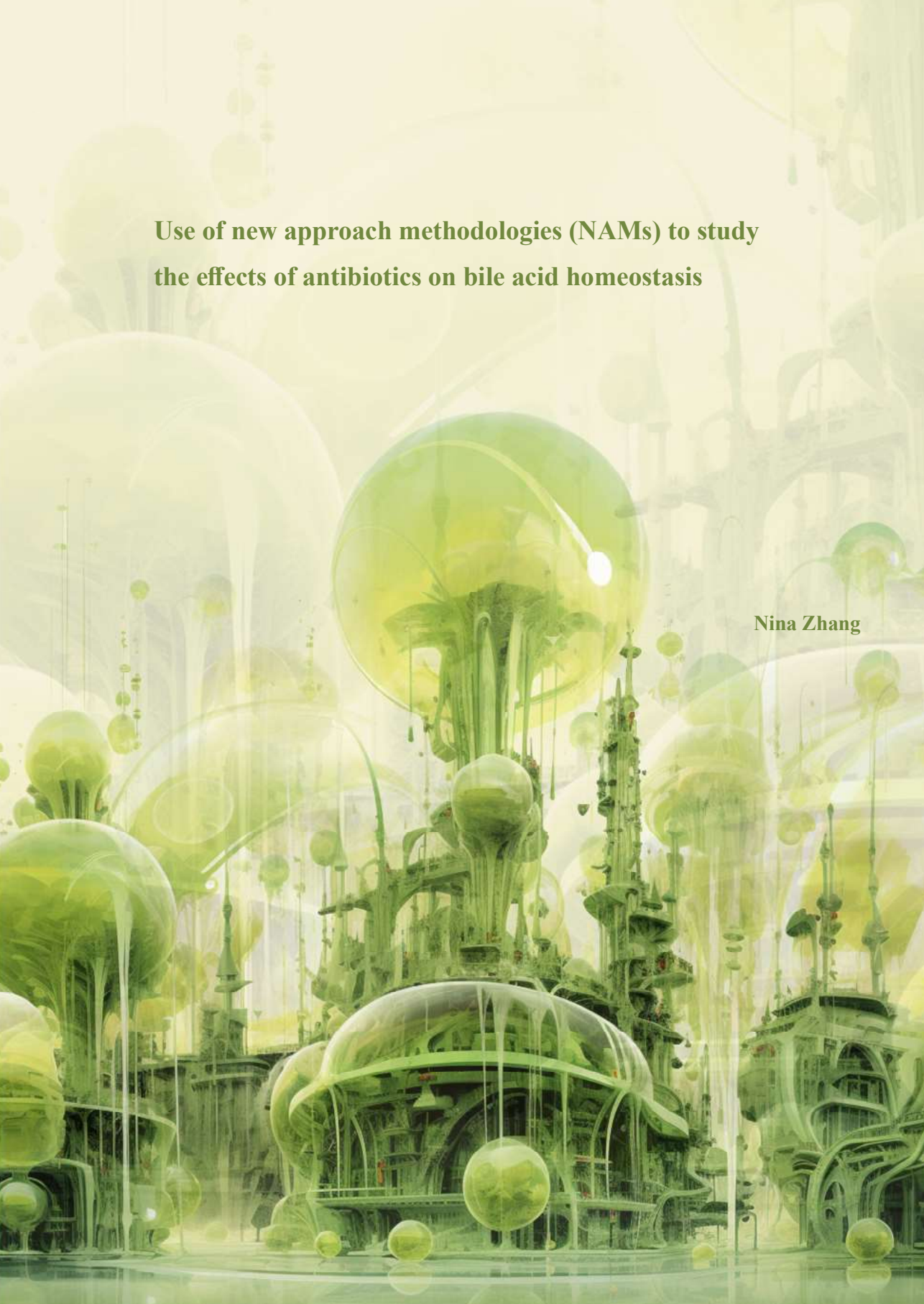
Use of new approach methodologies (NAMs) to study the effects of antibiotics on bile acid homeostasis

Nina Zhang



Propositions

1.New approach methodologies (NAMs) are able to elucidate the modes of action underlying the effects of antibiotics on host fecal and plasma metabolome patterns.
(this thesis)

2. Studying effects on bile acid homeostasis using *in vitro* models is best done with a mixture instead of individual bile acids.
(this thesis)

3. Artificial intelligence advances personalized drug therapy.

4. User friendly databases make life safer and easier.

5. It is better not to travel together with your good friends.

6. It is brave to choose a career based on intellectual interests instead of on social popularity.

Propositions belonging to the thesis, entitled

Use of new approach methodologies (NAMs) to study the effects of antibiotics on bile acid homeostasis

Nina Zhang

Wageningen, 16 October 2023

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Use of new approach methodologies (NAMs) to study the effects of antibiotics on bile acid homeostasis

Nina Zhang

Thesis

submitted in fulfilment of the requirements for the degree of doctor

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Chapter 1.

General introduction

1.1 Introduction and aim of the present thesis

The gut microbiota plays an important role in the health and wellbeing of its host organism and is also known to play a major role in the metabolism of molecules obtained from the diet. Such processes include amongst others: calorie production, synthesis of essential amino acids and certain vitamins, metabolism of bile acids and lipids. Other than their contribution to overall host metabolism, the microbiota also plays a role in the host immune system, maintaining host homeostasis and protecting the host from infections and disease (Cani and Delzenne 2009; Forbes et al. 2016; Gill et al. 2006; Hill 1997; Neis et al. 2015; Sagar et al. 2015). To better understand the contribution of the intestinal microbiota to the overall host metabolism and health, linking changes in the microbial community composition to changes in functionality is important. Advances in sequencing techniques have resulted in convenient tools that allow characterization of the gut microbial communities contributing to a better understanding of their importance (Gilbert et al. 2016).

Using these techniques, many studies characterized the effect of xenobiotics on gut microbial composition (Lindell et al. 2022; van Dongen et al. 2022). More recent studies did not only focus on the xenobiotic-induced changes in the gut microbial composition but also on consequences for its functionality, the latter by characterizing plasma or fecal metabolic patterns by metabolomics. Several *in vivo* rat studies for example investigated the interactions between gut microbiota and the fecal and plasma metabolome reporting effects of antibiotics on not only the intestinal bacterial composition but also on the fecal and plasma metabolome showing for example changes in bile acid profiles (Behr et al. 2018; Behr et al. 2019; Murali et al. 2023a; Murali et al. 2023b). In these studies, antibiotics which were known to have low or no systemic bioavailability and to be able to alter gut microbiota composition were orally administrated for 28 days to Wistar rats, after which fecal and plasma samples were collected for analysis of the gut microbial composition and the fecal and plasma metabolome. The results obtained demonstrated that fecal and plasma bile acid levels changed (Behr et al. 2019; Murali et al. 2023a; Murali et al. 2023b). Such *in vivo* animal studies, however, are expensive and time consuming, while they also bring ethical issues and raise the question of whether effects observed in experimental animals are relevant for human. Hence, in more recent years efforts are directed to reduce the use of experimental animals based on the 3Rs principle (Replacement, Reduction and Refinement) for use of experimental animals when evaluating the effects of chemicals to inform chemical risk and safety assessment. Application of new approach methodologies (NAMs) including *in silico*, *in chemico*, *in vitro* and *ex vivo* approaches to study chemical hazards and risks without use of experimental animals is gaining momentum (Parish et al. 2020; Stucki et al. 2022).

The aim of the present thesis was to investigate the use of new approach methodologies (NAMs) for studies on the effects of antibiotics on bile acid homoeostasis. Studying the same antibiotics as studied before in the 28 day rat studies will enable evaluation of the *in vitro* models and the results obtained. To this end selected *in vitro* model systems were evaluated

for their ability to simulate *in vivo* bile acid changes induced by antibiotics and to study the underlying modes of action. The results obtained were also used to define physiologically based kinetic (PBK) models, able to predict the effects of detected *in vitro* for the *in vivo* plasma bile acid concentrations in human. The *in vitro* model systems applied consisted of *in vitro* anaerobic fecal incubations and *in vitro* bile acid transport across Caco-2 cell layers in transwells. The bile acids were detected via LC-MS/MS, and 16S rRNA analysis was used to study effects of the antibiotics on gut microbiota composition. Altogether, the effects of a series of antibiotics (tobramycin, colistin sulfate, meropenem trihydrate, doripenem hydrate, lincomycin, streptomycin and vancomycin) on bile acid homeostasis were evaluated by the novel *in vitro* and *in silico* models. Validation of the new *in vitro* and *in silico* models was achieved by comparison of the *in vitro* results to reported outcomes of the effects of the selected antibiotics on bile acid homeostasis in the *in vivo* 28 days rat studies (Behr et al. 2018; Behr et al. 2019; Murali et al. 2023a; Murali et al. 2023b). A PBK model for bile acid kinetics in human was used to study the consequences of exposure to one of the studied antibiotics, tobramycin, on plasma bile acid levels in human.

1.2 Gut microbiota

The number of gut microbiota has been estimated to exceed 10^{13} in a recent revision, and the ratio between human and bacterial cells in the body has been estimated to be close to 1:1 (Sender et al. 2016). As a consequence of the large number of bacteria inhabiting the host, they are often regarded as a separate organ (Thursby and Juge 2017). The dominant microbiota in the human intestine belong to three main phyla: 30 - 52% Firmicutes, 9 - 42% Bacteroidetes, and 1 - 13% Actinobacteria, with other phyla making up only 2% (Rajoka et al. 2017). The gut microbiota plays an important role in human health by contributing to host metabolism via the activity of microbial enzymes. The intestinal microbiota can ferment dietary carbohydrates producing energy sources - short chain fatty acids (SCFAs) and gases (Cummings and Macfarlane 1991; Macfarlane et al. 1992; Rowland et al. 2018). The gut microbiota is also able to degrade proteins into amino acids, SCFAs, and gases (Macfarlane et al. 1986). It is well known that the gut microbiota can synthesize vitamin K and a variety of B vitamins such as biotin, cobalamin, riboflavin and thiamine (Hill 1997). The gut microbiota also changes bile acid structures and composition via microbial biotransformation (Begley et al. 2005). It has also been reported that the colonic microbiota metabolizes polyphenols thereby affecting their bioactivity, for example converting, in a subgroup of the population, the soy isoflavone daidzein to S-equol which is a more potent estrogen than its parent compound daidzein (Duda-Chodak et al. 2015; Russell et al. 2008). The microbial metabolites interact with the host immune system and facilitate immune responses under normal circumstances (Yoo et al. 2020). Dysbiosis of gut microbiota is associated with many diseases including metabolic diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and type 2 diabetes mellitus (Carding et al. 2015; Kaur et al. 2011; Principi et al. 2018; Sharma and Tripathi 2019). Gut microbiota dysbiosis has also been

associated with extra-intestinal disorders like allergy, asthma and cardiovascular disease (Hufnagl et al. 2020; Lau et al. 2017; Rachid and Chatila 2016). Overall, gut microbiota is crucial for host growth, development and health.

1.3 Bile acids

As indicated above the gut microbiota plays an important role in bile acid metabolism and homeostasis. Bile acids have important physiological roles related to conversion of cholesterol and intestinal absorption of dietary fats and vitamins (Chiang 1998). About 500 mg of cholesterol is catabolized into bile acids per day in the adult human liver (Russell 2003). Bile acid are synthetized via two major pathways: 1) the classic (neutral) pathway initiated by the rate-limiting conversion of cholesterol by the enzyme cholesterol 7α-hydroxylase (cytochome P450 (CYP) 7A1) and followed by a series of fourteen steps to produce the two main primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA); and 2) an alternative (acidic) pathway starting with CYP27A1 catalyzed sterol 27-hydroxylase activity and eventually also resulting in formation of CA and CDCA (Chiang 2004; Chiang 2017; Fuchs 2003). In subsequent steps CA and CDCA are conjugated with the amino acids taurine or glycine forming taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA). Figure 1 and 2 show a presentation of bile acid metabolic pathways in liver and large intestine of both rats and humans, including biosynthesis, conjugation, deconjugation, 7α- dehydroxylation, oxidation and epimerization. Conjugation increases water solubility and reduces the toxicity of CA and CDCA (Di Ciaula et al. 2018). Conjugated bile acids are secreted through the bile ducts and stored in the gall bladder (excluding rats, rabbits and horses that lack a gallbladder), and they will be released into the small intestine following meal ingestion (Winston and Theriot 2020). The majority of bile acids are reabsorbed in the ileum, while only a limited amount enters the colon. In the intestines gut microbiota convert primary bile acids to secondary and tertiary bile acids (Figure 2). Approximately 95 % of bile acids are reabsorbed from the intestine back to the liver, and only 5% is secreted into the feces (Chiang 2009). This highly efficient process where bile acids circulate between liver and intestine is called enterohepatic circulation.

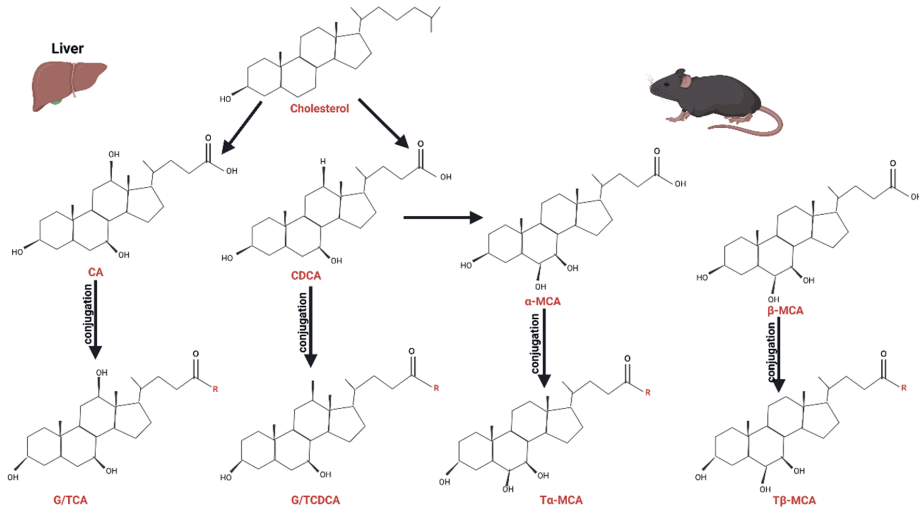


Figure 1 Bile acid synthesis in the liver for humans and rats. R means taurine/glycine.

There are several microbial biotransformation reactions by enzymes from the intestinal bacteria that modify bile acids. Deconjugation of primary conjugated bile acids upon their release from the liver into the gastrointestinal track occurs rapidly via bile salt hydrolases (BSH), present in several microbiota including the genus *Clostridium*, *Bifidobacterium*, *Bacteroides*, *Lactobacillus* and *enterococcus* (Begley et al. 2005; Patel et al. 2010)(Figure 2). Subsequently the unconjugated primary bile acids CA and CDCA can be converted to secondary bile acids like DCA and LCA respectively, via 7α-dehydroxylation, a reaction which is performed by only a few anaerobic microbial species including *Clostridium scindens*, *C. hiranonis*, *C. sordelli* and *C. hylemonae* which all belong to the *Firmicutes* phyla (Gérard 2014; Ridlon et al. 2013; Ridlon et al. 2006; Wells et al. 2003)(Figure 2). In addition, the deconjugated primary bile acids can be converted by oxidation and epimerization at the 3-, 7-, and/or 12- hydroxy groups by microbial hydroxysteroid dehydrogenases (HSDHs), resulting in formation of for example CDCA to UDCA (Doden et al. 2018)(Figure 2). In rodents, CDCA will convert to the primary bile acids alpha-muricholic acid (α-MCA) and beta-muricholic acid (β-MCA) and then form the secondary bile acid omega muricholic acid (ɯ-MCA) (Winston and Theriot 2020)(Figure 1 and 2). All together gut microbiota affects the bile acid pool size and composition, thereby playing a role in bile acid homeostasis.

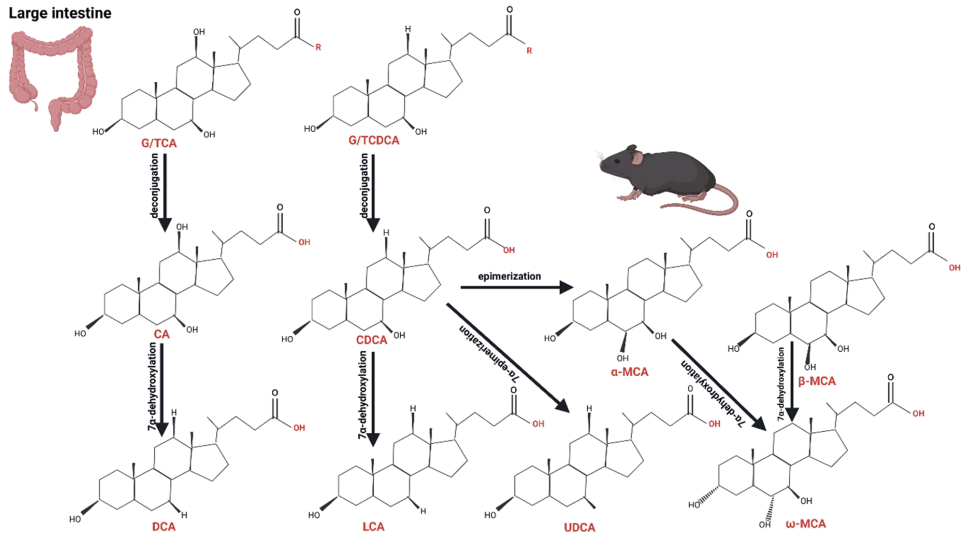


Figure 2 Bile acid deconjugation, 7α- dehydroxylation, oxidation and epimerization catalyzed by microbiota in the large intestine from humans and rats.

As already indicated, the majority of the intestinal bile acids enter the entero-hepatic circulation by being reabsorbed from the intestinal lumen. Active transport and passive diffusion work together to achieve this bile acid reabsorption, with the relative contribution of these processes varying with the bile acid of interest (Dietschy 1968).

In the overall homeostasis of bile acids, several transporters are involved. The Na^+ taurocholate co-transporting polypeptide (NTCP), the bile salt export pump (BSEP), the apical sodium-dependent bile acid transporter (ASBT), the ileal bile acid binding protein (IBABP) and the organic solute transporter OSTα-OSTβ are major bile acid transporters which control bile acid reuptake, enterohepatic circulation and excretion from the body (Alrefai and Gill 2007; Dawson et al. 2009). In the liver, NTCP is mainly responsible for absorbing sodium dependent bile acids from the portal blood into the liver while BSEP secretes bile acids into the bile across the canalicular membrane (Kosters and Karpen 2008). Most of the unconjugated bile acids are passively absorbed at any level of the gastrointestinal tract. Primary conjugated bile acids are actively transport at the terminal ileum by ASBT across the apical membrane of the intestinal cells and by IBABP across the cytoplasma followed by transport over the basolateral membrane and back into the portal blood by OSTα-OSTβ (Bahar and Stolz 1999; Dawson and Karpen 2015). Some conjugated bile acids are reabsorbed from the intestines back into the systemic circulation via passive diffusion at the proximal small intestine, by active and passive transport in the terminal ileum and via passive diffusion in the colon.

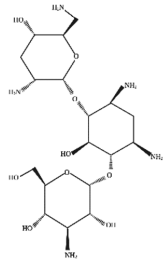
Bile acids are known to act as signaling molecules acting as ligands for several receptors including nuclear receptors such as farnesoid X receptor (FXR), the pregnane X receptor (PXR), the vitamin D receptor (VDR), the membrane receptor- G protein-coupled receptor (TGR5); and receptors of cell signaling pathways (c-*jun* N-terminal kinase ½, AKT, ERK ½) (Copple and Li 2016; Hylemon et al. 2009). Bile acids can activate these receptors to mediate gene expression thus regulating biosynthesis of many metabolites and physiological activities such as biosynthesis of bile acids, metabolism of glucose, fatty acids and lipoproteins, energy metabolism and immunity (Claudel and Trauner 2020; Di Ciaula et al. 2018). Given that some of these receptors, such as for example TGR5, are located around the whole body, it means that bile acids can have effects beyond the gut-liver axis.

Altogether, bile acids are multi-functional agents, not only promoting the intestinal absorption of lipids and vitamins, but also influencing glucose homeostasis, endocrine function, and inflammation (Evangelakos et al. 2021; Houten et al. 2006; Pârvu and Cătoi 2014). Bile acid disorders have been linked to many diseases including for example non-alcoholic fatty liver disease, obesity, cholestatic diseases, multiple sclerosis, and so on (Arab et al. 2017; McMillin and DeMorrow 2016; Shapiro et al. 2018). Thus, bile acid homeostasis, including bile acid synthesis, conversion, transport and function is important for human health and well-being.

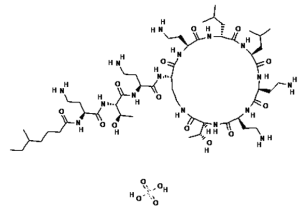
1.4 Antibiotics

Antibiotics are known to alter the composition and function of the host gut microbiota (Modi et al. 2014). The antibiotics in the present thesis were selected because they were shown previously to affect the fecal and plasma bile acid metabolome, upon oral administration for 28 days in *in vivo* rat studies (Behr et al. 2019; Murali et al. 2023a; Murali et al. 2023b). These antibiotics were selected to have low or no systemic availability except for lincomycin, therefore they have low/no systemic toxicity, which implies that potential effects on the plasma metabolome cannot be ascribed to systemic effects and may be the result from a change in the gut microbiota composition, or from other local effects of the antibiotics in the gastro intestinal tract, such as for example an effect on the transporters involved in bile acid reuptake. The antibiotics studied in this thesis were tobramycin, colistin sulfate, meropenem trihydrate, doripenem hydrate, lincomycin, streptomycin and vancomycin (Figure 3). Tobramycin and streptomycin belong to the group of aminoglycoside antibiotics which are broad spectrum antibiotics that are widely used against gram-negative bacteria. Their mode of action is based on inhibition of protein synthesis through interacting with the 30S ribosomal subunit providing a bactericidal effect (Becker and Cooper 2013; Neu 1976). It has also been reported that these aminoglycosides are able to get through the stop codon at ribosomes in human cells thereby hampering the synthesis of normal functional proteins also in human (Wangen and Green 2020). Colistin sulfate also called polymyxin E, is a polymyxin antibiotic, and usually applied for the treatment of infections by gram-negative bacteria. Colistin binds to the outer cell membrane of bacteria resulting in bacterial death via disruption

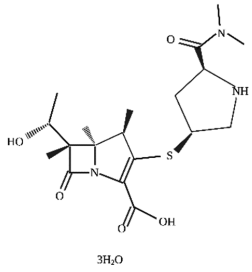
of the cell membrane and leaking of intracellular contents (Biswas et al. 2012). Meropenem trihydrate and doripenem hydrate are carbapenem antibiotics which are effective against gram-positive bacteria and to some extent also against gram-negative bacteria (Baldwin et al. 2008). They interfere with penicillin-binding proteins that are involved in peptidoglycan biosynthesis thus inhibiting synthesis of the bacterial cell wall (Codjoe and Donkor 2017; El-Gamal et al. 2017). Lincomycin is a lincosamide antibiotic and has been found to interfere with 50S ribosomal subunits thereby disrupting protein synthesis in bacteria (Josten and Allen 1964; Spížek and Řezanka 2004). Vancomycin is a glycopeptide antibiotic which suppresses peptidoglycan synthesis on the bacterial cell wall resulting in an antibacterial effect (Nagarajan 1991).



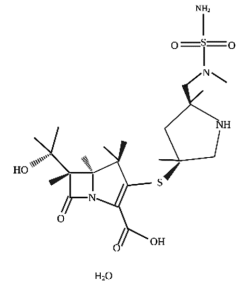
Tobramycin



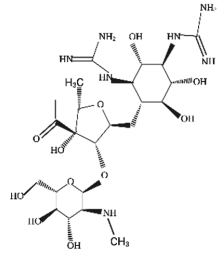
Colistin sulfate



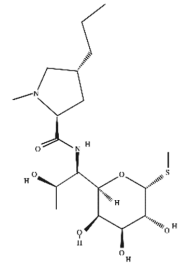
Meropenem trihydrate



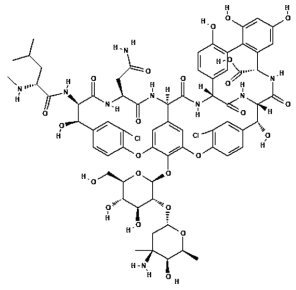
Doripenem hydrate



Streptomycin



Lincomycin



Vancomycin

Figure 3 Chemical name and structure of the antibiotics used in the thesis; colistin sulfate and vancomycin structures were taken from Pubchem.

1.5 *In vitro* model to study the intestinal deconjugation of bile acids

It is well known that gut microbiota is able to biotransform the primary bile acids to secondary and tertiary bile acids (Guzior and Quinn 2021). Microbial enzymes like for example bile salt hydrolases (BSH) are able to deconjugate conjugated bile acids removing the amino acid residue (taurine or glycine) thereby increasing the diversity of the bile acid pool (Bustos et al. 2018; Martin et al. 2018). To study the colonic metabolism of primary bile acids by the gut microbiota, *in vitro* anaerobic fecal incubation models are often used because they provide possibilities for time dependent dynamic and multiple sampling (Mortelé et al. 2019). These *in vitro* anaerobic fecal incubations could also be a reliable tool to assess intestinal microbial deconjugation of bile acids *in vitro*.

In the present thesis *in vitro* anaerobic fecal incubation models were used to study the effect of the selected antibiotics on intestinal microbial metabolism of bile acids. *In vitro* fermentation models include two groups: 1) *in vitro* batch fermentation models, that are appropriate for short-term experiments because of rapid depletion of substrate and reduction of pH hampering further microbial activities; 2) *in vitro* continuous fermentation models, which facilitate adjustment of pH and supplementation of nutrients in order to perform long-term experiments (El Houari et al. 2022; Pham and Mohajeri 2018). In the present thesis an *in vitro* batch fermentation model was used because it is simple, fast, cheap, easy to operate and reproducible. Additionally, an *in vitro* batch fermentation model is suitable for high-throughput studies and uses minimum amounts of media and test compounds. In the studies of the present thesis these batch fermentations were carried out for short period of time in PBS buffer so without the addition of extra nutrients and in the presence of only some residual nutrients originally present in the fecal samples and remaining after their centrifugation to remove big particles. This PBS incubation medium was used because these conditions were shown to 1) provide lower detection limits for the bile acids; 2) cause less variability between duplicate incubations; and considered to 3) keep the bacterial composition more close to the

in vivo intestinal situation by preventing rapid bacterial growth. The consequence of these nutrient poor conditions is that incubations can be carried out only for short periods of time.

1.6 *In vitro* models to study bile acid transport across Caco-2 cell layers

In addition to the effects of the antibiotics on the gut microbiota, there could also be effects on the intestinal cells and transporters involved in the reuptake of bile acids, as part of the mode of action underlying the changes in bile acid homeostasis induced by the antibiotics *in vivo*. To study this to some more extent, a transwell model with cells from the human intestinal Caco-2 cell line was used. The human intestinal Caco-2 cell line is widely used as a model of the intestinal barrier (Lea 2015; Sambuy et al. 2005; Sun et al. 2008). The cell line was originally obtained from a human colon adenocarcinoma, and the cells of this cell line are able to differentiate into a cell layer which represents the small intestinal epithelium structurally and functionally (Hilgers et al. 1990). The Caco-2 transwell model has been shown to be a useful *in vitro* model to assess drug and nutrient transport and metabolism (Meunier et al. 1995). Application of the *in vitro* Caco-2 transwell model to estimate apparent permeability (P_{app}) coefficients has been proven a reliable way to evaluate the kinetics of absorption/transport of drugs across the intestinal epithelium (Artursson and Karlsson 1991; Yee 1997a; Zhao et al. 2002). A robust quantitative structure-property relationship (QSAR) model has been described to convert Caco-2 apparent permeability (Papp) values to kinetic constants for intestinal reuptake (Zhao et al. 2002).

In this thesis, the *in vitro* Caco-2 cell models were applied to study the effect of the antibiotics (tobramycin, lincomycin, streptomycin and vancomycin) on bile acid transport. Pre-exposure and co-exposure of Caco-2 cell layers with these antibiotics and bile acids were investigated. Assessing the impact of antibiotics on the intestinal bile acid transport may provide insight in the role of effects on bile acid reuptake in the mode(s) of action underlying effects of the antibiotics on fecal and plasma bile acid profiles reported in *in vivo* studies (Behr et al. 2018; Behr et al. 2019; Murali et al. 2023a). Pre-exposure of the cells to an antibiotic can be expected to affect subsequent bile acids transport for example via changes at protein expression levels resulting in modified levels of transporters involved in the reuptake of conjugated bile acids (Li et al. 2020; Zhang et al. 2023). Co-exposure will reveal whether there are direct interactions between the antibiotics and bile acids affecting the bile acid transport. Because Caco-2 cell models only represent enterocytes, they do not simulate the complex interactions with other cells such as mucus producing cells which could be included in the *in vitro* cultures by cocultivation of the Caco-2 cells with for example HT-29-MTX cells. These cells secret mucus and cocultivation of the Caco-2 cells with these mucus producing cells would provide an *in vitro* model mimicking the human intestinal epithelium to an even better extent (Leonard et al. 2010; Martínez-Maqueda et al. 2015). Nevertheless, the *in vitro* Caco-2 cell model as such has been widely used to study the absorption of nutrients and the bioavailability and transport of drugs. The cells are enterocyte-like, well characterized, easily maintained and form adequate cell layers. Furthermore, several studies

have reported good correlations between data obtained in the *in vitro* Caco-2 cell model and *in vivo* intestinal absorption data (Angelis and Turco 2011; Yee 1997b) Therefore, the *in vitro* Caco-2 cell model systems is expected to contribute to a mechanistic understanding of the effects of antibiotics on the intestinal bile acid reuptake *in vivo*.

