related to microbial transformations of bile acids (Guzior et al., 2021). For example, the accumulation of CA could be caused by the weakening of deconjugation from TCA, and the deconjugation is associated with the effects on bile salt hydrolases (BSHs) linked to the microbial species belonging to *Bacteroidetes* and *Firmicutes* such as *Bacteroidaceae* and *Lactobacillaceae*, which was previously reported as well (Jia et. al., 2018).

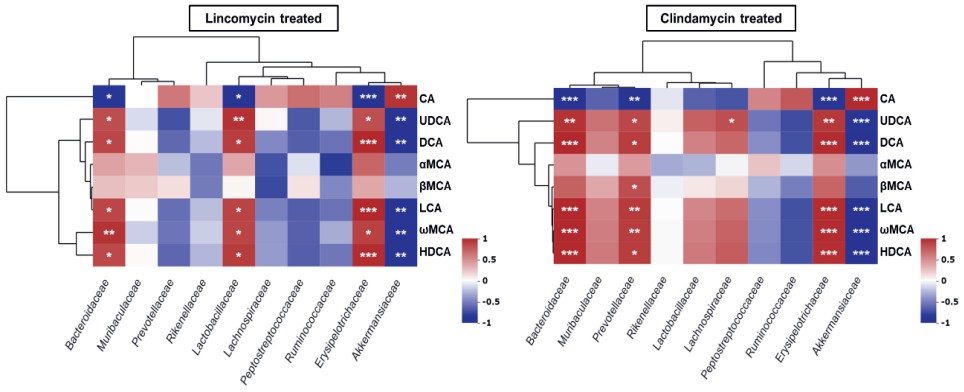


Figure 2.8 The heatmap presentation by the pearson correlation coefficients of the ten dominant families in rat fecal samples with the individual fecal bile acids including CA, UDCA, DCA, αMCA, βMCA, LCA, ωMCA, and HDCA is shown. All the fecal samples were taken at the 24 h of anaerobic incubation, and results are separately shown for lincomycin- and clindamycin- treated groups. The positive and negative correlation are presented by colours of red and blue respectively (*p <0.05, **p <0.01, ***p <0.001).

The changes observed in the primary and secondary bile acids after lincosamide administration following the 24 h incubation revealed that antibiotic treatment had an influence on the gut microbiome, subsequently resulting in effects on bile acid metabolism and profiles, with effects detected in the newly applied in vitro model being comparable to what has been observed in vivo.

2.4 Conclusion

In this study, we demonstrate the utility of using an anaerobic fecal batch culture system to model the effects of antibiotics on bacterial composition and the resulting metabolism of individual primary and secondary bile acids using 16S rRNA gene sequencing and LC–MS/MS. The consistent consequences for changes in fecal bile acid profiles as acquired from this in vitro model and a previous rat in vivo study, provide a proof of principle for the application of the developed 24 h in vitro fermentation batch model to study compound induced alterations in gut microbiota-dependent bile acid profiles. The method will facilitate elucidation of effects of other xenobiotics on the gut bacterial community and its consequences for bile acid metabolism, thereby contributing to the 3Rs (replacement, reduction and refinement) in animal testing.

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3

CHAPTER 3

Organophosphate pesticides modulate gut microbiota and influence bile acid metabolism in an in vitro fermentation model

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Ivonne M.C.M. Rietjens

Abstract

In this study, the effects of organophosphate (OP) pesticides on gut microbiota mediated bile acid metabolism were investigated using a simple batch fermentation in vitro model, in which mouse fecal samples were incubated with triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, or phorate and a mixture of bile acids. Samples were taken during the 24 h incubation and bile acid profiles were quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS). OP treatment induced microbiota dependent alterations of primary and secondary bile acid levels, including especially substantially increased production of ω-muricholate (ωMCA) and decreased levels of β-muricholate (βMCA). Among all OPs studied, exposure of the fecal microbiota to phorate led to the most wide and significant effects on the bile acid profile; as a result, phorate was selected as the OP for further determination of accompanying effects on the bacterial profile by 16S rRNA sequencing. Results showed that richness of the *Muribaculaceae spp.* significantly decreased after the exposure to phorate. Meanwhile, changes in the genera *Coriobacteriaceae UCG-002* and *Muribaculum* were found to be highly correlated with the altered bile acid profiles. In summary, OP treatment could lead to perturbation of gut microbiota resulting in correlated changes in related bile acid metabolism.

Key words: bile acid metabolism, organophosphate pesticides, gut microbiota, in vitro fermentation model

3.1 Introduction

Organophosphate (OP) pesticides are widely used in agriculture due to their high efficiency as insecticides (Gogol et al., 2000). Yearly, around 3 million people are exposed to these chemicals around the globe (Rusyniak and Nañagas 2004; Stallones and Beseler 2016; Eddleston et al. 2008). However, OPs have been shown to result in high levels of acute neurotoxicity (Kamanyire and Karalliedde, 2004) with the majority of the OPs being hazardous to both human health and to the environment (Jaga and Dharmani, 2003). Of all OPs, triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, and phorate (chemical structures shown in **Figure 3.1**) were the ones most frequently reported as pesticide residues in food such as fruits (Li et al., 2020), vegetables (Qin et al., 2016), and tea (Chen et al., 2016) in China. In a recent report of the European Food Safety Authority (EFSA), chlorpyrifos residues were detected in dried beans, pears as well as rice, and triazophos was detected in rice (EFSA, 2022). Considering the oral route for human exposure, it is of interest to focus on the impact of OPs on the intestinal barrier and gut microbiota. It has been demonstrated that exposure to chlorpyrifos and diazinon could induce dysbiosis in the microbial community in animals in vivo as well as in the SHINME (simulator of human intestinal microbial Ecosystem) in vitro model (Giambò et al., 2021).

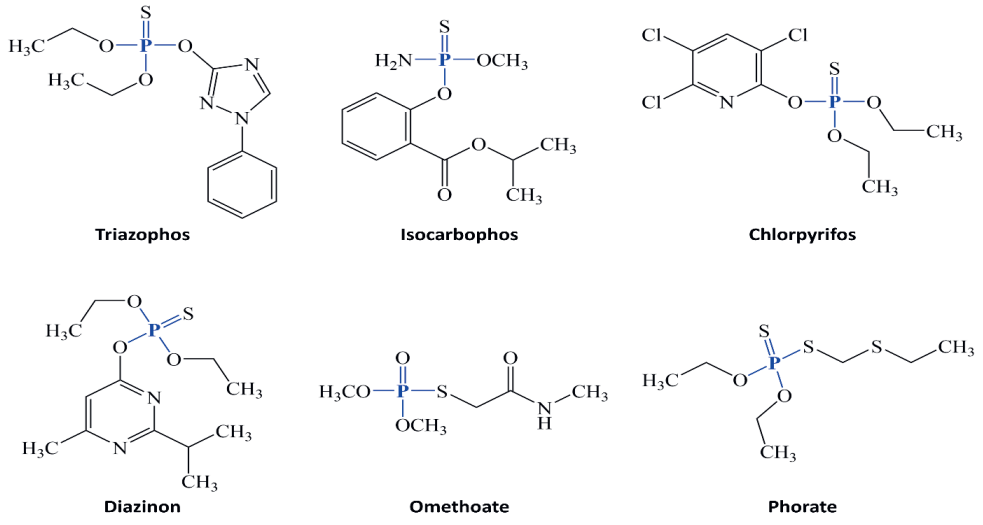

Figure 3.1 Chemical structures of the six OPs selected for the present study including triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, and phorate.

The gut microbiota consists of trillions of microorganisms residing in the intestine and has a mutual relationship with its host (Backhed et al., 2005). The gut microbiota plays an important role in maintaining host health, enhancing the immune system and regulating host metabolism (Turnbaugh et al., 2006; Tremaroli and Backhed, 2012; Nicholson et al. 2012). The host-microbe metabolic axis has been defined as a multidirectional interactive chemical communication highway between specific host cellular pathways and a series of microbial species, subecologies, and activities (Nicholson et al., 2012). For example, bile acids that are synthesized from cholesterol in the liver, excreted via the biliary tract into the intestinal tract, and further modified by gut microbes contribute to the host metabolic phenotype and hence to host health and disease (Gérard, 2013).

Metabolomics has been demonstrated to be valuable for revealing the mechanisms of toxicity (Robertson et al., 2011). Metabolic profiles such as bile acid profiles are also known to be sensitive to external environmental stimuli, since relative subtle changes in the metabolome can be detected in response to exposure to environmental or food-borne chemicals, including for example, OPs. To our knowledge, only a few studies report on the effects of OPs on human gut microbiota (Li et al., 2019; Joly Condette et al., 2015; Liang et al., 2019; Zhao et al., 2016) and little is known about OP pesticide induced gut microbiota-related metabolic changes in bile acid profiles. Only one metabolomics study evaluating in vivo effects of the OP chlorpyrifos on the gut microbial community and urinary bile acid profiles has been published (Zhao et al., 2016), and it revealed that the altered bile acid profile in urine was highly correlated with changes in the gut microbiota families induced by chlorpyrifos.

In this study, the effects of OP pesticides on bile acid profiles were determined using a simple batch in vitro fermentation model coupled with liquid chromatography tandem mass spectrometry (LC-MS/MS) and effects on bacterial profiles were determined by 16S rRNA gene sequencing analysis. The current study is not only the first in vitro study to evaluate the influence of OPs on gut microbial bile acid metabolism, but also the first study evaluating these effects for several OP pesticides including triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, and phorate (**Figure 3.1**) as well as the first study investigating the simultaneous effects of the OP phorate on gut microbiota and related bile acid profiles.

3.2 Materials and methods

3.2.1 Chemicals and solutions

Triazophos (CAS 24017-47-8), isocarbophos (CAS 24353-61-5), chlorpyrifos (CAS 2921-88-2), diazinon (CAS 333-41-5), omethoate (CAS 1113-02-6), phorate (CAS 298-02-2), and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Taurocholic acid (TCA), tauro-β-muricholate (TβMCA), tauro-α-muricholate (TαMCA), taurodeoxycholic acid (TDCA), taurolithocholic acid (TLCA), tauroursodeoxycholic acid (TUDCA), taurohyodeoxycholic acid (THDCA), tauro-ω-muricholate (TωMCA), cholic acid (CA), β-muricholate (βMCA), α-muricholate (αMCA), ω-muricholate (ωMCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), and hyodeoxycholic acid (HDCA) were supplied by Merck KGaA (Darmstadt, Germany), or Cambridge Isotope Laboratories (Tewksbury, USA). Methanol and acetonitrile (ACN) were obtained from Biosolve BV (Valkenswaard, The Netherlands), formic acid was ordered from VWR CHEMICA (Amsterdam, The Netherlands), and phosphate buffered saline (PBS) was purchased from Gibco (Paisley, UK). A mixture of taurine conjugated bile acids (T-BAs) composed of 500 μM TCA and 500 μM TβMCA was prepared in PBS, while individual stock solutions of OPs were first dissolved in DMSO at 100 times the targeted final concentration and then further diluted in PBS resulting in a final concentration of 1% DMSO in the incubations. Also the solvent control incubations contained 1% DMSO.

3.2.2 Concentrations of OPs to be tested in the in vitro fermentation model

To establish the in vitro concentrations to be used in the in vitro studies, information on the toxicity of the selected OPs including their no observed adverse effect level (NOAEL) and lethal dose for 50% of the population (LD50) were collected and summarized in **Table 3.1**. All LD50 values relate to acute toxicity in mice and originate from reports of the Joint Meeting on Pesticide Residues (JMPR), the EU pesticides database, or the China Food and Drug Administration (CFDA). Since 1/30 LD50 is normally utilized as the low dose in acute toxicity studies of OPs, values of 1/30 LD50 were used for determination of an exposure concentration when an LD50 was available. For isocarbophos, an LD50 value was not available and the concentration to be tested was determined based on the highest NOAEL reported amounting to 0.3 mg/kg bw (CFDA, 2007). The in vivo dose levels were converted to in vitro test concentrations assuming a mouse gut volume of 1.5 mL as

reported in a previous in vivo study (Davies and Morris, 1993) and a mouse body weight of approximately 0.02 kg, using the following equation:

$$\text{Exposure concentration in vitro (mM)} = \frac{\text{dose level in vivo (mg/kg bw)} * \text{body weight (kg)}}{\text{molecular weight (mg/mmol)} * \text{mouse gut volume (mL)}} \times 1000\ \text{(mL/L)}$$

This resulted in concentrations of 0.01, 0.01, 0.08, 0.47, 0.31, and 0.01 mM respectively selected as the in vitro test concentrations of triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, and phorate.

Table 3.1 Summary of literature data on toxicity and dose level selection for calculating the in vitro exposure concentrations for the six OPs used in this study including triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, and phorate.

OPs	NOAEL (mg/kg bw per day)	1/30 LD_{50} in mouse (mg/kg bw)	Oral dose selected (mg/kg bw per day)	Mouse body weight (kg)	Mouse gut volume (mL)	In vitro exposure to gut (mM)	Reference
Triazophos	0.1	0.25	0.25	0.02	1.5	0.01	(JMPR, 2002)
Isocarbophos	0.3	-	0.3			0.01	(CFDA, 2007)
Chlorpyrifos	1	2	2			0.08	(JMPR, 2004)
Diazinon	0.5	10.83	10.83			0.47	(JMPR, 2006)
Omethoate	0.03	5	5			0.31	(CFDA, 2007)
Phorate	0.07	0.22	0.22			0.01	(CFDA, 2007)

3.2.3 Fecal slurry preparation

Feces were obtained by physical massage of the rectum of C57BL/6N mice (30 males and 30 females), weighed and transferred immediately into anaerobic 10% (v/v) glycerol in PBS, pooled and diluted to a final fecal concentration of 20% (w/v) under an anaerobic atmosphere (85% N2, 10% CO2, and 5% H2)(BACTRON300 anaerobic chamber (Cornelius, USA)). Samples were then filtered using sterile gauze under anaerobic conditions, and aliquots of fecal slurry at the volume of 200 µL were prepared. Subsequently, a washing step was implemented so that added T-BAs together with the residual bile acids remaining in the fecal samples after washing, made up a bile acid profile similar to what would be expected to be excreted from the liver (Sayin et al., 2013). Thus, these prepared aliquots (200 µL) were twice washed using equal volumes of anaerobic PBS, followed by vortex-mixing for 1 min and centrifugation at 2,000 ×g for 5 min at 4 °C

under anaerobic conditions. Supernatants were removed, and PBS was supplied to 100 µL ensuring that the washed fecal slurries contain 20% feces (v/v) as before. All the washed slurries were collected and combined and new aliquoted samples of resulting fecal slurry were stored at -80°C until use.

3.2.4 Treatment of fecal samples with pesticides

In order to detect the effects of OPs on gut microbiota mediated bile acid metabolism, Eppendorf tubes containing 80 µL fecal slurry (final concentration: 160 mg feces/mL), 10 µL mixed solution of T-BAs (500 µM TCA and 500 µM TβMCA leading to final concentrations of 50 µM TCA as well as 50 µM TβMCA), and 10 µL control solvent or individual stock solutions of OPs were mixed. To the OP-treated samples, aliquots of 10 µL 10-times concentrated stock solutions of OPs were added resulting in final concentrations of 0.01 mM triazophos or 0.01 mM isocarbophos or 0.08 mM chlorpyrifos or 0.47 mM diazinon or 0.31 mM omethoate or 0.01 mM phorate. Samples were incubated under anaerobic conditions at 37oC. Incubations were performed in the BACTRON 300 anaerobic chamber (Sheldon, Cornelius, USA) with an atmosphere of 85% N2, 10% CO2, and 5% H2, at 37 °C. At 0 h, 6 h, 12 h, and 24 h of this batch fermentation, the reaction was stopped by adding a similar volume (100 µL) of acetonitrile. Samples were subsequently sonicated for 5 min, centrifuged at 21500 ×g for 15 min at 4°C, and the supernatants obtained were stored at -80°C overnight, followed by freeze drying for 8 h. Residuals obtained were then redissolved in methanol/water (1/1) acquiring the final volume of 100 µL for each aliquot. Subsequently, all samples were centrifuged at 21500 ×g for 15 min at 4°C, after which the supernatants were collected, filtered, and transferred to vials for measurement of bile acids by LC-MS/MS.

For further studies on the effect of the selected OP phorate (based on the results of Section 3.3.2) on the composition of the fecal microbiota, 240 µL mouse fecal slurry and 30 µL PBS, mixed with 30 µL control solvent or 30 µL solution of 0.1 mM phorate (final concentration 0.01 mM) were incubated under anerobic conditions for 24 h at 37°C. After 24 h, these samples were stored at -80°C overnight and shipped with dry ice for 16S rRNA analysis.

3.2.5 Bile acid profiling by LC-MS/MS analysis

Bile acid measurement was performed on a Nexera XR LC-20AD SR UPLC system coupled to a triple quadrupole LCMS 8050 mass spectrometer (Kyoto, Japan) with electrospray

ionization (ESI) interface, which was able to measure the 16 bile acids studied: TCA, TβMCA, TαMCA, TDCA, TLCA, TUDCA, THDCA, TωMCA, CA, βMCA, αMCA, ωMCA, DCA, LCA, UDCA, and HDCA. Bile acids in fecal samples and standards were separated on an Kinetex C18 column (1.7μm×100 A×50mm×2.1 mm, Phenomenex 00B-4475-AN) using an ultra-high performance liquid chromatography (UHPLC) system (Shimadzu) with mobile phases consisting of 0.01% formic acid in distilled water (solvent A), a mixture of methanol and acetonitrile (v/v=1/1)(solvent B), and acetonitrile containing 0.1% formic acid (solvent C). The total run time was 16 minutes with the following gradient profile: 95% A, 0% B and 5% C (0-2min), slowly changed to 30% A , 70% B, and 0% C from 2 to 7.5 min, rapidly reversed to 2% A, 98 % B, and 0% C in 0.1 min, then kept at 2% A, 98% B, and 0% C from 7.6 to 10 min, then changed to 70% A, 30% B, and 0% C in 0.5 min; in the end, slowly turned back to the initial condition of 95% A, 0% B and 5% C from 10.5 to 13 min, then maintained at these conditions till 16 min for equilibration. The column temperature was set at 40℃ and the sample tray temperature was set at 4℃. The mass spectrometer (MS) used electrospray ionization (ESI) in negative ion mode. The ESI parameters were as below: Nebulizing gas flow, 3L/min; drying gas flow and heating gas flow, 10L/min; Interface temperature, 300℃; Desolvation temperature, 526℃; heat block temperature, 400℃. Selective ion monitoring (SIM) and multiple reaction monitoring (MRM) used for the detection of the bile acids are shown in **Table S3.1**. The Postrun and Browser Analysis function from the LabSolutions software (Shimadzu, Kyoto, Japan) was employed to obtain the peak areas of the satisfied extracted ion chromatogram (EIC) for each target.

3.2.6 16S rRNA gene sequencing analysis

Fecal samples prepared (Section 3.2.4) were sent to an accreditated commercial laboratory (IMGM Laboratories GmbH, Martinsried, Germany) for DNA extraction, PCR, library preparation, and sequencing. Besides, quantification of the bacterial load was implemented by real-time qPCR. 16S V3-V4 primers (F-NXT-Bakt-341F: 5′-CCTACGGGNGGCWGCAG-3′ and R-NXT-Bakt-805R: 5′-GACTACHVGGGTATCTAATCC-3′) were used to amplify the PCR products. During an index PCR, barcodes for multiplexed sequencing were introduced using overhang tags. A sequencing library was prepared from barcoded PCR products and sequenced on the Illumuna○R MiSeq next generation sequencing system (Illumuna○R Inc.). Signals were processed to *.fastq-files and the

resulting 2×250 bp reads were demultiplexed. Microbiota identification was performed by clustering the operational taxonomic units (OTU).

3.2.7 Statistical analysis

Bile acid profile data acquisition and processing were implemented using the Lab solutions software in the LC-MS/MS system. Graphics were drawn using Graphpad Prism 5 (San Diego, USA). 16S rRNA analysis data were analyzed with R version 3.6.1 and QIIME 2 view. Statistical significance was determined by a one-way ANOVA with a Dunnett/Bonferroni correction for multiple tests and results were considered significant when $p < 0.05$. Chemical structures were drawn by using ChemDraw 18.0 (PerkinElmer, Waltham, USA). Results are shown as mean ± standard deviation (SD) of three independent measurements.

3.3 Results

3.3.1 Bile acid profile in fecal slurry preparation

Figure 3.2 presents the levels of T-BAs, primary bile acids, and secondary bile acids in samples during the preparation of the fecal slurry. The three pie charts show the composition of these bile acids in freshly isolated, washed, and T-BA supplemented fecal samples respectively. In original filtered isolated fecal samples, primary and secondary bile acids were present at a percentage of 70% and 30%, respectively, while T-BAs were hardly found. After washing the feces with PBS, primary and secondary bile acids were still dominant; however, the total amount of bile acids was reduced to no more than 20% of the original amount. Subsequently, a mixed T-BA solution was added aiming at a final concentration of 50 μM TCA and 50 μM TβMCA; as a result, levels of T-BAs increased from originally 0.17% to 57.5%, the levels of primary bile acids remained at a percentage similar to that in fresh isolated fecal samples, and levels of secondary bile acids significantly decreased from originally 72.7% to 17.9%, as shown in the right chart of **Figure 3.2**. The T-BA supplemented fecal samples have bile acid profiles that better match the bile acid profile in the liver and expected to be excreted from liver into the gastrointestinal tract (Sayin et al., 2013). These supplemented fecal samples with added T-BAs were used for further study on the effects of OPs on gut microbiota mediated bile acid metabolism.

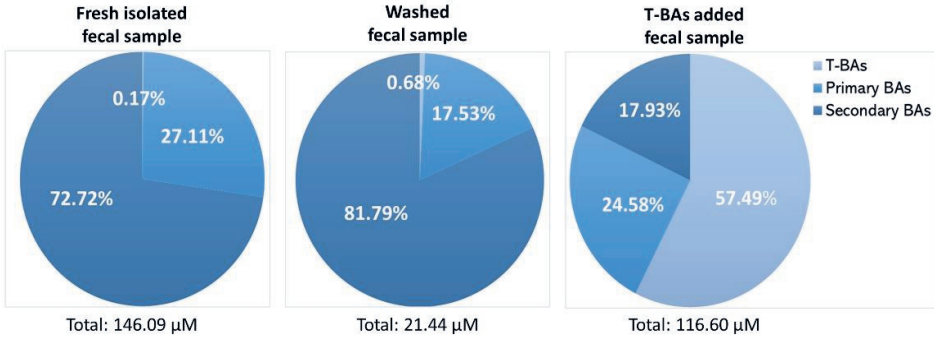

Figure 3.2 Composition of T-BAs, primary BAs, and secondary BAs in freshly isolated, washed, and T-BA supplemented fecal samples. T-BAs (light blue) mainly include TCA and TβMCA; primary BAs (blue) mainly include CA, αMCA, and βMCA; secondary BAs (dark blue) mainly include ωMCA, DCA, LCA, HDCA, and UDCA.

3.3.2 Effects of OPs on bile acid metabolism during the in vitro fermentation

The fecal bile acids detected at 24 h in control and OP-treated fecal samples are presented in **Figure 3.3**, and the time-dependent changes in these detected bile acids during the 24 h anaerobic incubation with OPs are shown in **Figure S3.1**. As shown by these results, a variety of alterations in the bile acid profiles emerged during the fermentation (6 h, 12 h, and 24 h) in the presence of the OPs resulting in substantial and significant changes at the final 24 h time point of the incubations. Among the primary bile acids, βMCA significantly decreased in all OP treated fecal samples compared with controls ($p < 0.0001$), whereas for another primary bile acid CA this reduction was observed in certain OP treated samples, with a statistical significance in triazophos ($0.05 < p < 0.01$), omethoate and phorate samples ($p < 0.001$). The primary bile acid αMCA showed differential effects in the OP-treated groups, showing a significantly raised level in the chlorpyrifos treated samples and a significantly reduced level in omethoate and phorate treated samples. For secondary bile acids, levels of ωMCA showed a significant increase in all OP-treated fecal samples compared with results of controls at 24 h of incubation ($p < 0.0001$), whereas levels of other secondary bile acids including LCA, UDCA, and HDCA showed both increases and decreases in OP-treated samples compared to controls. Notably, significant changes were observed for all the secondary bile acids in phorate-treated samples compared to controls, with a decreased level for LCA ($p < 0.001$) and UDCA ($p < 0.01$) and an increased level for HDCA ($p < 0.001$). Further, **Figure 3.3B** presents the changes in the

total amount of bile acids, primary bile acids, and secondary bile acids present in the fecal bile acid profiles acquired at 24 h. A significant increase in the total amount of secondary bile acids and an accompanying significant decrease in the overall amount of primary bile acids were observed for all six OP-treated fecal samples compared to controls; additionally, significant alterations in the total amount of bile acids were observed in diazinon- and omethoate- treated samples. The taurine conjugated bile acids added at the start of the incubations (TCA and TβMCA) were almost completely depleted at the end of the incubations, and are therefore not shown in **Figure 3.3A and B**. In addition to these changes in the bile acid profiles, the dominant pathways for bile acid transformation modulated by gut microbiota are described in **Figure 3.3C**. In general, levels of bile acids originating from the added TβMCA were altered by the OPs to a larger extent than those derived from the added TCA. Particularly for the transformation from βMCA to ωMCA, significant and substantial changes were observed in all OP-treated groups ($p < 0.0001$). The increased levels for ωMCA together with the reduced richness of βMCA revealed that the OP pesticides could result in a faster 6β-epimerization. Meanwhile, in phorate-treated samples the accumulation of ωMCA further led to the raised production of HDCA. In contrast, in chlorpyrifos and diazinon treated samples, the decreased level of HDCA observed at 24 h compared to the control could be related to an inhibition of 7β-dehydroxylation of ωMCA. Additionally, since the conversion of αMCA into βMCA is reversible, the effects on the production of αMCA induced by OPs were variable (**Figure S3.1**). The effects of the OPs on the bile acids involved in the TCA conversion route appeared less substantial than those in the TβMCA transformation. There was a different modifying effect on the production of DCA from CA in the different OP-treated groups. For example, exposure to isocarbophos, chlorpyrifos, and diazinon resulted in a reduced production of DCA, which indicates a slower 7α-dehydroxylation of CA to DCA. Conversely, an increased level of DCA with a simultaneously reduced level of CA were observed in phorate treated samples, which could be due to a faster 7α-dehydroxylation of CA to DCA. Additionally, a slower production of LCA than in the control was also observed in the phorate group, revealing the reduced oxidation of CA by the modulated gut microbial community. Characterization of the bile acid profile illustrates that the administration of all OPs studied affected the bile acid profile as compared to the control with the effects of phorate being most pronounced.