1.7 Applying a physiologically based kinetic (PBK) *in silico* model to predict *in vivo* plasma bile acid concentrations in human subjects

Physiologically based kinetic (PBK) models are a useful tool to study the absorption, distribution, metabolism, and excretion (ADME) of chemical compounds including drugs (Li et al. 2010). In a PBK model, organs and tissues are defined as separate or combined compartments, when they represent important organs in the ADME or toxicity of the drug or other xenobiotic under study, such as the liver for metabolism, the lungs in case of exposure via inhalation and the intestine to include microbial metabolism and intestinal reuptake and so on. Those organs or tissues not directly affecting ADME or toxicity of the compound under study can be combined in either a slowly or a richly perfused tissue compartment. To define the PBK model, three types of parameters are required, 1) physiological and anatomical parameters (e.g. weight of tissues and organs, cardiac output and blood flow to the tissues); 2) physiochemical parameters (e.g. the blood/ tissue partition coefficients); 3) kinetic parameters (e.g. the kinetic constants for metabolism or transport). Then differential equations for each compartment can be defined with the above three types of parameters. Subsequently, a PBK model can model physiologically relevant concentrations of the compound under study in any target organ for a certain dose, time point and route of administration by using these mathematical equations. For validation of the performance of the PBK model, PBK model based predictions can be compared to available *in vivo* literature data. PBK models are widely used in new approach methodologies (NAMs) for example for so-called reverse dosimetry to extrapolate results from *in vitro* studies to the *in vivo* situation (Rietjens et al. 2011).

Recently a successful PBK model was developed to describe the *in vivo* bile acid dynamics and homeostasis using GCDCA as an exemplary bile acid in human (de Bruijn et al.2022). The model included the role of two important bile acid transporters being the bile salt export pump (BSEP) and the apical sodium dependent bile salt transporter (ASBT). By including the role of BSEP in the PBK model also the effect of the BSEP inhibitor bosentan could be predicted. It was demonstrated that the PBK model adequately described the effect of BSEP abundance and resulting activity as well as of differences in the total bile acid pool between individuals on plasma bile acid concentration over time. The model also adequate described the effect of bosentan on these characteristics; bosentan treatment especially increased bile acid C_{max} values in individuals with a large total bile acid pool or low BSEP abundance. In another PBK modeling study the effects of the ASBT inhibitor odevixibat on bile acid plasma profiles were investigated (de Bruijin et al. submitted) . It was found that a functional ASBT was present in Caco-2 cell layers, so that kinetics for the effects of odevixibat could be defined and incorporated in the PBK model to show that oral odevixibat exposure would

reduce the level of conjugated bile acids in plasma. In the present thesis these previously developed PBK models were adapted to predict *in vivo* plasma bile acid concentrations in human upon tobramycin treatment, also using data from the in vitro anaerobic fecal incubations and the Caco-2 transport experiments to include the effect of tobramycin on bile acid kinetics in the PBK model.

1.8 Outline of the thesis

Gut microbiota has huge impact on health, well-being and disease of the host organism. The microbiota is crucial in many physiological processes including bile acid synthesis, xenobiotic and drug metabolism, antimicrobial protection and immunomodulation. Bile acids not only regulate the digestion and absorption of cholesterol, and dietary fat and vitamins, but also play an important role in host health and disease. Bile acid homeostasis is closely related to gut microbiota dynamics, and this homeostasis can be altered by xenobiotics such as for example antibiotics which are known to affect gut microbiota. The aim of the present thesis was to investigate the use of new approach methodologies (NAMs) for studies on the effects of antibiotics on bile acid homoeostasis.

Chapter 1 provides background information on gut microbiota and bile acid homeostasis, and also on the model compounds and new approach methodologies (NAMs) used in the thesis. The aim of the study is described and the outline of the thesis is presented.

Chapter 2 investigates if, and to what extent the potential effects of antibiotics on *in vivo* bile acid homeostasis could be detected in *in vitro* model systems including fecal anaerobic incubations and Caco-2 transwell transport experiments with a series of selected primary bile acids (TCA, TCDCA, GCA and GCDCA). It was also investigated whether such *in vitro* studies could provide additional insights into the mode(s) of action underlying effects of the antibiotics on bile acid homeostasis observed *in vivo*.

Chapter 3 investigates whether the effects on bile acid metabolism and transport upon treatment with tobramycin can also be studied in the *in vitro* model systems using mixtures of bile acids instead of individual bile acids, in order to i) elucidate the effect of tobramycin on the deconjugation and intestinal transport of other bile acids, ii) develop a more efficient testing strategy, iii) better mimic the *in vivo* situation where bile acids also do not occur in isolation, and iv) contribute to the development of new approach methodologies focusing on the 3Rs (replacement, reduction and refinement) of animal experiments.

Chapter 4 investigates whether other antibiotics, including the lincosamide lincomycin, the additional aminoglycoside streptomycin and the glycopeptide vancomycin, would exert the same inhibitory effect on bile acid transport as observed for tobramycin when studying transport of selected bile acids in the *in vitro* Caco-2 cell model.

Chapter 5 applies PBK modelling as an *in silico* tool to investigate the consequences of the effects of the antibiotic tobramycin on bile acid deconjugation and intestinal reuptake on bile

acid levels *in vivo* in human plasma. To this end kinetic data obtained in the *in vitro* models applied in the thesis were used to modify the relevant parameters in the PBK model that predicted human plasma bile acid levels.

Chapter 6 summarizes the main results of the above chapters and provides and overarching discussion including a comparison of the effects of the antibiotics on the microbiota in the *in vitro* and the *in vivo* study, an analysis of the interactions between gut microbiota and bile acids, and an analysis of the impacts of antibiotics on bile acid transport and prediction of the consequence for *in vivo* human plasma bile acid concentrations. The chapter also presents future directions and perspectives.

Finally, **Chapter 7** provides a concluding summary of the aim, results and conclusions of this thesis.

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Chapter 2.

In vitro models to detect *in vivo* bile acid changes induced by antibiotics

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Abstract

Bile acid homeostasis plays an important role in many biological activities through the bile-liver-gut axis. In this study, two *in vitro* models were applied to further elucidate the mode of action underlying reported *in vivo* bile acid changes induced by antibiotics (colistin sulfate, tobramycin, meropenem trihydrate and doripenem hydrate). 16S rRNA analysis of rat fecal samples anaerobically incubated with these antibiotics showed that especially tobramycin induced changes in the gut microbiota. Furthermore, tobramycin was shown to inhibit the microbial deconjugation of taurocholic acid (TCA) and the transport of TCA over an *in vitro* Caco-2 cell layer used as a model to mimic intestinal bile acid reuptake. The effects induced by the antibiotics in the *in vitro* model systems provide novel and complementary insight explaining the effects of the antibiotics on microbiota and fecal bile acid levels upon 28-day *in vivo* treatment of rats. In particular our results provide insight in the mode(s) of action underlying the increased levels of TCA in the feces upon tobramycin exposure. All together the results of the present study provide a proof-of-principle on how *in vitro* models can be used to elucidate *in vivo* effects on bile acid homeostasis, and to obtain insight in the mode(s) of action underlying the effect of an antibiotic, in this case tobramycin, on bile acid homeostasis via effects on intestinal bile acid metabolism and reuptake.

Keywords: antibiotics; tobramycin, fecal incubations; bile acid homeostasis; 16S rRNA analysis; bile acid reuptake;

Introduction

Bile acids play a key role in absorbing intestinal nutrients, emulsifying lipids, secreting toxic metabolites and xenobiotics. Additionally, bile acids are important signaling molecules that are able to modulate lipid, glucose and energy metabolism by reacting with diverse bile acid receptors (Houten et al. 2006; Thomas et al. 2008) .

Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the two primary bile acids synthesized from cholesterol in the liver and further conjugated with taurine or glycine to generate taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA) and glycochenodeoxycholic acid (GCDCA) increasing their solubility (Di Ciaula et al. 2018; Stamp and Jenkins 2008). Conjugated bile acids are secreted into the duodenum after a meal through the bile duct (Chiang and Ferrell 2018). Upon release into the intestinal tract typically, 95% of bile acids are actively reabsorbed in the ileum of the small intestine, resulting in enterohepatic circulation (Chiang 2009; Ridlon et al. 2016). A minor amount of bile acids escapes this reabsorption and enters the colon where the indigenous gut microbiota process their deconjugation, dehydrogenation and dihydroxylation converting the primary bile acids into secondary bile acids such as deoxycholic acid (DCA), lithocholic acid (LCA) and the tertiary BAs including for example ω-muricholic acid (ω-MCA) (Winston and Theriot 2020). Ursodeoxycholic acid (UDCA) is the primary bile acid in rodents being the 7β-hydroxy isomer of CDCA which is also converted to UDCA and subsequently to LCA by intestinal bacteria (Ridlon and Bajaj 2015). An overview of bile acid synthesis and conversion is presented in Figure 1. Disturbance of bile acid homeostasis has been related to several metabolic diseases such as obesity, type 2 diabetes mellitus and inflammatory bowel disease (IBD) (Duboc et al. 2013; Jones et al. 2014). In previous studies, it was shown that *in vivo* oral exposure to antibiotics may affect bile acid homeostasis resulting in changes in the bile acid metabolic profiles in intestinal tissues, feces and blood samples (Behr et al. 2019; Behr et al. 2018b). These effects were (in part) related to the effects of antibiotics on the host intestinal microbiota, known to contribute to bile acid metabolism.

The intestinal microbiota consists of approximately 10^{14} microbes including 500 to 1000 distinct bacterial species (Eckburg et al. 2005). It is estimated that gut microbiota includes more than 1000 phylotypes dividing into six phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* (Consortium 2012; Wang et al. 2005). The metabolic activity of the intestinal microbiome is critical not only in modulating host immune defense but also in maintaining host metabolism and health, as illustrated for example by studies using germ-free animals (Claus et al. 2008; Wikoff et al. 2009).

The gut microbiota participates in many metabolic processes such as vitamin synthesis, regulating dietary lipid metabolism, indigestible carbohydrates metabolism and secondary bile acids syntheis. A lot of bile acid studies show that the bile acid pool size, composition and

compartment concentrations are directly linked to the constitution of the gut microbiota and the microbial metabolism of bile acids in the intestine (Sayin et al. 2013).

Antibiotics are able to alter the diversity of the gut microbiota, subsequently also changing the metabolome, especially altering the composition and concentration of bile acids (Antunes et al. 2011; Behr et al. 2018a; Yap et al. 2008). Vrieze and colleagues orally administered vancomycin to male subjects for 7 days and provided evidence that the antibiotics influence gut microbiota composition and the makeup and concentrations of bile acids (Vrieze et al. 2014). Kuno and colleagues observed changes in secondary bile acids (lithocholic acid (LCA) and deoxycholic acid (DCA)) in mice upon oral administration of the non-absorbable antibiotics vancomycin and polymyxin B (Kuno et al. 2018). Behr and colleagues observed that antibiotic treatment in rats caused differences in bile acid metabolome profiles in plasma and feces especially significantly increasing taurine conjugated primary bile acids in both matrices (Behr et al. 2019). In recent studies, colistin sulfate and tobramycin which are also non-absorbable antibiotics appeared to also change the composition of the gut microbiota and influence bile acid metabolism, albeit to a different extent (Murali et al. in preparation).

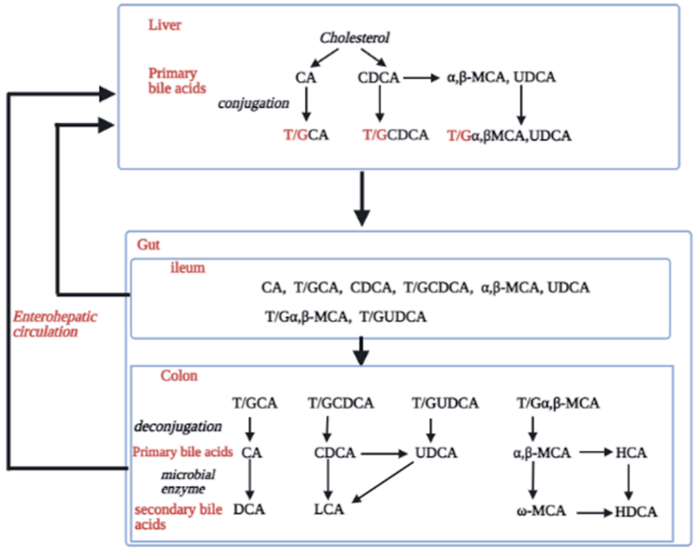


Figure 1 Bile acid synthesis and conversion. The primary bile acids α-MCA and β-MCA and their secondary bile acids are rodent specific.

In addition to effects on the microbiota, it might also be hypothesized that the effects of the antibiotics on the bile acid metabolome patterns may be related to an alteration of the bile acid reuptake into the liver in the ileum of the small intestine. Given that 95% of bile acids are actively reabsorbed even a limited inhibition of the transporters involved can be expected to

already substantially increase fecal levels of bile acids. The aim of the present study was to investigate if, and to what extent the potential effects of antibiotics on *in vivo* bile acid homeostasis could be detected in *in vitro* model systems, and whether such *in vitro* studies could provide additional insights into the mode(s) of action underlying effects of the antibiotics on bile acid homeostasis.

To this end, we analyzed the bile acid changes in rat fecal incubations, with and without adding conjugated primary bile acids, upon treatment with antibiotics. 16S rRNA sequencing was performed to estimate the effects of the antibiotics on the intestinal microbiota abundances and LC-MS/MS was used to quantify bile acid patterns. Additionally, Caco-2 cell layers in a transwell model were used to elucidate the potential impact of the studied antibiotics on the intestinal reuptake of bile acids.

Materials and Methods

Chemicals and Reagents

Chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA), hyodeoxycholic acid (HDCA), hyocholic acid (HCA), deoxycholic acid (DCA), cholic acid (CA), glycoursodeoxycholic acid (GUDCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycocholic acid (GCA), taurolithocholic acid (TLCA), tauroursodeoxycholic acid (TUDCA), taurohyodeoxycholic acid (THDCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and taurocholic acid (TCA) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Alpha-muricholic acid (α-MCA), beta-muricholic acid (β-MCA), and glycolithocholic acid (GLCA) were from Cambrige isotope laboratories (Massachusetts, USA). Tobramycin, colistin sulfate, meropenem trihydrate, doripenem hydrate were purchased from Sigma-Aldrich (Schnelldorf, Germany). AcroPrep™ 96-well filter plates were purchased from Pall Corporation (Amsterdam, Netherlands). Acetonitrile (ACN) and methanol were obtained from Biosolve BV (Valkenswaard, Netherlands). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (Darmstadt, Germany). Phosphate buffered saline (PBS) was purchased from Gibco (Paisley, UK). Fetal Bovine serum (FBS) was obtained from GE Healthcare Life Sciences Hyclone Laboratories (Logan, Utah, USA). 0.05% Trypsin-EDTA, minimum essential medium (MEM), penicillin-streptomycin-glutamine solution (PSG), sodium pyruvate, Hank's balanced salt solution (HBSS) and HEPES buffer solution were purchased from Gibco (Paisley, UK). Roche cell proliferation reagent WST-1 was purchased from Sigma-Aldrich (Schnelldorf, Germany). Corning Costar 12 well transwell plates were purchased from Corning Life Sciences (Schnelldorf, Germany). 96 Well cell culture plates were obtained from Greiner Bio-One B.V. (Alphen aan den Rijn, Netherlands).

Conversion of the *in vivo* dose levels to *in vitro* concentrations

To establish the *in vitro* concentrations of the antibiotics to be used in the *in vitro* studies, *in vivo* dose levels of the selected antibiotics (colistin sulfate, tobramycin, meropenem trihydrate and doripenem hydrate) used in rat metabolomics studies at BASF (Murali et al. in preparation), were converted to corresponding *in vitro* test concentrations using equation 1.

Equation 1: *In vitro* test concentration (in mM) = (*In vivo* daily exposure of compound (in mg/kg bw) x bw (in kg))/Volume of gastrointestinal tract (in mL)/Molecular weight of compound (mg/mmol) × 1000 (mL/L)

The *in vitro* test concentration thus derived from the *in vivo* dose levels are presented in Table S1 in the supplementary information. The volume of the gastrointestinal tract of rat was assumed to amount to 12 ml (McConnell et al. 2010), adding up the water content of a fed gastrointestinal tract of 10 g, and a solid content of 2 g, and assuming the density to be 1 g/ml. The concentrations used in the *in vitro* assays equaled the concentrations derived from the high dose levels, unless stated otherwise.

Anaerobic incubation of rat feces

Fecal slurry preparation

The fecal samples from tobramycin rats were provided by BASF and taken from an *in vivo* study in which Wistar rats were orally administrated a series of antibiotics, including tobramycin, for 28 days. The animal study was performed in an AAALAC-approved (Association for Assessment and Accreditation of Laboratory Animal Care International) laboratory in compliance with the German Animal Welfare Act and the effective European Council Directive. The study was approved by the BASF Animal Welfare Body, with the permission of the local authority, the Landesuntersuchungsamt Koblenz, Germany (approval number 23 177-07/G 18-3-098), and was carried out based on the OECD 407 Principles of Good laboratory Practice and the GLP provisions of the German Chemicals Act. Tobramycin was prepared in deionized water for oral administration at doses of 100 mg/kg body weight/day and 1000 mg/kg body weight/day respectively. Fecal samples were taken from respectively control rats (10 males and 10 females) and exposed rats (5 males and 5 females) receiving 1000 mg/kg body weight/day tobramycin at day 23 of the experiment. Blood and fecal samples of the animals were collected for a detailed metabolomics study, the results of which will be described in a separate paper (Murali et al. in preparation). Feces were obtained by physical massage of the rectum of rats, weighed and transferred immediately into anaerobic 10% (v/v) glycerol in PBS solution, pooled 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) (BACTRON300 anaerobic chamber (Cornelius, USA)). Subsequently, samples were filtered using sterile gauze under anaerobic conditions, and aliquoted samples of resulting fecal slurry were stored at -80°C until use.

Treatment of fecal samples with antibiotics

In order to detect the effects of antibiotics on the composition of the fecal microbiota, 280 µL control rat fecal slurry mixed with 70 µL MilliQ water or 70 µL colistin sulfate, tobramycin, meropenem trihydrate and doripenem hydrate (final concentration: 2 mM colistin sulfate, 45 mM tobramycin, 15 mM meropenem trihydrate, or 50 mM doripenem hydrate) were incubated under anerobic conditions for 24 h at 37°C. After 24h, these samples were stored at -80°C overnight and shipped with dry ice for 16S rRNA analysis. Based on the results obtained (see Result section) tobramycin and colistin sulfate were selected for further studies.

For further studies with the selected antibiotics on effects on bile acid metabolism, Eppendorf tubes containing 80 µl control fecal slurry (final concentration: 160 mg feces/ml) and 20 µl tobramycin or 20 µl colistin sulfate solution to give final concentrations of 45 mM and 2 mM, respectively, were incubated under anaerobic condition; 80 µl fecal slurry and 20 µl MilliQ water served as control group. 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 °C. At 0 h, 4 h, 8 h, and 24 h of incubation, the reaction was stopped by adding a similar volume (100 µl) of acetonitrile. Samples were subsequently sonicated for 5 min and centrifuged at 21500 g for 15 min at 4°C. Then the supernatants were transferred to a 96-well filter plate (Pall corporation, Amsterdam, Netherlands) and filtered, and samples thus obtained were stored at -80°C overnight, followed by freeze drying for 8h. The residuals thus obtained were dissolved in 100 µl (50 µl methanol: 50 µl water), and centrifuged at 21500 g for 15 min at 4°C, after which the supernatants were transferred to LC-MS/MS vials for bile acids measurement by LC-MS/MS.

Because parallel *in vivo* results showed that fecal conjugated bile acids increased upon tobramycin treatment of rats (Murali et al. in preparation), in addition to incubations studying the effect of antibiotics on the bile acids present in the fecal samples as such, the effects of the antibiotics on externally added conjugated bile acid conversion by the intestinal microbiota was studied. To this end, anaerobic fecal incubations (total volume 100 µl) contained: 1 µl control fecal slurry (final concentration: 2 mg/ml fecal samples), 10 µl of a 10 times concentrated solution containing conjugated bile acids (TCA, TCDCA, GCA or GCDCA) resulting in a final concentration of 500 µM for each of the bile acids tested, 79 µl PBS and 10 µl water (control) or 10 µl tobramycin or colistin sulfate from 10 times concentrated stock solutions in MilliQ water resulting in final concentrations of 45 mM and 2 mM respectively. Fermentation Eppendorf tubes were incubated 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°C. Incubations were ended after 0 h, 2 h, 4 h, 6 h, and 8 h, by adding a similar volume (100 µl) of acetonitrile. Samples were subsequently centrifuged at 21500 g for 15 min at 4°C, and the supernatants thus obtained were transferred to LC-MS/MS vials to store at -80°C until bile acids measurement by LC-MS/MS.

Furthermore, also incubations of fecal samples from tobramycin treated rats with TCA (final concentration: 500 µM) were performed and processed in a similar way.

16S rRNA gene sequencing analysis

Fecal samples 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® MiSeq next generation sequencing system (Illumuna® Inc.). Signals were processed to *.fastq-files and the resulting 2×250 bp reads were demultiplexed. Microbiota identification were performed by clustering the operational taxonomic units (OUT).

Caco-2 cell transport experiment

Cell cultures

Caco-2 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Passage 10 - 20 of Caco-2 cells were applied in this study. The cells were maintained in a culture medium consisting of MEM with 20% FBS, 1% sodium pyruvate and 1% penicillin-streptomycin-glutamine. The cells were kept in an incubator at 37°C, 5% CO_2 and 100% humidity. The cells were passaged twice each week upon detachment with trypsin-EDTA (0.05%).

WST-1 assay

Caco-2 cells were cultured in 75 cm^2 flasks in an incubator at 37°C, 5% CO_2 and 100% humidity to allow the cells to grow. Cells were collected from the flask at 50-60% confluence and seeded in the inner wells of a 96 well plate by adding 100 µl per well of a cell suspension containing of 4×10^5/ml cells. To limit evaporation, 100 µl PBS was added to the outer wells. The plate was incubated for 18 days in the incubator at 37°C, 5% CO_2 and 100% humidity to allow the cells to attach and differentiate, changing the medium to fresh medium every other day. After 18 days in culture, cells were exposed for 48 h to solvent control (0.5% DMSO in MEM medium), tobramycin (final concentrations: 10 µM, 100 µM, 1 mM, 5 mM, 10 mM, 20 mM, 45 mM, or 100 mM directly prepared in MEM medium with 0.5% DMSO) and positive control (10 mM potassium dichromate added to MEM from a 200 times concentrated stock solution in DMSO resulting in 0.5% DMSO and 50 µM final concentration). After 48 h, 5 µl WST-1 solution were added to each well and after 2 h further incubation, the absorbance was measured with a Softmax Pro 7.1 (California, US) at 440 nm and 620 nm. Data were acquired by deducting the 620 nm signal from the 440 nm signal and expressing the value for the treated samples as percentage of the control set at 100% viability.

The effect of tobramycin on TCA transport over a Caco-2 cell layer

To quantify the effect of tobramycin on transport of bile acids over an intestinal cell model, 0.5 ml of Caco-2 cell suspension in MEM medium containing 4×10^5 cells/ml were seeded in the apical chambers of a Corning 12 well transwell plate while 1.5 ml MEM medium was added to the basolateral chambers. The transwell plate was incubated for 18 days at 37℃, 5% CO_2 and 100% humidity to allow the cells to differentiate into a confluent cell layer. Medium was refreshed every other day. After this 18-day differentiation period cells were exposed to 45 mM tobramycin for 48 h. The TEER values of the cell layer were measured using a Millicell® ERS-2 Volt-Ohm Meter (Millipore, Amsterdam, Netherlands) and this was also done after exposure to tobramycin in order to confirm the integrity of the cell layer. Upon 48 h exposure to tobramycin, MEM medium was replaced by transport medium (HBSS supplemented with 10 mM HEPES solution) and the cells were incubated for 30 min, after which medium in the apical compartment was replaced with 0.5 ml transport medium containing 5 µM TCA (added from a 1 mM concentrated stock solution in DMSO) and medium in the basolateral compartment was replaced with 1.5 ml transport medium. Next the cells were incubated, taking 75 µl samples from the basolateral compartment at 1 h, 2 h and 3 h, refilling the volume with 75 µl transport medium.

Bile acid profiling by LC-MS/MS analysis

Bile acid analysis was performed on a triple quadrupole LC-MS/MS system, model LCMS-8045 (Shimadzu Corporation, Japan), using a method able to measure 20 bile acids: UDCA, HDCA, CDCA and DCA with values of Q1/Q3: 391.3/391.3; β-MCA, α-MCA, HCA and CA with values of Q1/Q3: 407.3/407.3; GLCA with values of Q1/Q3: 432.3/74; GUDCA,GDCA and GCDCA with values of Q1/Q3: 448.3/74; GCA with values of Q1/Q3: 464.3/74; TUDCA, THDCA, TCDCA and TDCA with values of Q1/Q3: 498.4/498.4; TCA with values of Q1/Q3: 514.4/514.4; TLCA with values of Q1/Q3: 482.3/482.3; and LCA with values of Q1/Q3: 375.3/375.3. 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 gradient elution using MilliQ water (0.01% formic acid) and methanol/acetonitrile (50% v/50% v) as mobile phase A and B, respectively. In order to enhance chromatographic performance, a C18 2.1 mm security guard (Phenomenex AJ0-8782) precolumn was used. Samples were injected (1 µL) onto the column equilibrated in 30% B at a flow rate of 0.4 mL/min. The following gradient was used: 0-10 min 30-70% B, 11-19 min70- 98% B and then 20-25 min 98-30% B with 10 min equilibration at 30% B before the next injection. 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, 3 L/min; drying gas flow and heating gas flow, 10 L/min; Interface temperature, 300℃; Disolvation temperature, 526 ℃; heat block temperature, 400℃. Selective ion monitoring (SIM) and multiple reaction monitoring (MRM) were used for the detection of the bile acids.

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). Statistical analysis was performed by one way analysis of variance with Dunnett's post-hoc test, or Student's t test. $P< 0.05$ was considered statistically significant. Results are shown as mean ± standard deviation (SD). The bile acid figure was drawn using Biorender (San Francisco, USA). 16S rRNA analysis data were analyzed with R version 3.6.1 and QIIME 2 view.

Results

16S rRNA microbial profile and correlation with metabolite formation

Figure 2 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 antibiotics (tobramycin, colistin sulfate, meropenem trihydrate and doripenem hydrate), showing the relative microbial profile for dominant families. The untreated *in vitro* fecal microbial community appeared to consist mainly of *Verrucomicrobiaceae*, followed by *Porphyromonadaceae* and *Erysipelotrichaceae*, *Lactobacillaceae*, *Ruminococcaceae* and *Lachnospiraceae*. Comparing the microbial communities in the control group to those observed for the fecal samples treated with the antibiotics revealed that among the four antibiotics tested, tobramycin had the most distinct impact on the gut microbiota composition after 24 h incubation. In the tobramycin treated group, *Verrucomicrobiaceae* and *Erysipelotrichaceae* decreased while *Lachnospiraceae* and *Ruminococcaceae* increased substantially. Data on the effect of the antibiotics on the fecal microbiota at phylum level are presented in Figure S1 in the supplementary materials. These data reveal that *Firmicutes*, *Verrucomicrobia* and *Bacteroidetes* are the main contributors while also at phylum level especially tobramycin appeared to affect the bacterial composition. Tobramycin treatment resulted in an increase in the relative abundance of *Firmicutes* and an accompanying decrease in the relative abundance of *Verrucomicrobia* (Figure S1 in supplementary information).

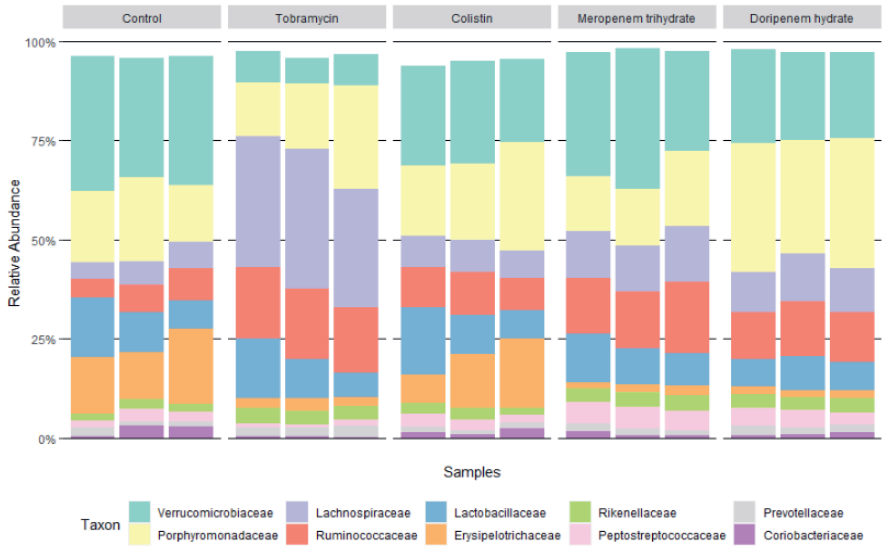


Figure 2 Relative microbial abundance at the dominant family level of the 24 h rat fecal samples from control rats either without (control) or with *in vitro* antibiotic treatment.