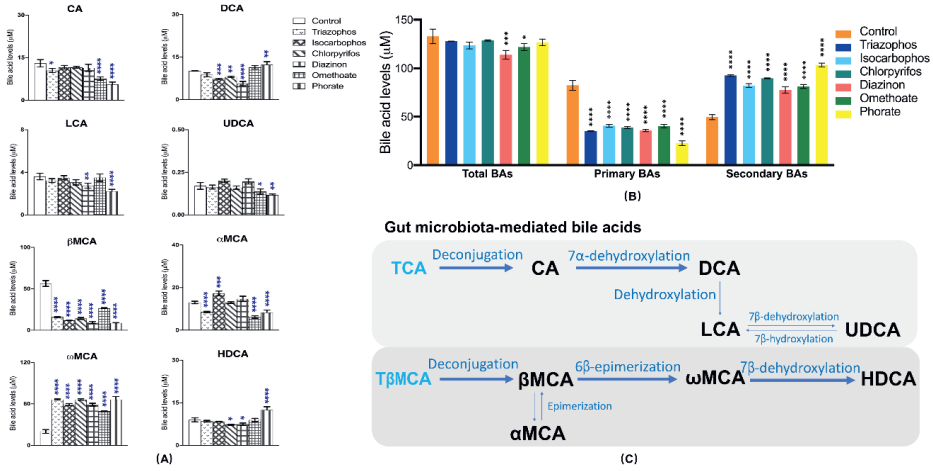

Figure 3.3 Bile acid profiles in control and OP exposed fecal samples upon 24 h in vitro anaerobic incubation (n=3) showing (A) the levels of individual fecal bile acids detected or (B) the total amount of bile acids (total BAs), primary bile acids (primary BAs), and secondary bile acids (secondary BAs), and (C) pathways for the dominant bile acid transformation modulated by gut microbiota (Wahlström et al, 2016; Tonin and Arends, 2018). Significance of altered bile acids or bile acid groups are marked (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001 indicates a difference from controls).

To further analyze the differences between the bile acid profiles in these fecal samples without and with the OP treatments (triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, and phorate), a principal coordinates analysis (PCoA) based on the Bray–Curtis distance (**Figure 3.4A**) and an analysis of principal coordinate 1 (PC1)(**Figure 3.4B**) of all groups were performed. The results obtained show that the controls as well as all OP treated samples cluster in their own group with 13.2% and 77.0% variation explained by PC1 and PC2, respectively. All OPs cluster apart from the control with chlorpyrifos, isocarbophos, and diazinon clustering close together, while the other three OPs including triazophos, omethoate, and phorate separated from one another and from the other OPs. Notably, phorate-treated fecal samples were the most clearly separated from the control group as shown by the PC1 analysis.

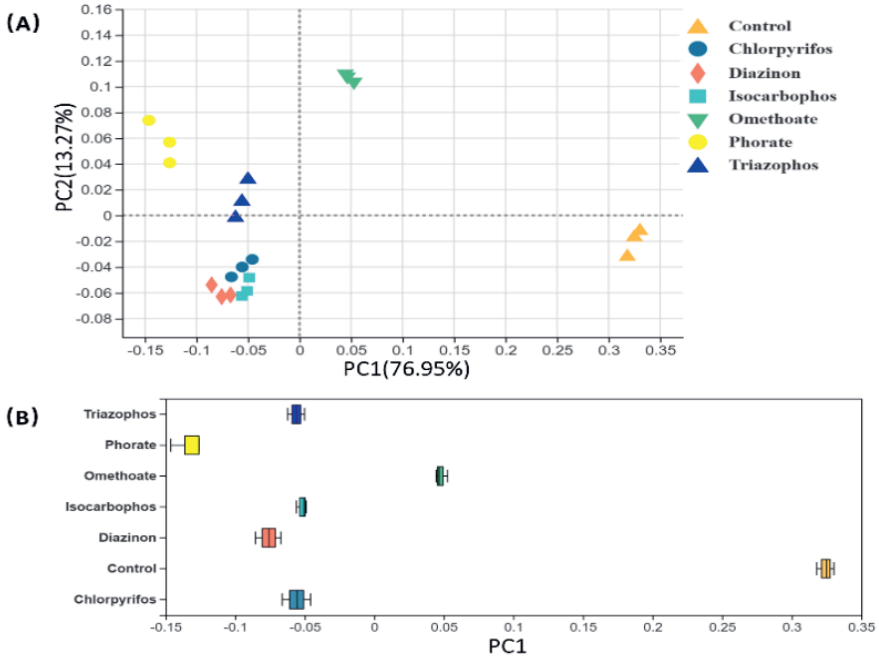

Figure 3.4 Principal coordinates analysis (PCoA) of all detected bile acids in fecal samples of untreated (control) and OP-treated groups (A), and analysis of all samples based on only principal coordinate 1 (PC1).

3.3.3 Bacterial alteration in phorate treated fecal samples

Since compared to other OPs the effects of phorate on bile acid profiles were most pronounced, phorate was selected for further studies on the effect of the OP pesticide on the bacterial profile. To this end, phorate-treated and control fecal samples were collected at 24 h of incubation and analysed by 16S rRNA analysis. **Figure 3.5** presents the relative abundance at phylum and family levels as well as results of the alpha diversity analysis using data from these fecal samples of control and phorate containing incubations at operational taxonomic unit (OTU) level. **Figure 3.5A** reveals that the mouse fecal bacterial community is dominantly composed of the phyla *Bacteroidetes* and *Firmicutes* (approximately 90%), and that upon phorate treatment the relative abundance of *Bacteroidetes* significantly increased ($0.01< p <0.05$) compared to the control at the cost of the relative abundance of the *Firmicutes*. The effects at the family level are summarized in **Figure 3.5C**, revealing that the proportions of the *Atopobiaceae* and *Muribaculaceae* separately showed a significant decrease ($p <0.0001$) and increase ($0.01< p <0.05$) in

phorate treated samples compared to controls. In the alpha diversity analysis (**Figure 3.5B**), the Chao 1 index shows a slightly decreased total amount of bacterial species upon phorate treatment, while the bacterial diversity presented by the Shannon index was similar in control and phorate treated samples.

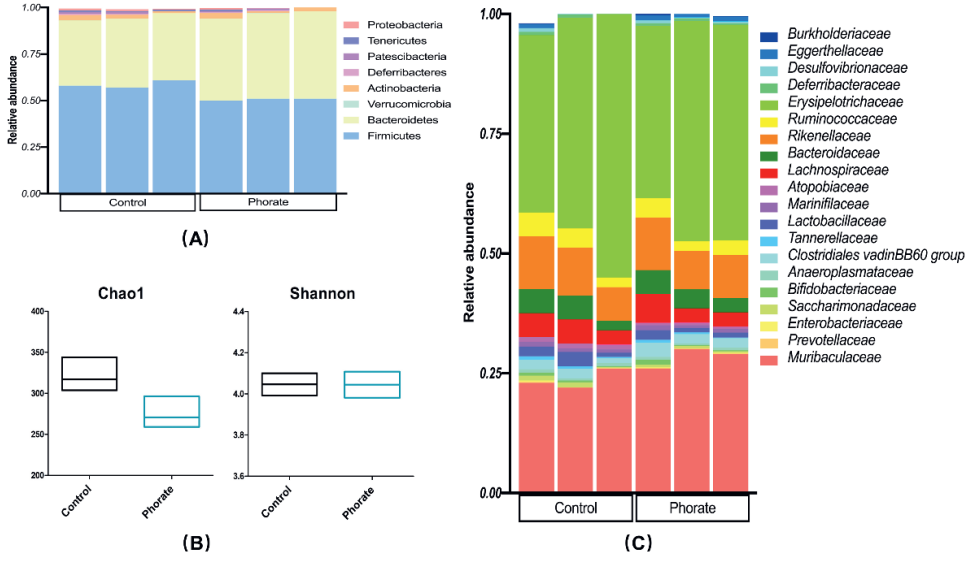

Figure 3.5 Relative abundance of gut bacteria on (A) phylum level and (C) family level and (B) alpha diversity as determined by Chao1 and Shannon Index in fecal samples from control and phorate treated samples (n=3). In (B) the line inside the box represents the median, while the whiskers represent the lowest and highest values within the 1.5 interquartile range.

Furthermore, **Figure 3.6** presents the pearson correlation coefficients to identify potential relevance between changes of dominant bacterial profiles and altered bile acids in the incubations with phorate versus control, with the correlations between bile acid profiles and the bacterial communities at the family and genus level presented in **Figure 3.6B and C**, respectively. In **Figure 3.6B**, the family *Atopobiaceae* showed significant correlations with the production of bile acids, including a positive relation to levels of the fecal primary bile acids CA, βMCA as well as αMCA, and the secondary bile acids LCA and UDCA but also a negative link to the secondary bile acids DCA, ωMCA and HDCA, showing the strongest correlations with the levels of βMCA (positive correlation) and ωMCA (negative correlation). The other family that showed phorate induced effects, *Muribaculaceae* was positively related to the ωMCA and negatively related to βMCA with significance, while the correlation with *Atopobiaceae* was negative. At the genus level

(**Figure 3.6C**), the genera *Coriobacteriaceae UCG-002* belonging to the *Atopobiaceae* and the genera *Muribaculum* belonging to the *Muribaculaceae* showed the most significant correlations with the altered bile acid profiles. Additionally, another genera named *uncultured bacterium-1* which also belongs to the *Muribaculaceae* and the genera *Enterorhabdus* classified to *Eggerthellaceae* appeared to be highly associated with the levels of βMCA and LCA respectively. Relative abundance for these genera that showed correlations to the bile acid profiles together with those which showed significant changes upon the treatment of phorate, are summarized in **Figure 3.6A**. According to the results shown in **Figure 3.6A and C**, the significantly reduced richness of *Coriobacteriaceae UCG-002* induced by the exposure to phorate could lead to the decreased levels of CA, αMCA, LCA, UDCA and especially βMCA accompanied by the raised richness of DCA, HDCA, especially ωMCA (see **Figure 3.3A**). Conversely, the raised proportion of *Muribaculaceae* upon the treatment with phorate could result in the totally opposite impact on these bile acids. Moreover, the deceased relative abundance of *Muribaculaceae uncultured bacterium-1* and *Enterorhabdus* could speed up the production of fecal βMCA and LCA respectively.

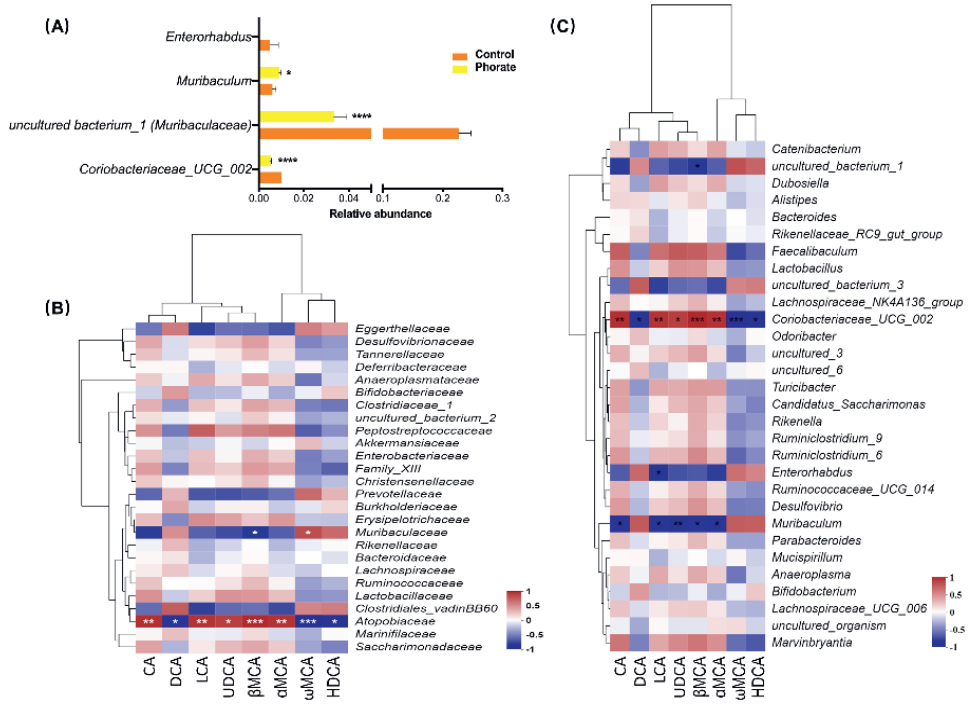

Figure 3.6 Data analysis for bacterial profiles including (A) the obviously altered genera upon the treatment of phorate, and a heatmap presentation for pearson correlations of the altered dominant bacterial species at (B) family or (C) genus level, with changes of the modified primary and secondary bile acids. Data were acquired from all samples of control and phorate groups at 24 h in vitro incubation. Red or blue heatmap squares indicate respectively a positive or negative correlation between the corresponding bile acids and the bacterial species, and the asterisks indicate significance (*p <0.05, **p <0.01, ***p <0.001)

3.4 Discussion

In the present study, for the first time, a simple batch fermentation in vitro model using mouse feces was applied to characterize the effects of OPs on gut microbiota mediated bile acid profiles. In vivo studies in which experimental animals were exposed to OPs have revealed potentially substantial effects of OPs on gut microbiota and the related metabolome of the host (Giambò et al., 2021). For example, oral administration of chlorpyrifos did not only result in changes of proinflammatory cytokines in rats (Li et al., 2019), alteration in serum amino acids and short-chain fatty acids in mice, but also caused a change in the composition of the gut/ fecal microbiota (Zhao et al., 2016). To date, there is not any in vitro or in vivo work studying effects of OPs on gut microbiota-mediated bile

acid profiles. The results obtained in the present study using an in vitro mouse fecal fermentation model clearly reveal that this model can be applied to efficiently detect potential effects. All OPs tested appeared to affect the bile acid profile and exposure of the fecal microbiota to phorate as the model compound, was shown to modulate the abundance and composition of the gut microbiota (**Figure 3.5** and **3.6A**), leading to correlated alterations in bile acid metabolism and the resulting profile (**Figure 3.3** and **S3.1**).

During the development and optimization of this in vitro fermentation batch model, many factors were considered such as pH, fecal sample used, duration of the anaerobic incubation, and also the initial bile acid profile at the beginning of the incubations. The bile acid profile in samples of liver, small intestine, cecum, colon and feces from germ-free and conventionally raised mice has been studied (Sayin et al., 2013). As shown in these reports, secondary bile acids formed by gut microbiota are abundant in feces, and a similar bile acid profile was obtained from the fresh isolated fecal samples in this study. Conjugated bile acids, including T-BAs and especially TCA and TβMCA were reported to be dominant in small intestine and liver (Sayin et al., 2013) but appeared almost absent in the fresh fecal samples. Thus, to better mimic the bile profile expected in the gastrointestinal tract in vivo upon release of the bile acids from the liver, the process of washing by PBS and subsequent supplementation of the fecal samples with a concentrated solution of TCA and TβMCA was applied. Results obtained indicate that the resulting composition of T-BAs, primary bile acids, and secondary bile acids present in the fecal incubations were in line with those reported for mouse small intestine (Sayin et al., 2013).

With respect to the changed bile acid metabolome as such, these effects can be ascribed to changes in bacterial composition; therefore, for control and phorate exposed samples 16S rRNA sequencing analysis was performed to investigate the alterations of intestinal microbial community induced by the selected OP and potential correlations of these alterations with the changes in the bile acid profile. The results obtained showed that the exposure to phorate significantly raised the relative abundance of the major phyla *Bacteroidetes* in mouse fecal samples, and this increase observed for phorate in the in vitro anaerobic fecal incubations was in line with the effect reported previously in in vivo studies focusing on the effects of the OP chlorpyrifos on rat gut microbiota (Li et al., 2019;

Condette et al., 2015) and on the intestinal community in mice (Zhao et al., 2016; Liang et al., 2019). Especially in Zhao's work (Zhao et al., 2016), such an OP induced shift with a significant increase in *Bacteroidetes* has been reported as well; additionally, these findings were found to be highly correlated with chronic intestinal inflammation (Walker et al., 2011). Further, *Muribaculaceae spp*. including *Muribaculum a*nd *uncultured bacterium-1* which showed significant alterations upon phorate treatment in the present study (**Figure 3.6A**) have already been reported as an indicator intestinal species, possessing a negative correlation with obesity indicators as well as with production of short-chain fatty acids, and contributing to glycolipid homeostasis (Ye et al., 2021). However, the relative abundance of these beneficial bacteria *Muribaculaceae spp*. significantly decreased upon the exposure to phorate.

Although *Muribaculaceae spp.* showed the most obvious changes in the bacterial composition, the genus of *Coriobacteriaceae UCG-002* belonging to the family of *Atopobiaceae* was observed to be the most strongly correlated to the bile acid profiling. Not only significant positive correlations between phorate induced changes in concentrations of CA, βMCA, αMCA, LCA as well as UDCA and *Coriobacteriaceae UCG-002* were observed, but also negative correlations of *Coriobacteriaceae UCG-002* with phorate induced changes in levels of DCA, ωMCA, and HDCA were detected. Linked to the bile acid transformation routes shown in **Figure 3.3C**, the positive and negative correlation of *Coriobacteriaceae UCG-002* with βMCA and ωMCA respectively suggests that *Coriobacteriaceae UCG-002* could substantially contribute to bile acid 6β-epimerization; similarly, its positive correlation with CA and positive correlation with DCA revealed that *Coriobacteriaceae UCG-002* could be involved in 7α-dehydroxylation as well. Recently, an important role for *Coriobacteriaceae UCG-002* in gut barrier function by modulating relevant metabolites such as bile acids has been reported as well (Li, S. et al., 2019; Guo et al., 2022). Besides, *Muribaculum* showed to be negatively related to the production of bile acids, which is in line with the previous suggestion that *Muribaculum* could provide a possible marker of bile acid metabolism disorders (Shang et al., 2022). Since *Muribaculum* has the capacity to provide bile salt hydrolase (BSH) activity in the small intestine of mice and also to support 7α-dehydroxylation (Marion et al., 2020), the markedly increased abundance of *Muribaculum* could speed up the 7α-dehydroxylation, exemplified by a faster conversion from CA to DCA which was shown by the significant decrease and increase in CA and DCA respectively (**Figure 3.6A**).

All together the results of the present work show that OPs affect the bile acid profiles probably by modulating gut bacteria. The results of bacterial profiles obtained in the in vitro fermentation model used were in line with limited data available from the only mouse in vivo study on evaluating the impact of the OP chlorpyrifos on the gut microbial community (Zhao et al., 2016). A major advantage of this in vitro fermentation model is that it is possible to characterize effects of a larger number of compounds of interest so that it provides a new approach methodology for studying the relevance between alterations of gut microbiota and related bile acid metabolism upon exposure to various xenobiotics.

3.5 Conclusion

In this study, an anaerobic mouse fecal batch culture system was utilized to characterize the bacterial metabolism of individual primary and secondary bile acids and to evaluate effects of OP exposure on gut microbiota and bile acid metabolism. Results obtained in this in vitro system showed that all six OPs studied (triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, and phorate) could lead to alterations of the bile acid profile especially resulting in a faster production of secondary bile acids at the expense of primary bile acids especially the production of ωMCA from βMCA. The bacterial profile of phorate exposed fecal samples was determined and altered in the abundance of the family of *Atopobiaceae* and *Muribaculaceae*, and the genera *Coriobacteriaceae UCG-002* belonging to *Atopobiaceae* was found to be the most highly correlated with bile acid profiling. Collectively, OPs could affect the bile acid metabolism by modulating the gut microbial community.

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4

CHAPTER 4

Impact of carbamate and pyrethroid pesticides on bile acid profiles in an in vitro gut microbiota model

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Ivonne M.C.M. Rietjens

Abstract

The impact on the microbial community following exposure to commonly used pesticides has recently gained increasing interest. In this study, effects of selected carbamates (carbofuran, aldicarb) and pyrethroids (cypermethrin and cyhalothrin) on gut microbiota and related bile acid metabolism were quantified using a 24 h in vitro fermentation model system with mouse feces. The results obtained reveal a pesticide induced significant increase the ratio of secondary over primary bile acids, particularly resulting from the enrichment of β-muricholate (βMCA) accompanied by the depletion of ω-muricholate (ωMCA). Besides, the bacterial profile showed significantly increased richness of *Eggerthellaceae* after 24 h exposure to carbofuran and cyhalothrin, and the genera *Enterorhabdus* belonging to *Eggerthellaceae* was found to be highly correlated to the fecal bile acid profile. In conclusion, in an in vitro gut microbial model carbamates and pyrethroids caused alterations of the gut microbial community resulting in the modulation of bile acid transformation. This illustrates that the gut microbiota and its metabolism may be a novel target to consider in future pesticide safety evaluations.

Keywords: fecal bile acid; gut microbiota; in vitro model; pyrethroid pesticides; carbamate pesticides

4.1 Introduction

In the past decades, the demand for food has risen significantly in relation to the world population's increase, and as a result pesticides are widely used worldwide to obtain better quality agricultural products and increase crop yields, bringing significant economic benefits but also increased health hazards (Jin et al., 2017). Carbamates and pyrethroids are two typical classes of insecticides which are frequently used to control pests in homes and agricultural crops (Rawn et al., 2006). The systemic N-methyl carbamate pesticides such as carbofuran and aldicarb (**Figure 4.1**) are also extensively applied as nematicide and acaricide for agricultural, domestic and industrial purposes (Mishra et al., 2020). Furthermore, synthetic and effective pyrethroids like cypermethrin and cyhalothrin (**Figure 4.1**) are largely replacing the use of organophosphorus insecticides due to the high risks of these organophosphorus to health and the environment (Muhamad et al., 2012). Recent work reported that residues of carbamates and pyrethroids in food may also cause neurotoxic, cytotoxic, reproductive, endocrine disrupting, embryo-toxic and dermal-skin problems (WHO, 2009). For example, it was indicated that oral administration of rats with carbamates could result in inhibition of cholinesterases (Silberman and Taylor, 2018), while accidental mammal exposure to pyrethroids could cause liver hypertrophy, mildly to severe irritation to skin and eyes, and neurotoxicity at high doses (Thatheyus and Selvam, 2013). Although regulation and control for use of carbamates and pyrethroids is being in place in many countries, it is inevitable that humans could be exposed to small amount of residues in a variety of food products (WHO, 2009), resulting particularly in the direct exposure of the gut and its microbiome.

The gut microbiome consists of trillions of microorganisms that colonize the mammalian gastrointestinal tract. The past few years have seen a surge in research on the gut microbiome, which has firmly established its critical role in host health (Shreiner et al., 2015; Tremlett et al., 2017; Rooks and Garrett, 2016; Halfvarson et al., 2017). The gut microbiome is involved in numerous aspects of host metabolism and physiology, from energy production to stress response, and can confer many benefits to the host (Deehan and Walter, 2016; Dinan and Cryan, 2012). One important role relates to modulating lipid metabolism potentially through bacterial-derived signalling molecules such as bile acids (Ghazalpour et al., 2016). Bile acids are a class of structurally diverse molecules with a

rich diversity and each of these entities may have different bioactive functions. In addition to their detergent-like properties and their use as substrates for microbial metabolism (Hylemon et al., 2018), bile acids act as hormones as well (Perino et al., 2021). As a result, the gut microbial community as well as the related bile acid profile play an important role in host health.

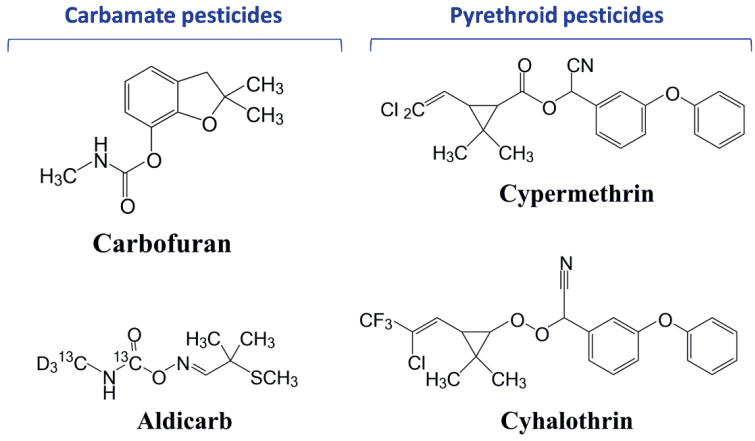

Figure 4.1 Chemical structures of the model pesticides of the present study including the carbamates carbofuran and aldicarb and the pyrethroids cypermethrin and cyhalothrin.

However, changes in the gut microbiota could be easily induced by various factors, for example, ageing (Leung and Thuret, 2015; Lynch et al., 2015), diet (Wu et al., 2016; Jeffery and O'Toole, 2013), diseases (Carding et al., 2015), and also exposure to chemicals including antibiotics, drugs and also some pesticides (Kang et al., 2013). Recently, the toxicity of pesticides towards non-target organisms such as the gut microbiota is gaining increased attention (Defois et al., 2018). Studies performed in vitro or in vivo on gut microbiota alterations and host outcomes induced by exposure to pesticides such as organochlorine pesticides, fungicides, and insecticides including carbamates have been reported (Giambò et al, 2021). In the only previous work evaluating the effects of carbamates on gut microbiota (Gao et al., 2018), the carbamate aldicarb showed effects on the gut microbiota including the increased abundance of *Erysipelotrichaceae* at the expense of *Christensenellaceae*, leading to changes in lipid profiling in C57BL/6J mice. The effects of pyrethroids including bifenthrin (Li et al., 2021; Li et al., 2022) and permethrin (Nasuti et al., 2016) causing gut microbiota dysbiosis have also been reported in recent years. However, scientific research for potential toxicity of cypermethrin, cyhalothrin, and

carbofuran to gut bacteria and the related metabolome are still absent. To date, no studies have reported on the consequences of the effects of these pesticides on the microbiome for bile acid homeostasis.

The aim of the present study was to assess the potential effects of selected carbamate and pyrethroid pesticides on the gut microbiota and the related consequences for bile acid metabolism and the resulting bile acid profile. To this end, an in vitro model was employed enabling studying of the influence of selected carbamates and pyrethroids on mouse intestinal microbiota and the resulting effects on processing of bile acids. The in vitro model consisted of anaerobic fecal incubations, in which altered bile acid profiles could be quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS) and bacterial profiles were determined by 16S rRNA gene sequencing analysis. Characterization of the effects of the exposure to carbamates and pyrethroids on the composition of gut microbiota and related bile acid metabolism will reveal whether the understanding of the interplay between oral administration of pesticides, gut microbiota and bile acid metabolism is an area of particular interest for future safety evaluations.

4.2 Materials and methods

4.2.1 Reagents and standards

Bile acid standards of taurocholic acid (TCA), tauro-β-muricholate (TβMCA), tauro-α-muricholate (TαMCA), taurodeoxycholic acid (TDCA), taurolithocholic acid (TLCA), tauroursodeoxycholic acid (TUDCA), taurohyodeoxycholic acid (THDCA), tauro-ω-muricholate (TωMCA), cholic acid (CA), β-muricholate (βMCA), α-muricholate (αMCA), ω-muricholate (ωMCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), and hyodeoxycholic acid (HDCA) were obtained from Merck KGaA (Darmstadt, Germany), or Cambridge Isotope Laboratories (Tewksbury, USA). The test pesticides carbofuran (CAS 1563-66-2), aldicarb (CAS 116-06-3), cypermethrin (CAS 52315-07-8), and cyhalothrin (CAS 68085-85-8), as well as dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Methanol and acetonitrile were supplied by Biosolve BV (Valkenswaard, The Netherlands), formic acid was ordered from VWR CHEMICA (Amsterdam, The Netherlands), and phosphate buffered saline (PBS) was purchased from Gibco (Paisley, UK). A mixture of taurine conjugated bile acids was prepared in PBS at final concentrations of 500 μM TCA and 500 μM TβMCA, and individual stock solutions of carbamates as well as pyrethroids were prepared in DMSO at 100 times

the targeted concentration and then further diluted in PBS for use resulting in a final concentration of 1% DMSO in the incubations. Also the solvent control incubations contained 1% DMSO.

4.2.2 Conversion of the in vivo dose levels to in vitro test concentrations

To establish the concentrations to be used in the in vitro studies, LD_{50} values reported for the respective pesticides in acute toxicity studies in mice were collected from the reports of the World Health Organization (WHO) and Joint Meeting on Pesticide Residues (JMPR). Since 1/10 LD50 is normally utilized as the low dose in toxicity studies of carbamate and pyrethroid pesticides, LD50 values of 30 mg/kg bw for carbofuran (WHO, 2003), 1.0 mg/kg bw for aldicarb (WHO, 2002), 250 mg/kg bw for cypermethrin (JMPR, 2006), and 20 mg/kg bw for cyhalothrin (JMPR, 2007) were converted to 3 mg/kg bw for carbofuran, 0.1 mg/kg bw for aldicarb, 25 mg/kg bw for cypermethrin and 2 mg/kg bw for cyhalothrin as the oral dose levels providing low but measurable effects without showing lethality. These dose levels were converted to the corresponding in vitro test concentrations as follows:

$$\text{Exposure concentration in vitro (mM)} = \frac{\text{dose level in vivo (mg/kg bw)} * \text{body weight (kg)}}{\text{molecular weight (mg/mmol)} * \text{mouse gut volume (mL)}} \times 1000\ \text{(mL/L)}$$

The volume of the gastrointestinal tract of mice was assumed to amount to approximately 1.5 mL (Davies and Morris, 1993) and the body weight of mouse was taken as 0.02 kg on average. Using the equation this resulted in test concentrations of 0.18 mM for carbofuran, 0.01 mM for aldicarb, 0.8 mM for cypermethrin, and 0.06 mM for cyhalothrin, concentrations that were employed as the test concentrations in the in vitro incubations.

4.2.3 Preparation of mouse fecal samples

Feces were obtained by physical massage of the rectum of C57BL/6N mice (30 male and 30 female mice), weighed and transferred immediately into anaerobic 10% (v/v) glycerol in PBS, pooled, homogenized and diluted to a final fecal concentration of 20% (w/v) under an anaerobic atmosphere (85% N_2, 10% CO_2, and 5% H_2) in a BACTRON300 anaerobic chamber (Cornelius, USA). Samples were then filtered using sterile gauze under the anaerobic conditions, and 200 μL aliquots of fecal slurry were prepared. Subsequently, a washing step was implemented to avoid effects of high levels of the residual endogenous fecal bile acids in the developed model; as a result, these prepared aliquots were twice

washed using an equal volume of anaerobic PBS, followed by vortex-mixing for 1 min and centrifugation at 2,000×g for 5 min at 4 °C under anaerobic conditions. Supernatants were removed and PBS was supplied to 200 μL ensuring that the washed fecal slurries contained 20% feces (v/v) as before. All the washed slurries were collected, mixed and newly aliquoted samples of 200 μL resulting fecal slurry were stored at -80℃ until use.

4.2.4 In vitro incubations

Eppendorf tubes contained incubations consisting of 80 μL fecal slurry prepared in Section 4.2.3., 10 μL mixed solution of taurine conjugated bile acids (500 μM TCA and 500 μM TβMCA), and 10 μL control solvent or individual stock solution of pesticides. Thus, aliquots of 100 μL mixture with final concentrations of 50 μM TCA as well as 50 μM TβMCA, and 0.18 mM carbofuran or 0.01 mM aldicarb or 0.8 mM cypermethrin or 0.06 mM cyhalothrin with 16% fecal slurry were incubated under anaerobic conditions. Incubations were performed in the BACTRON 300 anaerobic chamber (Sheldon, Cornelius, USA) with an atmosphere of 85% N_2, 10% CO_2, and 5% H_2, at 37 ℃. At 0 h, 6 h, 12 h, and 24 h of this batch fermentation, the reaction was stopped by adding a similar volume (100 μL) of acetonitrile. Samples were subsequently sonicated for 5 min, centrifuged at 21500 g for 15 min at 4℃, and the supernatants obtained were stored at -80℃ overnight, followed by freeze drying for 8h. Residuals obtained were then redissolved in methanol/water (1/1) acquiring the final volume of 100 μL for each aliquot. Subsequently, all samples were centrifuged at 21500 g for 15 min at 4℃, after which the supernatants were collected, filtered (PALL AcroPrep, PTFE 0.2 μm), and transferred to vials for measurement of bile acids by LC-MS/MS.

4.2.5 Bile acid profiling by LC-MS/MS analysis

Bile acid measurement was performed on a Nexera XR LC-20AD SR UPLC system coupled to a triple quadrupole LCMS 8050 mass spectrometer (Kyoto, Japan) with electrospray ionization (ESI) interface, which was able to measure the 16 bile acids studied herein: TCA, TβMCA, TαMCA, TCDCA, TDCA, TLCA, TUDCA, THDCA, TωMCA, CA, βMCA, αMCA, CDCA, ωMCA, DCA, LCA, UDCA, and HDCA. Bile acids in fecal samples, fecal incubations and standards were separated on a Kinetex C18 column (1.7μm×100 A×50mm×2.1mm; Phenomenex, Torrance, USA) using an ultra-high performance liquid chromatography (UHPLC) system (Shimadzu) with mobile phases consisting of 0.01% formic acid in distilled water (solvent A), a mixture of methanol and acetonitrile (v/v=1/1)(solvent B),

and acetonitrile containing 0.1% formic acid (solvent C). The total run time was 16 minutes with the following gradient profile: 95% A, 0% B and 5% C (0-2min), slowly changed to 30% A , 70% B, and 0% C from 2 to 7.5 min, rapidly reversed to 2% A, 98 % B, and 0% C in 0.1 min, then kept at 2% A, 98% B, and 0% C from 7.6 to 10 min, then changed to 70% A, 30% B, and 0% C in 0.5 min; in the end, slowly turned back to the initial condition of 95% A, 0% B and 5% C from 10.5 to 13 min, then maintained at these conditions till 16 min for equilibration. The column temperature was set at 40℃ and the sample tray temperature was set at 4℃. The mass spectrometer (MS) used electrospray ionization (ESI) in negative ion mode. The ESI parameters were as below: Nebulizing gas flow, 3L/min; drying gas flow and heating gas flow, 10L/min; Interface temperature, 300 ℃; Desolvation temperature, 526 ℃; heat block temperature, 400 ℃. Selective ion monitoring (SIM) as well as multiple reaction monitoring (MRM) were simultaneously used for the detection of the bile acids. Precursor and product ions were as flows: 407.3>407.3 *m/z* for αMCA, βMCA, ωMCA, and CA; 319.3>319.3 *m/z* for UDCA, HDCA, CDCA, and DCA; 498.4>498.4 *m/z* for TUDCA, THDCA, TCDCA, and TDCA; 514.4>514.4 *m/z* for TαMCA, TβMCA, TωMCA, and TCA; 375.3>375.3 *m/z* for LCA; 482.3>482.3 *m/z* for TLCA. The Postrun and Browser Analysis function from the LabSolutions software (Shimadzu, Kyoto, Japan) was employed to obtain the peak areas of the satisfied extracted ion chromatogram (EIC) for each target.

4.2.6 16S rRNA gene sequencing analysis

Based on the results of bile acid profiling (see Section 4.3.2), carbofuran and cyhalothrin were respectively selected as the representatives of carbamate and pyrethroid pesticides for further study of the effects on the microbial community by 16s rRNA analysis. To this end, mixtures comprising 240 µL prepared mouse fecal slurry (Section 4.2.3)(final concentration 16% feces) and 30 µL PBS, mixed with 30 µL control solvent or 30 µL solution of pesticides (final concentrations 0.18 mM carbofuran or 0.06 mM cyhalothrin) were incubated under anerobic conditions for 24 h at 37 ℃. After 24 h, the fecal samples with or without pesticides were stored at -80℃ overnight and subsequently delivered to an accredited commercial laboratory (IMGM Laboratories GmbH, Martinsried, Germany) for DNA extraction, PCR, library preparation, and sequencing. Besides, quantification of the bacterial load was implemented by real-time qPCR. 16S V3-V4 primers (F-NXT-Bakt-341F: 5′-CCTACGGGNGGCWGCAG-3′ and R-NXT-Bakt-805R: 5′-

GACTACHVGGGTATCTAATCC-3′) were used to amplify the PCR products. During an index PCR, barcodes for multiplexed sequencing were introduced using overhang tags. A sequencing library was prepared from barcoded PCR products and sequenced on the Illumuna® MiSeq next generation sequencing system (Illumuna® Inc.). Signals were processed to *.fastq-files and the resulting 2×250 bp reads were demultiplexed. Microbiota identification was performed by clustering the operational taxonomic units (OTUs).

4.2.7 Data analysis

Metabolic profile data acquisition and processing were implemented using the Labsolutions software in the LC-MS/MS system. Graphics were drawn using Graphpad Prism 5 (San Diego, USA). Chemical structures were drawn by using ChemDraw 18.0 (PerkinElmer, Waltham, USA). Results are shown as mean ± standard deviation (SD) of three independent measurements and statistical significance was determined by a one-way ANOVA with a Dunnett/ Bonferroni correction for multiple tests and results were considered significant when $p < 0.05$. 16S rRNA analysis data were analyzed with R version 3.6.1 and QIIME 2 view.

4.3 Results

4.3.1 Initial bile acid profile in the in vitro model

Figure 4.2 presents the initial bile acid profile at 0 h of incubation, including levels of all quantified bile acids consisting of conjugated bile acids, primary bile acids, and secondary bile acids. **Figure 4.2A** shows that following the optimized process of fresh isolation, filtration, washing with PBS, and addition of a mixture of taurine conjugated bile acids (final concentration of 50 µM TCA and 50 µM TβMCA), at t=0 h approximately 40 µM of TCA and TβMCA were detected in the samples together with a small amount of TαMCA detected as well. TCA and TβMCA were added to the incubations to better mimic intestinal bile acid profiles given that these conjugated bile acids are known to be excreted into the intestine from the liver, where they are present at high concentrations (Sayin et al., 2013). The primary and secondary BAs detected at t=0 mainly originate from the fecal samples, not being fully eliminated upon the washing procedure. This procedure of washing the fecal samples and adding TCA and TβMCA results in an initial bile acid profile that mimics the bile acid profile as excreted from the liver into the intestine (Sayin et al., 2013). The total amount of primary and secondary bile acids was quantified at a level of

approximately 40 µM each. In **Figure 4.2B**, it is shown that at the start of the incubations the conjugated and primary bile acids together make up of 83% of the overall bile acid profile, with the conjugated bile acids amounting to 50%. Secondary bile acids including ωMCA, DCA, LCA, and HDCA were quantified as well, and were present at concentrations below 10 µM each, together making up less than 20% of the total bile acid profile at the start of the incubations.

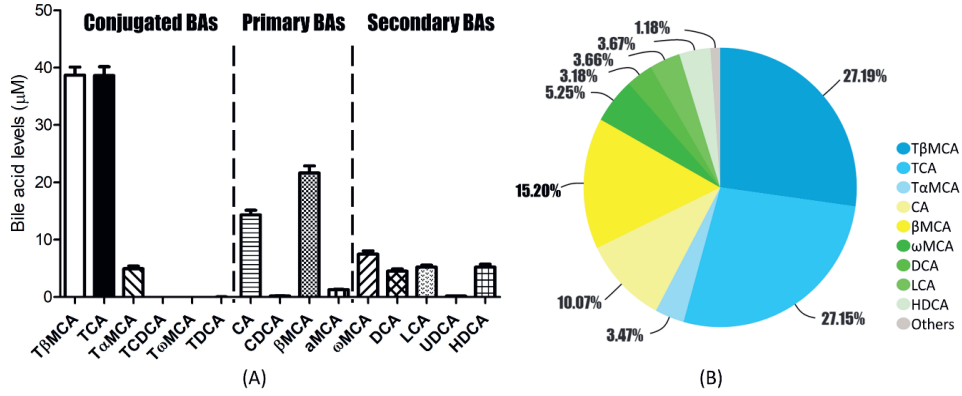

Figure 4.2 Initial bile acid profile at 0 h of incubation (n=3) represented as (A) absolute concentrations of the bile acids quantified including the conjugated bile acids TβMCA, TCA, TαMCA, TCDCA, TωMCA, and TDCA, the primary bile acids CA, CDCA, βMCA, and αMCA as well as the secondary bile acids ωMCA, DCA, LCA, UDCA, and HDCA and as (B) the relative percentage of the total bile acid profile showing the conjugated bile acids (blue), primary bile acids (yellow), secondary bile acids (green), and other bile acids at low concentrations (grey).

4.3.2 Bile acid profiles following 24 h incubation

Figure 4.3 presents bile acid profile characteristics detected during the 24 h of the in vitro fecal incubations in the absence (solvent control) or presence of the selected pesticides. As an example, **Figure 4.3** shows the LC-MS chromatogram (obtained at precursor and product ions: 407.3>407.3) of the control and cyhalothrin treated incubation at 24 h, clearly revealing an increase in the amount of ωMCA and a reduced peak height of βMCA together with a stable peak height of CA. **Figure 4.3B** presents the changes in the concentrations of the added taurine bile acids TCA and TβMCA during the 24 h anaerobic incubations; as observed, already upon 6 hours of incubation with or without pesticides, TCA as well as TβMCA were almost fully converted, reflecting fast deconjugation by the microbiota. Changes for other main fecal bile acids detected during the 24 h incubations

are presented in **Figure S4.1**, and the fecal bile acid profiles resulting at 24 h of incubation in the pesticide-treated samples and controls are shown in **Figure 4.3C**. Significantly increased ωMCA and decreased βMCA levels were observed at 24 h in all pesticide-treated samples compared to controls, fully in line with the chromatogram shown in **Figure 4.3A**. In addition, significantly decreased concentrations of αMCA were observed in carbofuran-, cypermethrin- and cyhalothrin- treated fecal incubations, while significantly reduced levels of DCA were observed in only the carbofuran and cyhalothrin groups compared to controls. Besides, at 24 h UDCA was detected at low concentrations <0.25 μM in all the pesticide-treated and control samples with a small but significant increase detected only in cypermethrin-treated samples ($p < 0.05$). No significant changes were found at 24 h of incubation in the levels of CA, LCA, and HDCA when comparing results from the pesticide-treated samples and controls. In addition to the evaluation of changes in the individual bile acids, the data were also analysed with respect to two important characteristics of bile acid profiles, including the proportion of conjugated bile acids (% conjugated BAs) and the ratio of secondary bile acids to primary bile acids (secondary/ primary BAs). **Figure 4.3D** presents the results obtained and reveals that the ratio of secondary/ primary BAs was significantly increased in all the pesticide-treated samples compared to controls, whereas significant changes in the % conjugated BAs were only observed in the pyrethroid-treated samples, showing decreases upon incubation with cypermethrin and cyhalothrin as compared to the control. Moreover in **Figure 4.3E**, the results of a principal coordinates analysis (PCoA) of the bile acid profiles obtained from all fecal samples is presented. It shows that the controls as well as all pesticide-treated samples cluster in their own groups, with all the pesticide groups clearly separating from the controls.

To facilitate interpretation of the results the metabolic pathways for the detected individual bile acids including conjugated, primary, and secondary bile acids is presented in **Figure 4.4**. The main bile acids detected can be divided into two groups, composed of the bile acids originating from the added TCA or from the added TβMCA. Combining these pathways with the results shown in **Figure 4.3**, reveals that the bile acids in the TβMCA pathway showed more significant alterations than those from the TCA based pathway. The combined data also indicate that conversions like bile salt hydrolase (BSH) deconjugation, epimerization, dehydroxylation, and hydroxylation were affected to a different extent upon the treatment of the fecal microbiota with different pesticides. The 6β-epimerization from βMCA to ωMCA was the most significantly affected conversion and

this conversion was obviously accelerated upon the exposure to pesticides, leading to the raised levels of ωMCA at the cost of βMCA and an increase in the ratio secondary/ primary bile acids. Meanwhile, in pyrethroid-treated samples, the decreased % conjugated BAs indicated an increase in the BSH deconjugation.

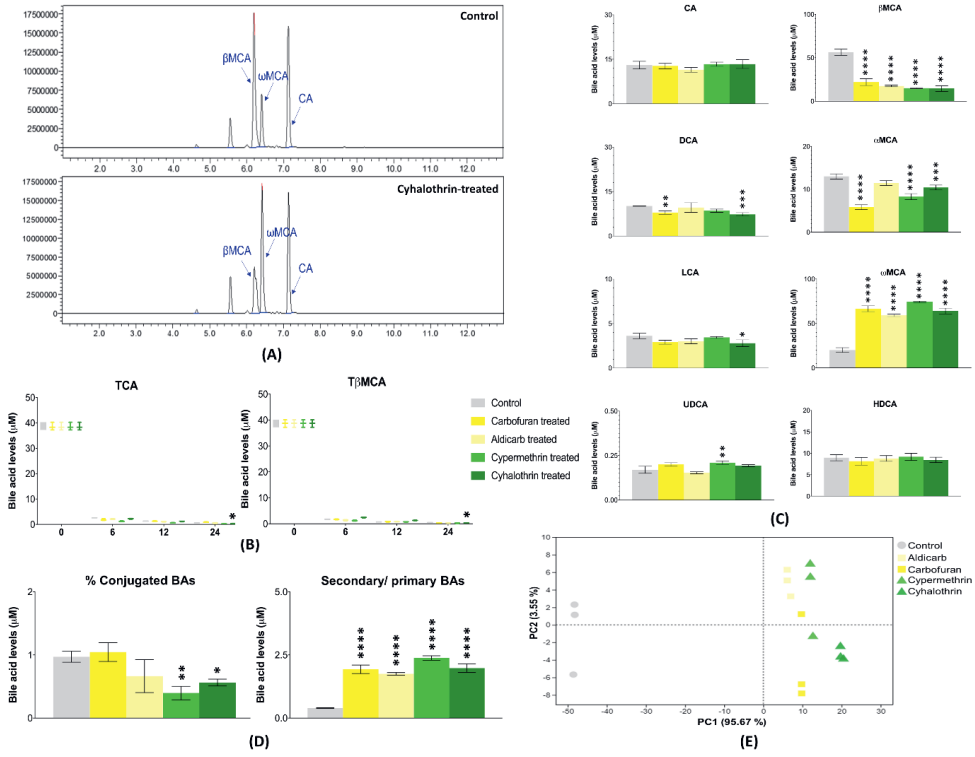

Figure 4.3 The altered bile acid profiles in the carbofuran, aldicarb, cypermethrin, or cyhalothrin -treated samples and controls, including (A) the chromatography of 407.3>407.3 m/z showing the altered peaks of βMCA, ωMCA, but not for CA in control and cyhalothrin (as example of pesticides) -treated fecal samples at 24 h of incubation, (B) the degradation of the added TCA and TβMCA at 6, 12, and 24 h following the incubations, (C) the changes of individual fecal bile acids detected in the pesticide-treated and control samples at 24 h of incubations, and (D) the altered bile acid profiles when expressed as the proportion of fecal conjugated bile acids (% conjugated BAs) and the ratio for the total amount of fecal secondary bile acids to primary bile acids (secondary/ primary BAs)(*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001; n=3). Moreover, (E) shows a principal coordinates analysis (PCoA) performed based on the bile acid profiles in the pesticide-treated and untreated fecal samples.

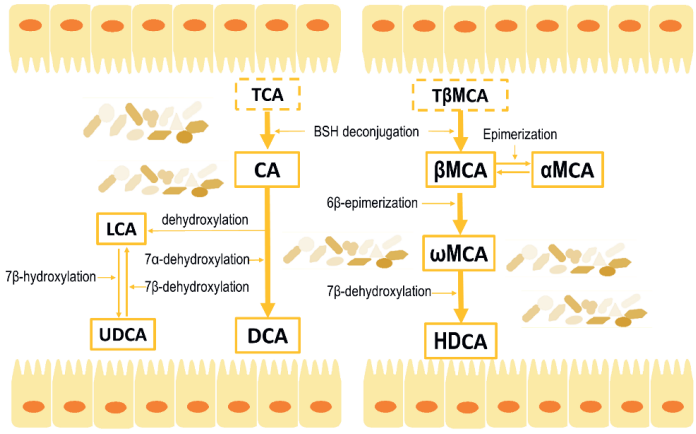

Figure 4.4 The bile acid transformation routes, starting from the added conjugated bile acids TCA and TβMCA, and including the formation of the primary bile acids CA, βMCA, and αMCA, and the secondary bile acids DCA, LCA, UDCA, ωMCA, and HDCA (Wahlström et al., 2016).

4.3.3 Alteration of the microbial community in anaerobic fecal incubations upon treatment with pesticides

Based on the results obtained for the bile acid profiles (Section 4.3.2), the carbamate carbofuran and the pyrethroid cyhalothrin were selected for further studies on their effects on the bacterial profiles, because these two pesticides resulted in the most pronounced effects on the bile acid profile. **Figure 4.5** presents the relative abundance of gut bacteria at phylum and family level as well as the analysis of alpha diversity in the fecal samples of the control and carbofuran or cyhalothrin treated groups. As shown in **Figure 4.5A**, the phyla *Bacteroidetes* and *Firmicutes* dominated in mouse feces, followed by a small amount of *Actinobacteria*. The relative abundance of the phyla *Actinobacteria* significantly increased in carbofuran ($p < 0.01$) and cyhalothrin ($p < 0.05$) treated groups compared to controls, whereas the richness of the phyla *Bacteroidetes* was significantly reduced only in the carbofuran treated samples ($p < 0.05$). At the family level (**Figure 4.5C**), *Erysipelotrichaceae* belonging to *Firmicutes* and *Muribaculaceae* belonging to *Bacteroidetes* were the most abundant bacterial species, accounting for more than 50% of the mouse gut microbial community. In addition, *Eggerthellaceae* was the most dominant family belonging to the phyla *Actinobacteria*, the relative abundance of which significantly increased in the carbofuran-treated ($p < 0.01$) and cyhalothrin-treated ($p < 0.01$) samples compared to controls. Besides, a raised richness of *Desulfovibrionaceae* was observed in

both pesticide-treated samples especially in those exposed to cyhalothrin. The richness of *Anaeroplasmataceae* was reduced and the proportion of *Enterobacteriaceae* was increased in carbofuran-treated and cyhalothrin-treated groups, respectively, albeit not to a significant extent. Further, the alpha diversity based on the data OTUs is described in **Figure 4.5B** utilizing the Chao1 and Shannon index, which separately indicate the total amount of bacterial species and the bacterial diversity. Results obtained show that the number of bacterial species as well as the diversity were reduced in fecal samples upon exposure to carbofuran, while both were slightly increased in the cyhalothrin-treated group, although these effects were not significant compared to the controls.

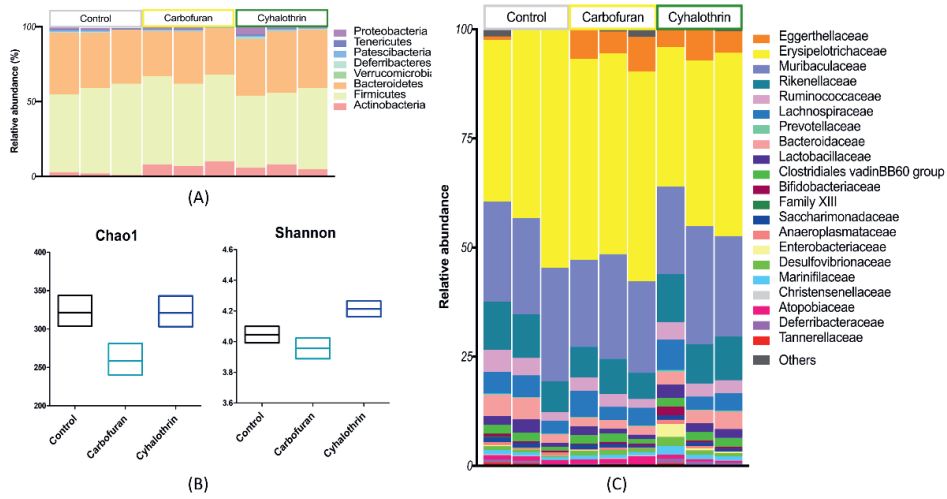

Figure 4.5 Composition of the microbiota community in untreated and pesticide treated fecal samples (n=3) after 24 h incubation in the absence (control) or presence of carbofuran or cyhalothrin, at (A) phylum level and (C) family level, and (B) the alpha diversity calculated based on the data of operational taxonomic units (OTUs) and shown by the index of Chao1 and Shannon.

4.3.4 Correlation of bile acid profile and microbiota

Figure 4.6A presents the results of a pearson correlation analysis to identify the relation between changes in the microbial community and the altered bile acid profiles upon 24 h incubation in carbofuran-treated and cyhalothrin-treated fecal samples, respectively. In general, the family of *Eggerthellaceae* showed to be the most highly related to the fecal bile acids, being positively correlated with ωMCA and negatively correlated with the primary bile acids βMCA and αMCA; additionally, it was also negatively related to TCA in the carbofuran-treated samples. Another family *Desulfovibrionaceae* also presented

strong correlations with fecal bile acid profiles, showing a negative association with TCA in the carbofuran group, and with TβMCA as well as LCA in the cyhalothrin group, and with αMCA in both pesticide-treated samples. Besides, *Anaeroplasmataceae* presented a highly negative correlation with CA in the carbofuran group, whereas *Enterobacteriaceae* appeared to be negatively correlated to UDCA in the cyhalothrin group. All the detected bacterial species involved in the four families that showed a link at genus level with the bile acid profiles are summarized in **Figure 4.6B**. As shown, the genera *Enterorhabdus* (>5% of the bacterial community), *Desulfovibrio* (>1% of the bacterial community), *Anaeroplasma* (<1% of the bacterial community), *Escherichia-Shigella* (<1% of the bacterial community) were the dominant bacterial species in the families *Eggerthellaceae*, *Desulfovibrionaceae*, *Anaeroplasmataceae*, and *Enterobacteriaceae*. In the pesticide-treated samples, the richness of *Enterorhabdus* significantly increased ($0.001 < p < 0.01$ in carbofuran; $0.01 < p < 0.05$ in cyhalothrin) at the expense of *Desulfovibrio*; additionally, the relative abundance of *Anaeroplasma* decreased in carbofuran-treated samples and that of *Escherichia-Shigella* markedly increased in cyhalothrin-treated samples. *Enterorhabdus* as well as *Desulfovibrio* appeared to be highly correlated to the bile acid profiles in both pesticide-treated samples. Further in **Figure 4.6C**, a linear regression model was utilized to reflect the correlation of these genera with the two crucial bile acid profile parameters including the % conjugated BAs and the ratio secondary/ primary BAs (**Figure 4.3D**) and the R square values were calculated. As shown, the genera *Enterorhabdus* was found to have a significant and strong correlation with the fecal ratio secondary/ primary BAs ($R^2=0.7$).