The effect of the antibiotics on the gut microbiome was also revealed by a principal coordinate analysis (PCoA) of Bray-Curtis distance matrix (Figure 3). In this analysis especially the tobramycin and the carbapenems antibiotics (meropenem trihydrate and doripenem hydrate which clustered together), appeared different from the control group, with tobramycin also being different from the carbapenems antibiotics, while the colistin sulfate samples did not clearly cluster different from the control. The bacterial load of the fecal samples incubated *in vitro* without (control) or with *in vitro* antibiotic treatment, determined as gene copy numbers per gram wet weight, is presented in supplementary information Figure S2. These results show that there were no significant effects of the antibiotic treatments on the bacterial load. The alpha diversity of the samples with or without (control) *in vitro* antibiotic treatment (supplementary material Figure S3) showed that the tobramycin samples had a higher alpha diversity compared to the control group and the other antibiotic treatment groups (supplementary information Figure S3).

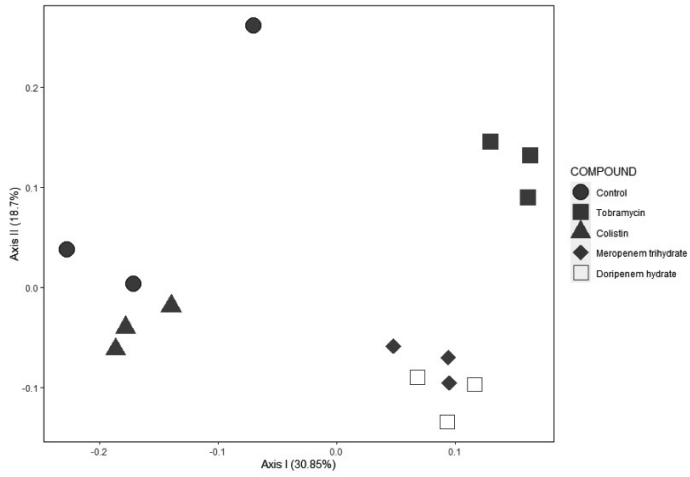


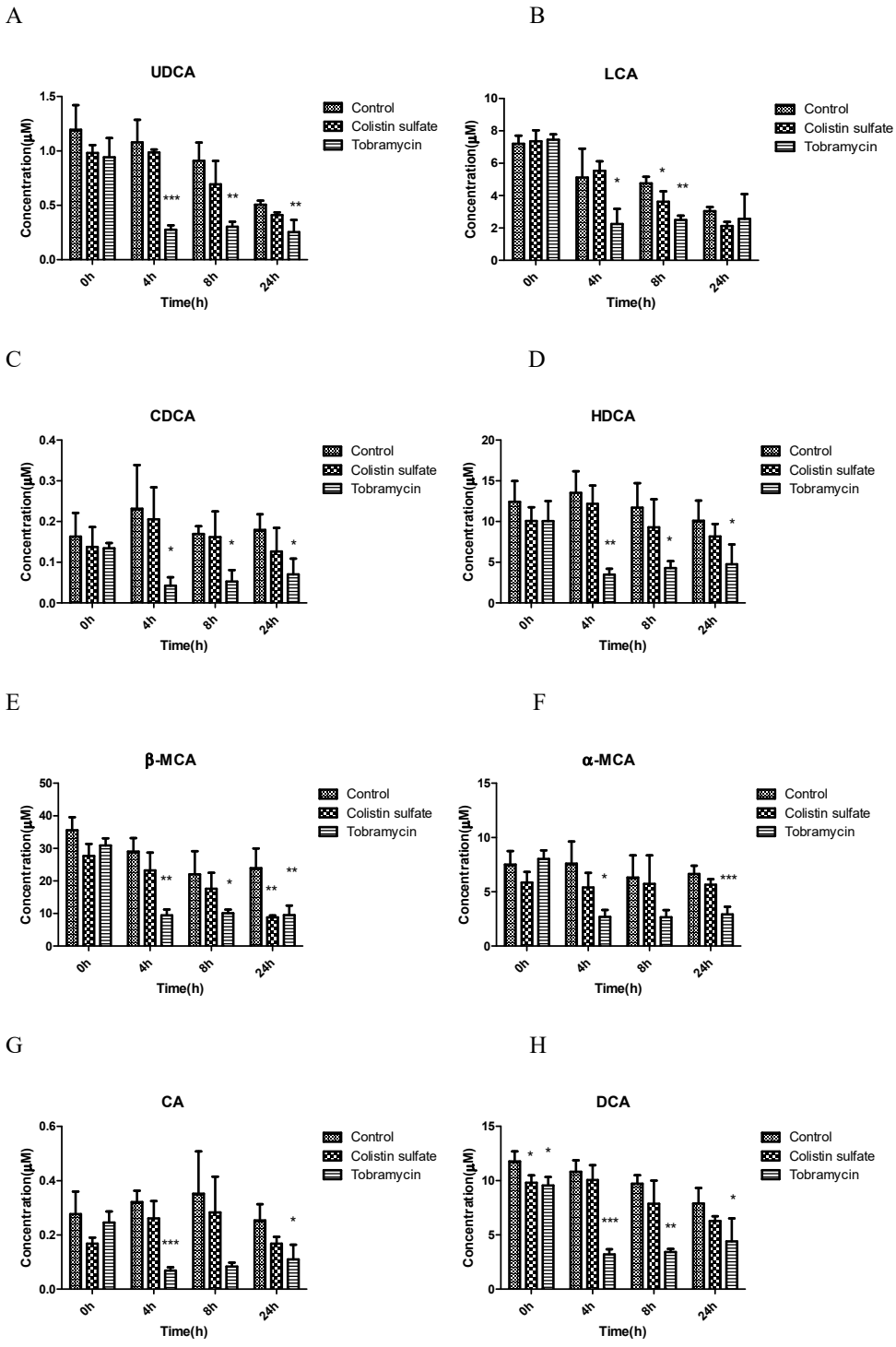
Figure 3 Principal coordinate analysis (PCoA) of the gut microbiota from control rat fecal samples incubated anaerobically *in vitro* for 24 h with or without (control) antibiotics.

Bile acid metabolites profiling

Based on the 16S rRNA sequencing results, tobramycin and colistin sulfate were chosen as the antibiotics to further study the effect of the antibiotics on the bile acid conversion by the gut microbiota. Colistin sulfate would represent a negative control whereas tobramycin was expected to affect the microbiota and thus potentially also the bile acid metabolism and profile. Figure 4 showed the time dependent bile acid profiles in supernatants collected over 24h from anaerobic rat fecal incubations in the absence (control) or presence of the antibiotics as quantified using LC-MS/MS.

At the start of the incubations, 9 bile acids originating from untreated rat fecal samples were detected, including 6 primary bile acids (α-MCA, β-MCA, UDCA, HCA, CA, CDCA) and 3 secondary bile acids (DCA, LCA, HDCA). The levels of these bile acids decreased in the order: b-MCA > HDCA > DCA > LCA = α-MCA > UDCA > HCA= CA > CDCA (Figure 4 A-I). The fecal sample incubations appeared not to contain detectable levels of conjugated primary bile acids, most likely because of their efficient deconjugation (see below).

The results presented in Figure 4 (A-I) also reveal that upon prolonged incubation especially the incubations with tobramycin showed a significant decrease in the levels of all bile acids, while the changes in the colistin sulfate group were less pronounced and matched those observed for the control. Some bile acids, including especially UDCA, LCA and DCA showed a significant time dependent decrease also in the control and colistin sulfate incubations albeit to a significantly lower extent than the decrease observed for the tobramycin incubations.


A
UDCA
Concentration(μM)
Time(h)
0h
4h
8h
24h
Control
Colistin sulfate
Tobramycin
B
LCA
C
CDCA
D
HDCA
E
β-MCA
F
α-MCA
G
CA
H
DCA

I

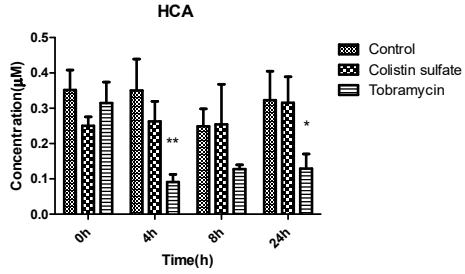
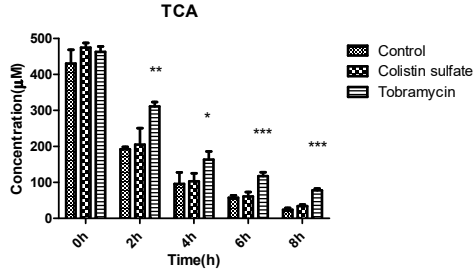


Figure 4 Intrinsic fecal bile acid composition and changes (A-I) over time (0 h, 4 h, 8 h, 24 h) in *in vitro* anaerobic rat fecal incubations with or without (control) treatment with the antibiotics colistin sulfate and tobramycin (**P*<0.05, ***P*<0.01, ****P*<0.001 indicates a difference from the control without antibiotic at the corresponding time point). Results are shown as mean ± SD from three independent incubations.

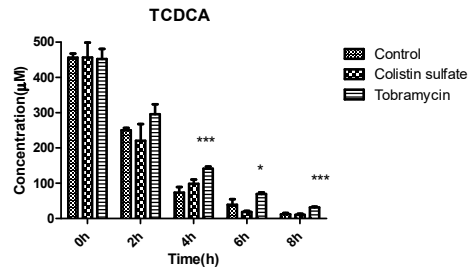
Given that the fecal incubation did not contain detectable levels of conjugated primary bile acids, in a next series of experiments, the fecal incubations were repeated with externally added TCA, TCDCA, GCA or GCDCA to also study the effects of the antibiotics on the potential of the fecal microbiota for deconjugation of these conjugated primary bile acids. The results thus obtained revealed deconjugation to be extremely fast so that in these incubations, 80-fold lower concentrations of fecal slurry had to be added to allow time dependent detection of the deconjugation. Figure 5 and 6 present the results obtained.

Deconjugation of TCA and TCDCA (Figure 5A and 5B) was readily detected and accompanied by formation of their corresponding unconjugated primary bile acids CA and CDCA (Figure 5C and 5D). Tobramycin significantly delayed the deconjugation of TCA and TCDCA and accompanying formation of CA and CDCA compared with the control group and the colistin sulfate group, the latter two again showing similar results.

A



B



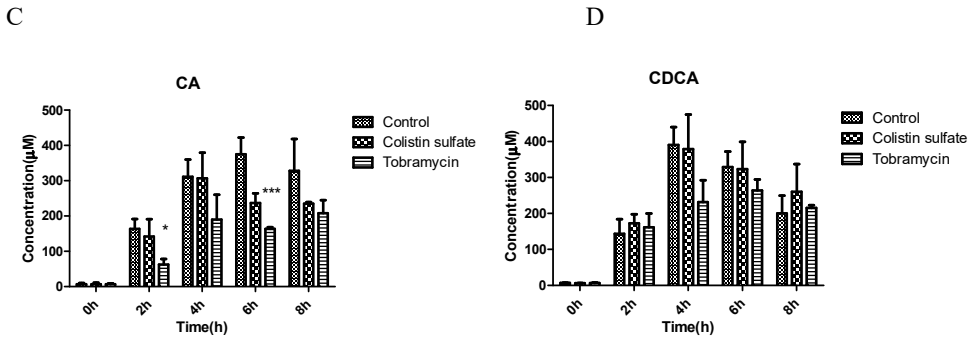


Figure 5 Time dependent deconjugation of taurine conjugated bile acids to their deconjugated metabolites in *in vitro* anaerobic rat fecal incubations (A-D) in the absence (control) and presence of the antibiotics colistin sulfate and tobramycin (**P*<0.05, ***P*<0.01, ****P*<0.001 indicates a difference from the control without antibiotic at the corresponding time point). Results are shown as mean ± SD from three independent incubations.

The deconjugation of GCA and GCDCA (Figure 6A and 6B) was also significantly reduced upon tobramycin treatment compared to the control and colistin sulfate groups, also reflected by relatively slower appearance of the corresponding deconjugated primary bile acids CA and CDCA (Figure 6C and 6D).

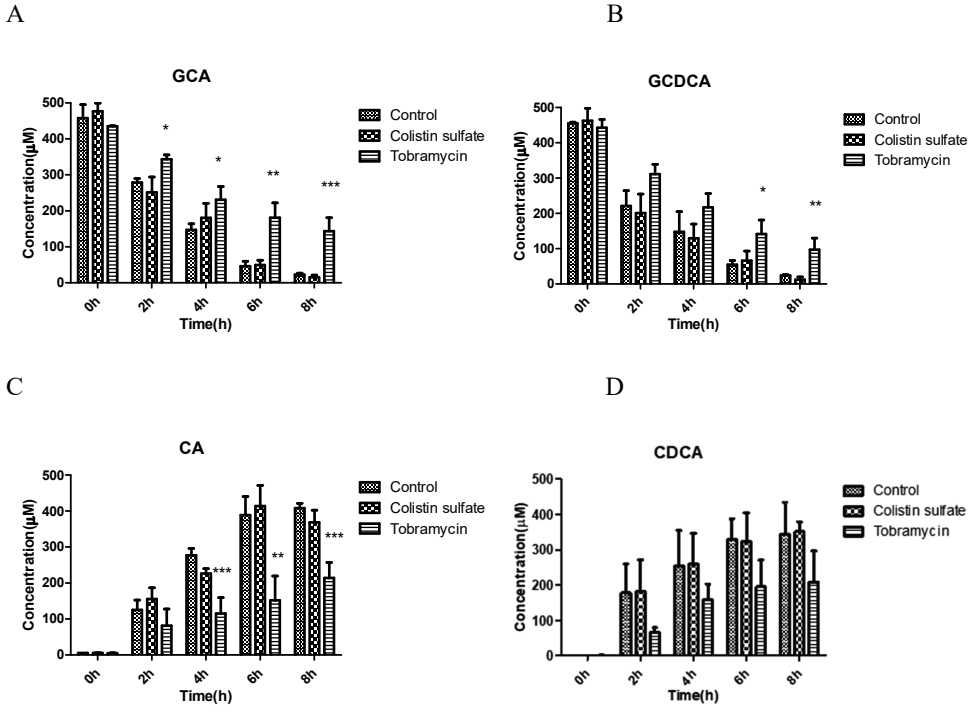


Figure 6 Time dependent deconjugation of glycine conjugated bile acids to their deconjugated metabolites in *in vitro* anaerobic rat fecal incubations (A-D) in the absence (control) and presence of the antibiotics colistin sulfate and tobramycin (**P*<0.05, ***P*<0.01, ****P*<0.001 indicates a difference from the control without antibiotic at the corresponding time point). Results are shown as mean ± SD from three independent incubations.

To enable comparison of the *in vitro* effects of tobramycin on the deconjugation of conjugated primary bile acids by fecal intestinal microbiota to effects induced by tobramycin *in vivo*, TCA as the model compound was incubated with fecal slurry from rats that were either untreated (control) or treated for 23 days with 1000 mg/kg bw/day tobramycin *in vivo*.

Figure 7A and 7B present the results of these incubations and reveals that fecal samples from rats treated for 23 days with tobramycin also show a significantly reduced activity for deconjugation of TCA to CA, as compared to fecal samples from control untreated rats.

A B

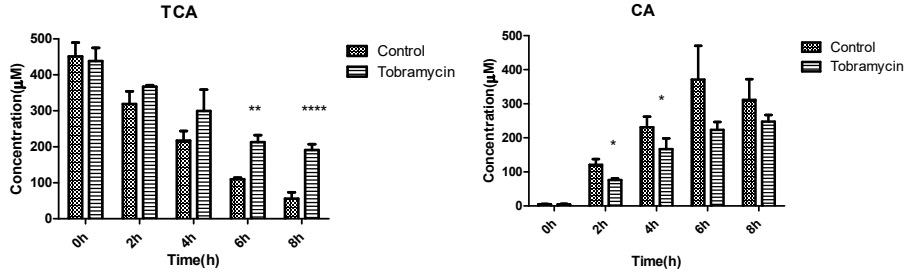


Figure 7 Time dependent deconjugation of TCA (A) and formation of its metabolite CA (B) in *in vitro* anaerobic incubations with fecal samples from rats exposed for 23 days to 0 (control) or 1000 mg/kg bw/day tobramycin *in vivo* (**P*<0.05, ***P*<0.01, *****P*<0.0001 indicates a difference from the control without antibiotic at the corresponding time point). Results are shown as mean ± SD from three independent incubations.

Effects of tobramycin on bile acid transport across a Caco-2 cell layer

In addition to an effect on TCA deconjugation, the increased fecal TCA levels observed upon *in vivo* treatment of rats with tobramycin (Murali et al. in preparation) may also be caused by and effect of tobramycin on bile acid reabsorption, Therefore, in addition to the *in vitro* studies on the effect of the selected antibiotics on microbiota composition and on bile acid metabolism by the gut intestinal fecal microbiota, additional studies aimed to characterize the effects of tobramycin on intestinal bile acid reuptake also using an *in vitro* model.

WST-1 assay to determine cell viability

A WST-1 assay was performed to identify non-cytotoxic concentrations of tobramycin. The results obtained (Figure S4 in supplementary information) revealed that the Caco-2 cells were

not affected by tobramycin up to concentrations of 45 mM tobramycin, the concentration selected for the transport experiments

Monolayer integrity

TEER values were measured before, during and after the TCA transport experiment to check whether the TEER value of the control group and the tobramycin treated cell layers remained unaffected during the whole experiment. The results confirmed that TEER values were consistent between the control group and tobramycin treated Caco-2 cell layers (Figure S5 in supplementary information).

Tobramycin inhibited TCA transport across a Caco-2 cell layer

Figure 8 shows the time dependent transport of TCA across the Caco-2 cell layer upon pre-treatment of the Caco-2 cells with tobramycin as compared to the control. The results reveal that pre-treatment of the Caco-2 cells with 45mM tobramycin significantly inhibited the translocation of TCA across the Caco-2 cell layer.

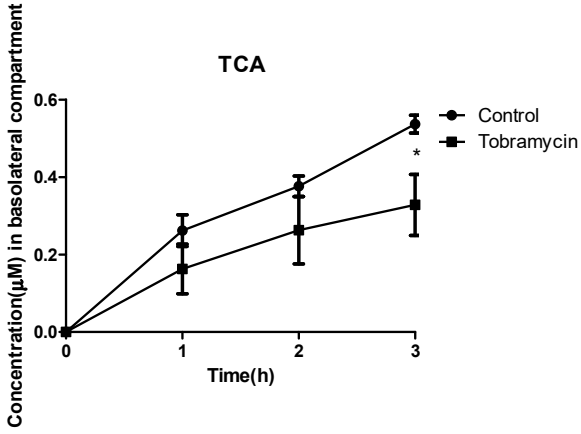


Figure 8 Time dependent translocation of TCA across a Caco-2 cell layer upon pre-exposure with 45 mM tobramycin or without tobramycin (control) (**P*<0.05 indicates a difference from the control without antibiotic at the corresponding time point). Results are shown as mean ± SD from four independent experiments.

Discussion

In vivo studies in which experimental animals are exposed to antibiotics have revealed potentially substantial effects of antibiotics on the host fecal and blood serum metabolome especially on bile acid homeostasis (Behr et al. 2018a). In these *in vivo* studies increased fecal levels of conjugated bile acids like for example TCA were frequently observed (Behr et al.

2019). In theory such increased fecal levels of conjugated bile acids upon exposure to antibiotics may be due to different underlying modes of action including especially an effect on bile acid metabolism by the intestinal microbiota and/or an effect on the process of bile acid reabsorption. Given that 95% of the bile acids are reabsorbed into the systemic circulation, it can be expected that an inhibition of this process will readily result in increased fecal bile acid levels, reflecting a potential disturbance of bile acid homeostasis.

Thus, in the framework of developing new approach methodologies (NAMs) replacing animal testing the aim of the present study was to evaluate two selected *in vitro* models for studying effects of antibiotics on intestinal bile acid homeostasis and obtain insight in the mode(s) of action underlying the *in vivo* effects of the antibiotics on bile acid homeostasis. To this end anaerobic fecal incubations as an *in vitro* model for studying effects of the antibiotics on gut microbiota and their bile acid metabolism as well as Caco-2 cell layers in a transwell model as an *in vitro* approach to study intestinal bile acid reabsorption were evaluated.

16S rRNA sequencing analysis was used to profile the composition of the intestinal microbiome after exposure with antibiotics (tobramycin, colistin sulfate, meropenem trihydrate, doripenem hydrate). Among the antibiotics tested tobramycin appeared to have the most obvious influence on microbial composition at family level, with reduced contributions of *Verrucomicrobiaceae* and *Erysipelotrichaceae* and increased amounts of *Lachnospiraceae* and *Ruminococcaceae*. *Verrucomicrobiaceae* belong to the *Verrucomicrobia* which is a phylum of gram-negative bacteria (Devos 2014; Szuróczki et al. 2020). *Erysipelotrichaceae* are part of the *Firmicutes* a phylum of gram-positive bacteria (Kaakoush 2015). *Lachnospiraceae* and *Ruminococcaceae* also belong to the *Firmicutes* (Gu et al. 2013). The overall increase of *Firmicutes* at the expense of *Verrucomicrobia* upon tobramycin treatment, is in accordance with its activities as an antibiotic affecting mainly gram-negative bacteria (Neu 1976). In an accompanying *in vivo* study (Murali et al. in preparation) tobramycin and colistin sulfate were also tested in 28 day oral toxicity studies at BASF. This enables comparison of the effects now detected in the *in vitro* anaerobic fecal incubations to those obtained *in vivo*. Comparison of the *in vitro* and *in vivo* 16S rRNA analysis results, revealed that both *in vitro* and *in vivo* tobramycin has the most substantial effects while the effects of colistin sulfate appeared limited. Similar to the PCoA of the present *in vitro* studies, an *in vivo* PCoA analysis of the 16S RNA data of fecal samples obtained upon 28-day treatment of rats with these antibiotics demonstrated that the tobramycin group separated from the control group, while the colistin sulfate samples did not clearly cluster different from the control. Furthermore, in control fecal samples in both the *in vivo* and *in vitro* study the dominant families were *Verrucomicrobiaceae*, *Porphyromonadaceae* followed by *Lachnospiraceae* and *Ruminococcaceae* while also the effect of tobramycin appeared to be similar resulting in a reduction of *Verrucomicrobiaceae* and an increase in *Lachnospiraceae* (Murali et al. in preparation), again corroborating the consistency between the *in vivo* and *in vitro* study. In both the *in vitro* and *in vivo* study, colistin sulfate treatment showed limited effects. The *in vitro* data revealed that meropenem trihydrate and doripenem hydrate were

different from controls as illustrated by similar effects on *Erysipelotrichaceae, Lactobacillaceae* and *Ruminococcaceae* as observed for tobramycin but less distinct.

The *in vivo* studies also reported effects on the fecal and plasma metabolome with substantial effects of tobramycin treatment on especially fecal levels of the bile acid TCA. Therefore, further experiments of the present paper focused on tobramycin and the potential modes(s) of action underlying this substantial increase in fecal TCA levels upon tobramycin treatment. In *in vitro* anaerobic incubations with and without extra added external bile acids tobramycin treatment significantly reduced degradation of conjugated bile acids including TCA, while in incubations with colistin sulfate the time dependent bile acid degradation matched that observed in the control without added antibiotics. These results revealed that tobramycin inhibits TCA deconjugation, an observation that was corroborated by incubating TCA with fecal samples obtained from control rats and with fecal samples from the rats treated for 23 days with tobramycin, which revealed significantly reduced rates of TCA deconjugation by fecal samples from tobramycin treated rats. This *in vitro* observation provides insight in a potential mode of action underlying the *in vivo* observed increases in fecal TCA levels, and indicates that upon treatment with tobramycin the intestinal microbiota become less efficient in deconjugation of this primary bile acid.

It is of interest to note that in the *in vitro* incubations without extra added external bile acids no conjugated bile acids were detected while the concentrations of all non-conjugated bile acids appeared to decrease in time. This first observations pointed at the highly efficient deconjugation by the fecal microbiota while the latter observation may best be explained by further conversion of these non-conjugated bile acid by the intestinal microbiota to products not included in the LC-MS/MS based analysis. For example the primary bile acids α-MCA and β-MCA known to be especially present in rodents, can be converted to secondary bile acids like ω-MCA (Chiang 2017), a bile acid not included in the LC-MS/MS method applied. The time dependent reduction in bile acids may also be ascribed to metabolism of bile acids regarding pathways enabling their use as energy source (Ma and Patti 2014). This latter explanation would be consistent with the upregulation of *Firmicutes* upon tobramycin treatment because some literature studies report on increased energy harvest induced by increased levels of *Firmicutes* (Figure S1 in supplementary information)(Turnbaugh et al. 2006).

In addition to inhibition of TCA deconjugation upon treatment of the intestinal microbiota by tobramycin it was also investigated whether the increased fecal levels of TCA upon tobramycin treatment of rats *in vivo*, may be due to an effect on intestinal reuptake. Given that 95% of bile acids are actively reabsorbed a limited effect on this reabsorption involved can be expected to substantially increase fecal levels of bile acids.

Upon pre-exposure of the Caco-2 cell monolayers with tobramycin, the transport of TCA across the intestinal monolayer was significantly reduced. The reduction transport activity upon pre-exposure may be related to the fact that tobramycin, being a aminoglycoside antibiotic, is

able to facilitate readthrough at stop codons at the ribosomes thus reducing the synthesis of normal function proteins (Prokhorova et al. 2017; Wangen and Green 2020). Hence, it is possible to hypothesize that the expression and thus activity of the apical sodium-dependent bile acid transporter (ASBT) was inhibited by tobramycin at the level of protein synthesis at the ribosomes. In addition, increased bile acid efflux from the liver and/or enhanced bile acid synthesis - the latter resulting from the induction of Cyp7a1 expression in the liver upon depletion of gut microbiota by antibiotics (Schneider et al. 2021) - may contribute to the increased intestinal and subsequent fecal TCA levels.Thus the increase in fecal TCA levels can be ascribed to a reduction in deconjugation capacity resulting from changes in the intestinal microbiome, an effect on the re-uptake via effects on synthesis and activity of the transport proteins involved, and/or an effect on de novo bile acid synthesis and efflux from the liver.

In addition to the effects of antibiotics on bile acid homeostasis at the levels investigated in the present study, it was reported that depletion of gut microbiota by antibiotics may result in induction of Cyp7a1 expression in the liver thereby enhancing bile acid synthesis (Awoniyi et al. 2022; Schneider et al. 2021). Development of a new approach methodology (NAM) to capture these effects is an interesting topic for future studies. Such studies may include identification of the antibiotic related changes in host blood or plasma bile acid metabolome profiles. Earlier *in vivo* studies already reported that antibiotics including lincomycin, clindamycin, roxithromycin, vancomycin (Behr et al. 2019) and also tobramycin (Murali et al in preparation) affected host blood or plasma metabolome patterns including bile acid concentrations resulting in an increase in the ratio of conjugated versus unconjugated bile acids .This finding is in line with results reported by Schneider et al. (Schneider et al. 2021). This relative increase in the ratio of conjugated versus deconjugated bile acids may provide a rationale for a potential effect on the expression of liver enzymes involved in bile acid synthesis. Including the effects observed in the present study in a physiologically based kinetic model (PBK) model for bile acid homeostasis, and use of the *in vitro* systems to define the relevant PBK model parameters for uptake and metabolism, may provide a NAM to elucidate to what extent the inhibition of bile acid deconjugation and reuptake by tobramycin in the intestine may affect systemic bile acid levels and provide a rationale for the effect on bile acid synthesis in the liver.

The *in vitro* studies of the present study also provide a basis to use the respective models to study the effect of combined antibiotic exposure (Worthington and Melander 2013). This is of relevance given that in daily practice often cocktails of broad spectrum antibiotics are applied. Use of *in vitro* models facilitates testing of a wider range of combinations providing insight in potential combination effects and/or enabling the definition of priorities for potential *in vivo* studies.

All together the results of the present paper provide a first proof-of-principle that *in vitro* studies can be of use to elucidate the effect of *in vivo* antibiotics, and thus also of other xenobiotics, on

the intestinal microbiota and their bile acid metabolism as well as on reuptake of bile acids, all resulting in effects on bile acid homeostasis.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary information

Supplementary data to this article can be found online at

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Chapter 3.