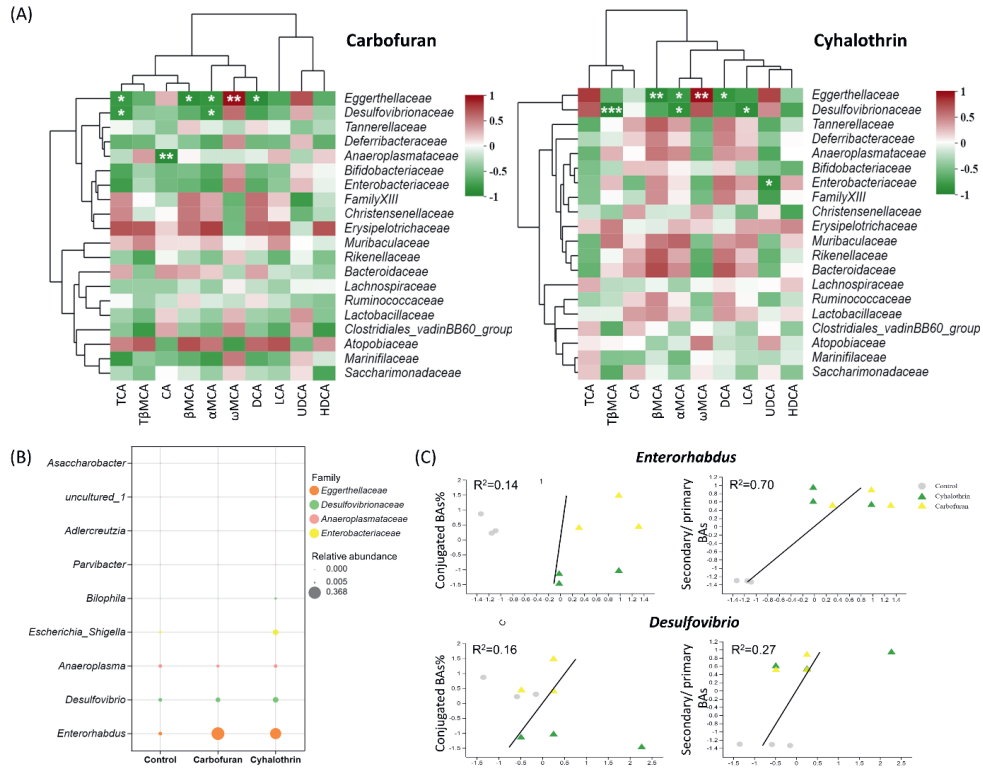

Figure 4.6 Correlations between the alterations within the bacterial community (family level) and the changes in bile acid profiles in carbofuran- and cyhalothrin- treated samples as reflected by (A) hierarchic clustering indicating statistical significance of the correlations found, (B) a bubble diagram presenting the changed richness of genera at the family level that showed high correlation with bile acid profiles and (C) linear regression plots of the correlations of the two mostly correlated genera shown in (B) with the bile acid profiles.

4.4 Discussion

The gut microbiota, in addition to their ability to process dietary derived material, are also capable of performing a range of bio-transformations on xenobiotics such as drugs and their metabolites; on the other hand, the gut microbiota can be affected by these compounds as well. In recent years studies on effects on the gut microbiota by xenobiotics, including for example antibiotics (Lange et al., 2016), antidiabetic drugs (Montandon and Jornayvaz, 2017) or some environmental pollutants including pesticides (Jin et al., 2017) have been reported. Particularly, some of the widely used pesticides were found to be able to alter the microbiota composition, which was suggested to lead to colon diseases and liver failure (Jin et al., 2017). However, very few studies have been published on the

consequences of pesticide exposure of the gut microbiota for the related metabolism and health. In the present study, using an in vitro anaerobic incubation model, we revealed, for the first time, that the exposure of the gut microbiota to selected carbamates and pyrethroids could modulate fecal bile acid profiles and additionally, it was shown that effects of carbofuran and cyhalothrin on the microbial community correlated with the alterations in bile acid profiles.

Results obtained for the altered bile acid profiles induced by the treatment of carbamates and pyrethroids (**Figure 4.3**) together with the bile acid transformation routes shown in **Figure 4.4** revealed that the 6β-epimerization of βMCA to ωMCA was significantly increased. In **Figure 4.6**, *Eggerthellaceae* simultaneously showed its negative correlation with βMCA and positive link to ωMCA; as a result, the raised richness of *Eggerthellaceae* could be correlated to the faster 6β-epimerization. Additionally, another epimerized metabolite of βMCA, αMCA was also reduced in its richness, which could originate from the decrease in βMCA and was also related to the increased abundance of *Eggerthellaceae*. In addition to the bile acid originating from the added TβMCA (**Figure 4.4**), the ones originating from TCA were also altered albeit to a less significant extent than the ones originating from TβMCA. For example, reduced levels of DCA were observed in both carbofuran and cyhalothrin treated samples, which could also be associated with raised richness of *Eggerthellaceae*. *Eggerthellaceae* belongs to the phyla *Actinobacteria* which has been previously reported for its beneficial effects on the degradation of toxic chemicals such as pesticides (Shivlata and Satyanarayana, 2017); meanwhile, *Eggerthellaceae* as an important member of *Actinobacteria* has also been found for its specific function on steroid metabolism (Hylemon et al., 2018). At the genus level, the dominant member of *Eggerthellaceae*, *Enterorhabdus* showed a significantly high correlation with the fecal bile acid profiles (**Figure 4.6B** and **C**) especially with the conversion of βMCA to ωMCA, thereby leading to an increased ratio of secondary/ primary bile acids. To date, few scientific research on *Enterorhabdus* has been published, however, it could be a biomarker for potential effects caused by exposure to xenobiotics. Some studies reported a significantly increased abundance of *Enterorhabdus* in case of rat small intestinal cell line IEC-18 cell damage (Chen et al., 2020), altered type of diet (Pagliai et al., 2020), and treatment with xylooligosaccharide (Li et al., 2015) in a mouse model. In the cyhalothrin group, the increased richness of *Desulfovibrionaceae* might have significantly raised the deconjugation of TβMCA leading to the obvious decrease of the %

conjugated BAs at 24 h of incubation. Although *Desulfovibrionaceae* only makes up a small proportion in the mouse gut microbial community, it has been found to be related to the bile acid metabolism (Hu et al., 2022). Meanwhile, for the genera *Desulfovibrio* dominating in *Desulfovibrionaceae* its raised abundance has been associated by others with gallstone disease and colorectal cancer in human (Ajouz et al., 2014; Ou et al., 2012).

On the other hand, not only the gut microbiota act on the bile acids, the altered bile acid profiles could also modulate the microbial community. For example, the conjugated bile acids were previously found to exhibit antibacterial activity (Jones et al., 2008; Inagaki et al., 2006); as a result, the observed reduced bacterial diversity in the carbofuran group at 24 h (**Figure 4.5B**) was shown to be related to the slightly increased proportion of conjugated bile acids (**Figure 4.3D**). Additionally, it has been reported that the ratio of secondary/ primary bile acids can be positively correlated to certain commensal genera (Kakiyama et al., 2013), hence the raised richness of *Enterorhabdus* may be ascribed to the increased ratio of secondary/ primary bile acids induced by the exposure to pesticides as well.

Collectively it can be concluded that, using the in vitro fermentation model it was shown that the exposure to pesticides significantly altered the gut microbial community resulting in changes of related bile acid profiles. A large amount of work has reported on the crucial role of bile acid signalling for host health (Perino et al., 2021); however to our knowledge, there is only one published study which revealed changes of gut microbiota-related bile acid profiles upon the exposure to pesticides. This study reported that the exposure to organochlorine pesticides induced an enhanced abundance of the genera *Lactobacillus* with BSH activity leading to altered bile acid metabolism. Thus, the present study provided a new proof for the effects of pesticides on gut microbiota and related bile acid metabolism using an in vitro model. To what extent the in vitro elucidated changes in the microbiome pattern and bile acid homeostasis are to be expected in the in vivo situation remains of interest for future studies.

4.5 Conclusion

In the current study, effects on gut microbiota and related bile acid metabolism caused by the exposure of mice fecal microbiota to carbamate and pyrethroids pesticides including carbofuran, aldicarb, cypermethrin and cyhalothrin were evaluated using an optimized in vitro fermentation model system with mouse feces. Results obtained revealed a

significant enrichment of secondary bile acids especially ωMCA with an accompanying decrease of the primary bile acid βMCA, indicating that the exposure of the microbiota to carbamate and pyrethroids could induce a faster transformation from primary bile acids to secondary bile acids. Meanwhile, an increased abundance of the bacterial family *Eggerthellaceae,* dominant in the phyla *Actinobacteria*, was observed in carbofuran and cyhalothrin treated samples; additionally, *Enterorhabdus* belonging to the *Eggerthellaceae* was found to be highly correlated to the fecal bile acid profiles. Our findings contribute to the issue on studying the altered gut microbial community and its related metabolism induced by pesticides, and point at potential effects on the host–microbial relationship upon pesticide exposure. The results also illustrate that the gut microbiota and its metabolism could be a novel target to consider in future pesticide safety evaluations.

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5

CHAPTER 5

Effects of lambda-cyhalothrin on gut microbiota and related bile acid metabolism in mice

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Abstract

Since the gut microbiota plays a crucial role in host metabolism and homeostasis, its alterations induced by xenobiotics, such as pesticides, could pose a risk to host health. Our previous in vitro fermentation study showed that pyrethroid pesticides could affect the mouse bacterial community and related bile acid profiles. Hence in the present study, the effects of the selected pyrethroid lambda-cyhalothrin on the intestinal microbial community and its related bile acid metabolism were evaluated in male and female mice. Results showed that the total amount of bile acids in plasma and fecal samples from lambda-cyhalothrin treated mice markedly increased compared to controls, which could be mainly ascribed to the significantly raised proportions of taurine conjugated bile acids in plasma, and the increase in fecal secondary bile acids. In gut microbial profiles, a significantly increased richness of *Prevotellacea* and a depletion of *Lachnospiraceae* were found at the family level upon the treatment with lambda-cyhalothrin. In conclusion, treatment of mice with lambda-cyhalothrin affected the gut microbiota with accompanying changes in bile acid homeostasis. The effects on fecal bile acid profiles were in line with those previously observed in our in vitro study, and corroborate that pyrethroid pesticides could affect gut microbiota and related bile acid profiles.

Keywords: bile acid metabolism; gut microbiota; pyrethroid pesticides; lambda-cyhalothrin

Abbreviations:

Bile acids: cholic acid (CA), taurocholic acid (TCA), glycocholic acid (GCA), chenodeoxycholic acid (CDCA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA), deoxycholic acid (DCA), taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), lithocholic acid (LCA), taurolithocholic acid (TLCA), glycolithocholic acid (GLCA), ursodeoxycholic acid (UDCA), tauroursodeoxycholic acid (TUDCA), glycoursodeoxycholic acid (GUDCA), hyodeoxycholic acid (HDCA), taurohyodeoxycholic acid (THDCA), glycohyodeoxycholic acid (GHDCA), α-muricholate (αMCA), tauro-α-muricholate (TαMCA), β-muricholate (βMCA), tauro-β-muricholate (TβMCA), ω-muricholate (ωMCA), tauro-ω-muricholate (TωMCA)

Others: lambda-cyhalothrin (LCT); bile salt hydrolase (BSH), dimethyl sulfoxide (DMSO), electrospray liquid chromatography mass spectrometry (LCMS), electrospray ionization (ESI), operational taxonomic units (OTUs), apical sodium-dependent bile acid transporter (ASBT), organic solute transporter subunit α/β (OSTα/β), multidrug resistance-associated protein (MRP), sodium taurocholate cotransporting polypeptide (NTCP), organic anion-transporting polypeptide 1 (OATP1)

5.1 Introduction

Bile acids, which are synthesized from cholesterol in hepatocytes, secreted into bile, stored in the gallbladder of human, and released into the intestine via the bile duct, are essential for intestinal solubilization, absorption, and metabolism of triglycerides, cholesterol, and fat-soluble vitamins (Fiorucci & Distrutti, 2015; Jia et al, 2018). Bile acids produced in the liver are referred to as primary bile acids, and may differ between species, with cholic acid (CA) as well as ursodeoxycholic acid (CDCA) being produced in humans, and CA, CDCA, α-muricholate (αMCA) as well as β-muricholate (βMCA) in rodents (Sayin et al., 2013). Further, these primary bile acids are conjugated to either mainly glycine in humans or taurine in rodents (Liu et al, 2015).

Upon release into the intestine, these conjugated bile acids are deconjugated by bacterial enzymes providing bile salt hydrolase (BSH) activity, and further metabolized to secondary bile acids by dehydroxylation, hydroxylation, or epimerization (Martin et al., 2007), resulting in formation of for example DCA and LCA by dehydroxylation of respectively CA and CDCA in humans or of ωMCA by 6β-epimerization of βMCA in rodents (Thomas et al, 2008). Despite trillions of bacteria in the gut, only a few species have been reported to have the capacity for transforming bile acids, such as *Clostridium* and *Bacteroides* which are able to support deconjugation by BSH as well as epimerization (Jia et al, 2018). Given this capacity of the gut microbiota for bile acid metabolism, the composition of the gut microbiota is a critical determinant in the ultimate bile acid profiles and related health aspects in the host (Fiorucci and Distrutti, 2015). It also implies that bile acid profiles may be disrupted by factors that affect the intestinal environment and thereby the gut microbiota (De Vadder et al., 2014).

Pyrethroids are extensively used pesticides which have been applied in agricultural and home formulations for three decades and account for approximately one-fourth of the worldwide insecticide market (Casida and Quistad, 1998). Pyrethroids can be classified into two large groups depending on their chemical structures without or with a cyano moiety (type I or type II), with cyhalothrin (**Figure 5.1**) being a classic type II pyrethroid. Cyhalothrin is a racemic mixture consisting of two pairs of enantiomeric isomers (**Figure 5.1**) with pair B being lambda-cyhalothrin (Birolli et al., 2018), which is the active component of cyhalothrin and commonly used. The Joint Meeting on Pesticides Residues has set the ARfD of 0.02 mg /kg bw for lambda-cyhalothrin (JMPR 2018). The adverse

health effects caused by these type II pyrethroids include neurotoxicity resulting in hypersensitivity, profuse salivation, choreoathetosis, tremor and paralysis (Vijverberg and vanden Bercken, 1990).

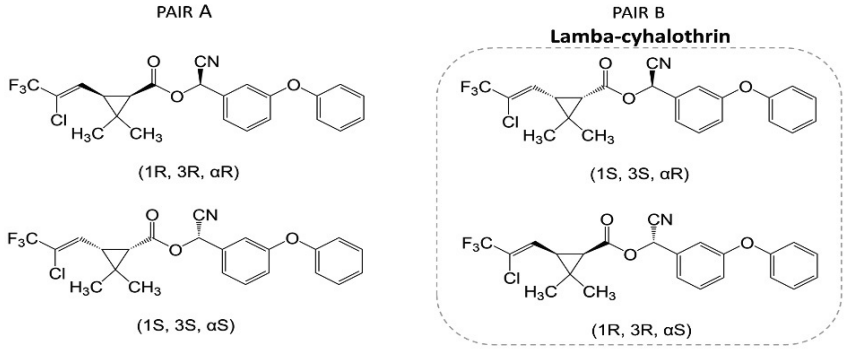

Figure 5.1 Chemical structure of the cyhalothrin stereoisomers including lambda-cyhalothrin (pair B).

The potential toxicity of pesticides towards the intestinal microbial community only started to gain attention in recent years (Giambò et al, 2021). Thus, studies published so far on gut microbiota toxicity of pyrethroids are limited. A study of Nasuti et al. (Nasuti et al., 2016) reported a decrease in the richness of the phyla of *Bacteroidota* in rat gut upon the administration of permethrin (a type I pyrethroid). Recently, another study reported that the exposure of small yellow croaker embryos to lambda-cyhalothrin markedly altered eight common genera of their gut microbial community, accompanied by significantly increased levels of the bile acids taurodeoxycholic acid (TDCA) and tauroursodeoxycholic acid (TUDCA) in water samples from their living surroundings (Zhan et al., 2022).

Moreover, in our previous work which utilized an optimized batch fermentation model system using mouse feces, effects of the pyrethroids cypermethrin and cyhalothrin on gut microbiota-mediated bile acid profiles were assessed. In that study all pesticides appeared to affect the fecal gut microbiota and related bile acid metabolism, with the exposure to cyhalothrin resulting in the most significant alteration of fecal bile acid profiles. The exposure of cyhalothrin caused a significant increase in fecal secondary bile acids and an accompanying decrease in fecal primary bile acids, resulting in a raised ratio of βMCA to ωMCA. Results obtained from that in vitro fermentation batch model indicated that pesticides may affect intestinal microbiota and the related bile aid profiles. Hence the

aim of the present study was to determine whether also in vivo such effects would occur. To that end C57BL/6 mice were exposed to lambda-cyhalothrin at two selected levels for a 28-day oral dose toxicity study to evaluate its influences on the intestinal microbial community, as well as on fecal and plasma bile acid profiles.

5.2 Materials and methods

5.2.1 Chemicals

Lambda-cyhalothrin was purchased from Sigma-Aldrich (St. Louis, MO, USA; CAS: 91465-08-6). Bile acid standards were supplied by Merck KGaA (Darmstadt, Germany), or Cambridge Isotope Laboratories (Tewksbury, USA). Formic acid and HPLC-grade acetonitrile were purchased from Merck (Darmstadt, Germany). Ultrapure water was acquired from a Milli-Q water system (Millipore, Billerica, MA, USA).

5.2.2 Animals and exposure

Eight-week-old male and female C57BL/6 mice were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal studies were performed in accordance with the Regulations for Care and Use of Laboratory Animals and Guideline for Ethical Review of Animals (China, GB/T 35892-2018) and the overall project protocols were approved by the Animal Ethics Committee of Beijing Institute of Technology. The accreditation number is SCXK-BIT-20210715010 promulgated by the Animal Ethics Committee of Beijing Institute of Technology. All animals were submitted to controlled temperature conditions (22–26 °C), humidity (50–60%), and light (12 h light/ 12 h dark) with the access to water and food ad libitum.

Dose levels equal to 1/20 and 1/10 of the reported LD50 of 19.9 mg/kg bw in mice (JMPR, 2007) for lambda-cyhalothrin were selected as the low- and high- oral doses for lambda-cyhalothrin respectively, resulting in dose levels of 1 and 2 mg/kg bw/day. Lambda-cyhalothrin was dissolved in dimethyl sulfoxide (DMSO) and further diluted in ultra-pure water to final concentrations of 1 and 2 mg/mL for dosing female mice, and 1.1 and 2.2 mg/mL for dosing males. Mice were divided over the different study groups based on similar weight distribution over the groups. The low dose of 1 mg/kg bw/day (named LCT group), the high dose of 2 mg/kg bw/day (named 2LCT group) or vehicle (control group) were administered by oral gavage (0.02 mL prepared lambda-cyhalothrin solution) once a day for 28 days (n=5 mice per sex per group).

5.2.3 Clinical examinations

The mice were observed and checked daily for clinically abnormal signs and mortalities. On study day 6, 13 and 27, body weight as well as food consumption were determined before the daily oral gavage. At the end of the 28 day treatment, all mice were sacrificed by decapitation under isoflurane anesthesia.

5.2.4 Sampling of blood and feces for bile acid profiling

On study day 7, 14 and 28 (morning 8:30 to 10:30 h), blood samples were taken from the tail vein of all mice (1.0 mL K-EDTA blood). The collected blood samples were centrifuged (12,000 ×g, 5 min, 4 °C), and the separated EDTA plasma samples (upper layers) were collected. Subsequently, 10 µL of each EDTA plasma sample was extracted with 400 µL acetonitrile, vortexed, and centrifuged (12,000 ×g, 5 min, 4 °C). Supernatants were collected in Eppendorf tubes, covered with nitrogen and frozen at −80 °C until bile acid analysis was performed.

On study day 28, after a fasting period of 16–20 h and one day after the last administration of lambda-cyhalothrin, feces were carefully removed during necropsy from the rectum of male and female mice. Fecal samples collected from individual mice were weighted, crushed, and extracted with acetonitrile (2 mL/ 1 mg feces) in an ultrasonic bath for 10 min following a 5 min centrifugation (21,5000 ×g, 4 °C). The supernatants were removed and stored at −80 °C until bile acid analysis was performed.

5.2.5 Bile acid measurement in plasma and feces

Bile acid measurement was performed on a Nexera XR LC-20AD SR UPLC system coupled to a triple quadrupole liquid chromatography mass spectrometry (LCMS) 8050 mass spectrometer (Kyoto, Japan) with electrospray ionization (ESI) interface, which was able to measure the following 24 bile acids: 15 conjugated bile acids including T/G-CA, T/G-CDCA, T/G-DCA, T/G-LCA, T/G-UDCA, T/G-HDCA, TαMCA, TβMCA, and TωMCA; 4 primary bile acids including CA, CDCA, αMCA, βMCA; 5 secondary bile acids including ωMCA, DCA, LCA, UDCA, and HDCA. Prior to analysis all the extracts of plasma and fecal samples were shaken briefly, centrifuged (21,5000 ×g, 4 °C), and filtered through a 0.2 µm syringe filter (MILLEX, Merck Millipore, Cork, Ireland).

Bile acids in all samples were separated on a Kinetex C18 column (1.7µm×100 A×50 mm×2.1mm; Phenomenex, USA) using an ultra-high performance liquid chromatography

(UHPLC) system (Shimadzu) with mobile phases consisting of 0.01% formic acid in distilled water (solvent A), a mixture of methanol and acetonitrile (v/v=1/1)(solvent B), and acetonitrile containing 0.1% formic acid (solvent C). The mass spectrometer (MS) used ESI in negative ion mode, and the parameters of ESI^- as well as the gradient profile are summarized in **Table S5.1**. Selective ion monitoring (SIM) and multiple reaction monitoring (MRM) were simultaneously employed for detection of the bile acids. The optimized precursor and product ions used for the detection of the bile acids are shown in **Table S5.1** as well. The Postrun and Browser Analysis function from the LabSolutions software (Shimadzu, Kyoto, Japan) was employed to obtain the peak areas of the extracted ion chromatogram (EIC) for each target.

5.2.6 Bacterial profiling of gut microbiome

Microbial DNA was extracted from fecal samples of C57BL/6 mice using the QIAamp DNA Fecal Microkit (Stras Qiagen GMBH, Germany). A NanoDrop 2000 spectrophotometer (Thermoelectric Science, Massachusetts, USA) and 1% agarose gel electrophoresis (AGE) was applied to measure the total DNA mass. Employing the extracted DNA as a template, an upstream primer 338F (5′-ACTCCTACGGGAGGCAGCAGCAG-3′) and a downstream primer 806R (5′ -GGACTACHVGGGTWTCTAAT-3′) with Barcode sequence were used to amplify the V3-V4 variable region of 16S rRNA genes (95 °C reaction for 3 min, one cycle; denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s, 27 cycles; stably extended at 72 °C for 10 min, ending at 10 °C). The recovered products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and detected by 2% AGE. The recovered products were detected and quantified using a Quantus™ Fluorometer (Promega, USA).

Purified amplicons were pooled in equimolar amounts and paired end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The data were then optimized by noise reduction processing to obtain ASV (Amplicon Sequence Variant) data representing the sequence and abundance information. The taxonomic identifications of 16S rRNA sequences were assigned to each representative sequence based on the silva138/16s_bacteria database with a confidence threshold of 70% using Naive bayes classifier in Qiime2.

5.2.7 Data analysis

GraphPad Prism 9.3.1. was adopted to analyze the data, and the continuous data conforming to the normal distribution were described using mean and standard deviation. Differences between the intervention groups and the control group were analyzed by one-way ANOVA. A multivariate statistical analysis was performed using ROPLS in R package from Bioconductor on Majorbio Cloud Platform (https://cloud.majorbio.com). The Alpha diversity indices including observed species and the Shannon index were calculated with Mothur v1.30.1 (Schloss et al., 2009).

5.3 Results

5.3.1 Clinical signs

No mortalities were found in any of the control or lambda-cyhalothrin exposed groups during the 28 day treatment period, and no abnormal symptoms were registered in the first two weeks. From the third week onwards, the activity of all female mice in the treated groups was slightly reduced, and especially females of the high-dose group (2LCT) showed temporary clinical signs of semi closed eyelids (2 animals). In addition, aggressive performance was observed for the treated male mice in both exposed groups in the second half of the experiments (week 3 and 4). In the fourth week, male mice of the 2LCT group (one cage) started to bite and fight with their group mates, leading to serious injury on the back of one animal.

Body weight and food consumption data are shown in **Table 5.1**. Body weight and food consumption of treated animals showed a decrease compared to the controls during the experimental period, although the decrease in food consumption did not reach statistical significance except for the last determination for the female 2LCT group. The reduction of mouse body weight was most pronounced in females, with the most significant reduction in body weight presented at the second determination (day 13). Collectively, the administration of lambda-cyhalothrin markedly reduced the body weight of studied animals in a dose dependent way.

Table 5.1 Body weight and food consumption of male and female C57BL/6 mice (n=5 per sex per group) dosed for 28 days with lambda-cyhalothrin or vehicle control. Data were collected on study days 6, 13, and 27 and values for exposed mice are compared to control (*p <0.05, **p <0.01, ***p <0.001)

Group	Day	Body weight				Food consumption			
		Male		Female		Male		Female	
		Acute values (gram)	vs. controls	Acute values (gram)	vs. controls	Average (gram/day)	vs. controls	Average (gram/day)	vs. controls
Control	6	23.82±0.81		20.32±0.51		2.73		2.79	
	13	26.08±0.70		21.84±0.25		3.32		3.17	
	27	27.92±1.09		23.00±0.65		3.89		3.94	
LCT	6	22.36±0.72	↓	19.44±0.83	↓	2.66	↓	2.53	↓
	13	23.96±0.71	↓*	20.10±0.85	↓*	3.23	↓	2.89	↓
	27	26.24±1.55	↓	21.70±0.72	↓	3.82	↓	3.49	↓
2LCT	6	21.74±1.08	↓*	18.58±1.16	↓*	2.64	↓	2.39	↓
	13	23.92±1.13	↓*	19.38±1.44	↓***	3.17	↓	2.77	↓
	27	26.18±1.57	↓	21.38±0.73	↓*	3.66	↓	3.31	↓*

5.3.2 Bile acid profiles in plasma

Figure 5.2 shows the results obtained for the bile acid profiles in plasma samples of control and lambda-cyhalothrin treated male and female mice. Of the twenty-four bile acids that could be detected by the method applied, in plasma samples only ten bile acids were present at levels that enabled their quantification. This included the conjugated bile acids TMCA (sum of TαMCA+TβMCA+TωMCA), TCA, TCDCA, THDCA, and TUDCA, the primary bile acids CA, αMCA, βMCA, and CDCA, as well as one secondary bile acid being DCA. Glycine-conjugated bile acids and other secondary bile acids, were not detected or detected below the limitation of quantification (0.01 µM).

Figure 5.2A shows in a heatmap the time-dependent mean concentrations of the bile acids detected in plasma samples from male and female mice of all groups on days of 7, 14, and 28. The most significant changes were detected in taurine conjugated bile acids that showed significant increases in a dose dependent manner in all plasma samples of treated mice compared to controls. Changes in the concentrations of primary and secondary bile acids were limited to significant decreases in βMCA in male and female mice exposed to lambda-cyhalothrin, and in CDCA for exposed female mice. The effects observed were dose and also time dependent with the largest changes detected in the 2LCT groups at the last experimental day (day 28). In addition, some substantial gender dependent differences were observed (**Fig. 5.2B**) especially for i) THDCA which increased more in male than female mice exposed to lambda cyhalothrin, ii) βMCA and CDCA which were present at lower levels and often below the limit of quantification in all male as

compared to female plasma samples and iii) DCA which was present at substantially higher concentrations in plasma samples of all female as compared to male animals. Collectively, significant dose dependent increases in all detected conjugated bile acids were observed in plasma samples from male and female lambda-cyhalothrin treated mice compared to controls (**Figure 5.2A** and **B**). Of the primary and secondary bile acids, dose dependent decreases were observed for βMCA and CDCA whereas for CA, αMCA and DCA there were no differences between the control and lambda-cyhalothrin exposed groups (**Figure 5.2A** and **B**). **Figure 5.2C** summarizes the bile acid profiles for the different groups at day 28 focusing on the total amount of bile acids as well as the total amount of taurine conjugated, primary, and secondary bile acids in the plasma of the mice. From this analysis it follows that there is a dose dependent increase in the plasma concentration of the total bile acids which can be mainly ascribed to a dose dependent increase in the amount of taurine conjugated bile acids.

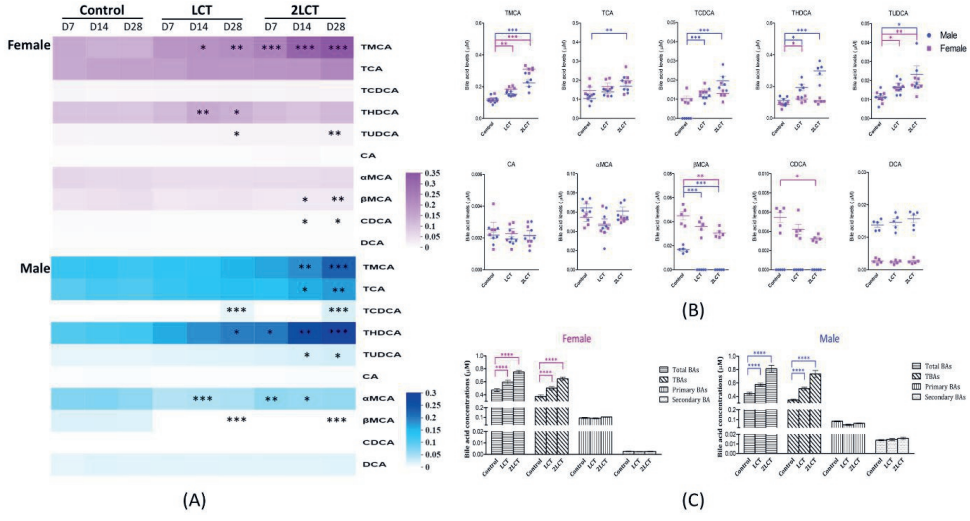

Figure 5.2 Bile acid profiles in plasma samples from male and female mice in untreated and lambda-cyhalothrin treated groups, shown as (A) a heatmap presenting mean concentrations of the detected bile acids in plasma samples from studied mice on days 7, 14, and 28 with shades of pink (female) and blue (male) indicating the concentrations; (B) the concentrations of individual bile acids detected in plasma samples on day 28, and (C) the total amount of bile acids (total BAs) in plasma samples at day 28 including also the total concentration of taurine conjugated bile acids (TBAs), primary bile acids (primary BAs), and secondary bile acids (secondary BAs)(n=5; *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001). The low and high dose groups are presented by LCT and 2LCT, respectively.

5.3.3 Bile acid profiles in feces

In addition to plasma bile acid concentrations, bile acid profiles were also quantified in fecal samples. **Figure 5.3** shows the results obtained. Fourteen bile acids including six taurine conjugated bile acids including TCA, TMCA (sum of TαMCA+TβMCA+TωMCA), TDCA, THDCA, TLCA, and TUDCA, three primary bile acids including CA, αMCA, and βMCA, and five secondary bile acids including DCA, LCA, UDCA, ωMCA, and HDCA could be quantified in the fecal samples from male and female mice of lambda-cyhalothrin treated and control groups collected on day 28.

The levels of fecal taurine conjugated bile acids and fecal unconjugated bile acids in samples from the studied male and female mice are presented in **Figure 5.3A** and **B**. For taurine conjugated bile acids (**Figure 5.3A**), significant dose dependent decreases were observed in TMCA and TCA in lambda-cyhalothrin treated male and female animals, whereas levels of THDCA showed substantial gender dependent differences with a significant decrease in feces of treated male mice and an increase in fecal samples of treated females, especially in the 2LCT group. For unconjugated bile acids (**Figure 5.3B**), significant dose dependent alterations were detected in lambda-cyhalothrin treated animals, with a marked decrease of the primary bile acid βMCA and an accompanying increase of the secondary bile acids ωMCA and HDCA. **Figure 5.3C** summarizes the fecal bile acid profiles of lambda-cyhalothrin treated animals and controls at day 28 based on the amount of total, taurine conjugated, primary and secondary bile acids. A significant dose dependent increase in the level of fecal secondary bile acids at the expense of fecal taurine conjugated and primary bile acids was observed, leading to a markedly increased size of the fecal bile acid pool. It has been reported that the majority of secondary bile acids in mice is composed of ωMCA and DCA that are formed by respectively 6β-epimerization of βMCA and 7α-hydroxylation of CA (Sayin et al., 2013). The ratio of βMCA over ωMCA and CA over DCA, as well as that of the total fecal primary bile acids over fecal secondary bile acids, together with another important ratio being the ratio between total fecal unconjugated bile acids and fecal conjugated bile acids (Sayin et al., 2013) are presented in **Figure 5.3D**. A markedly higher conversion rate from primary bile acids to secondary bile acids was observed in fecal samples from lambda-cyhalothrin treated animals, which could be ascribed to the significantly faster 6β-epimerization of βMCA to ωMCA in these treated animals than in control mice. The rate of deconjugation was also

obviously raised in the treated animals, whereas the transformation from CA to DCA did not show differences between lambda-cyhalothrin treated animals and controls.

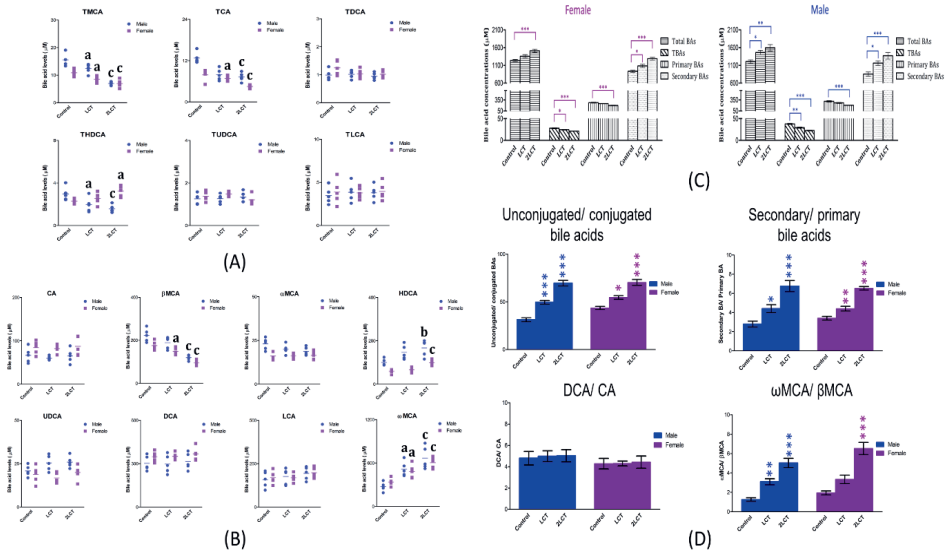

Figure 5.3 Bile acid profiles in fecal samples from male and female mice in untreated and lambda-cyhalothrin treated groups on day 28 as characterized by (A) concentrations of fecal taurine conjugated bile acids; (B) fecal unconjugated bile acids; (C) the total amount of fecal bile acids (total BAs), fecal taurine conjugated bile acids (TBAs), fecal primary bile acids (primary BAs), and fecal secondary bile acids (secondary BAs); and (D) the ratio of unconjugated to conjugated bile acids (unconjugated/ conjugated bile acids), the ratio of primary to secondary bile acids (secondary/ primary bile acids), the ratio of DCA to CA (DCA/ CA), and the ratio of ωMCA to βMCA (ωMCA/ βMCA). Data for male (blue) and female (pink) mice fecal samples are presented and significance of the differences in treated animals compared to controls is also indicated (a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$ in A and B; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ in C and D). The low and high dose groups are presented by LCT and 2LCT, respectively.

5.3.4 Bacterial profiling

Figure 5.4 describes the bacterial profiling of the fecal samples from lambda-cyhalothrin exposed mice and controls at the phylum and family level, as well as an analysis of alpha diversity performed based on the determined operational taxonomic units (OTUs). The results obtained reveal that the phyla of *Bacteroidota* and *Firmicutes* were dominant in fecal samples of the studied mice and no significant changes in the microbial community at phylum level were observed in lambda-cyhalothrin treated mice compared to controls.

Also the alpha diversity indicated by the index of total species observed (sobs) as well as the shannon diversity showed that no significant changes were found in the total amount or richness of the microbial community after the administration of lambda-cyhalothrin (**Figure 5.4B**). At the family level, the proportions of the main twenty-one bacterial families in the fecal samples from male and female mice are shown in **Figure S5.1**, and following from these results **Figure 5.4C** presents the proportions of the ten most common families of gut microbial species in male and female mice. As shown in **Figure 5.4C**, a significant dose dependent increase in relative abundance of *Prevotellacea* (phyla of *Bacteroidota*) and a dose dependent decrease in relative abundance of *Lachnospiraceae* (phyla of *Firmicutes*) were observed in male mice treated with lambda-cyhalothrin which were significant compared to control at the high dose level (2LCT group). In contrast, no significant bacterial changes at family level were observed in the lambda-cyhalothrin treated females.

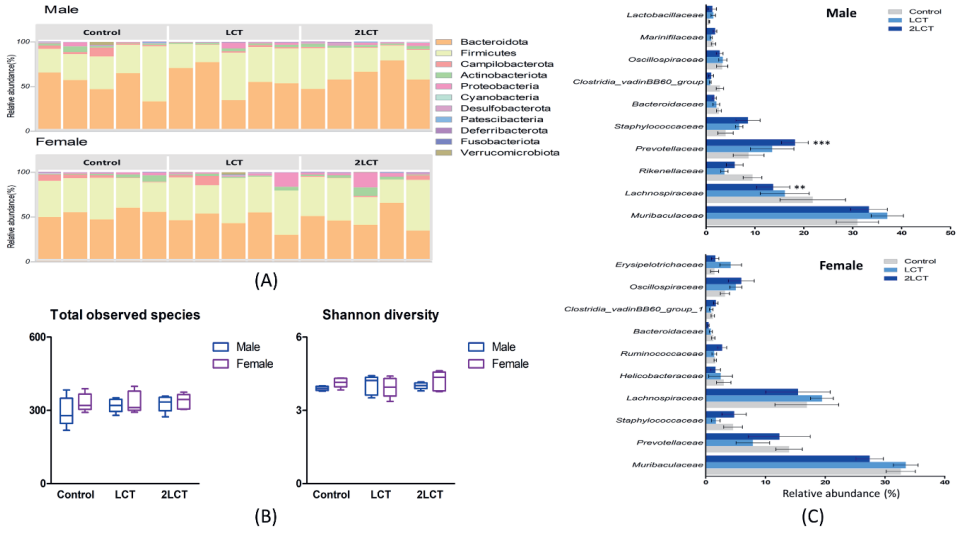

Figure 5.4 Gut bacterial profiles of fecal samples from untreated (control) and lambda-cyhalothrin treated male and female mice on day 28. LCT and 2LCT are the low and high dose groups. (A) shows the relative abundance of the gut microbial community in mice fecal samples at the phylum level (n=5 per sex per group), and (C) at the family level, presenting only the ten dominant families. (B) shows the alpha diversity by values of total observed species and shannon diversity (*p <0.05, **p <0.01, ***p <0.001).

5.3.5 Correlations between lambda-cyhalothrin induced changes in microbial and bile acid profiles

The changes in the gut microbial community upon exposure to lambda-cyhalothrin were also analyzed at genus level, and relationships between the changes in the bacterial and bile acid profile were analysed by a correlation analysis. Results obtained are shown in **Figure 5.5**. The bacterial species which were substantially different upon treatments were selected for this correlation analysis and are summarized in **Figure 5.5A**. The relative abundance of the genera named *Oscillospiraceae NK4A214 group* (class, Clostridia; phylum, Firmicutes) significantly increased in fecal samples from lambda-cyhalothrin treated male mice of the LCT and 2LCT groups, followed by the genera *Brevibacterium* and *Brachybacterium* that were increased albeit not significant ($p > 0.05$). In treated females, the relative abundance of the genera *Brachybacterium*and and *Brevibacterium* belonging to the phyla of *Actinobacteriota* was also increased, with statistical significance ($p < 0.05$) obtained for *Brachybacterium* in the female 2LCT group. **Figure 5.5B** presents the correlations and the statistical significance between the fifty dominant genera in mouse feces and the significantly altered fecal bile acid related endpoints including the ratios of total unconjugated bile acids and conjugated bile acids, total secondary and primary bile acids, as well as ωMCA and βMCA, and the significantly increased levels of HDCA (see **Figure 5.3**). In the heatmap presentation (**Figure 5.5B**), it can be seen that the genera of *Brachybacterium* and *Brevibacterium* showed a positive correlation with the ratio of unconjugated bile acids to conjugated bile acids ($p < 0.01$), revealing their relationship with bile acid deconjugation. Meanwhile, *Brachybacterium* and *Brevibacterium* were shown to be positively associated with the ratio of secondary and primary bile acids ($p < 0.05$) as well as the ratio between the secondary bile acid ωMCA and the primary bile acid βMCA ($p < 0.001$). The latter indicates the correlations of the raised richness of *Brachybacterium* and *Brevibacterium* with the markedly faster 6β-epimerization from βMCA to ωMCA leading to the significantly increased conversion of the total fecal primary bile acids to secondary bile acids. In contrast, the genera *Oscillospiraceae NK4A214 group* appeared to be only ones highly linked to the production of HDCA, the 7β-dehydroxylated metabolite of ωMCA (Sayin et al., 2013), as reflected by its significantly positive correlation with the 7β-hydroxylation of ωMCA. Besides, the genera *Prevotellceae UCG spp* (class, Bacteroidia; phylum, Bacteroidota) also showed a significant positive correlation with the deconjugation and the production of secondary bile acids, particularly ωMCA

produced from βMCA (p <0.01). Further, a linear regression model was utilized to study the correlation of the mouse fecal bile acid profiles with the dominant bacterial genera that showed significant changes in **Figure 5.5B**, and the analyzed correlations with R square values above 0.35 are summarized in **Figure 5.5C**. Results obtained corroborate the positive link of *Brachybacterium* as well as *Brevibacterium* with the 6β-epimerization of βMCA, and the correlation of the raised abundance of *Oscillospiraceae NK4A214 group* with the faster 7β-dehydroxylation of ωMCA in males. Moreover**,** the correlations between the concentrations of individual fecal bile acids and the relative abundance of fifty bacterial species are shown in **Figure S5.2**. In addition to the genera referred to above, *Bacteroides* (phylum Bacteroidota), *Staphylococcus* (phylum Firmicutes) and *Bacilli RF39 spp* (phylum Firmicutes) followed by *Rumminococcaceae spp*, *Oligella*, *Helicobacter* showed links to the transformation of individual fecal bile acids as well.

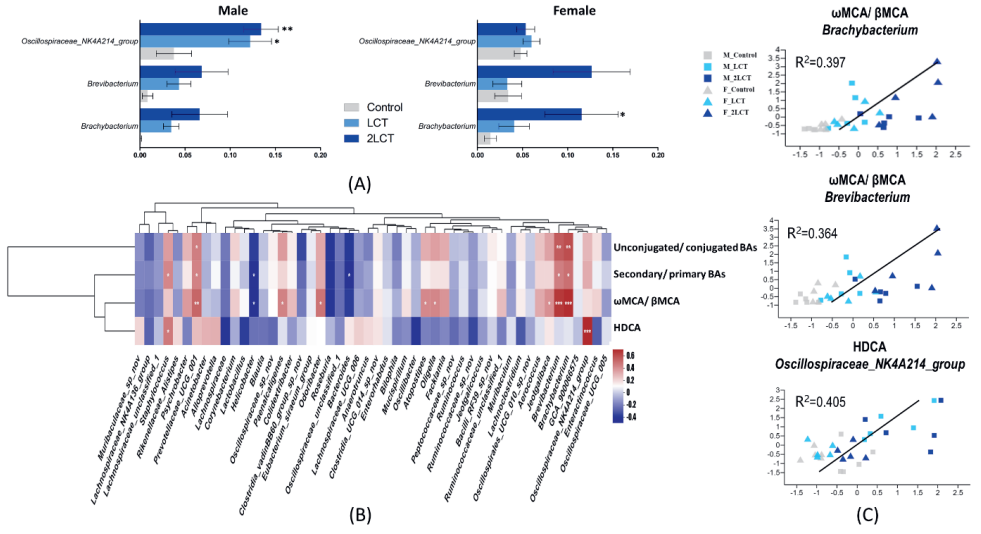

Figure 5.5 Gut bacterial genera in fecal samples of male and female control and lambda-cyhalothrin treated mice on day 28, as well as correlations between the bacterial and bile acid profiles. LCT and 2LCT are the low and high dose groups. (A) shows the significantly altered bacterial genera in lambda-cyhalothrin treated male and female mice of LCT (light blue), and 2LCT (blue) groups compared to controls (grey). (B) shows the heatmap presentation with pearson correlation coefficients of the main fifty genera in mouse fecal samples with the values of unconjugated/ conjugated bile acids, secondary/ primary bile acids, ωMCA/ βMCA, and the significant increased concentration of fecal HDCA. The positive and negative correlation are separately presented by colours of red and blue (*p <0.05, **p <0.01, ***p <0.001). The genera

significantly related to conversion of fecal bile acids (in B) were further analyzed using linear regression, and the correlations with R squares >0.3 between bacterial and bile acid profiles are shown in (C). The untreated and treated male (M) and female (F) mice of different groups were shown, namely, M_Control, M_LCT, M_2LCT, F_Control, F_LCT, and F_2LCT respectively (n=5).

5.3.6 Additional analysis of the impact of lambda-cyhalothrin on gut bacterial and bile acid profiles

Figure 5.6A and **B** separately show the results of a principal component analysis (PCA) for the bile acid profiles in respectively plasma and fecal samples from control and lambda-cyhalothrin treated mice. As shown in the two graphs, the bile acid profiles of untreated male and female mice cluster together and show a dose dependent separation from those of lambda-cyhalothrin treated mice, with the low-dose LCT group and high-dose 2LCT group clustering per group with a separation in between. The analysis also reveals the gender dependence of the effects as well as a more pronounced separation for the plasma than the fecal bile acid profiles. **Figure 5.6C** describes the effects of lambda-cyhalothrin on the gut microbial community based on data of OTUs in mice fecal samples, and shows no clear cluster of one gender or one group (treated or untreated) which is in line with the effects at phylum level and indicates the huge intra-species variations of bacterial profiles as compared to the detected lambda-cyhalothrin induced effects using data on OTUs. Moreover, **Figure 5.6D** describes the correlations between the bile acid profiles in plasma and fecal samples by groups of taurine conjugated, primary and secondary bile acids. The levels of taurine conjugated bile acids in plasma showed highly positive correlation with those of fecal secondary bile acids, and was negatively associated with the concentrations of fecal taurine conjugated and primary bile acids.

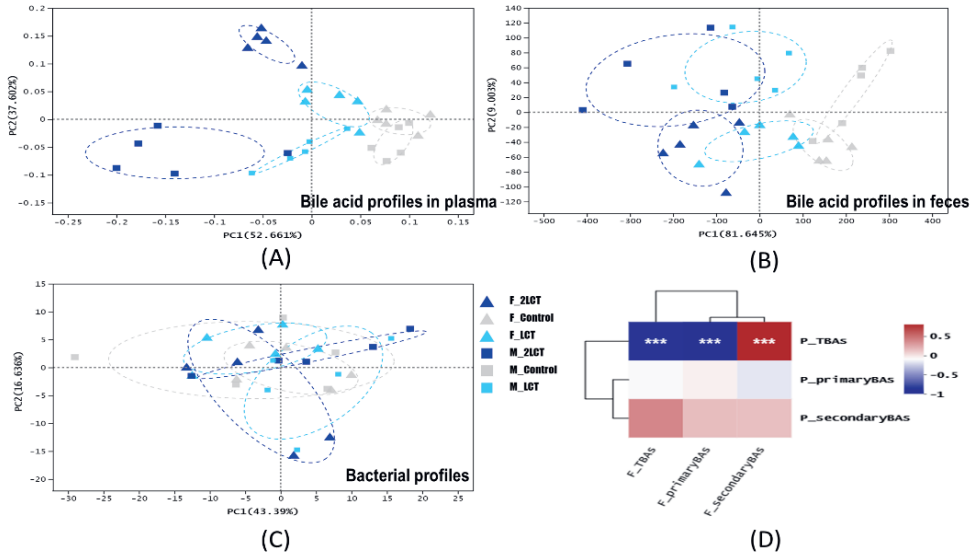

Figure 5.6 Principal component analysis (PCA) of bile acid and bacterial profiles, as well as the correlation between bile acid profiles in plasma and fecal samples. (A) and (B) show the PCA analyses for respectively the plasma and fecal bile acid profiles, and (C) shows the PCA analysis using OTUs for the fecal bacterial profiles. All plasma and fecal samples were obtained from control (grey) and lambda-cyhalothrin treated (LCT, low dose, light blue; 2LCT, high dose, blue) male (squares) and female (triangles) animals on day 28 (n=5 mice per group and sex). In (D), the correlation between bile acid profiles of plasma (P) and fecal (F) samples from all studied mice on day 28 was analyzed by pearson correlation coefficients, and is shown for groups of total taurine conjugated bile acids, total primary bile acids, and total secondary bile acids.

5.4 Discussion

In this study, the alterations in plasma and fecal bile acid profiles as well as in the gut microbial community induced by the exposure of male and female mice to lambda-cyhalothrin were characterized, showing significant lambda-cyhalothrin induced effects compared to controls (**Figure 5.6A**, **B**, and **C**).

A key finding of the current study is that the fecal bile acid profile was markedly altered upon the treatment of the mice with lambda-cyhalothrin, comprising not only a dose dependent increase in the amount of total and secondary bile acids and decreased levels of conjugated and primary bile acids (**Figure 5.3C**), but also dose-dependently raised ratios of unconjugated/ conjugated bile acids and secondary/ primary bile acids. Notably, the raised transformation from βMCA to ωMCA relating to an accelerated 6β-

epimerization (Thomas et al, 2008) was also found in our previous study with an optimized 24 h in vitro fermentation model in which mouse feces was incubated with cyhalothrin or another commonly used pyrethroid cypermethrin, leading also to an increased ratio of secondary/ primary bile acids (Zheng et al. submitted). In addition to changes in βMCA and ωMCA, the in vitro study also showed a significant decrease in αMCA upon exposure of the gut microbiota to cyhalothrin, while this was not shown in the present study. The reason for this discrepancy could be that in the current in vivo work the deconjugation was increased, as indicated by the raised ratio of unconjugated/ conjugated bile acids. Similar to the in vitro result, αMCA appeared to be efficiently metabolized, for example by epimerization to βMCA (de Boer et al., 2020), which, in combination with the faster deconjugation from TαMCA to αMCA could maintain the dynamic balance for the levels of αMCA in this in vivo study.

Although only approximately 5% of bile acids secreted by hepatocytes end up in feces (Hundt et al., 2017), the fecal bile acid profile was previously reported to largely reflect the intestinal bile acid composition which is dependent on activities of microbial enzymes (Joyce and Gahan et al., 2016) such as BSH, 7α-dehydroxylase, and 6β-epimerase present in the gut microbiota. The accelerated deconjugation related to the raised activities of microbial BSH (Sayin et al., 2013) could be linked to the significantly increased richness of the genera *Brachybacterium* and *Brevibacterium* upon the high-dose lambda-cyhalothrin treatment of female animals (**Figure 5.5A** and **B**). Meanwhile, the conversion of βMCA to ωMCA is carried out by 6β-epimerase (Chiang and Ferrell et al., 2020), which could also be provided by the genera *Brachybacterium* with *Brevibacterium* in the current study (**Figure 5.5B**). Conversely upon the administration of lambda-cyhalothrin, there were no obvious effects on 7α-dehydroxylation of CA to DCA, which indicates that the community of bacterial strains carrying 7α-dehydroxylase was not markedly affected. In addition to these microbial enzymes, the increase in fecal HDCA, the 7β-dehydroxylated metabolite of ωMCA could be ascribed to the highly active 7β-dehydroxylase (Sayin et al., 2013) provided by the enriched genera *Oscillospiraceae NK4A214 group* upon the treatment of male mice with lambda-cyhalothrin. Moreover, interactions between bile acids and intestinal microbiota have been previously described (Ramírez-Pérez et al., 2018), and the impact of bile acid composition in the gut on the intestinal microbiome has been examined. For example i) conjugated bile acids were reported to exhibit antibacterial activity (Jones et al., 2008; Inagaki et al., 2006); ii) the ratio of secondary

over primary bile acids was reported to be positively correlated to certain commensal genera (Kakiyama et al., 2013). Thus, the altered gut microbial community acquired upon the treatment with lambda-cyhalothrin could be caused by the changed fecal bile acid composition as well.

In addition to fecal bile acids, the bile acid profiles in plasma also significantly altered especially with respect to taurine conjugated bile acids (**Figure 5.3** and 5.**6A**). These levels of plasma taurine conjugated bile acids were positively correlated to the proportions of fecal secondary bile acids (**Figure 5.6D**). The raised amount of intestinal secondary bile acids are expected to be reabsorbed into enterocytes, secreted into the portal circulation, and delivered to hepatocytes (Thomas et al., 2008) in which the secondary bile acids are conjugated with taurine by specific enzymes to generate conjugated secondary bile acids leading to for example the increased levels of THDCA, TUDCA, and TωMCA (included in TMCA) detected in plasma (**Figure 5.2A** and **B**). Additionally, the significantly decreased levels of the fecal primary bile acid βMCA could directly lead to its reduction in plasma upon the treatment with lambda-cyhalothrin especially in male mice. However, the taurine conjugated primary bile acids such as TCA, were decreased in fecal samples but simultaneously increased in plasma, indicating that these effects may rather be ascribed to changes in bile acid transporters.

The enterohepatic circulation of bile acids is presented in **Figure 5.7** showing the flow of bile acids through the gut-liver axis and the transporters involved, while also presenting the changes of intestinal and plasma bile acid profiles induced by the treatment of the mice with lambda-cyhalothrin. As shown, except for the intestinal bacterial community, the hepatocytes and intestinal epithelial cells also contribute to the bile acid metabolism and transport, with an important role for the organic solute transporter subunit α/β (OSTα/ β). The crucial role of OSTα/ β in intestinal bile acid transport is well known, and its expression pattern highly correlates with that of the intestinal bile acid transporter ASBT (apical sodium-dependent bile acid transporter); additionally, the OSTα/ β is capable to protect the enterocytes from bile acid accumulation (Ferrebee et al., 2018). Previously, it has been shown that the expression of OSTα/ β could be not only affected by exposure to xenobiotics (Beaudoin et al., 2020), but also regulated by the bile acids such as CA or CDCA themselves (Frankenberg et al., 2006). As a result, it can be proposed that the raised proportions of taurine conjugated bile acids in plasma accompanied by

reduced levels in feces could be caused by an increase in the expression and activity of ASBT and hepatic/ intestinal OSTα/β, leading to more efficient reuptake of the conjugated bile acids from the intestinal tract into the portal vein and liver. In the last two decades, the role of OSTα/ β and ABST for the disposition of endogenous compounds particularly relating to the enterohepatic circulation of bile acids gained increasingly interest (Beaudoin et al., 2020; Vicens et al., 2007). Thus, such lambda-cyhalothrin induced effects on the expression and activity levels of ASBT and/or OSTα/β presents a topic of interest for future studies.