In vitro models to measure effects on intestinal deconjugation and transport of mixtures of bile acids

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Abstract

Bile acid metabolism and transport are critical to maintain bile acid homeostasis and host health. In this study, it was investigated if effects on intestinal bile acid deconjugation and transport can be quantified in *in vitro* model systems using mixtures of bile acids instead of studying individual bile acids. To this end deconjugation of mixtures of selected bile acids in anaerobic rat or human fecal incubations and the effect of the antibiotic tobramycin on these reactions was studied. In addition, the effect of tobramycin on the transport of the bile acids in isolation or in a mixture across Caco-2 cell layers was characterized. The results demonstrate that both the reduction of bile acid deconjugation and transport by tobramycin can be adequately detected in *in vitro* systems using a mixture of bile acids, thus eliminating the need to characterize the effects for each bile acid in separate experiments. Subtle differences between the experiments with single or combined bile acids point at mutual competitive interactions and indicate that the use of bile acid mixtures is preferred over use of single bile acid given that also *in vivo* bile acids occurs in mixtures.

Key words: tobramycin, fecal incubation, conjugated bile acids, Caco-2 cells, bile acid transport

1.Introduction

Bile acids are produced from cholesterol and then conjugated with glycine or taurine forming conjugated primary bile acids in the liver (de Aguiar Vallim et al. 2013; Di Ciaula et al. 2018). The conjugated primary bile acids are secreted into the small intestine through the bile duct, after which most of the conjugated primary bile acids are reabsorbed in the ileum and channeled back via the portal vein into the liver (Chiang 2009). Active reabsorption of bile acids in the ileum is important in sustaining bile acid homeostasis. More than 95% of conjugated primary bile acids are reabsorbed and returned to the liver (Besnard et al. 2004; Chiang 2013). This recycling is called enterohepatic circulation (Hofmann 2009; Pellicoro and Faber 2007), and helps to maintain bile acid homeostasis (Lefebvre et al. 2009; Thomas et al. 2008).A minor amount of conjugated primary bile acids escapes reuptake and is transferred to the large intestine, where intestinal microbiota perform deconjugation and 7α-dehydroxylation converting conjugated primary bile acids like for example taurocholic acid (TCA), via unconjugated primary bile acids like for example cholic acid (CA), into secondary bile acids like for example deoxycholic acid (DCA) (Urdaneta and Casadesús 2017; Winston and Theriot 2020). Figure 1 schematically presents the bile acid transformations in the large intestine of interest for the present study. Antibiotic treatment can cause profound changes in the composition and abundance of gut microbiota (Lange et al. 2016; Ramirez et al. 2020). Hence, several studies focused on the impact of antibiotics on gut microbiota and some studies also characterized the resulting influences on the bile acid pool. Most of these studies applied *in vivo* animal models measuring the bile acid composition and changes therein upon oral administration of antibiotics in different *ex vivo* matrices including feces, plasma and intestinal tissues (Behr et al. 2019; Selwyn et al. 2015; Vrieze et al. 2014). These studies elucidated the influence of antibiotics on bile acid profiles. In addition to the effects of antibiotics on the gut microbiota, there could also be effects on the intestinal cells and transporters involved in the reuptake underlying the changes in bile acid homeostasis induced by the antibiotics. In a previous study (Zhang et al. 2022b), we showed that the antibiotic tobramycin inhibits the microbial deconjugation of taurocholic acid (TCA) in anaerobic fecal incubations and also the transport of TCA over an *in vitro* Caco-2 cell layer used as a model to mimic intestinal bile acid reuptake. Thus, we showed that *in vitro* model systems could be used to study the effect of antibiotics on the gut microbial metabolism and intestinal transport using anaerobic fecal incubations and Caco-2 cell layers in a transwell system with an individual bile acid, taurocholic acid (TCA).

The aim of the present study was to investigate whether the effects on bile acid metabolism and transport upon treatment with tobramycin can also be studied in an *in vitro* model system using mixtures of bile acids instead of individual bile acids, in order to i) elucidate the effect of tobramycin on the deconjugation and intestinal transport of other bile acids, ii) develop a more efficient testing strategy, iii) better mimic the *in vivo* situation where bile acids also do

not occur in isolation, and iv) contribute to the development of new approach methodologies focusing on the 3Rs (replacement, reduction and refinement) of animal experiments.

In the current study, the two *in vitro* model systems previously used to obtain insight into how tobramycin would modulate TCA metabolism and transport were used to study the effect of tobramycin on deconjugation and transport of a series of (conjugated) primary bile acids. For deconjugation studies anaerobic fecal incubations were used while for transport studies the Caco-2 transwell model was applied. The Caco-2 cell line is able to differentiate into cells that resemble small intestinal cells not only showing morphological characteristics of intestinal cells but also expressing typical microvillus hydrolases and nutrient transporters (Hauri et al. 1985; Howell et al. 1992; Meunier et al. 1995). The Caco-2 cell line is a widely applied *in vitro* model to evaluate the transport of drugs and xenobiotics and has also been used to study the reuptake of bile acids (Sambuy et al. 2005; Yamashita et al. 2000; Zhang et al. 2022b).

Deconjugation and transport of the bile acids was studied for selected bile acids in isolation but also for a mixture of selected bile acids to further elucidate whether testing a mixture instead of single bile acids would provide an adequate way to study (effects on) bile acid transport. The results obtained provide insight into the applicability of the two *in vitro* model systems to study effects on bile acid homeostasis.

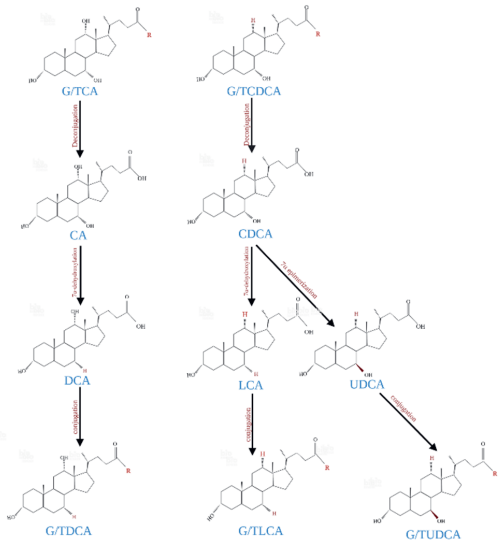


Figure 1. Bile acid transformations in the large intestine**.** R means taurine/glycine.

2.Materials and methods

2.1. Reagents

Tobramycin was purchased from Sigma-Aldrich (Schnelldorf, Germany). Chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA), hyodeoxycholic acid (HDCA), deoxycholic acid (DCA), cholic acid (CA), glycoursodeoxycholic acid (GUDCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycocholic acid (GCA), taurolithocholic acid (TLCA), tauroursodeoxycholic acid (TUDCA), taurohyodeoxycholic acid (THDCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and taurocholic acid (TCA) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Glycolithocholic acid (GLCA) was obtained from Cambrige isotope laboratories (Massachusetts, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Darmstadt, Germany). Acetonitrile (ACN) and methanol were obtained from Biosolve BV (Valkenswaard, Netherlands). Phosphate buffered saline (PBS) was purchased from Gibco (Paisley, UK). Fetal Bovine serum (FBS) was obtained from GE Healthcare Life Sciences Hyclone Laboratories (Logan, Utah, USA). 0.05% Trypsin-EDTA, minimum essential medium (MEM), penicillin-streptomycin-glutamine solution (PSG), sodium pyruvate, Hank's balanced salt solution (HBSS) and HEPES buffer solution were purchased from Gibco (Paisley, UK). Corning Costar 24 well transwell plates were purchased from Corning Life Sciences (Schnelldorf, Germany). 96 Well cell culture plates were obtained from Greiner Bio-One B.V. (Alphen aan den Rijn, Netherlands).

2.2 Anaerobic incubations of bile acids with rat or human feces

Fecal collection and processing

Fresh rat feces from control (10 males and 10 females) Wistar rats were provided by BASF SE (Ludwigshafen, Germany). Feces were obtained by physical massage of the rectum of rats, weighed and transferred immediately into anaerobic 10% (v/v) glycerol in PBS solution, pooled 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) (BACTRON300 anaerobic chamber (Cornelius, USA)). Subsequently, samples were filtered using sterile gauze under anaerobic conditions, and aliquoted samples of resulting fecal slurry were stored at -80°C until use. Human feces were collected from 12 healthy individuals without dietary limitations. They were from different gender, age and ethnicity. All of them had not taken antibiotics for at least 3 months prior to the study. After volunteers' donations, fecal samples were collected, weighed and immediately transferred to anaerobic 10% (v/v) glycerol in PBS solution, pooled 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) (BACTRON300 anaerobic chamber (Cornelius, USA)). A pooled sample was acquired by mixing of 12 fecal suspensions in equal amounts. The experimental protocol was evaluated and approved by the Medical Ethical Reviewing Committee of Wageningen

University (METC-WU) based on the Dutch Medical Research Involving Human Subjects Act. All volunteers gave their written consent.

Deconjugation of fecal bile acids

Incubations to study deconjugation of mixtures of bile acids and the effect of the antibiotic tobramycin on this deconjugation were performed essentially as described previously for isolated bile acids (Zhang et al. 2022b). Each incubation contained 1 µl rat fecal slurry (final concentration: 2 mg/ml fecal sample), 10 µl of a 10 times concentrated solution containing mixed conjugated bile acids (TCA, TCDCA, GCA and GCDCA) resulting in a final concentration of 500 µM for each of the bile acids tested, 79 µl PBS and 10 µl water (control) or 10 µl tobramycin from a 10 times concentrated stock solution in MilliQ water resulting in a final concentration of 45 mM tobramycin.

Anaerobic 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°C. Incubations were terminated by adding a similar volume (100 µL) acetonitrile after 0h, 2h, 4h, 6h and 8h. Samples were centrifuged at 21500 g for 15 min at 4 °C, and the supernatants were transferred to LC-MS/MS vials and stored at -80 °C until bile acid detection by LC-MS/MS.

Anaerobic incubations of human fecal slurry containing 45 mM tobramycin and mixed conjugated bile acids (TCA, TCDCA, GCA and GCDCA) at a final concentration of 500 µM for each bile acid were performed and processed in a similar way.

2.3. Cell culture

Human colon adenocarcinoma Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells (passage number 10-20) were cultured in the medium including MEM with 20% FBS, 1% sodium pyruvate and 1% penicillin-streptomycin-glutamine. The cells were grown in 75 cm^2 flask in an incubator at 37 °C, 5% CO_2 and 100% humidity. Cells were subcultured at 50%-60% confluence with 0.05% trypsin-EDTA.

2.4. Bile acid transport

To study bile acid transport across Caco-2 cell layers, 100 µl of a cell suspension containing 1.8×10^5 cells /cm^2 was seeded at the apical sides of a Corning 24 well transwell plate (Product no. CLS3413), and 600 µl MEM medium was added to the basolateral compartments. Cells were cultured for 18 days in order to differentiate into intestinal cell layers, while medium was changed every other day. MEM medium free of penicillin-streptomycin was applied when cells were seeded in the 24 well transwell plate in order to eliminate potential impact on the effects induced by the antibiotic tobramycin. After 18 days cultivation of the Caco-2 cells in the transwells, cell layers were exposed to 45 mM tobramycin or solvent control for 48 h. Following this pre-incubation, the integrity of the Caco-2 cell layers was measured by using transepithelial electrical resistance (TEER). The TEER was measured with a Millicell® ERS-2

Volt-Ohm Meter (Millipore, Amsterdam, Netherlands). The TEER value was detected before, during and after the transport assay, and expressed in $\Omega \times cm^2$. Only cell layers with a TEER value above 500 $\Omega \times cm^2$ were used for transport experiments and it was also checked that tobramycin pre-exposure did not affect the TEER value compared to the control, indicating there was no effect on cell layer integrity.

After the pre-exposure to tobramycin (or solvent control), the exposure medium was removed and the cell layers were cultured in transport medium (HBSS supplemented with 10 mM HEPES) for 30 min. After this 30 min incubation in transport medium, medium was removed and fresh transport medium containing 5 µM (final concentration) TCA, TCDCA, GCA or GCDCA, either individually or as a mixture, were added to the apical part of the wells. 50 µL samples were collected from the basolateral compartment at 1h, 2h and 3h, and the compartment was refilled with the same amount of transport medium after each collection. In the collected samples, the amount of the bile acids was quantified by LC-MS/MS.

Additionally, similar transport experiments for a mixture of 17 bile acids (UDCA, HDCA, CDCA, DCA, CA, GLCA, GUDCA, GDCA, GCDCA, GCA, TCDCA, TCA, TLCA, TUDCA, THDCA, TDCA and LCA) each at a final concentration of 5 µM were performed. Samples were collected as described above and measured by LC-MS/MS.

Concentrations of bile acids in the intestinal tract and plasma have been reported to be in the high micromolar or low millimolar range (Hamilton et al. 2007; Xiang et al. 2010). The overall concentrations used in the combined bile acid experiments of the present study amounted to 20 mM and 85 mM for the experiment with 4 or 17 bile acids combined and thus fall in the lower range of the physiological levels, while potential relative differences between different bile acids were not taken into account testing all bile acids at an equimolar concentration also to allow comparison.

2.5. Bile acid profiling by LC-MS/MS

A triple quadrupole LCMS-8045 (Shimadzu Corporation, Japan) was used to perform bile acid analysis. The detection method was able to measure 20 bile acids. A Kinetex C18 column (1.7µm×100 A×50mm×2.1 mm, Phenomenex 00B-4475-AN) was used to separate bile acids. Mobile phase A (0.01% formic acid in MilliQ water) and mobile phase B (50 % acetonitrile/50 % methanol) were applied. 1 µL sample was injected onto the column equilibrated with 70 % mobile phase A and 30 % mobile phase B at a flow rate of 0.4 mL/min. The following gradient was used: 0-10 min 30-70% B, kept at 70% B for 1 min; 11-19 min 70- 98% B, kept at 98% B for 1min; and then 20-25 min 98-30% B followed by 10 min equilibration at 30% B before the next injection. The mass spectrometer (MS) used electrospray ionization (ESI) in negative ion mode. The ESI parameters were as follows: Nebulizing gas flow 3 L/min; drying gas flow and heating gas flow 10 L/min; interface voltage 3 kV; interface current 0.9 uA; interface temperature 300 °C; Desolvation temperature 526 °C; DL temperature 250 °C; heat block temperature 400 °C; Conversion Dynode Voltage

10 kV; detector voltage 2.16 kV; IG Vacuum 1.8e-03 Pa; PG Vacuum 9.4e+01 Pa; CID Gas 230 kPa. Selective ion monitoring (SIM) and multiple reaction monitoring (MRM) were applied for the bile acid detection. Both SIM and MRM were used for detection of the bile acid to obtain adequate sensitivity for all the bile acids. Specific bile acid identification MS values are presented in Table S1 in the supplementary information.

2.6. Data processing and analysis

Bile acid profile data were analyzed using the Labsolutions software in the LC-MS/MS system. Graphical figures were drawn using Graphpad Prism 5 (San Diego, USA). Statistical analysis was performed by Student's t test. $P< 0.05$ was considered significant. Figure 1 was drawn by Biorender (San Francisco, USA). All the results were expressed as mean ± SD from three independent measurements.

3. Results

3.1 The impact of tobramycin on the deconjugation of conjugated bile acids when tested in isolation or in a mixture

In our previous study (Zhang et al. 2022a), the effect of tobramycin on the gut microbial deconjugation of TCA, TCDCA, GCA and GCDCA when tested in isolation was reported. Table 1 summarizes the rates of deconjugation derived from these previous data and reveals that the deconjugation of TCDCA and GCDCA was faster than that of TCA and GCA, and that tobramycin inhibited the deconjugation of all 4 conjugated bile acids. The results in Figure 2A-D show the effect of tobramycin compared to the control on the time dependent deconjugation of TCA, TCDCA, GCA and GCDCA when tested in a mixture. Figure 2E and F present the accompanying formation of the deconjugated metabolites CA, formed from TCA and GCA, and CDCA, formed form TCDA and GCDA. Table 1 also presents the rates of deconjugation derived from these data. From the results obtained it follows that when tested in a mixture, the rates of deconjugation were either comparable or at most up to about 2-fold lower than when testing the bile acids in isolation. Under both conditions the inhibition of bile acid deconjugation by tobramycin was readily observed with the effect being even somewhat more pronounced when the bile acids were tested in a mixture. Also when tested in a mixture deconjugation of TCDCA and GCDCA was faster than that of TCA and GCA which was also reflected by the faster rate of CDCA metabolite formation as compared to the rate of CA formation. The total mass balance for these rat fecal incubations at different timepoints is shown in supplementary information S1 A. The total mass recovery decreased from 94. 4% at 0 h to 56.8% at 8h, indicating that CA and CDCA were further degraded into other metabolites which were not included in the present LC-MS analyses.

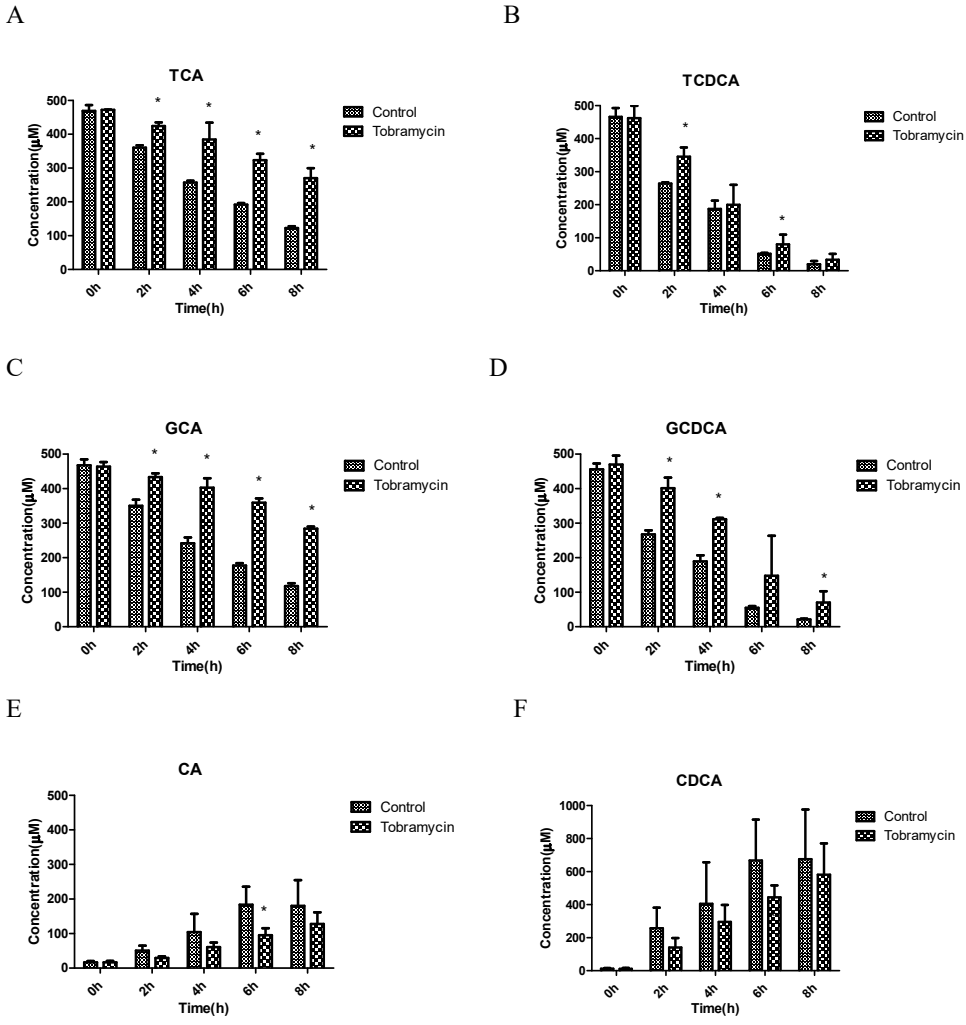


Figure 2. Time dependent deconjugation of a mixture of taurine and glycine conjugated bile acids (TCA, TCDCA, GCA and GDCA)(Figure A-D) to their unconjugated metabolites CA or CDCA (Figure E,F) in anaerobic rat fecal incubations with or without (control) tobramycin (* $P< 0.05$). Results are shown as mean ± SD, n=3.

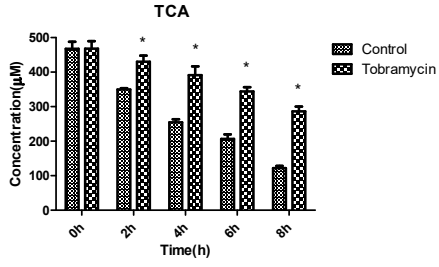
Table 1 Deconjugation rate (in μmol/h/g feces) of conjugated bile acids tested in isolation or in an equimolar mixture in anaerobic rat fecal slurry incubations in the absence (control) or presence of tobramycin (* $P< 0.05$). Data shown are mean ± SD, n=3.

	Deconjugation rate (µmol/h/g feces)			Deconjugation rate (µmol/h/g feces)		
	Rat feces, individual bile acids[1)]			Rat feces, mixture bile acids		
Bile acid	Control	Tobramycin	% of control	Control	Tobramycin	% of control
TCA	59.6±7.8	37.4±4.4*	62.8	26.5±1.6	10.9±6.1*	41.1
TCDCA	47.8±2.5	38.8±3.5*	81.2	50.5±5.8	29.1±10.8*	57.6
GCA	39.2±5.4	22.8±3.2*	58.2	28.2±4.2	7.6±3.2*	27.0
GCDCA	58.6±10.2	32.8±11.9*	56.0	47.1±6.1	17.1±1.1*	36.3

[1)] Data derived from figure 5 and figure 6 in (Zhang et al. 2022b).

After showing that the effect of tobramycin on bile acid deconjugation by rat gut microbiota can be elucidated in anaerobic fecal incubations with a mixture of the primary bile acids, the effects of tobramycin on bile acid deconjugation by human gut microbiota was investigated. Figure 3 shows the results obtained in anaerobic human fecal incubations with and without tobramycin and a mixture of the 4 conjugated bile acids (TCA, TCDCA, GCA and GCDCA). Table 2 presents the rate for bile acid deconjugation derived from these data. These data reveal that rates for deconjugation by rat and human feces were comparable and that, similar to the rat fecal incubations, the deconjugation of TCDCA and GCDCA by human feces was faster than that of TCA and GCA. Also, the inhibition of bile acid deconjugation by tobramycin was comparable for rat and human fecal incubations. The total mass recovery of the bile acids in the human fecal incubation at different incubation timepoints are presented in the supplementary information S1 B. The total mass recovery decreased from 93.14% at 0 h to 67.5% at 8h. This indicates that also in the anaerobic human fecal incubations CA and CDCA were further degraded into other metabolites which were not included in the present LC-MS analyses.

A



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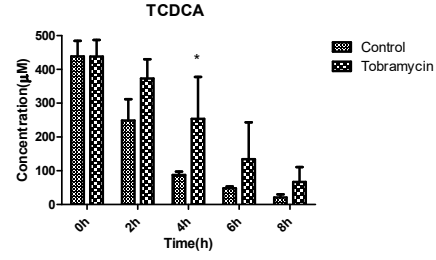


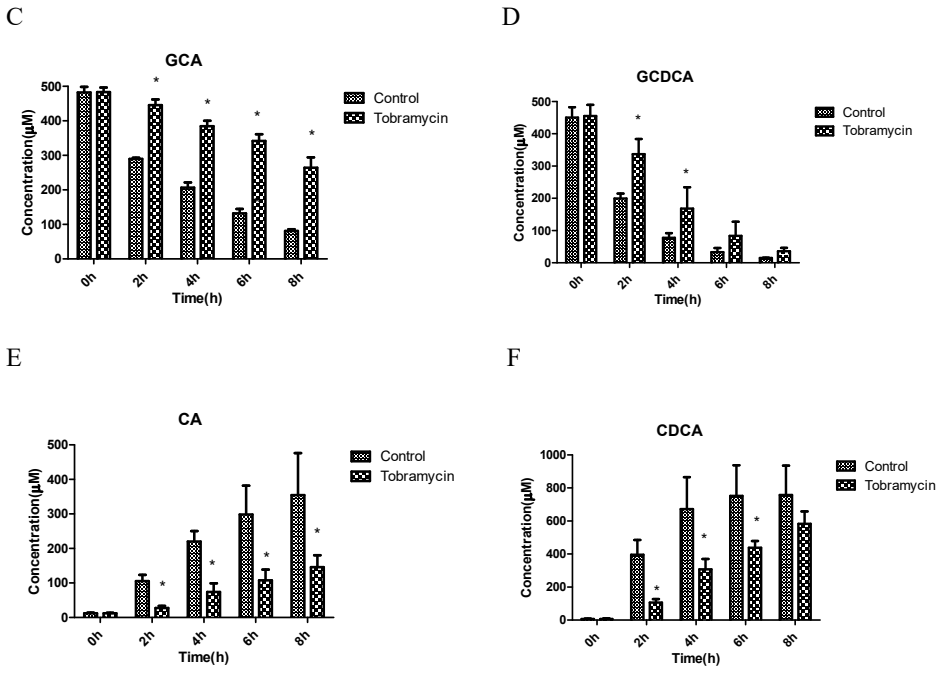


Figure 3. Time dependent deconjugation of an equimolar mixture of taurine and glycine conjugated bile acids (TCA, TCDCA, GCA and GCDCA) (Figure A-D) to their unconjugated metabolites CA or CDCA (Figure E,F) in anerobic human fecal incubations with or without (control) tobramycin (* $P< 0.05$). Results are shown as mean ± SD, n=3.

Table 2 Deconjugation rate of mixed conjugated bile acids in human fecal slurry in the absence (control) or presence of tobramycin (* $P< 0.05$) . Data shown are mean ± SD, n=3.

	Deconjugation rate (μmol/h/g feces)		
	Human feces, mixture bile acid		
Bile acid	Control	Tobramycin	% of control
TCA	26.7±2.4	9.6±1.2*	36.0
TCDCA	44.4±5.3	19.9±4.3*	44.8
GCA	33.8±3.6	9.5±1.4*	28.1
GCDCA	62.7±5.2	29.6±4.6*	47.2

3.2. Effect of tobramycin on bile acid transport across the Caco-2 cell layer when bile acids are tested in isolation or in a mixture

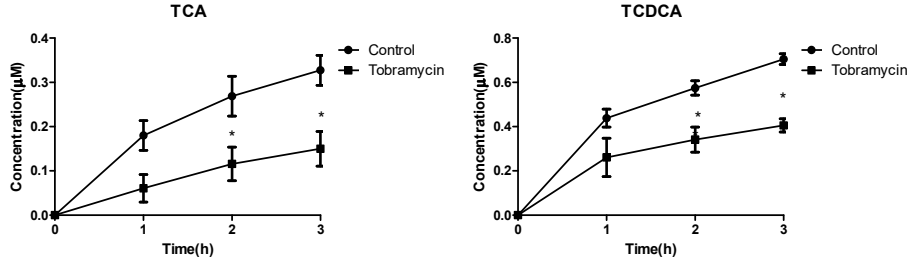
In further experiments, the effects of tobramycin pre-exposure on the transport of bile acids in a Caco-2 transwell system when tested in isolation or as a mixture was investigated. First, Caco-2 cell integrity was confirmed by measuring TEER values before, during and after the bile acid transport experiment to confirm the barrier integrity of Caco-2 cell layers. The TEER

values of the cell layers were consistent both in the control group and upon tobramycin pre-treatment and in the presence or absence of the bile acids (Figure S2-S4 in supplementary information).

Figure 4 shows the time dependent transport of the 4 selected primary bile acids tested in isolation across the Caco-2 cell layers with or without the tobramycin pre-treatment. The apparent permeability coefficient Papp expressed in cm/s was estimated based on the data at 1 h and the Papp values thus obtained are presented in table 3. From these data it follows that tobramycin pre-incubation results in a significant reduction of the transport of all 4 tested conjugated bile acid across the Caco-2 cell layers. The results also reveal that transport of GCDCA and TCDCA across the control as well as the tobramycin treated cell layers was about 2-fold higher than that of GCA and TCA. Transport of TCA and GCA was comparable and that also holds for the transport of TCDCA and GCDCA.

Figure 5 presents the results of the experiment when the bile acids were tested as a mixture. From these data it follows that both when testing bile acids in isolation or as an equimolar mixture, the Papp values for especially transport of GCA and GCDCA were comparable while those for TCA and TCDCA seemed somewhat lower when testing the mixture. Both conditions, however equally well detected the effect of tobramycin pre-treatment of the Caco-2 cells resulting in substantial and significantly decreased transport of all 4 conjugated bile acids compared with control. Also, when tested as a mixture the transport of TCA and GCA was comparable and the transport of TCDCA was comparable to that of GCDCA. Also similar to the results obtained with the individual bile acids, transport of GCDCA and TCDCA across the control as well as the tobramycin pre-treated cell layers appeared to be about 2- fold higher than that of GCA and TCA.

A B



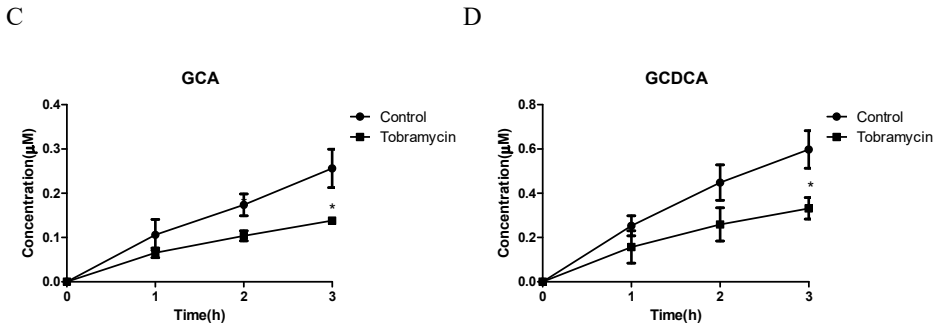


Figure 4. Time dependent transport of individual bile acids including A) TCA, B) TCDCA,C) GCA or D) GCDCA) across a Caco-2 cell layer upon pre-exposure with or without (control) tobramycin, when tested in isolation (* $P< 0.05$). Results are expressed as mean ± SD, n=3.