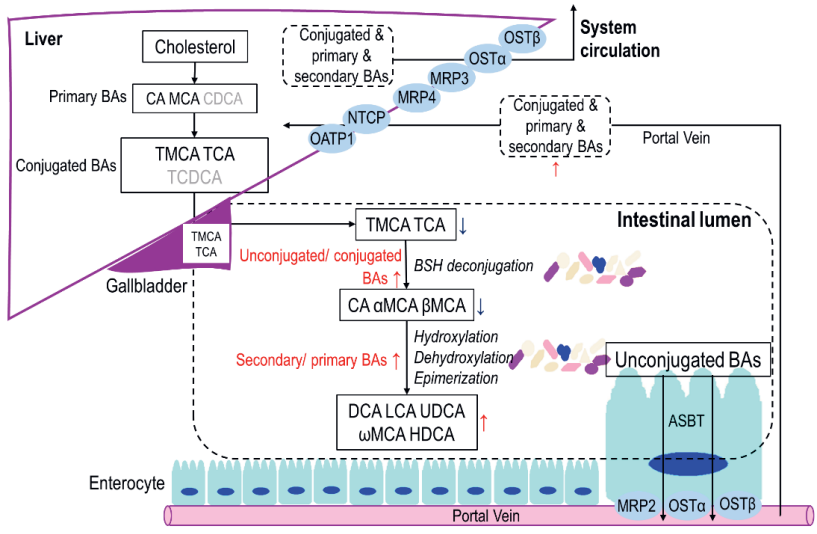

Figure 5.7 The biosynthesis, transport and metabolism of bile acids, as well as the alterations of bile acids in plasma and fecal samples of mice upon 28 days administration of lambda-cyhalothrin as reported in the present study. The upregulated and downregulated levels of bile acids were separately marked red and blue. Bile acids are produced by the liver, mainly conjugated to taurine (TMCA and TCA), excreted into the bile, released by the gallbladder into the intestine of mice via the bile duct. In the intestinal lumen, TMCA and TCA are deconjugated to CA, αMCA, and βMCA via BSH, and hydroxylated or dehydroxylated or epimerized to form the secondary bile acids of DCA, LCA, UDCA, ωMCA, and HDCA by mouse gut microbiota. At the terminal ileum, most unconjugated bile acids are reabsorbed by the transporter ASBT into enterocytes and secreted into the portal circulation via the basolateral bile acid transporters OSTα, OSTβ, and MRP2 (Trauner and Boyer, 2003). Bile acids are then taken up by NTCP and OATP1 into hepatocytes. Hepatic MRP3, MRP4 and the OSTα–OSTβ complex provide alternative excretion routes for bile acids into the systemic circulation (Meier and Stieger, 2002).

Furthermore, considering the important role of bile acid homeostasis in host health, it is of interest to note that increased levels of secondary bile acids as induced by the treatment of the mice with lambda-cyhalothrin in the present study, have been previously noted in the stool of hosts with ulcerative colitis and dysplasia or cancer (Bernstein et al., 2011; Ajouz et al., 2014); additionally, the secondary bile acids can also stimulate the bile acid receptor TGR5, which may result in suppression of the inflammatory response to microbial ligands, potentially leading to a lower degree of immunosurveillance against neoplasms (Vaughn et al., 2019). Besides, if our hypothesis for the increase in ASBT and/or hepatic/ intestinal OSTα/β holds, it can result in cholestatic disorders and other metabolic diseases posing a risk to host health as well (Beaudoin et al., 2020). Therefore, further studies on xenobiotic-gut microbiota-bile acid metabolism interactions and their consequences for host health are of interest for future studies.

5.5 Conclusion

In this study, the effects of lambda-cyhalothrin on gut bacterial and bile acid profiles were assessed in male and female mice. The key findings were that the treatment of the mice with lambda-cyhalothrin induced a markedly raised total amount of plasma and fecal bile acids, mainly caused by an increase in plasma conjugated bile acids and in fecal secondary bile acids. In addition, the gut microbial community showed a significantly increased richness of *Prevotellacea* and a depletion of *Lachnospiraceae* at the family level upon the treatment with lambda-cyhalothrin, and the genera *Brachybacterium*, *Brevibacterium* as well as *Oscillospiraceae NK4A214* were found to be highly correlated to the fecal bile acid levels. In summary, the alterations of bacterial and bile acid profiles obtained provide a proof-of-principle for effects of lambda-cyhalothrin on gut microbiota and related bile acid metabolism which may eventually influence host health.

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6

CHAPTER 6

General Discussion

6.1 Overview of the results and main findings

The gut microbiome plays a crucial role in host health and disease, and can be influenced by many factors such as for example diet, age, and chemical exposure (David et al., 2014; Modi, et al, 2014; Rosenfeld, 2017; Zhang et al., 2015). In the last decades, mechanistic understanding of the gut microbiome was advanced by the use of omics technologies like metagenomics, metaproteomics and metabolomics, which together provided a holistic view of the microbiome and the related metabolism as well as its interactions with host metabolism (Peters et al., 2019). Composition of the gut microbial community as well as its functionality could be elucidated by these integrative approaches unravelling the dynamics of host-microbiome-metabolism interactions (Bäumler and Sperandio, 2016). Hence it is now generally believed that gut microbiota is a critical factor contributing to host metabolism and health, by producing numbers of bioactive compounds that act as a signal to the host by activating cognate receptors in various cells (Holmes et al., 2012). Such signalling metabolites may include, for example, bile acids. In the present project, focus was on effects of antibiotics and pesticides on the gut microbial profiles and the consequences for bile acid metabolism.

Since the gut microbiota affects the size and composition of the bile acid pool, studying the bile acid profile and its modulation via effects on the gut bacterial community is of interest. So far, studies on the effect of chemical exposure on the intestinal bile acid pool have been done in vivo while there has been no study reporting on an in vitro model able to mimic and reproduce the alteration of intestinal bile acid profiles observed upon exposure to antibiotics or other chemicals in vivo. In **Chapter 2**, rat fecal samples and two lincosamides (lincomycin and clindamycin) were employed for the development of a novel in vitro fermentation model that would enable elucidation of compound-induced changes in bile acid and microbial community profiles, to identify key metabolites in the bile acid pool related to gut microbial changes, and to study the effect of xenobiotics on gut microbiota and the consequences for bile acid metabolism. Anaerobic, pH-controlled batch cultures were inoculated with rat fecal microbiota, and incubated with an optimized bile acid mixture and lincosamides as the model compounds. Samples were collected throughout the fermentation (0, 6, 12, and 24 h) and the bacterial composition was determined by 16S rRNA gene sequencing. Also a newly developed and optimized method was applied for sample preparation and quantification of 24 different bile acids using LC-

MS/MS. A correlation analysis identified that the treatment with lincosamides resulted in a significant decrease of most analyzed secondary bile acids while an increase of cholic acids was observed. Upon bacterial profiling, these changes could be related to the reduced abundance of *Lactobacillaceae* and *Erysipelotrichaceae* at the expense of *Akkermansiaceae*. Furthermore, comparison of the changes observed to literature reported effects of the lincosamides in an vivo metabolomic study in rats (Behr et al., 2019) revealed that the consequences of gut microbiome modulation for the alteration of unconjugated bile acids were consistent in the in vivo and in vitro model, providing a proof of principle for the use of this 24 h in vitro batch fermentation model to study the modulation of bile acid profiles via effects on the gut bacterial community.

Subsequently, a similar batch fermentation in vitro model was developed and applied using mice fecal samples in **Chapter 3**. To optimize this mice fecal sample-dependent model, a solution composed of TCA and TβMCA instead of the mixture containing TCA, TβMCA, and TUDCA used in **Chapter 2,** was employed to complement the residual fecal bile acids in order to imitate the mouse gut environment with respect to the bile acid pool expected to be released from the liver. In **Chapter 3** this batch fermentation in vitro model was applied to study the effect of some selected food-borne pesticide residues. To date, only a limited number of studies report on the toxicity of pesticides towards the gut microbiota whereas studies on potential consequences of these effects for bile acid profiles are almost absent. Therefore, in **Chapter 3** and **Chapter 4** some food-borne pesticides were selected for studying their impact on bacterial and bile acid profiles using the developed in vitro model. This in vitro model allows testing of larger number of compounds without the need for animal experiments. In **Chapter 3**, the toxic effects of six OP pesticides on bile acid profiles were investigated using the developed in vitro model. In anaerobic incubations, mouse fecal samples were exposed to 0.01 mM triazophos, 0.01 mM isocarbophos, 0.08 mM chlorpyrifos, 0.47 mM diazinon, 0.31 mM omethoate, or 0.01 mM phorate. Subsequently, in **Chapter 4**, effects of carbamates (carbofuran, aldicarb) and pyrethroids (cypermethrin and cyhalothrin) on gut microbiota and related bile acid profiles were also quantified. Samples were taken during the incubations at 0, 6, 12, and 24 h and bile acid measurement was performed by LC-MS/MS. In **Chapter 3**, alterations of primary and secondary bile acids were observed in OP-treated mouse fecal anaerobic incubations, including especially substantially increased production of ωMCA and decreased levels of βMCA. Among all OPs studied, exposure of the fecal microbiota to

phorate led to the most wide and significant effects on the bile acid profile; as a result, phorate was selected as the OP representative for further determination of accompanying effects on the bacterial profile by 16S rRNA sequencing. Results showed an increased and decreased relative abundance for bacterial species of *Muribaculaceae* and *Atopobiaceae* respectively; meanwhile, changes in *Muribaculum* (family, *Muribaculaceae*) as well as *Coriobacteriaceae UCG-002* (family, *Atopobiaceae*) were found to be highly correlated with changes in the bile acid profile. In **Chapter 4**, after 24 h exposure to carbamates or pyrethroids, results obtained revealed a pesticide-induced significant enrichment of secondary bile acids with the depletion of primary bile acids in the in vitro model. Bacterial profiling revealed that the abundance of the phyla *Actinobacteria* was markedly enhanced in fecal samples after the treatment with carbofuran and cyhalothrin, whereas the significantly reduced richness of *Bacteroidetes* was only found in the carbofuran-treated group. Diversity of the microbial community was slightly raised in cyhalothrin-exposed samples and reduced in the carbofuran group. Additionally, a strong correlation of the family *Eggerthellaceae* with the bile acid profiles was shown by a pearson correlation analysis of the data obtained by the bacterial and bile acid profiling in both cyhalothrin- and carbofuran- exposed samples. Results obtained reveal that the treatment of fecal bacterial samples with OPs, carbamates or pyrethroids caused alterations of the gut microbial community resulting in the modulation of bile acid conversions.

Hence, lambda-cyhalothrin, the representative toxic isomer of cyhalothrin was selected for a 28-day oral dose mouse in vivo model of **Chapter 5**, for not only a further study of its effects on the gut microbiota and bile acid profiles, but also for the further evaluation of the developed in vitro model system**.** Results of bile acid profiling in **Chapter 5** showed an increased amount of total bile acids and conjugated bile acids in plasma and fecal samples from lambda-cyhalothrin treated mice compared to controls. Higher levels of the secondary bile acids ωMCA and HDCA were observed in mouse feces accompanied by a decreased proportion of the primary bile acid βMCA. In addition, gut microbial profiling revealed a raised richness of the genera *Brachybacterium*, *Brevibacterium*, as well as of the *Oscillospiraceae NK4A214 group*, and the three bacterial strains showed to be strongly related to bile acid transformation in lambda-cyhalothrin treated mice. Besides, a significantly increased relative abundance of *Prevotellacea* (phyla of *Bacteroidota*) and a depletion of *Lachnospiraceae* were found at the family level of bacterial species. The results of this in vivo study revealed that bile acid and gut microbial profiles in mice were

significantly altered upon exposure to lambda-cyhalothrin. The changes observed were consistent with the results from the in vitro model described in **Chapter 4**. Both in the in vitro and in vivo studies, a faster ωMCA production accompanied by a corresponding depletion of βMCA was observed, leading to an increase in the relative amount of secondary bile acids in the bile acid profile compared to controls. Meanwhile, an obvious decrease of fecal conjugated bile acids (T-BAs) was observed in both in vitro and in vivo models upon treatment with cyhalothrin, whereas a significant increase of plasma T-BAs was additionally observed in the mouse in vivo study, which reveals an effect on the enterohepatic circulation of bile acids, most likely related to the expression and activity levels of ASBT (apical sodium-dependent bile acid transporter) and/or OSTα/β (organic solute transporter subunit α/β). Comparison of the in vitro and in vivo results indicates that effects of xenobiotics on intestinal bile acid metabolism can be evaluated by the developed in vitro fermentation model to present the altered gut microbiota-mediated bile acid transitions together with previously reported intestinal cell models (for example the Caco-2 cell transwell system) to determine the changes on intestinal bile acid resorption (Zhang et al., 2022).

6.2 General discussion and future perspectives

In this project, our aim was to develop an in vitro fermentation model for studying the effects of food-borne chemicals on the gut microbiome and related bile acid profiles, and to verify this model by comparison with data from previous and a newly performed in vivo study. To this end, effects of selected antibiotics (lincomycin and clindamycin) and pesticides (OP esters, carbamates and pyrethroids) on gut microbial and bile acid profiles were evaluated in this developed in vitro model; in addition, for comparison and model evaluation, an in vivo study was performed using a selected pesticide (the pyrethroid lambda-cyhalothrin) while data from an in vivo study on the respective antibiotics (lincosamides) were available in literature (Behr et al., 2019). The results obtained revealed effects of not only the antibiotics, but also of all selected groups of pesticides on gut microbiota and bile acid profiles. The results of the current study also presented challenges and limitations which can be the basis for suggestions for improvements of especially the in vitro batch fermentation model and/or a topic for future studies. These items are discussed in further detail in the following sections and involve:

- the advantages and disadvantages of intestinal in vitro and in vivo models

- the effects of xenobiotics on gut microbiota

- the effects on gut microbiota mediated bile acid metabolism

- the potential consequences of altered bile acid metabolism for host health

The last part of each section will present future perspectives for research and close with a conclusion.

6.2.1 The advantages and disadvantages of intestinal in vitro and in vivo models

Alternative approaches to in vivo animal models involve the use of a plethora of systems ranging from very simple models using human cells in mono- or co-culture, to whole tissue explants. Such in vitro/ex vivo models to study the intestine and gut microbiome and their interaction have been extensively reported in recent years. In addition to in vitro cell models to study intestinal metabolic process such as the commonly used cell lines including Caco-2, HT-29, T84, IEC, and IPEC, ex-vivo systems of intestinal enteroids and organoids were also utilized for the study of host-microbiome interactions, whereas fecal fermentation models are widely applied for studying the altered gut microbiome induced by acute stressors (Pearce et al., 2018). In the present thesis an in vitro batch fecal fermentation model was developed, optimized, and employed in **Chapter 2**, **3**, and **4**, for the evaluation of the impact of xenobiotics on the gut microbiome and related bile acid profiles.

These alternative in vitro approaches contribute to the 3Rs (replacement, reduction, and refinement) for studies in experimental animals. The in vitro model could link changes in the bacterial composition to changes in the bile acid composition. An advantage of the in vitro model is that sampling of the required feces in non-invasive so that it can easily be used for studies on mice, rat and also human, and/or for studying interindividual differences. Such inter- or intra- species variability is to be expected between rats, mice and human and also between individuals as reflected by the observation that even the mice at the same age, brought up in one cage, and fed with the same diet appeared to differ in their gut microbiota composition (**Chapter 5**). Moreover, the application of a combination of different “omics” approaches like metagenomics and metaproteomics for these in vitro model systems, can reveal not only the taxonomy but also the functional activity of gut microbiota. Results of the present thesis for the first time revealed that there are similarities in the antibiotic or pesticide induced effects on gut microbiota and

bile acid profiles observed in the in vitro model and in vivo. For example in **Chapter 2**, changes in the levels of fecal primary and secondary bile acids observed in the in vitro fermentation model were consistent with those of a previous in vivo study in lincosamides treated rats (Behr et al., 2019). Also, similar changes in the bile acid profile with an increase of secondary bile acids and an accompanying decrease of primary bile acids upon treatment with the selected pesticide (cyhalothrin) were observed in **Chapter 4** and **5**. There were also differences such as the significant increase in fecal taurine conjugated bile acids observed in the in vivo study (**Chapter 5**), but not observed in the in vitro model of **Chapter 4**. In the in vivo study exposure to lambda-cyhalothrin also resulted in an increased level of plasma conjugated bile acids that might have been due to changes in the apical sodium-dependent bile acid transporter (ASBT), an effect that cannot be studied in the in vitro fermentation model. To this end other in vitro models may be used, for example the Caco-2 transwell model that enables studying bile acid transport (Zhang et al., 2022). This in vitro model could provide a useful addition to the in vitro fermentation model of the present thesis to study effects of food-borne chemicals on bile acid homeostasis in the host, since the majority of bile acids are reabsorbed via enterocytes (approximately 95%) a process that may also influence fecal as well as plasma bile acid patterns (Fan et al., 2021). Thus to further enable studying the modulation of bile acid profiles by the combined gut microbial and host co-metabolism additional in vitro models should be combined with the current in vitro fermentation model system. This battery of in vitro models should enable analysis of effects on the bile acid enterohepatic circulation. These in vitro models should not only include the activity of transporters for re-uptake of the bile acids from the intestinal track such as ASBT and/or OSTα/β but also of the hepatic bile salt export pump (BSEP) and of biliary excretion mediated by multidrug resistance protein 2 (MRP2) and sinusoidal backflux regulated by MRP3 (Farooqui and Elhence, 2021).

In addition to the developed Caco-2 transwell model for revealing the altered expression of bile acid transporters (Zhang et al., 2022), also in silico approaches such as physiologically based kinetic (PBK) modelling can provide a tool to study the effect of exposure to compounds with BSEP-inhibitory properties on bile acid homeostasis (de Bruijn et al., 2022). Sofar these PBK models for bile acid homeostasis have not yet included the gut microbial metabolism. The in vitro model for gut microbial bile acid metabolism developed in the present thesis may be of use to define the required PBK

model parameters to include this gut microbial compartment and effects of chemical compounds on the related metabolism in the PBK models. To this end the in vitro model should be able to mimic the in vivo situation to the largest possible extent.

In the present work, performance of the model was optimised with respect to time and anaerobic conditions of incubation, growth medium, and procedures of sample preparation. At the initial stage of the development of this fermentation model, a variety of commonly used mediums with different pH were tested. However, ultimately PBS buffer was employed because i) this provided lower limits of detection (LODs) for the bile acids, ii) the variability between duplicate incubations was less, and iii) it was considered that medium without nutrients for bacterial growth would result in conditions closer to the in vivo intestinal situation. The improved LODs and reproducibility in PBS compared to complex media reflect stronger matrix effects on bile acid measurement and differences in bacterial responses after the addition of complex medium (Wang et al., 2020). The use of PBS without nutrients also implied that incubations should be limited to relatively shorter time periods of up to 24 hours. Optimisation also included the addition of conjugated bile acids like TCA and TβMCA in the mouse model and TCA, TβMCA, and TUDCA in the rat model, in order to complement the residual fecal bile acids in order to imitate the mouse or rat gut environment with respect to the bile acid pool expected to be released from the liver. Furthermore, when studying effects of food-borne chemicals in vitro another parameter to be considered and optimised was the concentration of the compounds to be tested. Herein, dose levels equal to the LD50s, acceptable daily intake (ADI), or acute reference dose (ARfD) were translated to expected concentrations in the gut using the reported animal gut volume calculating the test concentrations of xenobiotics expected to represent the exposure concentrations for the gut microbial community. Meanwhile, the fecal concentration and the incubation time were also optimized to ensure the amount of bacterial species and their activities would enable subsequent characterisation of the bacterial and bile acid profiles, and the changes therein. The batch fermentation model applied is different from the commonly used SHIME (Simulator of the Human Intestinal Microbial Ecosystem) model for intestinal and gut microbial conversions. Compared to the SHIME model which runs over one month, and is a large system in which only one samples at a time can be studied (Agans et al., 2018), the developed batch model needs less time, space, handling, and is cheaper and

suitable for short-term studies of up to 24 or 48 hours, while multiple samples can be incubated simultaneously (Karthikeyan et al., 1996).

In spite of the limitations of the batch fermentation model outlined above, its convenience and efficiency makes it a valuable tool for research on the microbial community or on individual bacterial species. It is not only a commonly used in vitro model for studying bacterial metabolic rates and growth kinetics (Mears et al., 2017), but also an effective method to obtain mechanistic insight for example by adjusting the model process parameters. In addition, as already outlined above, given the high throughput and non-invasive nature of the fecal sample collection the batch fermentation model also allows studies on inter- and intraspecies differences as illustrated for example by studies on the interspecies differences in metabolism of the food-borne mycotoxin deoxynivalenol by mouse, rat, pig, and human gut microbial communities, in which a 4 h in vitro fermentation model using the feces from mice, rats, pigs, and human was optimized and utilized (Jin et al., 2021), or the studies on the inter-individual diversity in the gut microbial conversion of green tea (-)-epigallocatechin-3-O-gallate by five male and nine female human volunteers (Liu et al., 2021). Application of a non-invasive in vitro fermentation model is especially an advantage for studies on the human gut microbiota, which has been reported to be highly linked to diseases, behaviour, and even brain disorders (Morais et al., 2021). In the present thesis the in vitro batch fermentation model enabled studying of the effect of a substantially larger number of antibiotics and pesticides than what could have been studied using in vivo models.

Collectively, the in vitro batch fermentation model is a valuable and effective tool to provide mechanistic understanding of host microbiota-metabolism interactions, while to obtain insight in the complete enterohepatic circulation the model should in future be combined with other in vitro models and even PBK modelling to obtain a full picture of the effects of food-borne chemicals on bile acid homeostasis. For example, the newly developed PBK models for predicting the dose dependent accumulation of bile acids in humans upon treatment with a BSEP inhibitor (de Bruijn et al., 2022) can be extended to include intestinal microbial metabolism using the in vitro fermentation model to define the parameters for this part of the PBK model. In a recent study (Zhang et al., 2022), it was already shown that use of the Caco-2 cell line in a transwell model in addition to the fermentation batch model enables elucidation of the modes of action underlying the

altered levels of fecal conjugated bile acids induced by exposure of rats to tobramycin, showing that this antibiotic inhibits both bacterial deconjugation of TCA as well as the transport of TCA through the Caco-2 cell layer, the latter most likely via an effect of tobramycin on protein synthesis thus resulting in reduced expression levels of the ASBT transporter responsible for re-uptake of conjugated bile acids (Zhang et al., 2022). Thus, the batch fermentation model should be part of a battery of in vitro tests to mimic the in vivo situation, and this provides an important goal for future research.

6.2.2 The effects of xenobiotics on gut microbiota

Previous studies have reported that several classes of xenobiotic chemicals could interfere with the biochemical and enzymatic activity of gut microbes, resulting in alterations of the gut microbial community and overall homeostasis, further affecting host health (Claus et al., 2017). Focussing on the chemicals studied in the present thesis, the impact of antibiotics as well as pesticides on gut microbiota are discussed to a further extent in the following sections.

Antibiotics

Antibiotics have been commonly used for decades to prevent the proliferation of bacterial pathogens and thus treatment of bacterial infections. They are also used to improve the efficiency of animal feeds (Looft et al., 2012). However, it is well known that antibiotics are able to shift the gut microbiota to temporally alternative stable states, which itself may become resilient against external influences (Dethlefsen et al., 2007). Antibiotic treatment has tremendous impact on the overall taxonomic composition of the gut microbiota; appearing fast and being surprisingly long-lasting. Within days after antibiotic treatment, profound compositional effects on luminal and mucosal bacteria occur resulting in decreased taxonomic richness (Dethlefsen et al., 2008; Heinsen et al., 2015). And although antibiotics may be prescribed only for a short-term use, they may lead to long-lasting changes of the gut microbiota and consequently altered interaction with the host which may be relevant especially in the first 2 years of childhood (Vangay et al., 2015). Even a 24 h exposure of rat fecal gut microbiota to antibiotics in the developed in vitro model of **Chapter 2**, resulted in a significantly reduced abundance of the bacterial families *Erysipelotrichaceae, Bacteroidacea* and *Lactobacillaceae* (lincomycin treated) or *Prevotellaceae* (clindamycin treated). Also in in vivo studies within days after antibiotic treatment, profound compositional effects on luminal and mucosal bacteria have been

reported with decreased taxonomic richness (Heinsen et al., 2015). In a 28-day oral dose in vivo rat study (Behr et al., 2018) for example the relative abundance of *Bacteroidetes* was substantially reduced upon 28-day treatment of the animals with lincosamides.

Meanwhile, different classes of antibiotics could result in different impact on and consequences for the gut microbiota, while differences in dosage, duration, spectrum and route of administration of the antibiotics can lead to different effects as well (Lange et al., 2016). For example, the treatment of mice with clindamycin resulted in an increased richness of gram-positive bacteria and a decreased richness of anaerobes (Almeida et al., 2016), effects that were also observed in the in vitro study in **Chapter 2**. Conversely, the administration of ciprofloxacin did not show effects on the microbial composition of gram-positive bacteria or anaerobes, but led to a reduced abundance of entero-bacteria (Van De Leur et al., 1997). Based on the altered fecal and plasma metabolic profiles by the administration of six antibiotics in a previous in vivo study (Behr et al., 2019), the class of lincosamides involving lincomycin and clindamycin were found to have the most significant impact on gut microbiota and related bile acid profiles. This was the reason why these lincosamides were selected to be applied for the evaluation of the developed in vitro model in **Chapter 2**. Particularly for clindamycin, it has been reported in another study (Rashid et al., 2015) that the compound disrupts the gut microbiota, resulting in a shift in bacterial colonization with overgrowth of *C. difficile*, putting the gut at risk of pseudo membranous colitis in contrast to the gut of healthy individuals (Rashid et al., 2015). The symptoms of diarrhea and gastritis could further cause disturbance of normal bowel function thereby loss of short chain fatty acids, consequently bloating. Meanwhile, a long-term effect of clindamycin was seen on the gut microbiota reflected by a reduced level of colonic *Bacteriodes* (Jernberg et al., 2010), which also is in line with the decreased richness of the phyla *Bacteroidetes* reported in another in vivo study (Behr et al., 2018) and in the in vitro model of the present project (**Chapter 2**).

Collectively, effects that may impact host health induced by the altered gut microbial community after the treatment with antibiotics primarily include the reduced microbiota diversity, an altered metabolome, and antibiotic resistance (Ramirez et al., 2020). For example, by causing a decrease in species diversity, antibiotics can result in the overgrowth of pathogens, such as toxigenic *C. difficile* (Ianiro et al., 2020). In addition the effects on the microbiota may reflect enhanced antibiotic resistance of specific subspecies,

a phenomenon that is of interest since it reflects the capacity of a bacterial species to survive antibiotic concentrations that inhibit or kill other bacteria (Sabtu et al., 2015). 35,000 deaths in the US and 25,000 deaths in Europe each year are estimated because of antibiotic resistance (European Medicines Agency and European Centre for Disease Prevention and Control, 2009; Centers for Disease Control and Prevention, 2019), and it is reported that the number of related deaths could reach 10 million by 2050 (World Health Organization, 2019). A range of processes to elude the effects of antibiotics are developed by the gut bacteria, including protection against the uptake of antibiotics through their cell membranes, developing enzymatic processes that modify or degrade the antibiotic, altering the molecules that antibiotics target, and actively removing antibiotics from the cell via specialized efflux proteins (Giedraitiene et al., 2011). Although novel approaches to avoid drug resistance are researched continually, the development of new antimicrobials or modifications of the current arsenal may not be able to effectively address the trends in resistance. Instead, it has been reported that antibiotic resistance may better be addressed with the delivery systems and the innovative combinations of biologically inspired molecules, for example, quorum sensing inhibitors, biosurfactants, bacteriophage, and enzymes (Brooks and Brooks, 2014).

Hence, knowledge not only on the antibiotic activity spectra at the level of individual bacterial strains, but also on modes of action and the modification of metabolic profiles such as bile acid profiles as well as the potential antibiotic resistance remain to be further elucidated. It is likely that these important research areas will and already do benefit from in vitro model systems as applied in the present thesis.

Pesticides

In contrast to antibiotics, the toxicity of pesticides is not generally directed against bacteria. Nevertheless they are intrinsically toxic raising increasing concerns over the last several decades due to their wide use and the resulting environmental pollution and residues in the food chain (Jin et al., 2010, 2014, and 2017). Previously, side effects induced by exposure to pesticides including metabolic diseases (such as obesity and type 2 diabetes)(Foley et al., 2018), dysregulation of the immune system (Daisley et al., 2017; Oliveira et al., 2018), neurotoxicity (Liu et al., 2016), endocrine alterations, reproductive disorders (Hocine et al., 2016), and even tumours (Vivarelli et al., 2019) haven been widely reported. Effects on gut microbiota induced by pesticide exposure has gained

increasing attention in recent years (Defois et al., 2018). Among all the pesticides which have been reported to cause gut microbiota dysbiosis, the organophosphate (OP) and carbamate (CBM) pesticides with acute effects of AChE-inhibition (Gordon et al., 2006; Padilla, 1995), as well as the pyrethroid (PYR) pesticides with acute neurobehavioral effects were selected and separately studied in **Chapter 3**, **4**, and **5** of this thesis. Below, the available evidence on the effects of various OP, CBM and PYR pesticides on gut microbiota, including the results obtained in the present study, are discussed in some further detail.

Several studies have characterized the action of OPs including chlorpyrifos, diazinon, and monocrotophos on the gut microbiome. Zhao et al. (2016) reported that a 30-day exposure of adult male mice to chlorpyrifos via the oral (gavage) route caused a dysbiotic effect on the pattern of fecal microbial communities. In another study, Fang et al. (2018) administered chlorpyrifos to young adult male Wistar rats in a 9-week oral-dose study resulting in a biologically significant divergence in the gut microbiota between chlorpyrifos-treated and control animals. The effects of OPs on gut microbiota were also shown in a recent 25-week exposure study in adult male rats, with the observed disruption of endocrine as well as inflammatory-control pathways caused by the altered gut bacteria after the administration of chlorpyrifos (Li et al., 2019). In **Chapter 3**, the effects of chlorpyrifos were evaluated using the in vitro fermentation model, and the significantly altered bile acid profiles pointed at changes of the gut microbial community as well. In a 13- week exposure of spf C57BL/6 mice to the OP diazinon also effects on the gut bacterial community were observed (Gao et al., 2017), an effect also observed in our 24 h in vitro incubation reported in **Chapter 3.** In addition, the OP monocrotophos induced changes in rat gut related to the activation of inflammatory factors leading to increased gut motility, although the study did not relate these effects of monocrotophos to the gut microbial community (Rajini, 2014). It appears that the studies mentioned above were the only work published to date on the effects of OPs on the gut microbial community. The studies reported in the present thesis in **Chapter 3** add to this knowledge and confirm that these OP pesticides may target the gut microbiota and influence microbial profiles as well as the related metabolism. Results in the current study showed an arising richness of *Bacteroidetes* at the expense of *Firmicutes* in the phorate-treated fecal samples compared to controls in the in vitro fermentation model, indicating that the OP phorate can induce gut microbial disorder. Besides, the other three OPs tested

including triazophos, isocarbophos, and omethoate, which were not studied before for their potential effects on the gut microbiota and/or related microbial metabolism were shown to have similar effects on the bile acid profile as phorate, suggesting that they may also have similar effects on the gut microbiota.

In addition to OPs, the carbamates were also found to affect the gut microbiota. In a study on the consequences of administration of the carbamate aldicarb to C57BL/6 male mice, alterations in maturation of gut microbiota, promoted expansion of bacterial pathogens, modulated lipid metabolism, and induced oxidative stress as well as DNA damage were reported (Gao et al., 2018). Moreover, the effects of the carbamate propamocarb on the gut microbiota and related metabolism were assessed in a 4-week male mouse in vivo study, and results revealed effects on the colonic-cecum gut microbiota resulting in changes of fecal microbial metabolites as well (Wu et al., 2018). In **Chapter 4** of this project, the impact of the carbamate carbofuran was evaluated in the in vitro fermentation model using mouse feces, and it was found that the relative abundance of the phyla *Actinobacteria* significantly increased accompanied by the enhanced production of secondary bile acids. Also the widely used pyrethroids were assessed for their effects on the gut microbiota. After the administration of the pyrethroid permethrin, various bacterial genera showed enduring changes in their relative abundance in adult rats; meanwhile, a reduction in the levels of specific gram-positive and gram-negative bacteria and two strains of yeast were observed in the permethrin-treated animals (Nasuti et al., 2016). In **Chapter 4**, the pyrethroid cyhalothrin was evaluated in the in vitro fermentation model and resulted in a significantly increased abundance of the families *Eggerthellaceae* (phylum, Actinobacteria) and *Desulfovibrionaceae* (phylum, Proteobacteria) and a decreased richness of *Enterobacteriaceae* (phylum, Proteobacteria). While in the subsequent in vivo study of **Chapter 5**, an enhanced richness of the family *Prevotellacea* (phylum, Bacteroidota) and a dose dependent decrease in the abundance of *Lachnospiraceae* (phylum, Firmicutes) were observed after the 28-day oral treatment of mice with cyhalothrin. The differences in the bacterial profiles obtained in the control samples of the in vitro and in vivo models of **Chapter 4** and **5** are most likely due to the fact that the gut microbial community can be affected by many factors including for example species, sex (Valeri and Endres, 2021), diet, and a variety of environmental factors (Flint et al., 2017). Nevertheless in the in vivo and in vitro models, the majority of the alterations in the bile acid profile induced by treatment with the model compounds

were similar, for example consistent and significantly increased fecal secondary bile acid levels at the cost of primary bile acids were obtained in both the in vivo as well as the in vitro models after the treatment with cyhalothrin.

It is also relevant to consider the concentrations of the pesticides that were tested in the in vitro studies of the present thesis. These in vitro test concentrations were calculated based on oral doses derived from the available LD50 values obtained in acute toxicity studies in rats or mice selecting 1/10 of the LD50 values for the carbamates and pyrethroids and 1/30 of the LD50s for the OPs as the oral dose levels providing low but measurable effects without showing lethality. These dose levels (**Table 6.1**) were converted to the corresponding in vitro concentrations which were tested in **Chapter 3** and **4** of this thesis. Additionally, the no observed adverse effect levels (NOAELs) which were calculated by multiplying the acceptable daily intake (ADIs) reported by the official organizations (JMFR, 2002, 2004, 2006, 2007; CFDA, 2007) by a commonly used safety factor of 100, are also summarized in **Table 6.1**. As shown, some of the NOAELs calculated for the compounds including for example isocarbophos, aldicarb, and cyhalothrin, were in the range of the oral doses used to determine the in vitro concentrations tested and shown to have effects in the present project, indicating that at the reported NOAELs of these pesticides there might be an effect on the microbiome and related metabolism. To what extent such effects can be considered adverse remains to be established.

Table 6.1 ADIs, NOAELs, and oral dose levels used for the pesticides studied in this project.

Classification	Pesticides	ADIs reported for human (mg/kg bw per day)	[a]NOAELs calculated for rat/ mouse IN VIVO (mg/kg bw per day)	[b]Oral dose levels used for conversion to concentrations tested IN VITRO (this project) (mg/kg bw per day)
Organophosphates (Chapter 3)	Triazophos	0.001	0.1	0.25
	Isocarbophos	0.003	0.3	0.3
	Chlorpyrifos	0.01	1	2
	Diazinon	0.05	5	10.83
	Omethoate	0.0003	0.03	5
	Phorate	0.0007	0.07	0.22
Carbamates (Chapter 4)	Carbofuran	0.001	1	3
	Aldicarb	0.003	0.3	0.1
Pyrethroids (Chapter 4 & Chapter 5)	Cypermethrin	0.02	2	25
	Cyhalothrin	0.02	2	2
	λ-Cyhalothrin	0.02	2	1, 2

a. NOAELs, no observed adverse effect levels; NOAELs calculated by ADIs*safety factor (100)
b. 1/30 of the LD50s for the organophosphates and 1/10 of the LD50 values for the carbamates and pyrethroids

Thus, the results of the present studies point at the potential importance of considering the gut microbiota as an unintended target upon pesticide exposure. Particularly, the long-term consequences of chronically consuming pesticides at a realistic dietary level (via dietary exposure) on the microbial diversity should be addressed. In the future, research should focus on the mechanisms of pesticide pollution-induced gut microbiota dysbiosis and its long-term effects on host health. Hence the gut microbiota provides a new target for assessing pesticide toxicity and the consequences of pesticide contamination of the food chain.

6.2.3 The effects of xenobiotics on gut microbiota mediated bile acid metabolism

In the present thesis, effects of antibiotics and pesticides on the microbiota were studied in combination with potential metabolomic consequences focusing on the effects on bile acid profiles. Bile acids are synthesized from cholesterol primarily in pericentral hepatocytes through a series of sterol ring hydroxylations and side chain oxidation and conjugation steps to form the conjugated bile acids (**Figure 1.1** in Chapter 1). Following their release into the intestine, the conjugated bile acids are deconjugation by the microbiota generating the corresponding primary bile acids, and these primary bile acids are subsequently converted to secondary bile acids by a variety of microbial reactions including dehydroxylation, dehydrogenation, and epimerization (Dawson and Karpen, 2015)(**Figure 1.2** in Chapter 1). Collectively, the contribution of the gut microbiota to bile acid metabolism and the resulting bile acid profile may relate to a variety of reactions catalyzed by microbial enzymes, including for example, the microbial bile salt hydrolase (BSH) enzymes which catalyze bile acid deconjugation, and the microbial dehydroxylases which are involved in the transformation of deconjugated primary bile acids to secondary bile acids (Enright et al, 2018). BSH activity is common to all major bacterial strains in the gut (Jones et al., 2008). However, although well represented among gut bacteria there is huge variation in BSH deconjugation ability even at the bacterial species level (Fang et al., 2009). For example, in *Lactobacillus johnsonii* and *Lactobacillus acidophilus*, the cbsT1 and cbsT2 genes encode for members of the major families that facilitate transformation of TCA to CA by BSH (Elkins et al., 2001; Elkins and Savage, 2003). In addition, the microbial capacity of BSH may also differ between host species, for example, a metagenomic analysis revealed BSH enzyme differences between the murine and human microbiota, which likely reflect responses to the bile acid pool composition of the host species (Jones

et al., 2008). Following their deconjugation, the majority of deconjugated primary bile acids undergo additional transformations including 7-dehydroxylation performed by microbial 7α-dehydroxylases, which are primarily encoded in members of the *Firmicutes* phylum such as the *Clostridium cluster XVI* (Molinero et al., 2019). Except for dehydroxylation, epimerization is another notable transformation including the formation of the bile acid UDCA through the 7α/β-epimerization of CDCA (MacDonald et al., 1982)(**Figure 1.2** in Chapter 1). This epimerization arises due to successive reactions catalysed by 7α- and 7β- hydroxysteroid dehydrogenases (HSD) produced by the gut bacteria (Lepercq et al., 2004). This biotransformation can be performed by a single species possessing both enzymes, for example, *Clostridium absonum* (Sutherland and Macdonald, 1982). In addition, there are substantial differences in gut bacterial community between host species (Moran et al., 2019) and the different communities present different microbial capacities for deconjugation, dihydroxylation and epimerization for bile acid metabolism, leading to a different bile acid pattern between species (Thakare et al., 2018). This explains the different bile acid profiles obtained in the control groups of **Chapter 2** (rat feces), and **Chapter 3** & **4** (mouse feces). The results of the present thesis indicate that species dependent differences as well as antibiotic or pesticide induced changes in the gut microbial profile with consequences for bile acid metabolism can also be detected in an in vitro batch fermentation system.

Given that the batch fermentation model elucidated effects of the compounds tested on both the microbial as well as the bile acid profiles the model system also supports correlation analyses to elucidate relationships between the changes in microbial communities and the alterations in the bile acid profile. Thus, studies on the impact of OPs on gut microbiota and the related bile acid profiles in **Chapter 3**, revealed that the bacterial families *Coriobacteriaceae UCG-002* and *Muribaculum* were related to bile acid production, particularly, *Coriobacteriaceae UCG-002* showed a high correlation to the transformation of primary bile acids to secondary bile acids. In **Chapter 4**, the genera *Enterorhabdus* was shown to be positively correlated to the bile acid epimerization in our study on the effects of carbamates and pyrethroids. Moreover, in **Chapter 5**, the genera *Brachybacterium*, *Brevibacterium*, as well as the *Oscillospiraceae NK4A214 group* showed a strongly correlation to bile acid transformation, and their increased richness led to a significant increase in formation of secondary bile acids especially ωMCA and HDCA. In future research, these genera showing high correlation with bile acid profiles could be

isolated and further evaluated for their capacity for bile acid metabolism. Also, future studies should consider the bi-directional nature of the relationship between gut microbiota and bile acids: gut bacteria could not only benefit from metabolizing bile acids by acquiring glycine and taurine for subsequent metabolism (Philipp, 2011), but may also be injured by the antimicrobial properties of bile acids or stimulated to produce antimicrobial factors themselves (Hofmann and Eckmann, 2006; Inagaki et al., 2006; Merritt and Donaldson, 2009). Meanwhile, mechanisms responsible for the antimicrobial actions of bile acids have been investigated, including direct interaction with cell membranes (Kutdi et al., 2006), acidification of the cytoplasm, and induction of DNA damage (Merritt and Donaldson, 2009). A detergent role for conjugated bile acids and dietary long chain fatty acids derived from the digestive process has also been proposed to explain the antimicrobial actions of endogenous and exogenously administered conjugated bile acids (Hofmann and Eckmann, 2006).

Understandably, much of the research to date has focused on identifying the bacterial genes and their related biological functions. It is of interest to note that the expression of the bacterial transporters responsible for bile acid uptake and efflux also play an important role in controlling the transformation of bile acids, which would in turn affect the size, composition, and hydrophilicity of the host's bile acid pool. Also, these transporters may be affected by exposure to pesticides, an effect that remains to be investigated.

6.2.4 Potential consequences of altered bile acid metabolism for host health

Given the effects of the model compounds tested in the present thesis on microbiota and related bile acid profiles it is of interest to consider in some more detail in what way bile acids and changes in bile acid profiles may influence host health. Bile acids are known to act as ligands for cellular receptors that include a family of bile-responsive nuclear receptors such as farnesoid X receptor (FXR), vitamin D receptor (VDR), and pregnane X receptor (PXR) as well as G-protein-coupled receptors including takeda G-protein coupled receptor 5 (TGR5) and muscarinic receptors (Li and Chiang 2014; Zhou and Hylemon, 2014). The bile acid signalling network provides a feedback mechanism that regulates bile acid synthesis along a gut-microbiota-liver axis. Moreover, bile acid signalling also directly regulates important gastrointestinal and systemic metabolic functions including cholesterol, triglyceride (TG) and glucose metabolism, energy expenditure, electrolyte

transport, and immune function (Ichikawa et al., 2012; Inagaki et al., 2006; Li and Chiang, 2014; Ward et al., 2013). The most important receptors in these bile acid signalling pathways, FXR and TGR5 are discussed in some more detail in the following section.

The FXR is a nuclear receptor of bile acids which is expressed in gastrointestinal epithelial cells and in the liver (Li and Chiang, 2014). Generation of unconjugated bile acids through microbial BSH activity has significant potential to locally influence FXR signalling in the gut (Joyce et al., 2014; Sayin et al., 2013). Furthermore, it is known that the murine bile acid tauro-β-muricholic acid (TβMCA) is a potent antagonist of FXR (Sayin et al., 2013). Notably, an increased production of fecal unconjugated bile acids as well as increased levels of TβMCA, as observed in cyhalothrin treated mice in **Chapter 6**, may impact the function of FXR. In addition, conjugated bile acids could also be potent activators of the TGR5, with TLCA being the most potent agonist (Li and Chiang, 2014). This suggests that hydrolase and dehydroxylase activities are crucial microbial conversions of bile acids, which have significant potential to influence the signalling capacities of bile acids with downstream effects on host cellular responses. Moreover, FXR also plays a significant role in the maintenance of host TG and cholesterol homeostasis, since mechanistic studies have reported the role of FXR in reducing hepatic lipogenesis, increasing fatty acid oxidation, and promoting the clearance of plasma TG (Li and Chiang, 2014). Given that elevated plasma TG and cholesterol are markers of increased risk of metabolic disease it would be of interest for future studies to investigate whether the reported antibiotic and pesticide induced changes in bile acid profiles, result in increased host TG and cholesterol levels as risk factors for cardiovacular disease. In addition to playing a significant role in lipid metabolism, bile acid signalling via FXR also influences glucose homeostasis and energy regulation. As observed in the 28-day oral dose in vivo study of **Chapter 6**, the cyhalothrin-treated mice presented a significant decrease in body weight as well as food consumption especially in female mice which may be related to this gluconeogenesis. Additionally, TGR5 modulated by bile acid signalling was also highlighted to be crucial in the control of weight gain and metabolic rate in mice (Watanabe et al., 2006). Moreover, FXR and TGR5 play important roles in immune regulation on the basis of bile acid signalling. FXR is thought to have anti-inflammatory capacity in the host as demonstrated in models of liver injury (Zhang et al., 2009). In contrast, TGR5 is likely to be able to regulate macrophage activity via bile acid signalling (Calmus and Poupon, 2014). Considering potential beneficial effects of TLCA and other TGR5 ligands it has been

reported that they could reduce the expression of proinflammatory cytokines and induce the anti-inflammatory cytokine IL-10 in response to LPS in primary human macrophages (Haselow et al., 2013).

Macroscopically, changes in the bile acid profiles and size of the bile acid pool also show associations with diseases such as obesity, bile acid diarrhea (BAD), irritable bowel syndrome (IBS), type 2 diabetes (T2D), and *C. difficile* infection (CDI)(Joyce and Gahan, 2016). Obesity has been associated with an increase in the secondary bile acid DCA and a decrease in the level of the primary bile acid CA (Hara, 2015). In addition, predisposition to obesity is found to be correlated with low levels of gut microbial richness and diversity, and strongly related to high plasma bile acid concentrations (Prinz et al., 2015). Meanwhile, elevations in specific plasma bile acids are found to be associated with type 2 diabetes (T2D)(Wewalka et al., 2014). BAD occurs as a result of dysfunctional bile acid absorption in the ileum linked to underlying pathologies (Camilleri, 2015). IBS is a common gut functional disorder and IBS-diarrhea is associated with increased fecal levels of unconjugated bile acids CA, DCA, and CDCA, whereas the secondary bile acid LCA has been linked to IBS-constipation (Duboc et al., 2012). In another previous work, it has been reported that the consumption of dietary polyphenols could lead to the increase of fecal secondary bile acids DCA and LCA, relating to a raised risk of colon cancer as well (Han et al., 2009). Meanwhile, bile acids also play a significant role in the pathogenesis of *C. difficile* infection (CDI) which can result in effects that range from mild diarrhea to pseudomembranous colitis (PMC) and even death (Bartlett and Gerding, 2008). And it has been reported that the secondary bile acid DCA is capable of inhibiting growth of vegetative cells of *C. difficile* (Sorg and Sonenshein, 2008), thereby inhibiting infection (Buffie et al., 2015). Indeed, broad-spectrum antibiotic therapy reduces bacteria particularly, *C. scindens* that are involved in the generation of secondary bile acids (DCA), thereby reducing DCA and inducing patients sensitivity to *C. difficile* infection (Buffie et al., 2015).

Overall, bile acids clearly mediate important functions in the host that extend beyond their role as biological detergents. The gut microbiota undoubtedly plays a central role in bile acid metabolism, and bile acids have therefore emerged as major effectors in microbe-host signalling events that influence host energy metabolism, weight gain, and inflammation. Clearly this indicates that the potential consequences for human health of

the changes in gut microbiota and related bile acid profiles induced by the antibiotics and pesticides studied in the present thesis provide an interesting topic for further research.

6.3 Overall conclusion

Compared to other signalling hormones, bile acids have the capacity to act as the signalling agents beyond the gut due to their ability of influencing a diverse range of physiological processes (Zhou and Hylemon, 2014). They interact either locally or systemically with specific cellular receptors, in particular the FXR and TGR5. These signalling functions influence systemic lipid and cholesterol metabolism, energy metabolism, immune homeostasis, and intestinal electrolyte balance. Through defined enzymatic activities, the gut microbiota can significantly modify the signalling properties of bile acids and therefore can have an impact on host health. The gut microbiota-mediated bile acid metabolism has been related to metabolic disease, obesity, diarrhea, inflammatory bowel disease (IBD), *Clostridium difficile* infection, colorectal cancer, and hepatocellular carcinoma. The results of the present thesis not only provide a proof of principle for the application of a 24 h in vitro batch fermentation model to study effects on the gut bacterial community and related bile acid metabolism induced by antibiotics and pesticides, but also provides evidence for the impact of these xenobiotics on gut microbiota-mediated bile acid profiling, involving an increase in primary bile acids and a decrease in secondary bile acids upon treatment with lincosamides, and, conversely, raised levels of secondary bile acids at the cost of primary bile acids upon exposure to the tested pesticides.

It is concluded that the gut microbiome and its related bile acid metabolism, which are both crucial for host heath, can be affected by orally ingested antibiotics and pesticides, and may provide a novel target to be considered in future safety evaluations.

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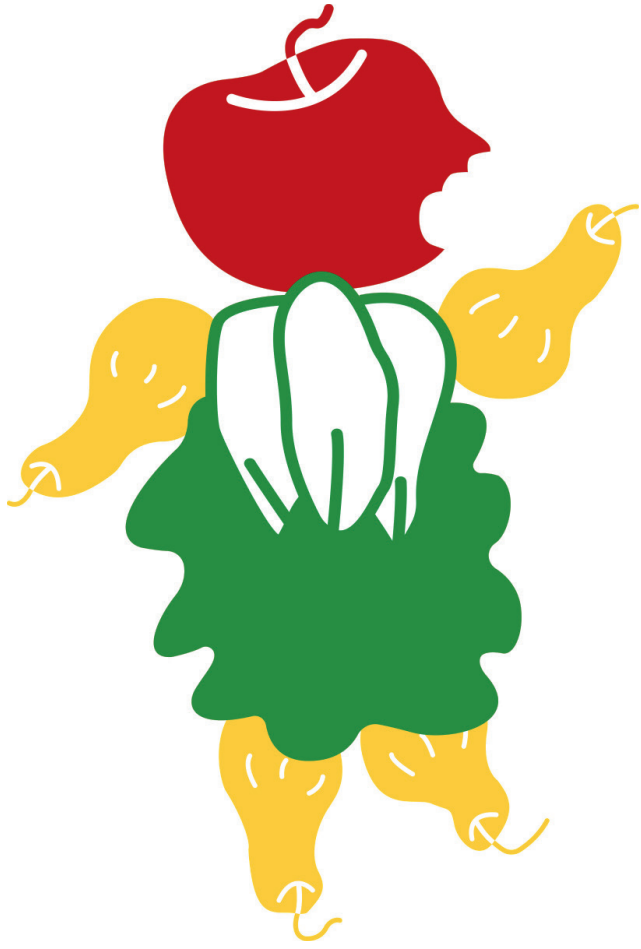


APPENDIX

Table S2.1 Initial bile acid levels in freshly isolated, washed and T-BA-treated fecal samples (mean± SD, n=3).

Bile acids	In freshly isolated feces (μM)	In washed feces (μM)	In T-BAs added feces (μM)
T-BAs			
TCA	0.09±0.01	0.05±0.01	30.77±0.25
TUDCA	0.02±0.01	0.03±0.01	4.74±0.68
TβMCA	0.07±0.02	0.02±0.00	4.91±0.03
TαMCA	0.04±0.01	0.02±0.01	1.43±0.31
Total	0.22±0.03	0.12±0.00	41.58±0.89
Unconjugated bile acids			
CA	5.10±0.23	0.07±0.02	17.32±0.81
CDCA	0.51±0.03	0.35±0.43	0.51±0.03
αMCA	8.32±0.30	0.83±0.07	1.88±0.14
βMCA	25.68±0.96	2.50±0.23	2.41±0.47
UDCA	1.91±0.15	0.16±0.03	0.21±0.03
ωMCA	52.26±1.65	4.48±0.34	3.37±0.50
HDCA	28.88±1.65	3.66±0.54	4.99±0.20
DCA	15.36±0.39	3.97±0.16	4.52±0.38
LCA	7.81±0.37	5.27±0.13	5.66±0.33
Total	145.84±4.58	21.29±0.68	40.87±0.36

Table S3.1 MS parameters and limits of detection for bile acids studied.

Analytes	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision energy (eV)	LOD* (nM)
Cholic acid (CA)	407.3	407.3	25	5
Taurocholic acid (TCA)	514.4	514.4	30	5
Deoxycholic acid (DCA)	391.3	391.3	15	5
Lithocholic acid (LCA)	375.3	375.3	30	10
Ursodeoxycholic acid (UDCA)	391.3	391.3	15	40
Hyodeoxycholic acid (HDCA)	391.3	391.3	15	40
α-Muricholate (αMCA)	407.3	407.3	25	5
β-Muricholate (βMCA)	407.3	407.3	25	5
Tauro-β-muricholate (TβMCA)	514.4	514.4	30	10
ω-Muricholate (ωMCA)	407.3	407.3	25	5

*LOD, limit of detection

Table S5.1 MRM data acquisition parameters, ESI parameters, and the gradient profile of the LC-MS/MS procedure for twenty-four targeted bile acids.