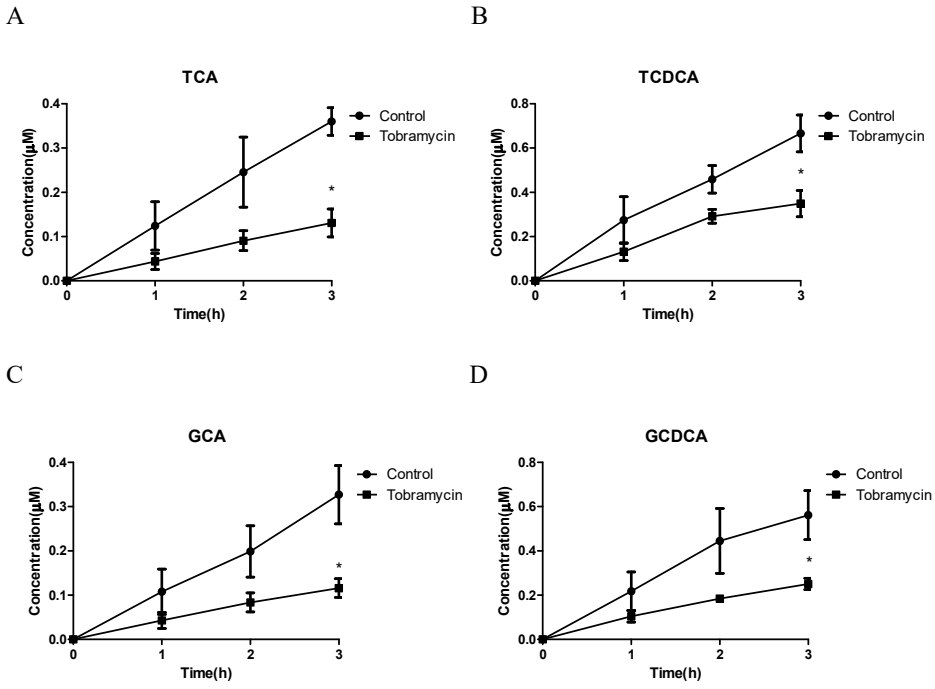
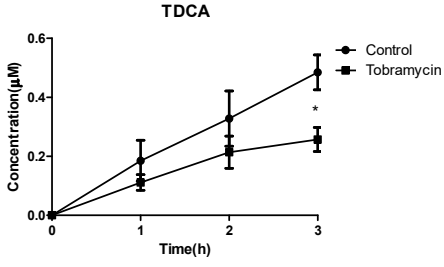


Figure 5. Time dependent transport of bile acids including A) TCA, B) TCDCA, C) GCA and D) GCDCA) across a Caco-2 cell layer upon pre-exposure with or without (control) tobramycin, when tested as an equimolar mixture (* $P< 0.05$). Results are expressed as mean ± SD, n=3.

After it was shown that the effect of tobramycin pre-exposure on bile acid transport could be equally well detected by testing 4 selected bile acids in a mixture, the effect of tobramycin on the transport of a mixture of 17 conjugated and unconjugated bile acids was tested. Figure 6

shows the results obtained for a selected number of representative bile acids while the remaining figures are presented in Figure S5 in the supplementary information. From the results obtained it follows that, in line with what was observed when testing the conjugated bile acids in isolation or in a mixture of 4, tobramycin pre-treatment significantly affected the transport of the conjugated bile acids TCA, TCDCA, GCA, GCDCA and -not tested in the mixture of 4 bile acids- TDCA. It also appeared that, in contrast to an effect on the transport of the conjugated bile acids, tobramycin did not have an effect on transport of the unconjugated bile acids including UDCA, CA, CDCA, HDCA, DCA, and LCA. For the other conjugated bile acids tested including GLCA, GUDCA, GDCA TUDCA and THDCA (with the exception of TLCA), there was a decrease in transport upon tobramycin pre-treatment of the Caco-2 cell layer, albeit not significant. Table 3 presents the Papp values derived from this experiment for the 4 conjugated bile acids that were also tested in isolation and in a mixture of 4. Comparison of the Papp values obtained when testing these bile acids in a mixture of 17 bile acids reveals that especially for GCA and GCDCA the results are similar to the results obtained when testing the bile acids in isolation or in a mixture of 4, while for TCA and TCDCA testing in a mixture of 17 resulted in transport that was comparable to what was observed when the bile acids were tested in a mixture of 4 while transport was about 2-fold lower than when testing these two bile acids in isolation. Similar to data obtained when testing bile acids in a mixture of 4, also when testing them in a mixture of 17, the transport of TCDCA and GCDCA was similar and about 2-fold higher than that of TCA and GCA, which were also transported to a similar extent. Also the effect of tobramycin pre-treatment of the Caco-2 cells was similar resulting in a significant about 2-fold reduction of the transport.

A



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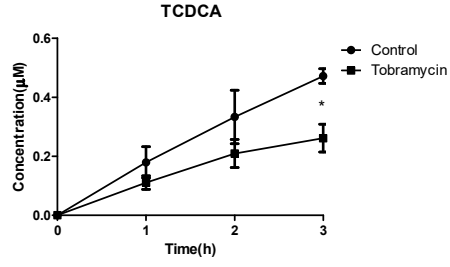

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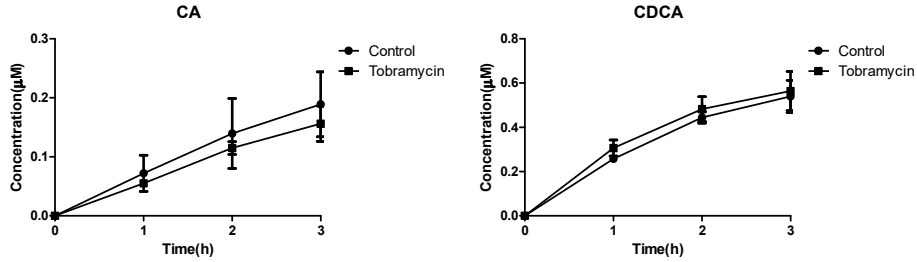

Figure 6. Time dependent transport of 4 selected bile acids (TDCA, TCDCA, CA and CDCA) when tested in a mixture of 17 bile acids, across a Caco-2 cell layer upon pre-exposure without (control) or with tobramycin (* $P< 0.05$). Results are expressed as mean ± SD, n=3.

Table 3 Transport rate (Papp) of 4 selected conjugated bile acids when tested in isolation or in a mixture of 4 or 17 bile acids across Caco-2 cell layers upon pre-exposure without (control) or with tobramycin (* $P< 0.05$). Data shown are mean ± SD, n=3.

	Papp ($\times 10^{-5}$ cm/s)			Papp ($\times 10^{-5}$ cm/s)			Papp ($\times 10^{-5}$ cm/s)		
	Individual bile acids			Mixture of 4 bile acids			4 from a mixture of 17 bile acids		
Bile acid	Control	Tobramycin	% of control	Control	Tobramycin	% of control	Control	Tobramycin	% of control
TCA	1.8±0.3	0.5±0.1*	27.8	1.2±0.1	0.5±0.1*	41.7	0.8±0	0.4±0.1*	50.0
TCDCA	4.4±0.4	2.7±0.4*	61.4	2.2±0.1	1.4±0*	63.6	1.6±0.1	1±0.2*	62.5
GCA	0.9±0.2	0.7±0.1*	77.7	1.1±0.1	0.4±0.1*	36.4	0.9±0.1	0.5±0.1*	55.6
GCDCA	2±0.3	1.3±0.4*	65.0	2.3±0.3	0.9±0.1*	39.1	1.6±0.1	1±0.2*	62.5

4. Discussion

In our previous study, it was shown in *in vitro* model systems that tobramycin significantly inhibited the deconjugation of the individual bile acids TCA, TCDCA, GCA or GCDCA and the intestinal transport of TCA (Zhang et al. 2022b). To facilitate further studies on the effect of oral exposure to antibiotics and other drugs or food-borne chemicals on bile acid homeostasis, it is of interest to investigate whether the effects can also be detected when testing bile acids in a mixture. This does not only enable the concomitant detection of effects on a large number of bile acids but also can be expected to more closely mimic the *in vivo* situation where bile acids will also not occur in isolation. The results of the present study reveal that in a qualitative way the experiments with mixtures of bile acids give results that are similar to those obtained when testing individual bile acids. When testing mixtures subtle

differences compared to the results obtained with isolated bile acids were also observed. Deconjugation of the 4 conjugated primary bile acids in anaerobic fecal incubations was 1.5- to 2-fold slower when tested as a mixture, and their transport across a Caco-2 cell layer was 1.5- to 3-fold slower when tested as a mixture. These differences can best be ascribed to the involvement of enzymes and active transporters for which different bile acids may act in competition with one another thereby resulting in mutual inhibition of the deconjugation and transport.

Deconjugation of taurine or glycine conjugated bile acids is known to be rapid and to proceed via bile salt hydrolases (BSH) provided by a wide variety of gut microbiota such as for example *Bacteroides fragilis*, *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Enterococcus* and others (Foley et al. 2019; Gérard 2013; Winston and Theriot 2020). Conjugated bile acids when present as a mixture may compete with each other at the active site of these enzymes performing the deconjugation. The results also show that deconjugation of glycine conjugated GCA and GCDCA is faster than that of taurine conjugated TCA and TCDCA in anaerobic incubations with both human and rat gut microbiota, while there was no species difference in these deconjugations. This observation can likely be ascribed to the fact that a wide range of gut microbiota and/or enzymes may be involved in the deconjugation of these primary bile acids by human and rat gut microbiota

Studying transport of the bile acids in isolation or in mixtures in a Caco-2 transwell system revealed that under both conditions the transport of GCDCA and TCDCA was faster than that of GCA and TCA. This may be due to the fact that GCDCA and TCDCA are dihydroxy bile acids while TCA and GCA are trihydroxy bile acids (Binder and Rawlins 1973; Iga and Klaassen 1982). The apical sodium-dependent bile acid transporter (ASBT) is located at the apical membrane of enterocytes and is responsible for reabsorption of the bile acids from the intestine back to the liver (Chen et al. 2012; Xiao and Pan 2017). Also, in Caco-2 cells the ASBT transporter is responsible for bile acid transport across the cell layer (Takashima et al. 2021; van der Mark et al. 2014). It is known that dihydroxy bile acids have a greater affinity to the apical sodium-dependent bile acid transporter (ASBT) than trihydroxy bile acids (Balakrishnan and Polli 2006; Balakrishnan et al. 2006a; Dawson 2011). It is also known that the passive diffusion for these conjugated bile acids is inversely related to the number of hydroxyl groups of bile acids (Dietschy 1968). Given that the bile acids were all tested at a similar concentration the greater affinity of the dihydroxy bile acids can be expected to result in faster transport by the ASBT transporter of GCDCA and TCDCA than what is observed for the trihydroxy bile acids TCA and GCA, while for GCDCA and TCDCA also passive diffusion is expected to be higher than for GCA and TCA. Together these characteristics may explain the faster rate of transport for GCDCA and TCDCA than what is observed for the trihydroxy bile acids TCA and GCA.

The experiments also showed that pre-incubation of the Caco-2 cells with tobramycin resulted in substantial reduction of intestinal transport for the 4 conjugated bile acids (TCA, TCDCA,

GCA or GCDCA) compared to the transport across the control Caco-2 cell layers, either when tested in isolation or in a mixture. In a quantitative way also in these transport experiments testing of the bile acids as a mixture resulted in somewhat lower transport rates likely due to mutual competition.

In the normal *in vivo* situation, more than 50 bile acids are present in the systemic and the enterohepatic circulation (Dawson and Karpen 2015; Dosedělová et al. 2021). Thus, we also studied the effect of tobramycin on the transport of a mixture of more than the 4 primary conjugated bile acids, including 17 common bile acids, including the 4 conjugated primary bile acids tested in the earlier mixtures. Thus, the mixture consisted of TCA, GCA, GCDCA and TCDCA, as well as 7 other conjugated secondary bile acids including GLCA, GUDCA, GDCA, TDCA, TLCA, TUDCA and THDCA and 6 unconjugated bile acids including UDCA, HDCA, CDCA, DCA, CA, LCA. It was of interest to note that for the unconjugated bile acids there was no difference in transport across the Caco-2 cell layer either with or without tobramycin pre-treatment. This observation is consistent with the consensus that unconjugated bile acids are reabsorbed via passive diffusion (Alrefai and Gill 2007; Bahar and Stolz 1999; Dawson et al. 2009). For the 6 conjugated secondary bile acids including GLCA, GUDCA, GDCA, TLCA, TUDCA and THDCA the transport upon the tobramycin pre-treatment of the Caco-2 cells was lower than in the control group albeit not significant. This observation is in line with the consideration that also some conjugated bile acids are known to be mainly transported via passive or facilitated diffusion (Love and Dawson 1998). Thus, only the transport of the 4 conjugated primary bile acids TCA, TCDCA, GCA and GCDCA and 1 taurine conjugated secondary bile acid TDCA appeared to be significantly inhibited upon the pre-treatment of the cells with tobramycin, an observation that can be related to an effect of tobramycin on the activity of the ASBT transporter, which has been reported to represent the rate-limiting step in bile acid reuptake (Balakrishnan et al. 2006a). Tobramycin is a bactericidal drug able to affect protein synthesis inside the bacterial cells (Davis 1987; Davis et al. 1986; Mingeot-Leclercq et al. 1999). It is also reported that aminoglycoside antibiotics affect protein synthesis in human cells thus leading to a reduction in normal functional protein formation (Wangen and Green 2020). Tobramycin belongs to the aminoglycosides, hence, it may inhibit ASBT synthesis at the protein level thus resulting in decreased bile acid transport. The synthesis of another important bile acid transporter which is located in the basolateral cell membrane – the organic solute transporter OSTα-OSTβ may also be inhibited by tobramycin further reducing the active transport of the bile acids across the intestinal cell layer (Aldini et al. 1992; Haeusler et al. 2016; Schiff et al. 1972).

Taking all together the results obtained support that the effects of the selected model compound tobramycin on bile acid deconjugation and transport can be adequately detected in the *in vitro* systems using a mixture of bile acids, thus eliminating the need to characterize the effects for each bile acid in separate experiments. Subtle differences between the experiments with single or combined bile acids point at mutual competitive interactions and indicate that the use of bile

acid mixtures is preferred over use of single bile acid given that also *in vivo* bile acids occur in mixtures.

Credit authorship contribution statement

Nina Zhang: Methodology, Experiments, Measurement, Formal analysis, Writing original draft, Visualization. **Weijia Zheng:** Methodology, Software, Measurement, Writing-review & editing. **Bakker Wouter:** Software, Measurement, Writing-review & editing. **Bennard van Ravenzwaay:** Writing-review & editing. **Ivonne M.C.M. Rietjens:** Conceptualization, Methodology, Writing-review & editing, Supervision, Funding acquisition.

Declaration of competing interest

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

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Supplementary information

Supplementary data to this article can be found online at

https://www.sciencedirect.com/science/article/pii/S0009279723001126?via%3Dihub

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Chapter 4.

Antibiotics reduce intestinal bile acid reuptake in an *in vitro* model system

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Abstract

Enterohepatic circulation of bile acids is a highly efficient process that is important for bile acid homeostasis. The aim of the present study was to characterize the impact of a series of antibiotics (lincomycin, streptomycin, vancomycin and tobramycin) on the intestinal reuptake of conjugated bile acids (TCA, TCDCA, GCA and GCDCA) using a Caco-2 cell layer *in vitro* transwell model system. The results obtained demonstrate that both pre-exposure and co-exposure of the cells to an antibiotic and the bile acids, affected bile acid transport, to an extent that depended on the antibiotic, its concentration and the type of conjugated bile acid tested. Tobramycin, at concentrations in line with dose levels at which this antibiotic induced effects on bile acid homeostasis *in vivo*, appeared able to inhibit bile acid transport after pre-exposure of the cells, likely resulting from an effect on the expression of bile acid transporters via its effects on protein synthesis at the ribosome level. Upon co-exposure of the Caco-2 cells to an antibiotic and the bile acids, all four antibiotics appeared to significantly reduce the transport of especially the conjugated bile acids TCDCA and GCDCA with a potency that decreased in the order vancomycin > tobramycin = streptomycin > lincomycin. The effects observed contribute to the effects reported for these antibiotics on bile acid homeostasis *in vivo*, and illustrate the possibility of using a new approach methodology (NAM) to study effects on intestinal bile acid reuptake.

Key words: Antibiotics[1], conjugated bile acid[2], bile acid transport[3], Caco-2 cells[4], *In vitro* intestinal model[5]

1.Introduction

Bile acid synthesis from cholesterol in the liver is a multistep process (Russell and Setchell 1992; Vaz and Ferdinandusse 2017). Cholic acid (CA) and chenodeoxycholic acid (CDCA) are two primary bile acids produced in the liver from cholesterol by *de novo* synthesis (Stamp and Jenkins 2008). They are conjugated with glycine or taurine to generate taurine/glycine conjugated bile acids such as taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA) (Chen and Cassaro 2019; Di Ciaula et al. 2018). Upon release into the intestinal tract, conjugated bile acids enhance the digestion of dietary proteins as well as the absorption of fat-soluble vitamins (Dawson 2018; Gass et al. 2007). Additionally, bile acids have antimicrobial effects thus avoiding the overgrowth of gut microbiota which has been related to for example inflammatory bowel disease (IBD) (Fiorucci et al. 2021; Hofmann and Eckmann 2006).

In the small intestine about 95% of the bile acids are reabsorbed contributing substantially to the enterohepatic circulation and bile acid homeostasis (Hofmann 1977; Hofmann 2009; Roberts et al. 2002). Bile acids are able to recycle 4-12 times daily through the liver and intestine, limiting the amount of bile acids that need to be synthesized de novo per day (Dietschy 1968; Mok et al. 1977).

In our previous study, it was shown that the aminoglycoside antibiotic tobramycin especially inhibited the transport of conjugated bile acids across a Caco-2 cell layer pointing at reduced intestinal re-uptake (Zhang et al. 2022). Application of the *in vitro* Caco-2 cell model to estimate apparent permeability (P_{app}) coefficients has been proven a reliable way to evaluate absorption/transport of drugs across the intestinal epithelium (Artursson and Karlsson 1991; Yee 1997). The model was previously shown to also enable characterization of the intestinal reuptake of bile acids (Wang et al. 2022). Assessing the impact of antibiotics on this intestinal bile acid transport may provide insight in the mode(s) of action underlying effects of the antibiotics on fecal and plasma bile acid profiles reported in *in vivo* studies (Behr et al. 2018; Behr et al. 2019; de Bruijn et al. 2020; Murali et al. 2023). The aim of the present study was to investigate whether other antibiotics, including the lincosamide lincomycin, the additional aminoglycoside streptomycin and the glycopeptide vancomycin, would exert the same inhibitory effect on bile acid transport when studying transport of selected bile acids in the *in vitro* Caco-2 cell model. To this end in the present study the effects of the antibiotics on bile acid transport were studied using pre-exposure as well as co-exposure of the Caco-2 cell layers to one of the antibiotics tested and the bile acids. Pre-exposure of the cells to an antibiotic can be expected to affect subsequent bile acids transport for example via changes at protein expression levels resulting in modified levels of transporters involved in the reuptake of conjugated bile acids (Li et al. 2020; Zhang et al. 2023). Co-exposure will reveal whether there are direct interactions between the antibiotics and bile acid transport. In addition bile acids were tested either individually or as a mixture in order to detect whether testing as a mixture would enable higher throughput and still reveal the same effects. The results contribute to a mechanistic understanding of the effects of the selected antibiotics on bile acid homeostasis *in vivo*.

2.Materials and methods

2.1. Chemicals and reagents

Tobramycin, lincomycin hydrochloride, and vancomycin hydrochloride were purchased from Sigma-Aldrich (Schnelldorf, Germany). Streptomycin sulfate was purchased from Santa Cruz

Biotechnology (Texas, USA). Taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA) were obtained from Sigma-Aldrich (Schnelldorf, Germany). Dimethyl sulfoxide (DMSO) and potassium dichromate were purchased from Sigma-Aldrich (Darmstadt, Germany). Methanol was purchased from Biosolve BV (Valkenswaard, Netherlands). Phosphate buffered saline (PBS) was purchased from Gibco (Paisley, UK). Fetal Bovine Serum (FBS) was obtained from GE Healthcare Life Sciences Hyclone Laboratories (Logan, Utah, USA). 0.05% Trypsin-EDTA, minimum essential medium (MEM), penicillin-streptomycin-glutamine solution (PSG), sodium pyruvate, Hank's balanced salt solution (HBSS) and HEPES buffer solution were purchased from Gibco (Paisley, UK). Roche cell proliferation reagent WST-1 was purchased from Sigma-Aldrich (Schnelldorf, Germany). Corning Costar 24 well transwell plates were purchased from Corning Life Sciences (Schnelldorf, Germany). 96 Well cell culture plates were obtained from Greiner Bio-One B.V. (Alphen aan den Rijn, Netherlands).

2.2. Cell culture

Human colon adenocarcinoma Caco-2 cells (passage number 10-25) were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in MEM with 20% FBS, 1% sodium pyruvate and 1% PSG. The cells were grown in 75 cm^2 flasks in an incubator at 37 °C, 5% CO_2 and 100% humidity. Cells were subcultured at 50%-60% confluence with 0.05% trypsin-EDTA.

2.3 Calculation of *in vitro* test concentrations

The concentrations of the antibiotics to be tested were determined based on exposure levels used in 28 day *in vivo* rat studies in which effect of the antibiotics on the plasma and fecal metabolome, including bile acid profiles, were quantified (Behr et al. 2019; Murali et al. 2023). In these *in vivo* rat studies the high dose levels were selected in such a way that, based on available literature, they were expected to induce gut dysbiosis without inducing organ toxicity and the low doses were ten times lower than the high dose (Behr et al. 2019; Murali et al. 2023). To establish the *in vitro* concentrations to be tested the high dose levels from the *in vivo* studies were converted to corresponding *in vitro* test concentrations in the same way as done before for tobramycin (Zhang et al. 2022). In short, the concentrations (in mM) were calculated by dividing the daily *in vivo* dose level (in mg/kg bw) times the bodyweight (in kg) by the volume of the gastrointestinal tract (in L) and the molecular weight of the compound (mg/mmol). The volume of the gastrointestinal tract of rat was assumed to amount to 12 mL (= 0.012 L) (McConnell et al. 2010), adding up the water content of a fed gastrointestinal tract of 10 g, and a solid content of 2 g, and assuming the density to be 1 g/mL

In this way, *in vivo* dose levels of 300 mg/kg bw per day for lincomycin, 100 mg/kg bw per day for streptomycin, 50 mg/kg bw per day for vancomycin and 1000 mg/kg bw per day for tobramycin were converted to concentrations used for the transport studies of 14 mM lincomycin, 1.4 mM streptomycin, 0.7 mM vancomycin and 45 mM tobramycin respectively (supplementary information Table S1). For cytotoxicity studies a dose range of the antibiotics was tested to ascertain that the concentrations of the antibiotics used in the transport studies did not affect cell viability (see section 2.4).

2.4 Cell viability and exposure

For cell viability experiments, Caco-2 cells were collected from 75 cm^2 flasks at 50%-60% confluence and seeded in the inner wells of a 96 well plate by filling each inner well with 100 µL at a density of 4×10^5 cells /mL so at 4×10^4 cells/well. 100 µL PBS was added to the outer

wells in order to limit evaporation from the inner wells. The plates were incubated for 18 days in the incubator under 37 °C, 5% CO_2 and 100% humidity for cells to differentiate, changing the medium every other day. At day 18, cells were exposed to lincomycin, streptomycin, vancomycin, the positive control or the solvent control for 48h. 50 µM potassium dichromate was used as a positive control. Exposure solutions were prepared from 200 times concentrated stock solutions in DMSO diluted in supplemented MEM medium (final concentration 0.5% v/v DMSO).

After 48h, 5 µl WST-1 reagent was added to each well followed by 2 h incubation, after which the absorbance was detected at 440 nm and 620 nm using a Softmax Pro 7.1 (California, US). Results were obtained by subtracting the 620 nm signal from the 440 nm signal and calculating the percentage viability relative to the control set at 100% viability. The cell viability of tobramycin was determined in a previous study, showing 45 mM not to affect cell viability (Zhang et al. 2022).

2.5. Bile acid transport across Caco-2 cell layers

For the transport experiments, 1.8×10^5 cells /cm^2 were seeded at the apical part of the inserts of Corning 24 well transwell plates and 600 µl supplemented MEM was added to the basolateral compartment of each well. Cells were cultured for 18 days in order to differentiate into a cell monolayer, while medium was changed every other day. MEM without PSG was applied when cells were seeded in 24 well transwell plates in order to eliminate potential disturbance of the effects of the targeted antibiotics. Two types of experiments were performed including pre-exposure and co-exposure to an antibiotic and the conjugated bile acids. For the pre-exposure transport studies, after 18 days cultivation, cell layers were exposed for 48 h to 14 mM lincomycin, 1.4 mM streptomycin, 0.7 mM vancomycin or 45 mM tobramycin in MEM, respectively, all added from 200 times concentrated stock solutions in DMSO. Then the integrity of the cell layers was measured by using transepithelial electrical resistance (TEER). The TEER value was measured with a Millicell® ERS-2 Volt-Ohm Meter (Millipore, Amsterdam, Netherlands). The TEER value was detected before, during and after the transport experiment and is expressed in $\Omega\times cm^2$. Only Caco-2 cell layers with a TEER value above 500 $\Omega\times cm^2$ were used for transport experiments. After checking the TEER value the exposure medium was removed and the cell layers were cultured in transport medium (HBSS supplemented with 10 mM HEPES, pH=7.4) for 30 min. After 30 min incubation in transport medium, transport medium containing 5 µM of each of the bile acids tested (TCA, TCDCA, GCA or GCDCA) was added to the apical part of the transwell plates followed by continued incubation at 37˚C under 5% CO_2 and 100% humidity. Samples from the basolateral compartment were obtained at 1h, 2h and 3h, for bile acid analysis by LC-MS/MS. For the co-exposure transport studies, cells were cultured for 20 days, the TEER value was measured to ascertain the integrity of the cell layers, and also for these experiments the TEER value was measured before, during and after the transport experiment. After checking the TEER value MEM was removed and cell layers were incubated in transport medium for 30 min. Subsequently, transport medium containing 5 µM of TCA, TCDCA, GCA and GCDCA (individually or as a mixture) and 14 mM lincomycin, 1.4 mM streptomycin, 0.7 mM vancomycin or 45 mM tobramycin respectively were added to the apical part of the wells. Samples were collected from the basolateral compartment at 1h, 2h and 3h for bile acid analysis by LC-MS/MS.

Additionally, pre- and co-exposure transport studies were performed testing the effect of 5 mM of each antibiotic on the transport of a mixture of bile acids (TCA, TCDCA, GCA and GCDCA) each at 5 µM concentration. The incubation, sampling and analysis were performed as described above.

Finally, to investigate the nature of the transport, active and/or passive, the transport of a mixture of bile acids (TCA, TCDCA, GCA and GCDCA) each at 5 µM concentration across the Caco-2 cell layers was quantified at either 37 ℃ or 4℃ during 3 h incubation time, Sampling and analysis were performed as described for the other transport experiments.

2.6. Bile acid profiling by LC-MS/MS

Bile acid analysis was performed using a triple quadrupole LCMS-8045 (Shimadzu Corporation, Japan). The method applied was able to detect and quantify 20 bile acids. Bile acid samples were separated on a Kinetex C18 column (1.7µm×100 A×50mm×2.1 mm, Phenomenex 00B-4475-AN). Mobile phase A was MilliQ water with 0.01% formic acid and mobile phase B was 50% v/50% v acetonitrile/ methanol. An injection volume of 1 µL was used. Briefly, the column was equilibrated with 70 % mobile phase A and 30 % mobile phase B at a flow rate of 0.4 mL/min. The gradient started with 0-10 min from 30 to 70% B, was kept at 70% B for 1 min then modified from 11-19 min from 70 to 98% B, kept at 98% B for 1 min; and then from 20-25 min reduced from 98 to 30% B followed by 10 min equilibration at 30% B before the next injection. The mass spectrometer (MS) was operated with electrospray ionization (ESI) in negative ion mode. The ESI parameters included; Nebulizing gas flow 3 L/min; drying gas flow and heating gas flow 10 L/min; interface voltage 3 kV; interface current 0.9 uA; interface temperature 300 ℃; Desolvation temperature 526 ℃; DL temperature 250 ℃; heat block temperature 400 ℃; Conversion Dynode Voltage 10 kV; detector voltage 2.16 kV; IG Vacuum 1.8e-03 Pa; PG Vacuum 9.4e+01 Pa; CID Gas 230 kPa. Selective ion monitoring (SIM) and multiple reaction monitoring (MRM) were applied in the bile acid detection. A combination of SIM and MRM was used for bile acid detection and quantification in order to reach optimal sensitivity for all bile acids. Bile acid identification and quantification were performed as previously described (Wang et al. 2022; Zhang et al. 2022; Zhang et al. 2023).