Bile acids	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision energy (eV)	Bile acids	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision energy (eV)
TCA	514.4	514.4	30	GLCA	432.3	74	55
TCDCA	498.4	498.4	55	GHDCA	448.3	74.0	43
TαMCA	514.4	514.4	30	GUDCA	448.3	74.0	43
TβMCA	514.4	514.4	30	CA	407.3	407.3	25
TDCA	498.4	498.4	55	CDCA	391.3	391.3	15
TLCA	482.3	482.3	54	αMCA	407.3	407.3	25
TωMCA	514.4	514.4	30	βMCA	407.3	407.3	25
THDCA	498.4	498.4	55	DCA	391.3	391.3	15
TUDCA	498.4	498.4	55	LCA	375.3	375.3	30
GCA	464.3	74.0	43	ωMCA	407.3	407.3	25
GCDCA	448.3	74.0	43	HDCA	391.3	391.3	15
GDCA	448.3	74.0	43	UDCA	391.3	391.3	15

ESI parameters:

Nebulizing gas flow: 3 L/min; Drying gas flow: 10 L/min; Heating gas flow: 10 L/min

Interface temperature: 300°C; Desolvation temperature: 526°C; Heat block temperature: 400°C

Gradient profile:

Time	Solvent A%	Solvent B%	Solvent C%	Time	Solvent A%	Solvent B%	Solvent C%
0 min	95	0	5	10 min	2	98	0
2 min	95	0	5	10.5 min	70	30	0
7.5 min	30	70	0	13 min	95	0	5
7.6 min	2	98	0	16 min	95	0	5

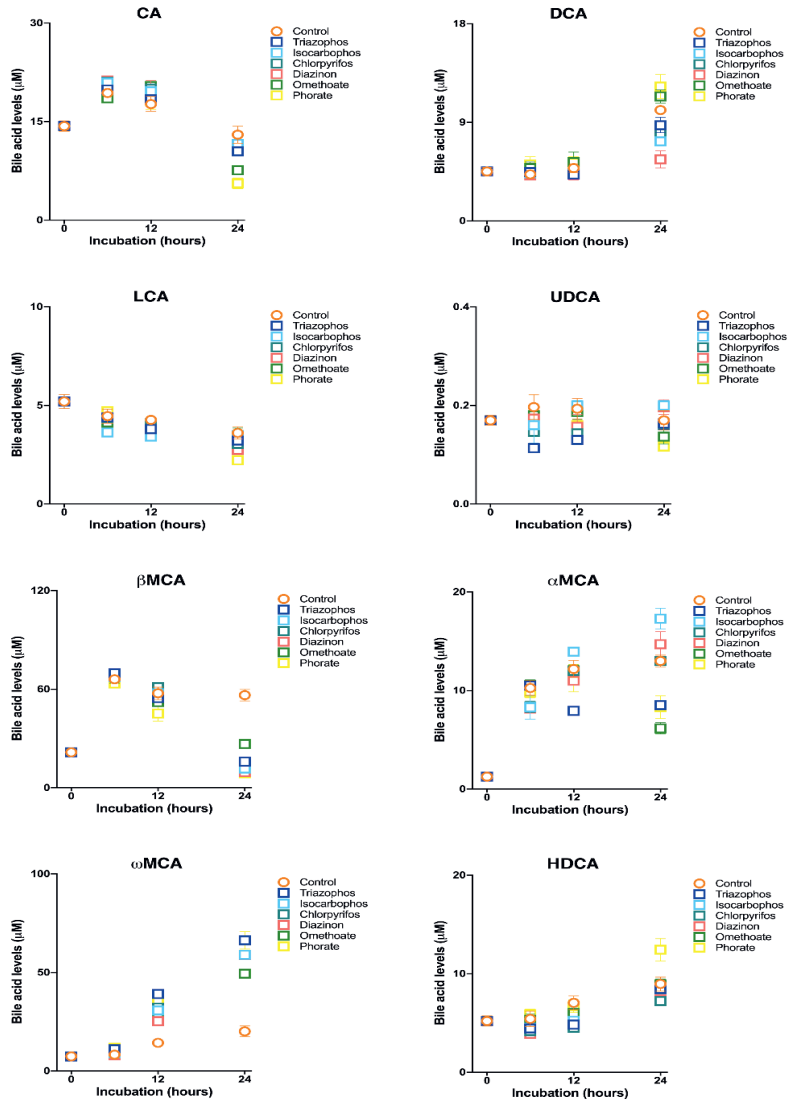


Figure S3.1 Time dependent changes in bile acid profiles in control and OP treated fecal samples up to 24 h in vitro anaerobic incubations. Samples were taken at 0, 6, 12, and 24 h during the fermentation separately from control, triazophos or isocarbophos or chlorpyrifos or diazinon or omethoate or phorate treated groups (n=3).

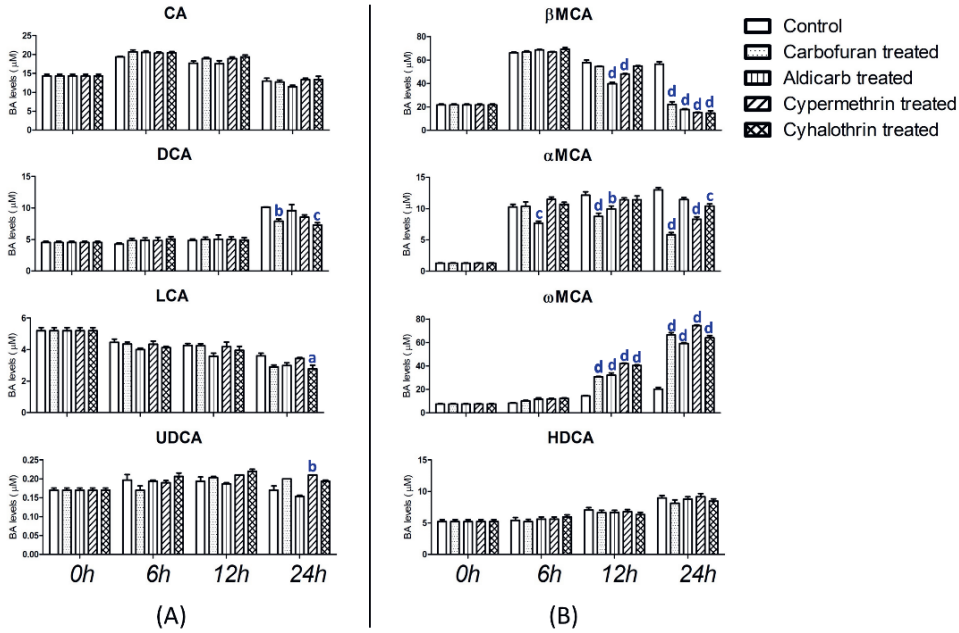


Figure S4.1 Bile acid changes in in vitro anaerobic mouse fecal incubations with or without (control) treatment with carbamates (carbofuran and aldicarb) and pyrethroids (cypermethrin and cyhalothrin) following the 24 h incubations (n=3). Transformation of (A) the added TCA, and the resulting changes in TCA, CA, DCA, LCA, and UDCA, as well as (B) the added TβMCA and the resulting changes in βMCA, αMCA, ωMCA, and HDCA. Samples were taken at 0 h, 6 h, 12 h, and 24 h during the incubation, and significance was marked using a (*p <0.05), b (**p <0.01), c (***p <0.001), d (****p <0.0001), which indicates a difference with control.

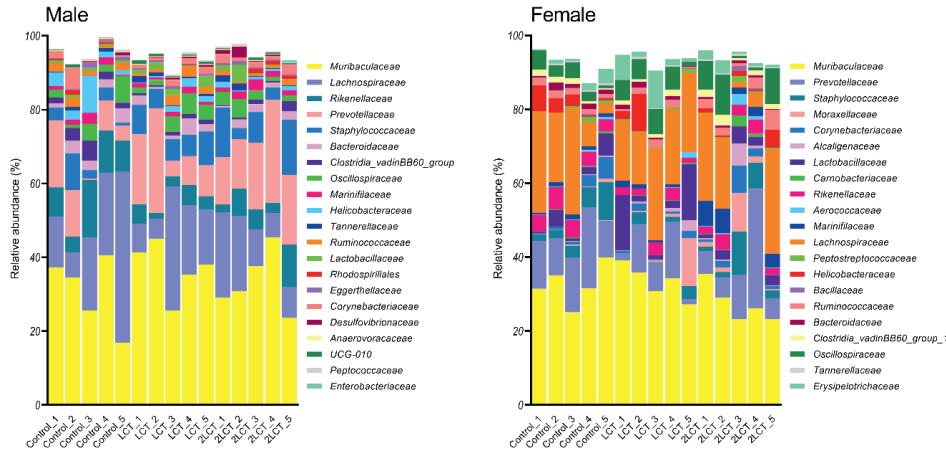


Figure S5.1 Relative abundance of gut microbial community at family level in fecal samples from male and female mice of LCT, 2LCT, and control groups on day 28. The dominant twenty-one bacterial families in fecal samples from male and female mice are shown (n=5 per sex per group).

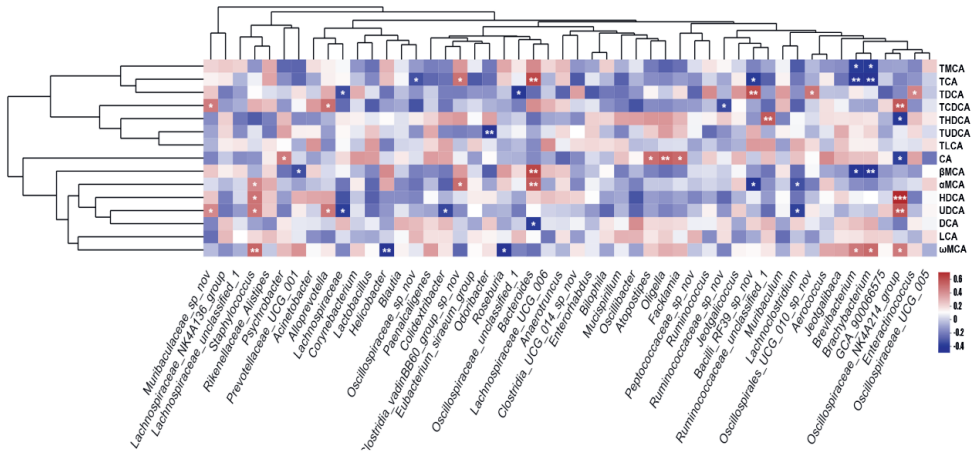


Figure S5.2 The heatmap presentation with pearson correlation coefficients between the main fifty genera in mouse fecal samples and levels of the detected bile acids in fecal samples from male and female control and lambda-cyhalothrin treated mice on day 28. The positive and negative correlation are separately presented by colours of red and blue (*p <0.05, **p <0.01, ***p <0.001).

SUMMARY

The gut microbiota consists of trillions of microorganisms residing in the intestine and has a mutual relationship with its host. It plays a crucial role in maintaining host health, enhancing the immune system and regulating host homeostasis and metabolism. The metabolic patterns modulated by the gut microbiota such as bile acid metabolism are also reported to be sensitive to external stimuli including environmental or food-borne xenobiotics, for example antibiotics and pesticides. Additionally, altered bile acid signalling has been reported to be associated with multiple adverse health effects, such as obesity, diabetes and colon cancers. However, the changes in bile acid profiling due to the altered gut microbial community induced by exposure to antibiotics and pesticides remain unclear. In line with the 3R principles (replacement, refinement and reduction of animal experiments), an alternative in vitro fermentation batch model for studying changes in the gut microbiota-dependent bile acid profiles induced by xenobiotics was developed in **Chapter 2**. Sample processing and bile acid incubation conditions were developed and optimized using feces from rats and the prepared fecal samples were incubated for 24 h with or without lincosamide antibiotics (lincomycin and clindamycin), which have been reported for their effects on the gut bacterial community and related fecal bile acid profiles in a previous rat in vivo study. Upon treatment of the fecal gut microbiota with lincosamides, a reduced richness of the families *Erysipelotrichaceae* and *Bacteroidacea* was shown, resulting in significantly increased and decreased levels of fecal primary and secondary bile acids respectively, as quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS). Further, results obtained in this in vitro model were evaluated by comparison to those from the previous in vivo study. Similarities in the effects of the lincosamide antibiotics on the bacterial and related bile acid profiles in vivo and in the present in vitro work, provide a proof of principle for application of the model to detect such effects in vitro. Hence in the subsequent two chapters, this in vitro fermentation model was utilized for evaluating effects of some commonly used pesticides on the gut bacteria and related bile acid profiles.

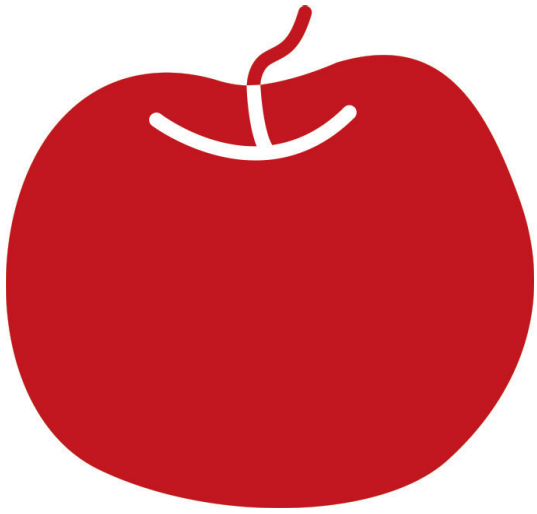
In **Chapter 3**, effects of organophosphate pesticides on gut microbiota mediated bile acid metabolism were investigated using this simple batch fermentation in vitro model, in this case using mouse fecal samples that were incubated with triazophos, isocarbophos, chlorpyrifos, diazinon, omethoate, or phorate and a bile acid mixture optimized for bile aid profiles expected in mice. Results showed that treatment with the organophosphates induced microbiota dependent alterations of primary and secondary bile acid levels,

including especially substantially increased production of ω-muricholate (ωMCA) and decreased levels of β-muricholate (βMCA). Additionally, of all the organophosphates exposure of the fecal microbiota to phorate led to the most wide and significant effects on the bile acid profile. As a result, phorate was selected for further determination of accompanying effects on the bacterial profile by 16S rRNA sequencing. After exposure to phorate, the related richness of the *Muribaculaceae spp*. was found to be significantly decreased in the mouse fecal microbial community, and the genera *Coriobacteriaceae UCG-002* and *Muribaculum* were shown to be highly correlated with the altered bile acid profiles. In addition to organophosphates, effects of carbamates (carbofuran, aldicarb) and pyrethroids (cypermethrin and cyhalothrin) on gut microbiota and related bile acid metabolism were studied in **Chapter 4**. After exposure to carbamates and pyrethroids, a significantly increased ratio of secondary over primary bile acids, particularly resulting from the enrichment of βMCA accompanied by the depletion of ωMCA, was observed. Besides, the bacterial profile showed significantly increased richness of *Eggerthellaceae* after 24 h exposure to carbofuran or cyhalothrin, and the genera *Enterorhabdus* belonging to *Eggerthellaceae* were found to be highly related to the fecal bile acid profile. A principal coordinates analysis (PCoA) of the altered bile acid profiles was performed and bile acid profiles observed in fecal samples exposed to cyhalothrin were found to be the most clearly separated from controls, revealing a substantial impact on the gut microbiota-mediated bile acid metabolism. As a result, the toxic paired enantiomeric isomers of the pyrethroid cyhalothrin, lambda-cyhalothrin was selected to be further studied in an in vivo mouse study.

In **Chapter 5**, changes of the intestinal microbial community and its related bile acid metabolism in plasma and feces induced by the treatment with lambda-cyhalothrin were evaluated in male and female mice. Results obtained showed that the total amount of bile acids in plasma and fecal samples from lambda-cyhalothrin treated mice markedly increased compared to controls, and these changes could be ascribed to the significantly raised proportion of taurine conjugated bile acids in plasma together with an increase in fecal secondary bile acids. In gut microbial profiles, a significantly increased richness of *Prevotellacea* at the cost of *Lachnospiraceae* were found at the family level upon exposure to lambda-cyhalothrin. It was concluded that treatment of mice with lambda-cyhalothrin affected the gut microbiota with accompanying changes in bile acid homeostasis, and that

the effects on fecal bile acid profiles were in line with those acquired in the in vitro model system in **Chapter 4**.

Overall, the current project provided a proof of principle for the application of the developed 24 h in vitro fermentation batch model, including the method for sample preparation and bile acid analysis, for studying the modulation of bile acid metabolism via effects on the gut bacterial community. Based on this in vitro model as well as a further in vivo study, treatment with antibiotics and pesticides were found to be able to result in changes in gut microbial and bile acid profiles. It is concluded that the gut microbiome and its related bile acid metabolism, which are both crucial for host heath, can be affected by orally ingested antibiotics and pesticides, and may provide a novel target to be considered in future safety evaluations.



ACKNOWLEDGEMENTS

Delightedly and sadly, my PhD journey ends! Herein, I would like to thank many many people who were accompanying me and supporting me in various ways.

First, I would like to express my very great appreciation to my promotor **Ivonne Rietjens**, co-promotor **Jing Wang**, daily supervisor **Jacques Vervoort** and **Maojun Jin**.

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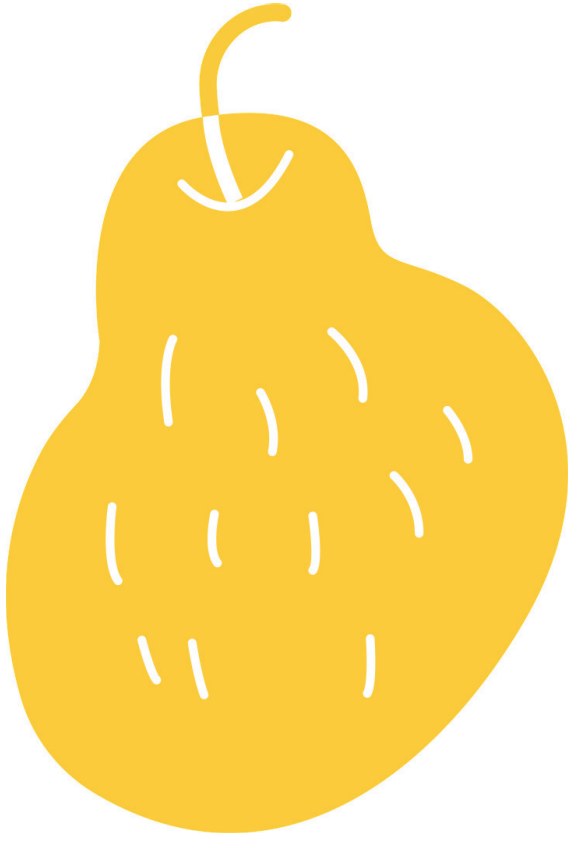

ABOUT THE AUTHOR

Curriculum Vitae

Weijia Zheng was born in Harbin, China. In 2015, Weijia completed her 5-year Bachelor Degree programme in Veterinary Medicine from Northeast Agricultural University (NEAU). In the second year of her undergraduate study, she studied at Konkuk University (KU) in Seoul, South Korea, and at the end of her bachelor she went back to China and did her thesis in the Animal Hospital of NEAU. In 2015, Weijia moved to Seoul and continued her education with a major in Pharmacology and Toxicology, at the department of Veterinary Medicine at KU. During her master study, she was working for the Korean Ministry of Food and Drug Safety (MFDS) and doing many research projects related to the method development for detection of various drug residues in animal-derived food products as well as to the ADME (absorption, distribution, metabolism and excretion) of chemicals. In August 2018, she obtained her Master of Science in Veterinary Medicine, moved to Beijing, and started to arrange her PhD project at the Chinese Academy of Agricultural Sciences (CAAS). Her PhD project was supported by CAAS in collaboration with chair groups of Toxicology and Biochemistry in Wageningen University and Research (WUR). In 2019, she moved to Netherlands and started her PhD research on studying the effects of xenobiotics on gut microbiota and related bile acid metabolism by in vitro and in vivo models. During her PhD, she followed the postgraduate education in toxicology (PET) required for the registration as European Registered Toxicologist (ERT) and also obtained the Chinese animal testing license.

List of publications

PhD Period

Zheng, W., Bakker, W., Baccaro, M., Jin, M., Wang, J., Rietjens, I.M.C.M. (2022). A simple in vitro fermentation model to detect alterations in microbiota dependent bile acid metabolism. *Submitted*.

Zheng, W., Bakker, W., Jin, M., Wang, J., Rietjens, I.M.C.M. (2022). Organophosphate pesticides modulate gut microbiota and influence bile acid metabolism in an in vitro fermentation model. *Submitted*.

Zheng, W., Bakker, W., Jin, M., Wang, J., Rietjens, I.M.C.M. (2022). Impact of carbamate and pyrethroid pesticides on bile acid profiles in an in vitro gut microbiota model. *Submitted*.

Zheng, W., Xu, L., Jin, M., Wang, J., Rietjens, I.M.C.M. (2023). Effects of lamba-cyhalothrin on gut microbiota and related bile acid metabolism in mice. *Submitted*.

Zhang, N., **Zheng, W**., Bakker, W., van Ravenzwaay, B., & Rietjens, I.M.C.M. (2023). In vitro models to measure effects on intestinal deconjugation and transport of mixtures of bile acids. *Chemico-Biological Interactions*, 110445.

Wang, J., Bakker, W., **Zheng, W.**, de Haan, L., Rietjens, I. M., & Bouwmeester, H. (2022). Exposure to the mycotoxin deoxynivalenol reduces the transport of conjugated bile acids by intestinal Caco-2 cells. *Archives of Toxicology*, 96(5), 1473-1482.

De Bruijn, V. M., Wang, Z., Bakker, W., **Zheng, W.**, Spee, B., & Bouwmeester, H. (2022). Hepatic bile acid synthesis and secretion: Comparison of in vitro methods. *Toxicology Letters*, 365, 46-60.

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Bachelor and Master Period (only 1st, 2nd, and 3rd author included)

Zheng, W., Yoo, K.-H., Choi, J.-M., Park, D.-H., Kim, S.-K., Kang, Y.-S., . . . Bekhit, A. E.-D. (2019). A modified QuEChERS method coupled with liquid chromatography-tandem mass spectrometry for the simultaneous detection and quantification of scopolamine, L-

hyoscyamine, and sparteine residues in animal-derived food products. *Journal of Advanced Research*, 15, 95-102.

Zheng, W., Yoo, K.-H., Abd El-Aty, A., Park, D.-H., Choi, J.-M., Kim, S.-K., . . . Bekhit, A. E.-D. (2019). Quantitative determination of carbasalate calcium derived metabolites, acetylsalicylic acid and salicylic acid, in six animal foods using liquid-liquid extraction method coupled with liquid chromatography-tandem mass spectrometry. *Food chemistry*, 278, 744-750.

Zheng, W., Yoo, K. H., Choi, J. M., Park, D. H., Kim, S. K., Kang, Y. S., . . . Shim, J. H. (2019). Residual detection of naproxen, methyltestosterone and 17α-hydroxyprogesterone caproate in aquatic products by simple liquid–liquid extraction method coupled with liquid chromatography–tandem mass spectrometry. *Biomedical Chromatography*, 33(1), e4396.

Zheng, W., Choi, J. M., Abd El-Aty, A., Yoo, K. H., Park, D. H., Kim, S. K., . . . Shim, J. H. (2019). Simultaneous determination of spinosad, temephos, and piperonyl butoxide in animal-derived foods using LC–MS/MS. *Biomedical Chromatography*, 33(6), e4493.

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Zheng, W., Abd El-Aty, A., Kim, S. K., Choi, J. M., Hacımüftüoğlu, A., Shim, J. H., . . . Shin, H. C. (2018). Quantification of artesunate and its metabolite, dihydroartemisinin, in animal products using liquid chromatography–tandem mass spectrometry. *Journal of separation science*, 41(18), 3538-3546.

Zheng, W., Park, J.-A., Abd El-Aty, A., Kim, S.-K., Cho, S.-H., Choi, J.-m., . . . Jeong, J. H. (2018). Development and validation of modified QuEChERS method coupled with LC–MS/MS for simultaneous determination of cymiazole, fipronil, coumaphos, fluvalinate, amitraz, and its metabolite in various types of honey and royal jelly. *Journal of Chromatography B*, 1072, 60-69.

Zheng, W., Park, J. A., Abd El-Aty, A., Kim, S. K., Cho, S. H., Choi, J. M., . . . Shin, H. C. (2018). Development and validation of a simple solid-phase extraction method coupled with

liquid chromatography–triple quadrupole tandem mass spectrometry for simultaneous determination of lincomycin, tylosin A and tylosin B in royal jelly. *Biomedical Chromatography*, 32(4), e4145.

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Zheng, W., Park, J.-A., Zhang, D., Abd El-Aty, A., Kim, S.-K., Cho, S.-H., . . . Kim, J.-S. (2017). Determination of fenobucarb residues in animal and aquatic food products using liquid chromatography-tandem mass spectrometry coupled with a QuEChERS extraction method. *Journal of Chromatography B*, 1058, 1-7.

Zheng, W., Park, J.-A., Abd El-Aty, A., Kim, S.-K., Cho, S.-H., Choi, J.-M., . . . Shim, J.-H. (2017). Bithionol residue analysis in animal-derived food products by an effective and rugged extraction method coupled with liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B*, 1064, 100-108.

Choi, J. M., **Zheng, W.**, Abd El-Aty, A., Kim, S. K., Park, D. H., Yoo, K. H., . . . Jeong, J. H. (2019). Residue analysis of tebufenozide and indoxacarb in chicken muscle, milk, egg and aquatic animal products using liquid chromatography–tandem mass spectrometry. *Biomedical Chromatography*, 33(7), e4522.

Cho, S.-H., Park, J.-A., **Zheng, W.**, Abd El-Aty, A., Kim, S.-K., Choi, J.-M., . . . Shim, J.-H. (2017). Quantification of bupivacaine hydrochloride and isoflupredone acetate residues in porcine muscle, beef, milk, egg, shrimp, flatfish, and eel using a simplified extraction method coupled with liquid chromatography–triple quadrupole tandem mass spectrometry. *Journal of Chromatography B*, 1065, 29-34.

Yang, Z., Liu, C., **Zheng, W.**, Teng, X., & Li, S. (2016). The functions of antioxidants and heat shock proteins are altered in the immune organs of selenium-deficient broiler chickens. *Biological trace element research*, 169, 341-351.

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Overview of completed training activities

Discipline specific activities		
Courses		
Molecular Toxicology	PET, Amsterdam	2019
Cellular Toxicology	PET, Leiden	2019
Laboratory Animal Science	PET, Utrecht	2019
Risk Assessment	PET, Wageningen	2019
Mutagenesis & Carcinogenesis	PET, Leiden	2019
Medical and Forensic Toxicology	PET, Utrecht	2019
Organ Toxicology	PET, Nijmegen	2020
Epidemiology	PET, Online	2020
Pathobiology	PET, Online	2021
Meetings		
16th International Food Safety & Technologies	CBIFS, Hangzhou, China	2021
61th Society Of Toxicology Annual Meeting	SOT, online	2022
American Society for Microbiology Annual Meeting (registered)	ASM, Houston, USA	2023
General courses		
Effective Academic Development	WGS, Beijing, China	2018
VLAG PhD week	VLAG, Wageningen	2019
Project and Time Management	WGS, Wageningen	2019
Reviewing a Scientific Paper	WGS, Wageningen	2019
Other activities		
Preparing research proposal	VLAG, Wageningen	2019
Environmental Toxicology (TOX30806)	WGS, Wageningen	2019
Food Toxicology (TOX30306)	WGS, Wageningen	2019
General Toxicology (TOX20303)	WGS, Wageningen	2019
Reviewing scientific articles	Food Chemistry & Analytical Biochemistry	2021 -2023

Approved by Graduate School VLAG.

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