2.7. Data analysis including statistical analysis

Bile acid profiles were analyzed using the Labsolution software in the LC-MS/MS system. Graphical figures were drawn using Graphpad Prism 5 (San Diego, USA). Statistical analysis was performed by one way analysis of variance with Dunnett's post-hoc test. $P< 0.05$ was considered significant. All the results are expressed as mean ±SD.

3. Results

3.1 Cell viability

To determine cell viability upon incubation of the Caco-2 cells with increasing concentrations of the antibiotics to be tested a WST-1 assay was performed. The results obtained indicate that the concentrations to be tested derived from the *in vivo* dose levels as described in the Materials and method section, amounting to 14 mM lincomycin, 1.4 mM streptomycin, 0.7 mM vancomycin or 45 mM tobramycin, did not affect cell viability (Figure S1 in supplementary information).

3.2 Caco-2 cell layer integrity

TEER values were detected before, during and after the bile acid transport experiment to confirm that the barrier integrity of the Caco-2 cell layers was not influenced by exposure to the antibiotics. TEER values of the cell layers were >500 $\Omega \times cm^2$ and this pointed at intact cell layers and confirmed that the integrity of the cell layers was not affected by the exposure for both control and antibiotic treated cell layers (Figure S2-S7 in supplementary information).

3.3. Effect of pre-exposure of the cells to antibiotics at *in vivo* dose-derived concentrations on the transport of individual bile acids across Caco-2 cell layers

After pre-exposure of the differentiated Caco-2 cell layers to 14 mM lincomycin, 1.4 mM streptomycin, 0.7 mM vancomycin or 45 mM tobramycin for 48h, the transport of the selected conjugated individual bile acids was assessed. Figure 1 shows the time dependent transport of the bile acids (TCA, TCDCA, GCA and GCDCA) across the Caco-2 cell layers with or without pre-exposure to the antibiotics. Table 1 presents the apparent permeability coefficients (P_{app} values)(expressed in cm/s) derived from the data at 1 h incubation. The results obtained reveal that preincubation of the Caco-2 cell layers with tobramycin statistically significantly inhibited the transport of TCA, TCDCA, GCA and GCDCA while upon pre-exposure to lincomycin, streptomycin and vancomycin, the bile acid transport was similar to that observed for the control group. In all samples the amount of transported TCDCA and GCDCA appeared to be about 2 times higher than that of TCA and GCA, while the transport of TCA and GCA was comparable and so was that of TCDCA and GCDCA.

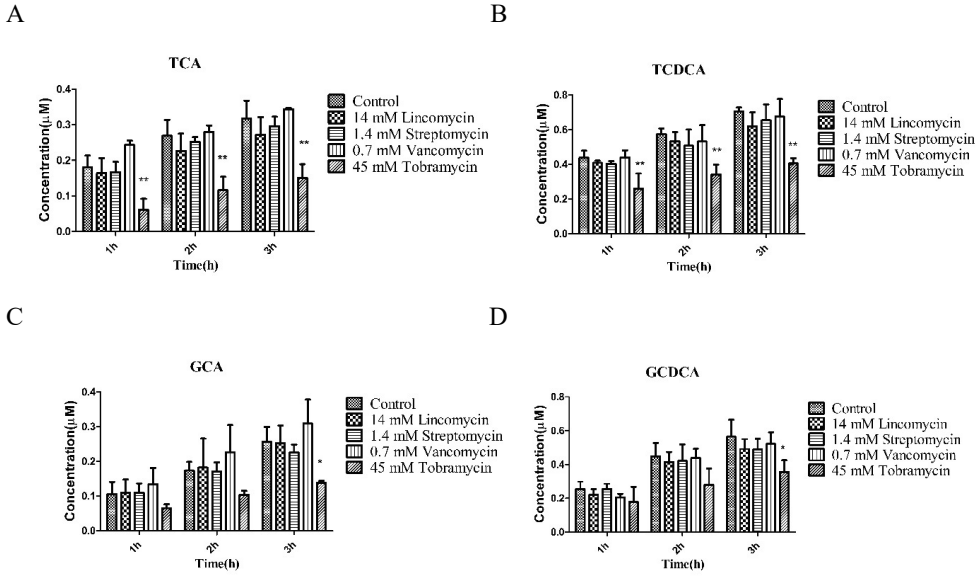

Figure 1. Time dependent transport of individual bile acids (TCA,TCDCA, GCA and GCDCA) across a Caco-2 cell layer upon pre-exposure of the cells without (control) or with an antibiotic, the latter at a concentration based on the *in vivo* dose level from a 28 day rat study on effects on the plasma and fecal metabolome (Behr et al. 2019; Murali et al. 2023) (*P< 0.05,**P<0.01, ***P<0.001 for the difference with respect to the control at the same time point). Results are expressed as mean ± SD, n=3.

Table 1 Permeability constants (P_{app} values) for the transport of individual bile acids (TCA, TCDCA, GCA and GCDCA) across a Caco-2 cell layer upon pre-exposure of the cells without (control) or with an antibiotic, the latter at a concentration based on the *in vivo* dose level from a 28 day rat study on effects on the plasma and fecal metabolome (Behr et al. 2019; Murali et al. 2023) (* *P*< 0.05 for the difference with respect to the control). Data shown are mean ± SD, n=3.

	P_{app} (in10^{-5} cm/s)				
	Sample				
Bile acid	Control	Lincomycin	Streptomycin	Vancomycin	Tobramycin
TCA	1.8±0.3	1.6±0.4	1.7±0.3	2.5±0.1	0.6±0.2*
TCDCA	4.4±0.4	4.1±0.1	4.0±0.2	4.4±0.4	2.6±0.8*
GCA	1.0±0.1	0.9±0.2	0.8±0.1	1.0±0.2	0.7±0.1*
GCDCA	2.5±0.2	2.1±0.3	2.1±0.5	2.2±0.3	1.8±0.1*

3.4. Effect of pre-exposure of the cells to antibiotics at *in vivo* dose-derived concentrations on transport of a mixture of bile acids across Caco-2 cell layers

Figure 2 displays the results of the experiment in which the effects of the pre-exposure of the cells to the antibiotics on the transport of the bile acids (TCA, TCDCA, GCA and GCDCA) was studied using the bile acids as a mixture. Table 2 presents the P_{app} values derived from the data at 1 h of incubation. Similar to what was observed when testing the effect of the pre-exposure with antibiotics on the transport of the bile acids in isolation, tobramycin appeared to inhibit the transport of all four bile acids (TCA, TCDCA, GCA and GCDCA) significantly, whereas lincomycin, streptomycin and vancomycin seemed not to affect bile acid transport. The P_{app} values for each bile acid appeared somewhat (12.0 % to 38.6 %) lower when transport was studied using a mixture of bile acids than when the bile acids were studied in isolation, while the effects of the antibiotics on the bile acid transport were in line with what was observed when testing the bile acids in isolation. Also in line with the study using the isolated bile acids, when tested in a mixture the transport of TCA and GCA was comparable and so was the transport of TCDCA and GCDCA while the transport of TCDCA and GCDCA appeared to be about 2 times higher than that of TCA and GCA. Based on these results in further experiments the effects of the antibiotics on bile acid transport was studied testing the bile acids in combination.

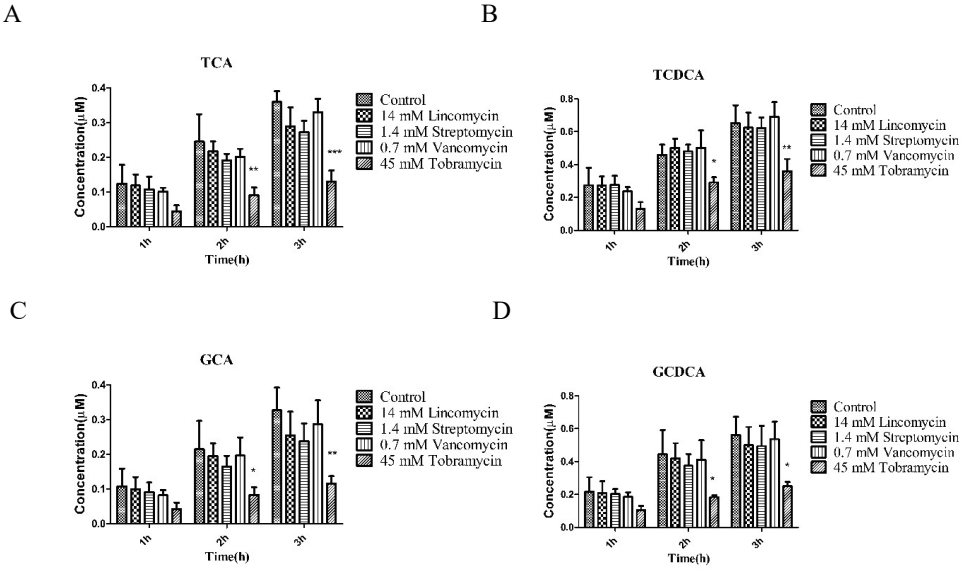

Figure 2. Time dependent transport of a mixture of bile acids (TCA,TCDCA, GCA and GCDCA) across a Caco-2 cell layer upon pre-exposure of the cells without (control) or with

an antibiotic, the latter at a concentration based on the *in vivo* dose level from a 28 day rat study on effects on the plasma and fecal metabolome (Behr et al. 2019; Murali et al. 2023) (*P< 0.05,**P<0.01, ***P<0.001 for the difference with respect to the control). Results are expressed as mean ± SD, n=3.

Table 2 Permeability constants (P_{app} values) for the transport of a mixture of bile acids (TCA, TCDCA, GCA and GCDCA) across a Caco-2 cell layer upon pre-exposure of the cells without (control) or with an antibiotic, the latter at a concentration based on the *in vivo* dose level from a 28 day rat study on effects on the plasma and fecal metabolome (Behr et al. 2019; Murali et al. 2023) (* *P*< 0.05 for the difference with respect to the control). Data shown are mean ± SD, n=3.

	P_{app} (in10^{-5} cm/s)				
	Sample				
Bile acid	Control	Lincomycin	Streptomycin	Vancomycin	Tobramycin
TCA	1.3±0.2	1.1±0.1	1.1±0.3	1.0±0.1	0.5±0.1*
TCDCA	2.7±0.4	2.5±0.3	2.8±0.5	2.5±0.5	1.5±0.2*
GCA	1.0±0.4	1.0±0.2	0.9±0.2	1.0±0.2	0.5±0.1*
GCDCA	2.2±0.5	2.1±0.5	2.0±0.3	2.1±0.3	0.9±0.1*

3.5. Effect of pre-exposure of the cells to a similar concentration (5 mM) of antibiotics, on the transport of a mixture of bile acids across Caco-2 cell layers

Given that the concentrations of the antibiotics tested differed substantially when they were based on the dose levels selected for the 28 day *in vivo* studies to characterize their effects on the fecal and plasma metabolome (Behr et al. 2019; Murali et al. 2023), differences between the effects of the different antibiotics may be due to the differences in the concentrations tested. Therefore, it was also of interest to test the antibiotics at a similar concentration. To this end the effect of pre-exposure of the cells to 5 mM of each one of the antibiotics on the bile acid transport across the Caco-2 cell layers was studied. Testing a higher concentration was limited by the solubility of vancomycin. Figure 3 presents the results obtained while the P_{app} values for the bile acids derived from these data are shown in Table 3. At 5 mM all four antibiotics, including tobramycin, had no effect on bile acid transport. As in the previous experiments the P_{app} values of TCA and GCA were comparable and so were those of TCDCA and GCDCA. Also, transport of TCDCA and GCDCA was higher than that of TCA and GCA.

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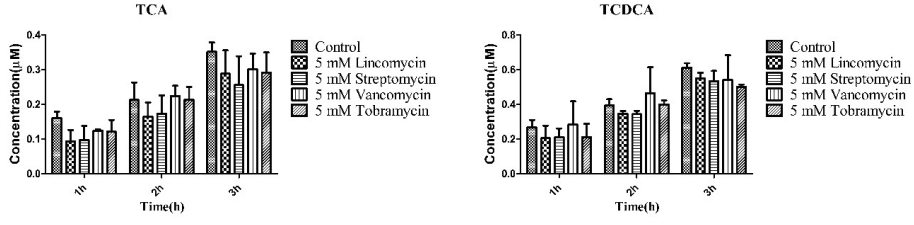

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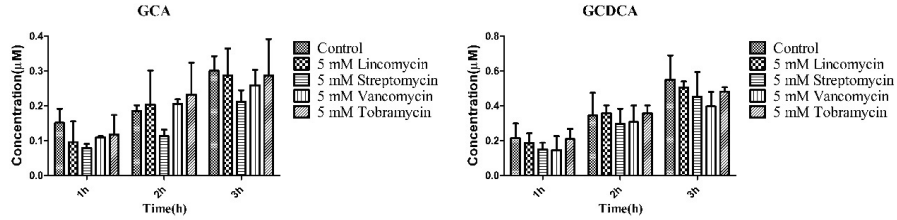

Figure 3. Time dependent transport of a mixture of bile acid (TCA, TCDCA, GCA and GCDCA) across a Caco-2 cell layer upon pre-exposure of the cells without (control) or with a similar concentration of one of the antibiotics (5 mM) (*P< 0.05,**P<0.01, ***P<0.001 for the difference with respect to the control). Results are expressed as mean ± SD, n=3.

Table 3 Permeability constants (P_{app} values) for the transport of a mixture of bile acids (TCA, TCDCA, GCA and GCDCA) across a Caco-2 cell layer upon pre-exposure of the cells without (control) or with similar concentration (5 mM) of one of the antibiotics (* *P*< 0.05 for the difference with respect to the control). Data shown are mean ± SD, n=3.

	P_{app} (in10^{-5} cm/s)				
	Sample				
Bile acid	Control	Lincomycin	Streptomycin	Vancomycin	Tobramycin
TCA	1.1±0.2	0.8±0.2	0.9±0.3	1.1±0.1	1.0±0.2
TCDCA	2.7±0.4	1.8±0.1	1.8±0.1	2.4±0.3	2.0±0.1
GCA	1.5±0.4	1.0±0.3	0.8±0.1	0.9±0.1	1.2±0.5
GCDCA	1.7±0.2	1.8±0.2	1.5±0.4	1.6±0.5	1.8±0.2

3.6. Effect of co-exposure of the cells to bile acids and antibiotics at *in vivo* dose-derived concentrations on transport of a mixture of bile acid across Caco-2 cell layers

To investigate whether antibiotics would interfere with bile acid transport in a direct way, the effect of the antibiotics on bile acid transport across the Caco-2 cell layers was tested using co-exposure to an antibiotic and the bile acids. The results obtained are presented in Figure 4 and Table 4 and reveal that all four antibiotics reduced the transport of especially TCDCA and GCDCA in a significant way upon co-exposure, whereas the reduction in transport was not significant, or only significant at 1h incubation in case of transport of GCA, upon treatment with streptomycin and tobramycin, respectively. As in the other experiments the P_{app} values of TCA and GCA were comparable as were those of TCDCA and GCDCA that were again higher than those of TCA and GCA.

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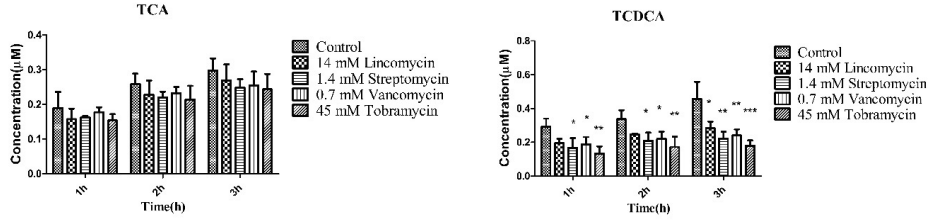

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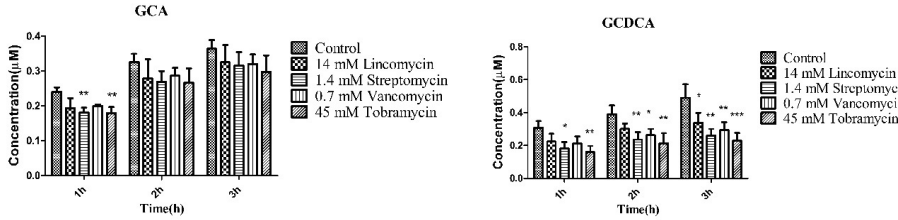

Figure 4. Time dependent transport of a mixture of bile acids (TCA, TCDCA, GCA and GCDCA) across a Caco-2 cell layer upon co-exposure of the cells to bile acids without (control) or with an antibiotic, the latter at a concentration based on the *in vivo* dose level from a 28 day rat study on effects on the plasma and fecal metabolome (Behr et al. 2019; Murali et al. 2023) (*P< 0.05,**P<0.01, ***P<0.001 for the difference with respect to the control). Results are expressed as mean ± SD, n=3.

Table 4 Permeability constants (P_{app} values) for the transport of a mixture of bile acids (TCA, TCDCA, GCA and GCDCA) across a Caco-2 cell layer upon co-exposure of the cells to bile acids without (control) or with an antibiotic, the latter at a concentration based on the *in vivo* dose level from a 28 day rat study on effects on the plasma and fecal metabolome (Behr et al. 2019; Murali et al. 2023) (* *P*< 0.05 for the difference with respect to the control). Data shown are mean ± SD, n=3.

	P_{app} (in10^{-5} cm/s)				
	Sample				
Bile acid	Control	Lincomycin	Streptomycin	Vancomycin	Tobramycin
TCA	1.3±0.2	1.1±0.2	1.1±0.1	1.2±0.1	1.1±0.2
TCDCA	3.0±0.5	1.9±0.1*	1.7±0.3*	1.9±0.2*	1.3±0.4*
GCA	1.6±0.1	1.4±0.3	1.4±0.2	1.4±0.1	1.3±0.2
GCDCA	3.1±0.4	2.3±0.1*	1.8±0.4*	2.1±0.5*	1.6±0.3*

3.7. Effect of co-exposure of the cells to bile acids and a similar concentration (5 mM) of antibiotics, on the transport of a mixture of bile acids across Caco-2 cell layers

The effect of co-exposure with antibiotics on the bile acid transport was also tested using all four antibiotics at the concentration of 5 mM. Figure 5 and Table 5 show the results obtained. Also at 5 mM all four antibiotics appeared to significantly inhibit the transport of TCDCA and GCDCA across the Caco-2 cell layers while the effects on transport of TCA and GCA were limited with only a significant effect of vancomycin at 5 mM on the transport of GCA detected at 3 h of incubation. Similar to the other experiments transport of TCDCA and GCDCA was comparable and about two times higher than that of TCA and GCA.

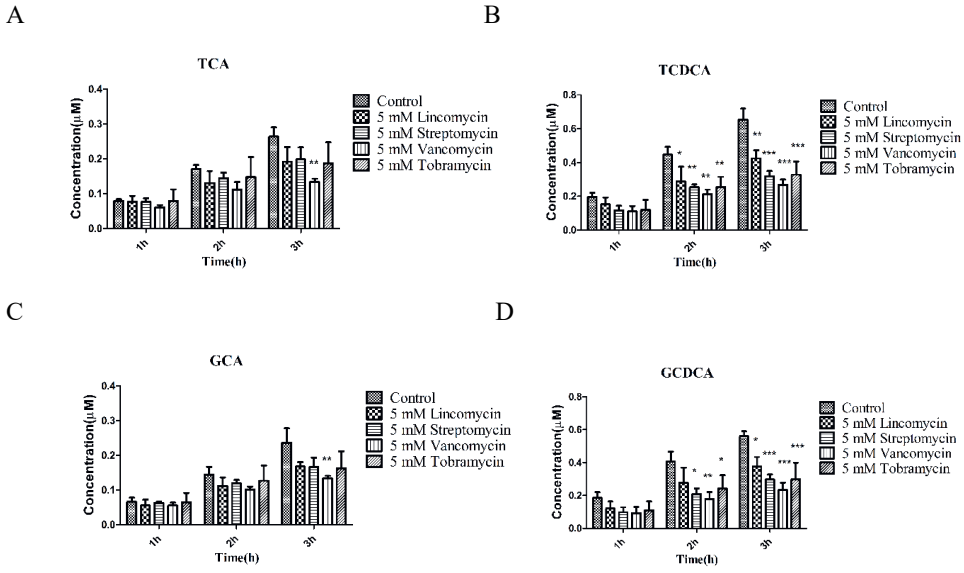

Figure 5. Time dependent transport of a mixture of bile acid (TCA, TCDCA, GCA and GCDCA) across a Caco-2 cell layer upon co-exposure of the cells to bile acids without (control) or with a similar concentration of one of the antibiotics (5 mM) (*P< 0.05,**P<0.01, ***P<0.001 for the difference with respect to the control). Results are expressed as mean ± SD, n=3.

Table 5 Permeability constants (P_{app} values) for the transport of a mixture of bile acid (TCA, TCDCA, GCA and GCDCA) across a Caco-2 cell layer upon co-exposure of the cells to bile acids without (control) or with a similar concentration of one of the antibiotics (5 mM) (* *P*< 0.05 for the difference with respect to the control). Data shown are mean ± SD, n=3.

	P_{app} (in10^{-5} cm/s)				
	Sample				
Bile acid	Control	Lincomycin	Streptomycin	Vancomycin	Tobramycin
TCA	0.9±0.1	0.7±0.2	0.7±0.1	0.6±0.1	0.6±0.2
TCDCA	2.3±0.3	1.5±0.1*	1.3±0.1*	1.1±0.2*	1.2±0.2*
GCA	0.7±0.1	0.6±0	0.6±0.1	0.5±0	0.6±0.2
GCDCA	1.9±0.2	1.4±0*	1±0.1*	0.8±0.2*	1.2±0.4*

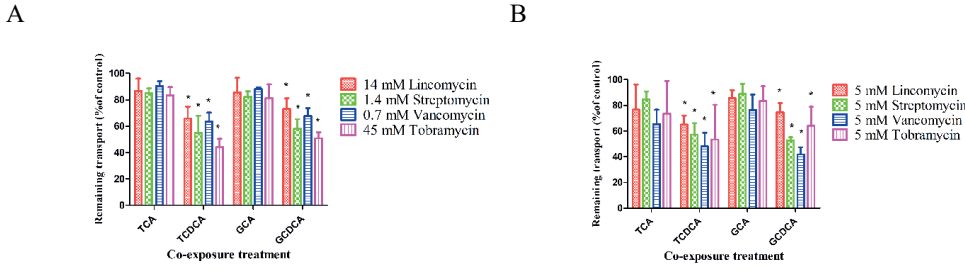

Figure 6. Transport (% of control) of a mixture of bile acids (TCA, TCDCA, GCA and GCDCA) across a Caco-2 cell layer upon co-exposure of the cells to bile acids and an antibiotic at a concentration based on the *in vivo* dose level from a 28 day rat study on effects on the plasma and fecal metabolome (Behr et al. 2019; Murali et al. 2023) (6A) or at a similar concentration of the antibiotics (5 mM) (6B). (* $P < 0.05$ for the difference with respect to the control). Data shown are mean ± SD, n=3.

Figure 6 presents an overview of the remaining transport activity (%) in the co-exposure experiments. These results show that the inhibition of bile acid transport is higher for TCDCA and GCDCA compared to TCA and GCA, but also that the inhibitor effect of the four antibiotics at 5 mM shows differences in potency, with a decreasing order: vancomycin > tobramycin = streptomycin > lincomycin.

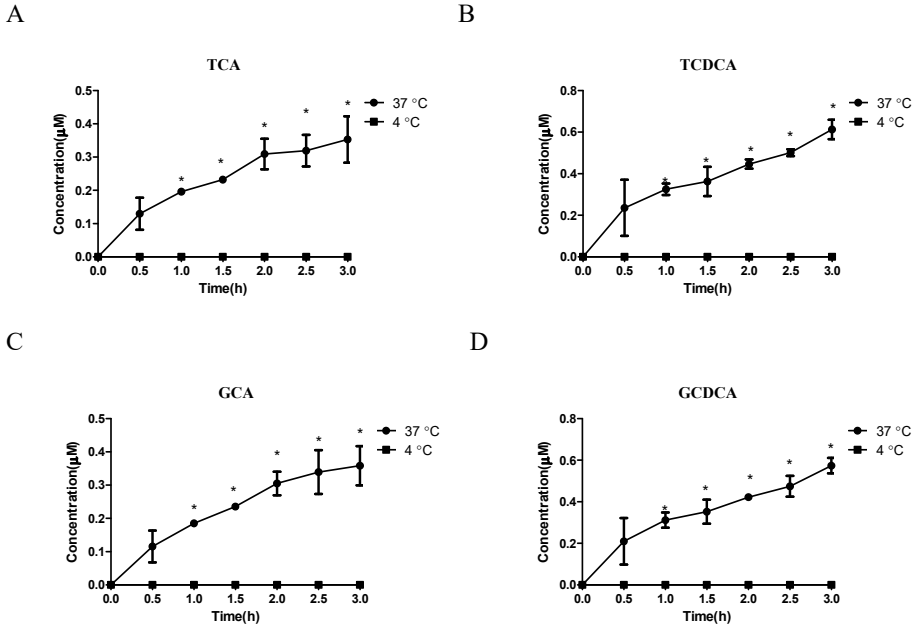

Figure 7. Time dependent transport of a mixture of bile acid (TCA, TCDCA, GCA and GCDCA) across a Caco-2 cell layer without (control) during 3 h at 37 °C or 4 °C. (* $P < 0.05$ for the difference with respect to the control). Data shown are mean ± SD, n=3.

Finally, figure 7 presents the transport of bile acids in the absence of antibiotics during 3 h incubation at 37 °C or at 4 °C. The transport of TCA, TCDCA, GCA and GCDCA appeared to be absent at 4°C while readily observed at 37°C indicating that these bile acids are transported mainly via active transport.

4. Discussion

In a previous study it was found that the aminoglycoside antibiotic tobramycin when tested at an *in vitro* concentration of 45 mM, a concentration derived from an *in vivo* dose of 1000 mg/kg bw, significantly inhibited both the intestinal deconjugation and transport of conjugated bile acids including TCA, GCA, TCDCA and GCDCA (Zhang et al. 2022; Zhang et al. 2023). These *in vitro* effects of tobramycin provided further insight in the mode of action underlying increased fecal levels of TCA and TCDCA in a 28 day oral rat study

(Murali et al. 2023; Zhang et al. 2022). The fact that pre-exposure of the Caco-2 cells to tobramycin caused a decrease in bile acid transport in a transwell model system pointed at an effect on the expression level of the responsible apical sodium dependent bile acid transporter (ASBT). Such an effect would match the known activity of tobramycin as an inhibitor of protein synthesis in human cells (Wangen and Green 2020). The aim of the present study was to investigate the potential effect of a series of additional antibiotics, including the lincosamide lincomycin, the additional aminoglycoside streptomycin and the glycopeptide vancomycin, on bile acid transport using the *in vitro* Caco-2 transwell model. Previously, 28 day *in vivo* studies reported effects of these antibiotics on bile acid profiles (Behr et al. 2019; Murali et al. 2023). Similar to what was observed for tobramycin, exposure of the rats to lincomycin showed substantial effects on *in vivo* bile acid profiles, while vancomycin and streptomycin also showed effects albeit somewhat less pronounced (Behr et al. 2018; Behr et al. 2019). In these *in vivo* rat studies the high dose levels were selected in such a way that, based on available literature, they were expected to induce gut dysbiosis without inducing organ toxicity and the low doses were ten times lower than the high dose (Behr et al. 2019). The LD50 of these antibiotics upon oral dosing in rodents were reported to amount to > 7000 mg/kg for tobramycin (Welles et al. 1973), > 4000 mg/kg bw for lincomycin (Gray et al. 1964), > 5000 mg/kg bw for vancomycin (Wold and Turnipseed 1981) and 700-1000 mg/kg bw for streptomycin (Gray et al. 1966). The high *in vivo* dose levels in the reported 28 day studies, based on which the *in vitro* test concentrations of the present study were determined, were substantially lower than these LD50 values and also the cytotoxicity results of the present study corroborating that differences in the effects of the antibiotics on bile acid transport in the Caco-2 model system were not due to cellular toxicity. The results of the present study showed that effects of the antibiotics on transport of bile acids across the Caco-2 cell layers could be detected when testing a mixture of the four bile acids under study, thus eliminating the need to characterize the effects for each bile acid in isolation in subsequent experiments.

In the pre-exposure studies, when testing the four antibiotics in line with the high dose levels from the *in vivo* studies, it turned out that tobramycin was the only antibiotic that reduced the bile acid transport significantly while pre-treatment of the Caco-2 cells with lincomycin, streptomycin and vancomycin did not affect bile acid transport. Bile acid transport/ reabsorption of these conjugated bile acids from the intestine is known to be mediated by specific bile acid transporters such as the apical sodium-dependent bile acid transporter (ASBT), the ileal bile acid binding protein (IBABP) and the organic solute transporter OSTα-OSTβ (Ballatori et al. 2009; Dawson et al. 2005; Hagenbuch and Dawson 2004; Nakahara et al. 2005; Shneider 2001). ASBT is located on the apical membrane, IBABP is located in the cytoplasm and OSTα-OSTβ is located in the basolateral membrane of intestinal cells. The results of the present study showed that in the Caco-2 transwell model the conjugated bile acids were transported at 37°C while transport no longer occurred at 4 °C, corroborating the role of active transport. Since the antibiotics tested are from different classes of antibiotics subtle differences in their potential for inhibiting the expression or activities of these bile acid transporters may explain their differential effects on bile acid transport, although differences may also originate from the differences in the concentrations tested. Tobramycin and streptomycin are from the same class of antibiotics, the aminoglycosides, while lincomycin is from the class of lincosamides and vancomycin is from the class of glycopeptides (Barna and Williams 1984; Becker and Cooper 2013; Spížek and Řezanka 2004).

Tobramycin and streptomycin belong to the same class of antibiotics which may exert antibacterial effects by affecting bacterial ribosomes and also preventing the detection of stop codons at ribosomes in human cells leading to disruption of the synthesis of normal functional

proteins (Fosso et al. 2015; Wangen and Green 2020). The potency of tobramycin to reduce bile acid transport upon pre-incubation of the Caco-2 cells is most likely related to the higher concentration that was tested (45 as compared to 1.4 mM for streptomycin). This is confirmed by the observation that upon pre-incubation of the cells at a similar concentration of 5 mM both aminoglycoside did not affect bile acid transport. Testing of streptomycin at 45 mM was hampered by its cytotoxicity (Figure S1 in supplementary information). It is of interest to note that the results obtained are in line with the more pronounced effect of tobramycin than streptomycin on *in vivo* bile acid profiles at the dose levels tested (Behr et al. 2019; Murali et al. 2023). Lincomycin, tested up to 14 mM, is reported to react with 50S ribosomal subunits thus blocking protein synthesis in bacteria but there is no clear evidence showing that it may also disrupt protein synthesis in human cells (Josten and Allen 1964; Spížek and Řezanka 2004). This could explain the absence of an effect on bile acid transport upon pre-exposure of the Caco-2 cells with this antibiotic. Vancomycin, may have an antibacterial effect by suppressing peptidoglycan formation on the cell wall (Nagarajan 1991), an effect that would not be expected to interfere with the synthesis of bile acid transporters, thus explaining the lack of an effect of the pre-exposure.

In addition to testing the effects of pre-incubation of the Caco-2 cells with the selected antibiotics, the antibiotics were also tested for a potential effect on bile acid transport while being simultaneously present with the bile acids. Also in these experiments the antibiotics were tested at the concentrations derived from the *in vivo* dose levels, as well as at a similar concentration of 5 mM the highest concentration for testing at a similar concentration given the limited solubility of vancomycin.

Co-exposure of the Caco-2 cells to the antibiotics and bile acids revealed that when the bile acids were tested as a mixture in the presence of each individual antibiotic, all the antibiotics significantly inhibited TCDCA and GCDCA transport compared to control while at 3 h incubation the transport of TCA and GCA was reduced by 7.7 % to 28.6 % albeit not significant. At a similar test concentration of 5 mM the potency of the individual antibiotics to inhibit transport of TCDCA and GCDCA decreased in the order vancomycin > tobramycin = streptomycin > lincomycin, showing the effect of the two aminoglycosides to be similar. The mode of action underlying this inhibition upon co-exposure remains to be elucidated. It might be ascribed to competition of antibiotics with bile acids for the active transporters and also to an effect of the antibiotics on passive diffusion involved in transport of the bile acids across the Caco-2 cell layers. It is reported that bile acid binding to the hASBT (human apical sodium-dependent bile acid transporter) may be the rate-limiting step in the apical transport of bile acids and may also be inversely related to the number of hydroxyl moieties in the bile acid (Balakrishnan et al. 2006). This would explain why in all Caco-2 transport experiments TCDCA and GCDCA which are dihydroxy bile acids are transported at a higher rate than TCA and DCA which are trihydroxy bile acids. A higher contribution of this hASBT mediated transport to the overall translocation may also explain why the transport of TCDCA and GCDCA is inhibited to a larger extent than that of TCA and GCA.

It is also of interest to note that the effects of co-exposure to the antibiotics on bile transport were more pronounced than that of pre-exposure where only high concentrations of tobramycin affected the transport.

Altogether, our results reveal that especially tobramycin may affect intestinal bile acid transport via an indirect mode of action inhibiting protein synthesis thereby potentially reducing expression levels of bile acid transport proteins, as well as via a direct inhibiting effect on these transport activities and /or on passive diffusion. Lincomycin, streptomycin and vancomycin also appeared to affect bile acid transport but mainly via a direct inhibiting effect with a potency

that decreased in the order vancomycin > tobramycin = streptomycin > lincomycin. The effects observed likely contribute to the effects reported for these antibiotics on bile acid homeostasis *in vivo*.

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Conflict of interest

The authors declare that they have no conflict of interest.

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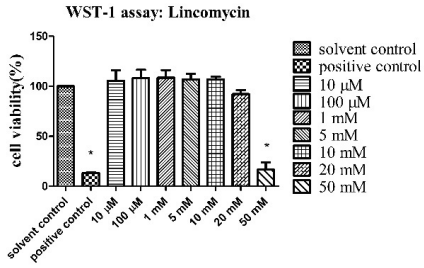
Supplementary information

Table S1. Conversion of *in vivo* antibiotic dose levels to *in vitro* antibiotic concentrations level

Antibiotic	*In vivo* dose level	*In vivo* reference	*In vitro* exposure concentration[1]
lincomycin	300 mg/kg bw/d	(Behr et al. 2019)	14 mM
streptomycin	100 mg/kg bw/d	(Behr et al. 2019)	1.4 mM
vancomycin	50 mg/kg bw/d	(Behr et al. 2019)	0.7 mM
tobramycin	1000 mg/kg bw/d	(Murali et al. 2023)	45 mM

A



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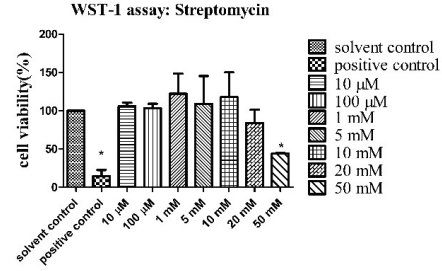

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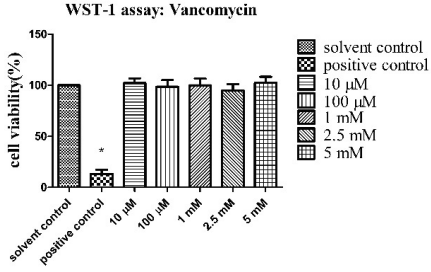

[1] *In vitro* exposure concentration (in mM) = (*In vivo* daily dose level of compound (in mg/kg bw) x bw (in kg))/Volume of gastrointestinal tract (in L)/Molecular weight of compound (mg/mmol)

Figure S1. Concentration dependent effect of lincomycin, streptomycin and vancomycin on cell viability as determined by the WST-1 assay. (* $P < 0.05$ for the difference with respect to the control). Results are shown as mean ± SD, n=3.

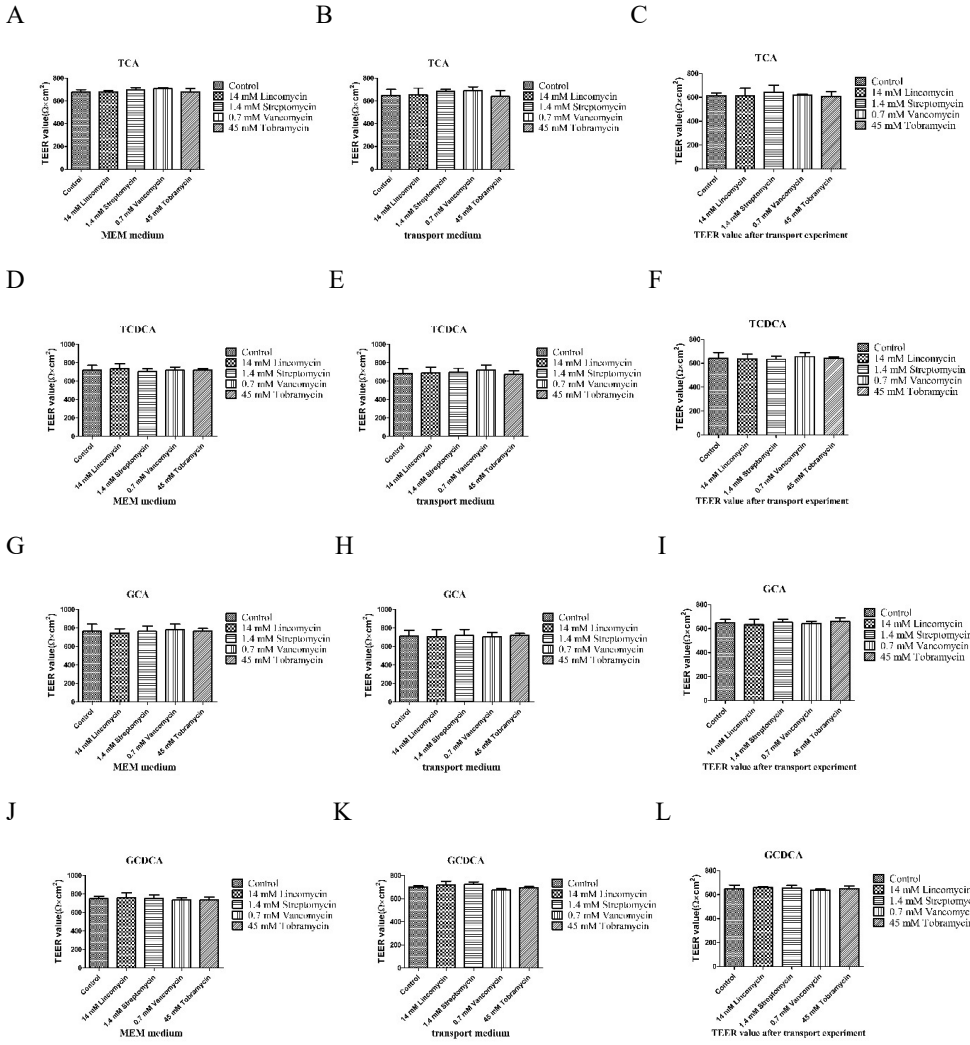

Figure S2. TEER values before (MEM medium), during (transport medium) and after the individual bile acid transport experiment across Caco-2 cell layers upon pre-exposure of the cells without (control) or with an antibiotic, the latter at a concentration based on the *in vivo* dose level from a 28 day rat study on effects on the plasma and fecal metabolome (Behr et al. 2019; Murali et al. 2023). Results are shown as mean ± SD, n=3.

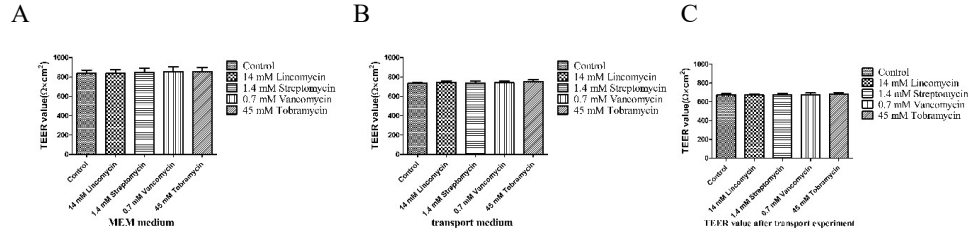

Figure S3. TEER values before (MEM medium), during (transport medium) and after a transport experiment across Caco-2 cell layers for a mixture of bile acid (TCA, TCDCA, GCA and GCDCA) upon pre-exposure of the cells without (control) or with an antibiotic, the latter at a concentration based on the *in vivo* dose level from a 28 day rat study on effects on the plasma and fecal metabolome (Behr et al. 2019; Murali et al. 2023). Results are shown as mean ± SD, n=3.

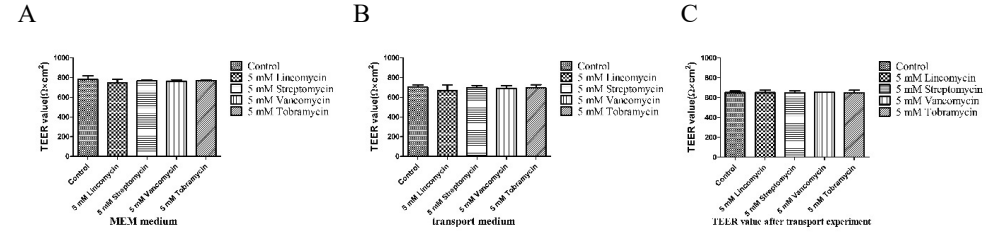

Figure S4. TEER values before (MEM medium), during (transport medium) and after a transport experiment across Caco-2 cell layers upon pre-exposure of the cells without (control) or with a similar concentration of the antibiotics (5 mM) and a mixture of bile acids (TCA, TCDCA, GCA and GCDCA). Results are shown as mean ± SD, n=3.

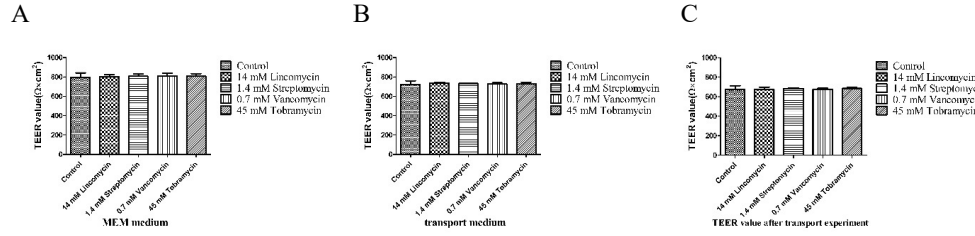

Figure S5. TEER values before (MEM medium), during (transport medium) and after a transport experiment across Caco-2 cell layers upon co-exposure of the cells to a mixture of bile acids (TCA, TCDCA, GCA and GCDCA) bile acids without (control) or with an antibiotic, the latter at a concentration based on the *in vivo* dose level from a 28 day rat study on effects on the plasma and fecal metabolome (Behr et al. 2019; Murali et al. 2023). Results are shown as mean ± SD, n=3.

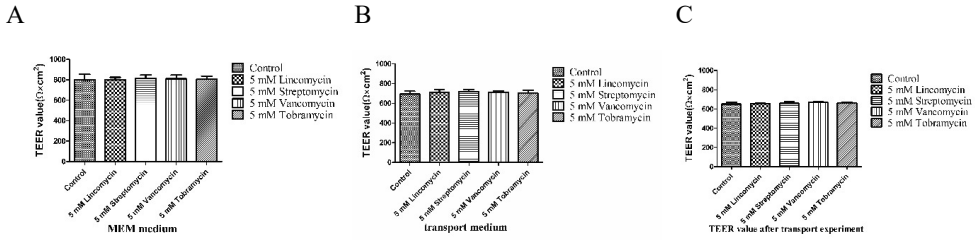

Figure S6. TEER values before (MEM medium), during (transport medium) and after a transport experiment across Caco-2 cell layers of a mixture of bile acids (TCA, TCDCA, GCA and GCDCA) upon co-exposure of the cells to bile acids without (control) or with a similar concentration of antibiotics (5 mM). Results are shown as mean ± SD, n=3.

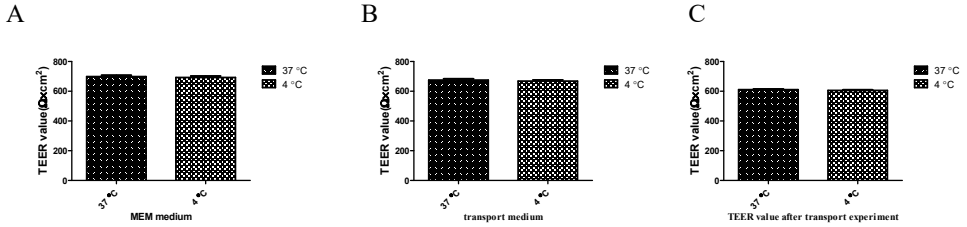

Figure S7. TEER values before (MEM medium), during (transport medium) and after a transport experiment across Caco-2 cell layers of a mixture of bile acids (TCA, TCDCA, GCA and GCDCA) without (control) at 37 °C or 4 °C. Results are shown as mean ± SD, n=3.

Chapter 5.

Application of physiologically based kinetic (PBK) modeling to quantify the effect of the antibiotic tobramycin on bile acid levels in human plasma

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Manuscript in preparation

Abstract

Systemic bile acid homeostasis plays an important role in human health. In this study, a physiologically based kinetic (PBK) model that included microbial deconjugation and intestinal reuptake via the apical sodium dependent bile acid transporter (ASBT) was applied to predict the systemic plasma bile acid concentrations in human upon oral treatment with the antibiotic tobramycin. Tobramycin was previously shown to inhibit intestinal deconjugation and reuptake of bile acids and to affect bile acid homeostasis upon oral exposure of rats. Kinetic parameters to define the effects of tobramycin on intestinal bile acid transport were determined *in vitro* using a Caco-2 cell layer transwell model for studying the intestinal translocation of 4 model bile acids including glycochenodeoxycholic acid (GCDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA) and deoxycholic acid (DCA) the latter as a model for unconjugated bile acids (uBA). Kinetic constants for the effect of tobramycin on intestinal microbial deconjugation were taken from previous *in vitro* studies using anaerobic fecal incubations. The PBK model simulations predicted that exposure to tobramycin at the dose level also used in the previous 28 day rat study of 1000 mg/kg bw would reduce human plasma C_{max} levels of GCA, GCDCA, GDCA and uBA by 36.1 %, 18.7 %, 14.5 % and 33.8 %, especially via an effect on ASBT mediated intestinal reuptake with for GDCA and GCDCA the effect being less pronounced when basal bile acid levels are lower. The effect of tobramycin on intestinal deconjugation did not substantially affect plasma C_{max} levels, likely because deconjugation happens to a large extent in the colon which has limited subsequent bile acid reuptake. The results reflect that oral exposure to xenobiotics that are not or poorly bioavailable can affect systemic bile acid homeostasis. Altogether, the PBK model appears to provide a 3R compliant tool to evaluate the effect of oral exposure to xenobiotics on host bile acid homeostasis via effects on intestinal bile acid deconjugation and reuptake.

Key words: Bile acids, Caco-2 cell, apical sodium dependent bile acid transporter (ASBT), Physiologically based kinetic (PBK) model, tobramycin

1.Introduction

Cholesterol conversion to bile acids is an important metabolic pathway in humans (Di Ciaula et al. 2018; Hofmann 1999a). Bile acids play a key role in inducing bile flow and biliary lipid secretion, assimilating the dietary fat and lipid-soluble vitamins in the intestinal tract (Chiang 1998). Two major primary bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA) which are synthetized from cholesterol in the liver (Russell and Setchell 1992). Before secretion from liver into bile canaliculi, CA and CDCA are conjugated with taurine or glycine to form the corresponding taurine and glycine conjugated bile acid (Bahar and Stolz 1999; Vlahcevic et al. 1999). Upon bile acid secretion through the bile duct into the gastrointestinal tract, about 95 % of the bile acids are reabsorbed from ileum and colon via the portal vein back to the liver, with only a minor amount being excreted into feces (Dawson 2018; Dowling 1972; Hofmann 1999b). This so-called enterohepatic circulation is a highly effective process sustaining bile acid homeostasis.

Many transporters participate in this enterohepatic circulation including the Na^+ taurocholate co-transporting polypeptide (NTCP) and the bile salt export pump (BSEP) in the liver, and the apical sodium-dependent bile acid transporter (ASBT) and the organic solute transporter OST α/β in the intestine (Balakrishnan and Polli 2006; Dawson et al. 2005; Halilbasic et al. 2013; Stieger et al. 2007). These bile acid transporters facilitate bile acid absorption and excretion within the body, and contribute to bile acid homeostasis under physiological conditions. Gut microbiota also participates in the bile acid metabolism. In the colon, efficient deconjugation of conjugated primary bile acids is catalyzed by bile salt hydrolases (BSH) present in a variety of gut microbiota such as *Clostridium*, *Bacteroides*, *Lactobacillus* and *Enterococcus* and others (Begley et al. 2005; Jones et al. 2008; Ridlon et al. 2006).

Recently we developed a physiologically based kinetic (PBK) model to describe bile acid metabolism in humans (de Bruijn et al, 2022). This model included the activity of the bile salt export pump (BSEP) in the liver enabling modeling of the effect of the BSEP inhibitor bosentan on bile acid profiles in human plasma. In a subsequent study also the activity of the ASBT responsible for intestinal reuptake of bile acids was included in the PBK model enabling characterization of the effect of the ASBT inhibitor Odevixibat on human bile acid plasma levels (de Bruijn et al. submitted). The aim of the present study was to apply the model to investigate the effect of the antibiotic tobramycin on bile acid homeostasis. Tobramycin is an aminoglycoside antibiotic known to affect protein synthesis in bacterial as well as human cells thus leading to a reduction in normal functional protein formation (Neu 1976; Wangen and Green 2020). A previous 28 day study in young adult Wistar rats reported that oral exposure to tobramycin at dose levels affecting the intestinal microbiota but not causing systemic toxicity, substantially affected bile acid levels in host feces and plasma samples (Murali et al. 2023). Previous studies in an *in vitro* transwell intestinal transport model with Caco-2 cell layers revealed that pre-exposure of the Caco-2 cells to tobramycin significantly reduced the transport of taurochenodeoxycholic acid (TCDCA) and

glycochenodeoxycholic acid (GCDCA) across the Caco-2 cell layer (Zhang et al. 2023). Given that both *in vivo* but also in Caco-2 cells the ASBT is responsible for the transport of conjugated bile acids across the intestinal cell layer (Balakrishnan and Polli 2006; Balakrishnan et al. 2006; Chen et al. 2012; Dawson 2011; Xiao and Pan 2017), this effect of tobramycin is likely mediated via an effect on the expression level of ASBT. To predict the consequences of this effect of tobramycin for bile acid plasma levels in human, the previously developed PBK model was used to predict the effects of tobramycin on plasma levels of three model conjugated bile acids including glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), and glycodeoxycholic acid (GDCA). Effects on deoxycholic acid (DCA) were also characterized, because DCA is known to be the most abundant unconjugated bile acid (uBA) in human plasma (Rodrigues et al. 2014) and thus representative for the behavior of unconjugated bile acids (uBAs). To define the PBK model parameters, transport of a mixture of these bile acids across Caco-2 cell layers upon pre-exposure and co-exposure of the Caco-2 cells with tobramycin was quantified. Kinetic data for the effect of tobramycin on deconjugation of conjugated bile acids were taken from a previous study with human fecal incubations (Zhang et al. 2023). Thus, data generated *in vitro* were used as input in the PBK model to predict the effects of tobramycin on *in vivo* plasma bile acid levels in humans.

2. Materials and methods

2.1 Chemicals and reagents

Tobramycin was obtained from Sigma-Aldrich (Schnelldorf, Germany). Glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA) and deoxycholic acid (DCA) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Phosphate buffered saline (PBS) was purchased from Gibco (Paisley, UK). Fetal Bovine Serum (FBS) was obtained from GE Healthcare Life Sciences Hyclone Laboratories (Logan, Utah, USA). 0.05% Trypsin-EDTA, minimum essential medium (MEM), penicillin-streptomycin-glutamine solution (PSG), sodium pyruvate, Hank's balanced salt solution (HBSS) and HEPES buffer solution were purchased from Gibco (Paisley, UK). Corning Costar 24 well transwell plates were purchased from Corning Life Sciences (Schnelldorf, Germany). Acetonitrile (ACN) and methanol were obtained from Biosolve BV (Valkenswaard, Netherlands). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Darmstadt, Germany).

2.2 Cell culture

The human epithelial Caco-2 cell line (used in passage number 15-22) was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultivated in MEM with 20% FBS, 1% sodium pyruvate and 1% PSG. The cells were grown in 75 cm^2 flasks in an incubator at 37°C, 5% CO_2 and 100% humidity. Cells were subcultured when reaching 50%-60% confluence with 0.05% trypsin-EDTA.

2.3 *In vitro* bile acid transport

For the transport assay, 1.8×10^5 Caco-2 cells/cm^2 in 100 µL medium were seeded at the apical side of the wells of a Corning 24 well transwell plate and 600 µL MEM medium was added to the basolateral side. Cells were cultivated for 18 days to form intestinal cell layers, with fresh medium provided every other day. In order to exclude effects of other antibiotics than tobramycin, MEM medium without PSG was used when cells were cultured in the 24 well transwell plates. Caco-2 cells were exposed to 45 mM tobramycin or solvent control for 48 h after 18 days cultivation in the transwell plate. The concentration of tobramycin was based on the dose level that was shown in a reported *in vivo* rat bioassay to affect bile acid homeostasis (Murali et al. 2023), converting the dose of 1000 mg/kg bw/day to a concentration of 45 mM as reported previously (Zhang et al. 2022). Integrity of the cell layers in the wells was checked by measurement of the transepithelial electrical resistance (TEER) values, before and after the pre-exposure or co-exposure of the cells to tobramycin or control in transport buffer, as well as at the end of the transport experiment. The TEER value was measured with a Millicell® ERS-2 Volt-Ohm Meter (Millipore, Amsterdam, Netherlands) and expressed Ω×cm^2 and only cell layers with a TEER value above 500 Ω×cm^2 were used for transport experiments.

After pre-exposure with tobramycin or solvent control, the exposure medium was removed and the cell layers were cultured in transport medium (HBSS supplemented with 10 mM HEPES, pH 7.4) for 30 min. Following this 30 min incubation in transport medium, 5 µM GCA, 5 µM GCDCA, 5 µM GDCA and 5 µM DCA as a mixture dissolved in transport medium, were added to the apical part of the transwell plate, and incubation was continued. Samples of 50 µL were taken and replenished from the basolateral compartment at 1h, 2h and 3h, following addition of the bile acid mixture, and analyzed by LC-MS/MS for bile acid levels. Co-exposure of the cells to a mixture of GCA, GCDCA, GDCA and DCA each at 5 µM and 45 mM tobramycin was performed in a similar way starting at day 20 of culture.

2.4 Bile acid profiling by LC-MS/MS

Bile acid profiling was performed using a triple quadrupole LCMS-8045 instrument (Shimadzu Corporation, Japan). The method applied was able to measure 20 different bile acids which were separated on a Kinetex C18 column (1.7µm×100 A×50mm×2.1 mm, Phenomenex 00B-4475-AN). For the LC-MS/MS runs, mobile phase A (MilliQ water containing 0.01% formic acid) and mobile phase B (50% acetonitrile/ 50% methanol) were used. The injection volume was 1 µL and the flow rate 0.4 mL/min. The gradient applied was as follows: 0-10 min 30-70% B, kept at 70% B for 1 min, changed from 70-98% B from 11-19 min, kept at 98% B for 1 min; and then changed from 98 to 30% B from 20- 25 min followed by 10 min equilibration at 30% B before the next injection. Electrospray ionization (ESI) was used in a negative ion mode within the mass spectrometer. The ESI parameters included Nebulizing gas flow 3 L/min; drying gas flow and heating gas flow 10 L/min;

interface voltage 3 kV; interface current 0.9 uA; interface temperature 300 °C; Desolvation temperature 526 °C; DL temperature 250 °C; heat block temperature 400 °C; Conversion Dynode Voltage 10 kV; detector voltage 2.16 kV; IG Vacuum 1.8e-03 Pa; PG Vacuum 9.4e+01 Pa; CID Gas 230 kPa. Both selective ion monitoring (SIM) and multiple reaction monitoring (MRM) were used for the bile acid detection in order to obtain optimal sensitivity for each bile acid. Detailed bile acid identification and quantification was achieved using the characteristics previously described (Zhang et al. 2022; Zhang et al. 2023).

2.5 Physiologically based kinetic (PBK) model of bile acid homeostasis

PBK modeling was used to predict *in vivo* human plasma concentrations of GCA, GCDCA, GDCA and uBA with or without tobramycin treatment, using the PBK model previously described (de Bruijn et al. submitted). uBA represents a lumped pool of unconjugated bile acids, including DCA, but also lithocholic, cholic and chenodeoxycholic acid. The unconjugated bile acids were lumped to reduce model complexity. DCA was used as a model bile acid to obtain kinetic rate constants and tissue:partition coefficients for the uBAs, because it is the most abundant secondary uBA in human serum (Rodrigues et al. 2014). GCA, GCDCA and GDCA deconjugation in the intestine to their corresponding uBAs was assumed to be rapid and complete by setting the deconjugation rate to 1000 hr^{-1}. After hepatic reuptake, uBAs were rapidly conjugated with a rate constant of 1000 hr^{-1}. The fraction of GCA, GDCA and GCDCA formed in the liver was derived from the fraction of these bile acids present in human liver biopsies (Rodrigues et al. 2014). The Michaelis-Menten equation in the PBK model that represents ASBT-mediated bile acid transport across the intestine is as follows:

Equation 1: $$v = \frac{Vmax,ASBT \times [S]}{Km,ASBT + S}$$

Where v is the velocity of transport in µmol/h, $V_{max,ASBT}$ is the maximum velocity of transport in µmol/h, $K_{m,ASBT}$ is the Michaelis-Menten constant of transport in µmol/L and S is the concentration of the respective bile acid expressed in µmol/L. Tobramycin pre-exposure of the Caco-2 cells inhibited bile acid transport most likely by downregulating the protein expression of the bile acid transporter ASBT. Co-exposure of the cells to tobramycin and the bile acids inhibited bile acid transport by direct interactions. The inhibition of the ASBT mediated transport was modelled by reducing the V_{max} to include the effect of pre-exposure and by reducing the absorption rate constant k_s to include the effect of co-exposure, with the level of reduction being derived from the data obtained in the *in vitro* Caco-2 model system to set the % of reduction of the V_{max} or k_s respectively. The model code can be found in the supplementary information.

2.6 Data analysis and Statistics

Bile acid profile data were analyzed using the Labsolutions software within the LC-MS/MS system. Graphical figures were drawn by using Graphpad Prism 5 (San Diego, USA) and R

version 4.1.0. Statistical analysis was performed by Student's t test. The effects were considered significant when $P<0.05$. All the data are presented as mean ± SD from at least three independent measurements.

3. Results

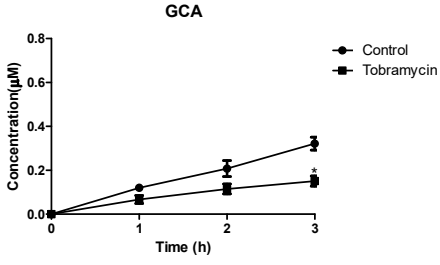
3.1 Caco-2 cell layer integrity

TEER values were detected before, during and after the bile acid transport assays in order to ensure that the barrier integrity of the cell layers was not affected by the pre-exposure with tobramycin or the co-exposure with tobramycin during the transport experiment. The TEER values of the cell layers confirmed that these cell layers were not affected by the pre-exposure and co-exposure for both control and tobramycin treatment conditions (supplementary information Figure S1 and S2). Also the presence of the bile acids did not affect the TEER value (supplementary information Figure S1 and S2).

3.2 Transport of a mixture of bile acids across Caco-2 cell layers pre-exposed to tobramycin

Figure 2 presents the time dependent transport of a mixture of the 4 bile acids (GCA, GCDCA, GDDCA, and DCA as the model compound for the uBAs) across the Caco-2 cell layers without or with pre-exposure of the cell layers to tobramycin. Table 2 presents the apparent permeability coefficients (P_{app} values) (expressed in cm/s) derived from the data at 1h incubation. Pre-exposure of the cells with tobramycin reduced the transport of GCA and GCDCA to a significant extent while reduction of the transport of GDCA was not significant and there was no effect on DCA transport. The data also reveal that transport of GCDCA, GDDCA and DCA was comparable and at least 2 fold higher than that of GCA.

A



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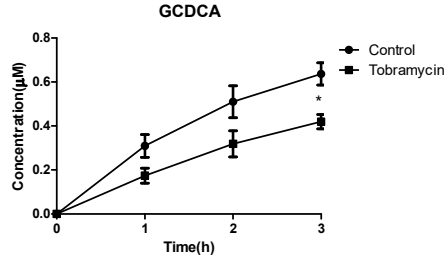

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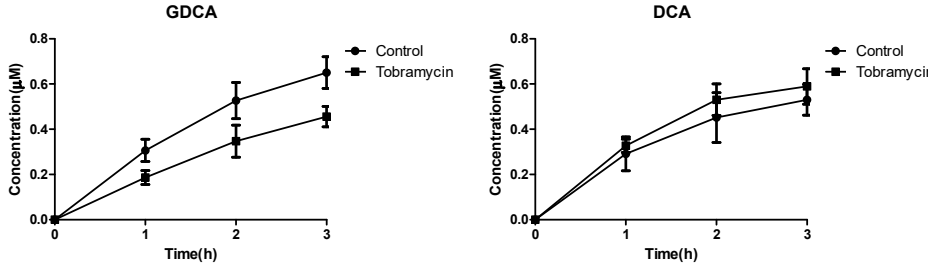

Figure 1. Time dependent transport of a mixture of bile acids (GCA, GCDCA, GDCA and DCA) across the Caco-2 cell layers upon **pre-exposure** of the cell layers without (control) or with 45 mM tobramycin (**P*< 0.05). Results are expressed as mean ± SD, n=3.

Table 1 Permeability constants (P_{app} values) for the transport of a mixture of bile acids (GCA, GCDCA, GDCA and DCA) across Caco-2 cell layers upon **pre-exposure** of the cell layers without (control) or with 45 mM tobramycin (**P*< 0.05). Results are expressed as mean ± SD, n=3.

	P_{app} (in 10^{-5} cm/s)		
	Sample		
Bile acid	Control	Tobramycin	Residual transport activity (% of control)
GCA	1.0±0.1	0.5±0.1*	50.0
GCDCA	2.1±0.2	1.4±0.1*	66.7
GDCA	2.2±0.1	1.8±0.3	82.0
DCA	2.9±0.7	3.3±0.3	113.8

From the results presented in table 1, it follows that the effect of pre-exposure of the Caco-2 cells to tobramycin on bile acid transport is due to an effect on active transport. This follows from the observation that the transport of DCA which is known to be predominantly transported via passive diffusion (Dietschy 1968) is not inhibited, while that of GCA, a bile acid that predominantly crosses the intestinal barrier via ASBT mediated active transport (Dawson et al. 2009) is inhibited to the largest extent. The % reduction appeared to vary between the different bile acids, which can best be ascribed to other transport mechanisms such as for example passive diffusion, contributing to a variable extent to the transport of the respective bile acids.

Based on these considerations the consequences of the effect of tobramycin on bile acid transport via an effect on the ASBT transporter expression levels was investigated by reducing the V_{max} for ASBT mediated transport in the PBK model to reflect the % residual transport activity presented in table 1.

3.3 Transport of a mixture of bile acids across the Caco-2 cell layers upon co-exposure with tobramycin

Since tobramycin appeared also able to disturb bile acid transport via direct effects, bile acid transport upon co-exposure of a mixture of bile acids with tobramycin across the Caco-2 cell layers was quantified. Figure 2 and table 2 present the results obtained and demonstrate that tobramycin co-exposure reduced the transport of GCDCA and DCA significantly, while it did not affect the transport of GCA, and had only a limited effect on transport of GDCA. The P_{app} values for DCA, GCDCA, GDCA were again at least 2 fold higher than that of GCA.

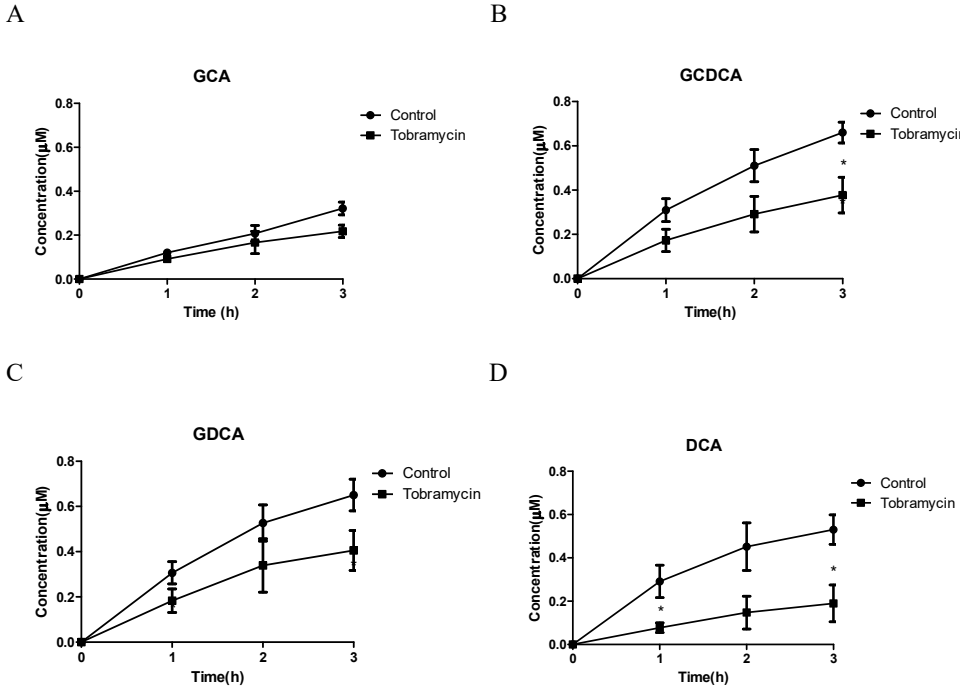

Figure 2. Time dependent transport of a mixture of bile acids (GCA, GCDCA, GDCA and DCA) across the Caco-2 cell layers upon **co-exposure** without (control) or with 45 mM tobramycin (*P< 0.05). Results are expressed as mean ± SD, n=3.

Table 2 Permeability constants (P_{app} values) for the transport of a mixture of bile acids (GCA, GCDCA, GDCA and DCA) across Caco-2 cell layers upon **co-exposure** without (control) or with 45mM tobramycin (*P< 0.05). Results are expressed as mean ± SD, n=3.

	P_{app} (in 10^{-5} cm/s)		
	Sample		
Bile acid	Control	Tobramycin	Residual transport activity (% of control)
GCA	1.0±0.1	0.9±0.1	90.0
GCDCA	2.2±0.2	1.3±0.1*	59.0
GDCA	2.1±0.1	1.7±0.3	81.0
DCA	3.0±0.6	0.8±0.1*	27.0

The P_{app} values in table 2 reveal that inhibition of bile acid transport upon co-exposure of the Caco-2 cells to tobramycin and the bile acids most likely results from an effect on passive diffusion and not from an effect on the ASBT transporter. This follows from the fact that the reduction is most substantial for DCA which is transported mainly by passive diffusion, while the effect is smallest for GCA which is predominantly transported by active ASBT mediated transport. Thus, to investigate the consequences of this effect of tobramycin for the plasma bile acid level, the absorption rate constants for intestinal uptake (k_s) in the PBK model were reduced to reflect the % residual transport activity presented in table 2.

3.4 Prediction of the consequences of inhibition of bile acid reuptake by tobramycin for the *in vivo* plasma bile acid concentrations

Figure 3 presents the PBK model simulations for *in vivo* plasma GCA, GCDCA, GDCA and uBA concentrations with and without tobramycin treatment. The curves reflect the consequences of 3 meals a day at 8:00, 12:00 and 16:00 respectively and are based on V_{max} and k_s values that are adapted in line with the results from table 1 and 2. The results obtained reveal that, in line with the *in vitro* bile acid transport results, systemic plasma levels of especially the 3 conjugated bile acids are reduced by effects of tobramycin on ASBT expression levels while plasma uBA levels are especially sensitive to effects of the co-exposure on passive diffusion and not affected by the effects on ASBT expression levels. Given that upon *in vivo* exposure to tobramycin both effects of pre- and co-exposure are likely to occur simultaneously, the purple lines in Figure 3 present the PBK model predictions where both the effect on V_{max} and k_s were taken into account.

These data reveal that for all four bile acids tobramycin exposure at an oral dose level similar to what was applied in the 28 day rats studies is predicted to result in a substantial reduction of the plasma C_{max} concentrations by 36.1 %, 18.7 %, 14.5 % and 33.8 % of the concentrations without tobramycin for GCA, GCDCA, GDCA and uBA respectively.

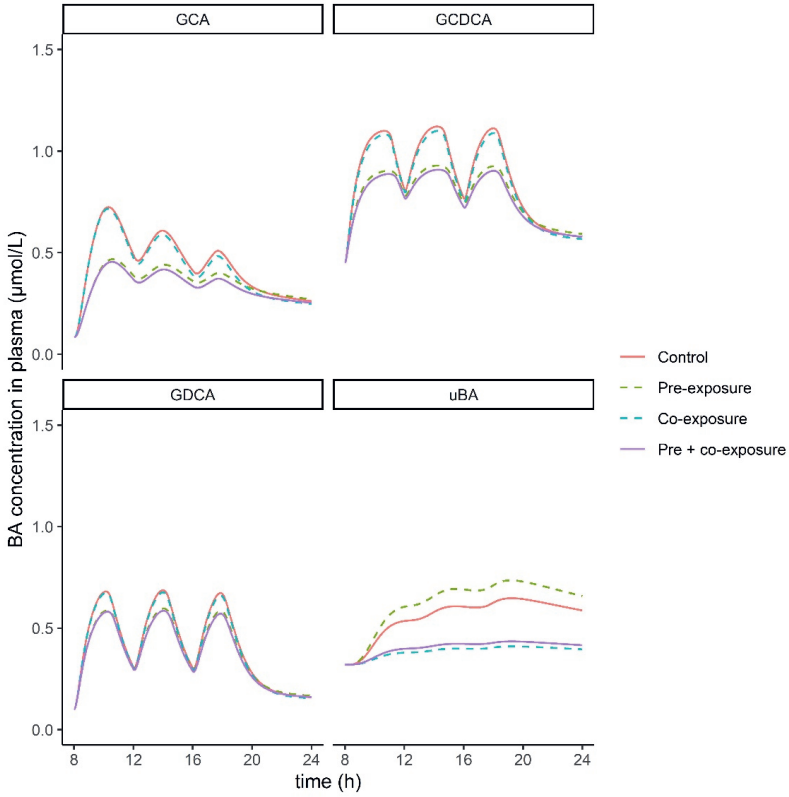

Figure 3 PBK model based predictions for the effects of an oral dose of 1000 mg/kg bw tobramycin on the systemic plasma levels of the bile acids (GCA, GCDCA, GDCA and uBA). The curves present simulations assuming 3 meals (at 8:00, 12:00 and 16:00) per day. The curves present the predicted plasma levels without tobramycin (control; red solid lines), with the effect of tobramycin on ASBT expression levels (based on pre-exposure to tobramycin) (green dotted lines), with the direct effect on bile acid absorption (based on co-exposure to tobramycin) (blue dotted lines) and with taking both effects of tobramycin into account (based on pre-exposure and co-exposure to tobramycin) (purple solid lines).

3.5 Prediction of the consequences of the effect of tobramycin on intestinal bile acid deconjugation for the *in vivo* plasma bile acid concentrations

Previously tobramycin was also shown to inhibit intestinal bile acid deconjugation by the microbiota (Zhang et al. 2023). To further elucidate the consequences of this effect of tobramycin on bile acid metabolism further PBK modeling was performed. *In vitro* anaerobic incubations with human fecal slurry and a mixture of bile acids demonstrated that tobramycin inhibited deconjugation of GCA and GCDCA resulting in 28.1 and 47.2 % residual activity (Zhang et al. 2023). To include this effect in the PBK modeling the deconjugation constant for the model that predicted the effect of tobramycin treatment was assumed to decrease from 1000 hr^{-1} to 281 hr^{-1} for GCA and 472 hr^{-1} for GCDCA. This change however, appeared not to

influence the PBK model predictions for the plasma levels of GCA and GCDCA (Table 3). This outcome is in line with previous results from a sensitivity analysis that revealed the kdec for intestinal deconjugation not to be an influential parameter for the calculated plasma bile acid C_{max} (de Bruijn et al. submitted).

Table 3 PBK model based predicted residual C_{max} of the bile acids (GCA, GCDCA, GDCA and uBA) in human plasma when simulated without and with the effects of tobramycin on intestinal deconjugation while also including the effects of tobramycin on intestinal reuptake of the bile acids compared to control (without tobramycin)

	Residual C_{max} (%)			
	GCA	GCDCA	GDCA	uBA
Without intestinal deconjugation	63.9	81.3	85.5	66.2
With intestinal deconjugation	63.9	81.3	85.5	66.2

3.6 Interindividual variation in bile acid pool size

The total bile acid pool size is determined by the amount of bile acids in the gallbladder, the systemic fasting concentrations and the *de novo* synthesis. Previous PBK modeling results revealed that the amount of bile acids in a full bladder (Gdose) and systemic fasting concentrations (CBfs) have the largest impact on the systemic maximal bile acid concentrations C_{max} (de Bruijn et al. 2022). To reflect differences in this total bile acid pool known to exist between individuals, the total bile acid pool size in the model can be adapted by the scaling factor sensitivity (sens). Setting the sens value at 0.5, 1 and 1.5, results in PBK models that reflect individuals with different total bile acid pools. Figure 4 shows the effects of this change in the total bile acid pool on the consequences for the effect of tobramycin on the systemic bile acid concentrations. From this it follows that the effect of tobramycin on C_{max} levels for GCA and GCDCA is predicted to be substantial at all sens values, amounting to 30 % and 17 % residual C_{max} at a sens value of 1.5, to 35 % and 20 % residual C_{max} at a sens value of 1.0 and to 38 % and 20 % residual C_{max} at a sens value of 0.5 respectively. For GDCA and uBA the effect seems to decrease with a decrease in the sens value, amounting to 15 % and 40 % at a sens value of 1.5, to 8 % and 17 % at a sens value of 1.0 and to 0 % and 8 % at a sens value of 0.5 respectively.

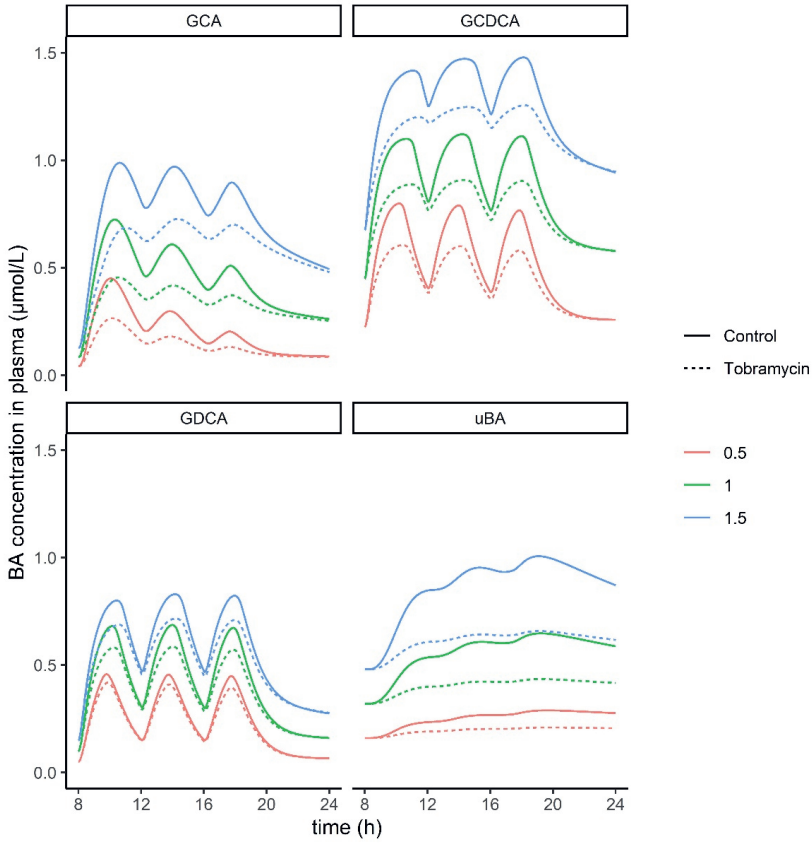

Figure 4 PBK model based predictions for the effect of tobramycin on the systemic plasma levels of the bile acids (GCA, GCDCA, GDCA, uBA) for individuals with different total bile acid pools. The curves present simulations assuming 3 meals (at 8:00, 12:00 and 16:00) per day. The curves present the plasma levels without tobramycin (control: solid lines) and with tobramycin (dotted lines) including both an effect on the V_{max} for ASBT mediated transport (based on pre-exposure caco-2 cell experiments), and a direct effect on ks for intestinal reuptake (based on co-exposure Caco-2 cell experiments). The total bile acid pool size in the model was multiplied with a certain sens value (0.5, 1 or 1.5). Red=sens value 0.5, green=sens value 1, and blue=sens value 1.5.

4. Discussion

The antibiotic tobramycin was shown to inhibit intestinal deconjugation and reuptake of bile acids in previous studies (Zhang et al. 2022; Zhang et al. 2023). These *in vitro* results were in line with the observation of increased fecal bile acid levels and reduced plasma levels in an *in vivo* 28 day rat study (Murali et al. 2023). In the present study we used the PBK model previously developed to predict the effects on the systemic plasma bile acid levels in human induced by the ASBT inhibitor Odevixibat (de Bruijn et al. submitted) to study the effects of

exposure to tobramycin on human plasma bile acid levels. In these previous studies, it was also shown that the transport of the bile acids in the Caco-2 cell model was mediated by active transport via the ASBT since the transport was substantially lower at 4ºC as compared to 37ºC (Chapter 4 Zhang et al. in preparation) and also in the absence of sodium (de Bruijn et al. submitted). In this study, a PBK model that included microbial deconjugation and intestinal reuptake via the ASBT was applied to predict the systemic plasma bile acid concentrations in human upon treatment with the antibiotic tobramycin. Results obtained can be compared to results from the 28 day rat study showing effects of tobramycin on bile acid homeostasis (Murali et al. 2023) and results from a study in which the effects of the approved ASBT inhibitor drug Odevixibat on plasma and fecal bile acid levels in human volunteers were reported (Graffner et al. 2016). The predicted plasma concentrations of conjugated bile acids (GCA, GCDCA, GDCA) decreased while that of the unconjugated bile acid (DCA) was not significantly increased upon Odevixibat treatment. This is in line with the effects of Odevixibat on systemic plasma data in human reported in the literature (Graffner et al. 2016). Also, the relative amount of glycine conjugated bile acids in human serum was reported to be about 45% of the total bile acid pool, for unconjugated bile acids this values amounted to about 40% and for taurine conjugated bile acids it was about 15% (Bathena et al. 2013). Therefore, these bile acids were considered as the target bile acids in the PBK model.
To obtain kinetic parameters to define the effects of tobramycin on intestinal bile acid transport, *in vitro* Caco-2 cell layer transwell experiments were performed to quantify the effect of tobramycin on the intestinal translocation of 4 model bile acids. After pre-incubation and co-incubation of the Caco-2 cells to tobramycin and a mixture of GCA, GCDCA, GDCA and DCA, tobramycin was shown to inhibit the transport of these bile acids, and these effects were included in the model via reducing the V_{max} and k_s parameters. The PBK model simulated predictions for the time dependent plasma concentrations of the bile acids thus obtained show that tobramycin exposure was predicted to reduce the plasma levels of GCA, GCDCA and uBA significantly while the level of GDCA was affected to a lesser extent. The results of our PBK model predictions can also be compared to reported concentrations of GCDCA in human serum (control) which are about 5 fold higher than those of GCA (García-Cañaveras et al. 2012), while in our simulations GCDCA plasma concentrations were also predicted to be higher (2 fold) than those of GCA.
Kinetic constants for the effect of tobramycin on intestinal deconjugation were taken from previous *in vitro* studies using anaerobic fecal incubations (Zhang et al. 2022; Zhang et al. 2023). Tobramycin is an aminoglycoside antibiotic, acting against gram-negative bacteria by performing bactericidal effects (Brogden et al. 1976; Reyhanoglu and Reddivari 2019). In our previous studies, 16S rRNA analysis revealed that tobramycin treatment changed gut microbiota composition significantly (Murali et al. 2023; Zhang et al. 2022); it also affected the deconjugation of fecal bile acids showing significant suppression of deconjugation of GCA and GCDCA in both rat and human fecal incubations (Zhang et al. 2022; Zhang et al. 2023). About 95 % of bile acids are actively reabsorbed in the small intestine, while the remaining 5 % of bile acids enters the colon where the majority is recaptured and only a minor amount is excreted in the feces (Chiang 2009). The effect of tobramycin on intestinal deconjugation appeared not to affect plasma C_{max} levels likely because deconjugation mainly occurs in the colon for only a limited portion of the total bile acid pool.

The PBK model simulations of the present study revealed that oral exposure to tobramycin at the high dose level tested before in the 28 day rat study of 1000 mg/kg bw (Murali et al. 2023) is predicted to reduce human plasma C_{max} levels of GCA, GCDCA, GDCA and uBA by 36.1 %, 18.7 %, 14.5 % and 33.8 %, so levels that were predicted to amount to about 0.64, 0.81, 0.86 and 0.66 times the control values, with especially for GDCA and GCDCA the effect being less pronounced when basal bile acid levels are lower. Human data to evaluate the PBK model predictions are not available but a comparison can be made to results from the 28 days *in vivo* rat study (Murali et al 2023). In this 28 day study rats were exposed to tobramycin at a dose level that was selected to affect the intestinal microbiome without causing systemic toxicity. In the rat study tobramycin exposure also resulted in a reduction of plasma levels of GCA, GCDCA, GDCA and uBA (Murali et al. 2023). In the rat study the reductions in bile acid levels were especially seen in female rats where exposure to tobramycin resulted in plasma levels of GCA, GCDCA, GDCA and DCA (uBA) that were reduced to 0.35, 0.38, 0.04 times and below of detection limit the values in control animals, respectively (Murali et al. 2023). Thus, the tobramycin induced reduction of bile acid plasma levels occurred in the order: uBA > GDCA >GCA ≥ GCDCA in the *in vivo* rat study. For human the PBK model predicted the tobramycin induced reduction in plasma bile acids in human to vary in the order: GCA> uBA > GCDCA > GDCA. The less pronounced effect for GDCA in the PBK model predictions for human than in the rat *in vivo* study, may originate from a species difference in the (inhibition of) the active transporters involved, being more pronounced in rat than in human. The less pronounced effect for uBA in the PBK model simulations for human than in the *in vivo* rat study may be related to the fact that DCA used in the *in silico* modelling was assumed to represent all uBAs.

It is also important to note that tobramycin is poorly absorbed from the gastrointestinal tract thus showing poor bioavailability when orally administration (Reyhanoglu and Reddivari 2019). This implies that the current PBK simulation reflect the effects of an orally administered xenobiotic on systemic plasma levels without a need for systemic bioavailability of the xenobiotic itself. It is also relevant to note that the effects on human plasma levels of bile acids upon oral exposure to tobramycin predicted in the present study, do not reflect effects expected upon intravenous or intramuscular exposure when using tobramycin at lower dose levels as an antibiotic drug in human. The results of the present study rather reflect that oral exposure to xenobiotics that are not or poorly bioavailable can affect systemic bile acid homeostasis.

Altogether, the PBK model appears to provide a 3R compliant tool to evaluate the effect of oral exposure to xenobiotics on host bile acid homeostasis via effects on intestinal bile acid deconjugation and reuptake.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary information

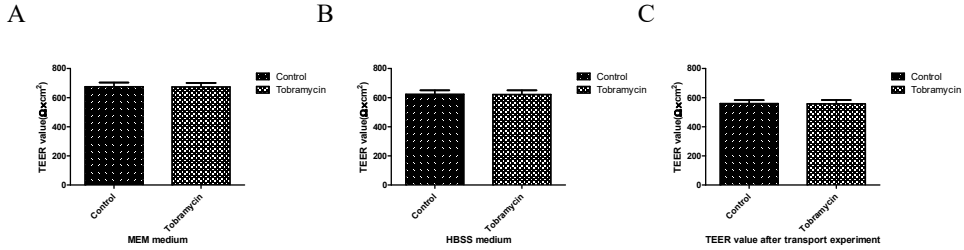

Figure S1 TEER values before (MEM medium), during (HBSS medium) and after pre-exposure a mixture of bile acids (GCA, GCDCA, GDCA and DCA) transport without (control) and with tobramycin across Caco-2 cell layers. Results are shown as mean ± SD, n=3.

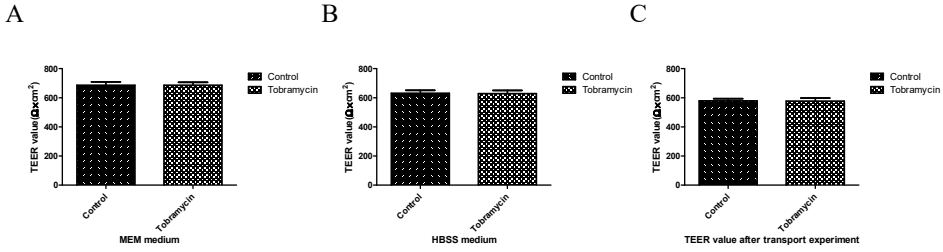

Figure S2 TEER values before (MEM medium), during (HBSS medium) and after co-exposure a mixture of bile acids (GCA, GCDCA, GDCA and DCA) transport without (control) and with tobramycin across Caco-2 cell layers. Results are shown as mean ± SD, n=3.

#PBK model 4 bile acids

#GCDCA, GCA, GDCA + DCA

#species: human

#author: Véronique de Bruijn

#date: april 2023

.libPaths("C:/ProgramData/R/win-library/4.1")

#install.packages("RxODE")

library(RxODE)

```
library(tidyverse)
library(readxl)
library(data.table)
library(pmxTools)

##Set working directory
setwd()

#Simulations
amount.units="umol"       #unit for the amount
time.units="h"            #unit for time
nbr.doses=1               #number of doses
time.0=8                  #time start dosing
time.end=32               #time end of simulation
time.frame=0.01           #time steps of simulation
N=1                 #Number of individuals

#step 1: read in parameters
parms <- read_excel("parms_4BA_Nina.xlsx") %>%
  dplyr::select(1:6)

parms_long <- melt(setDT(parms), id.vars=1, variable.name="parm") %>%
  mutate(name=paste(parm, parm.1, sep="_"))%>% dplyr::select(3,4)%>% drop_na()

colnames <- parms_long$name
par_var <- length(colnames)
Mean <- parms_long$value

#create dataframe for 1) control, 2) pre exposure, 3) co exposure, 4) pre and co exposure, 5) pre and co exposure
+ reduced faecal deconjugation
# 2: pre exposure affects Vmax, 3: co exposure modifies the absorption rate constant (ka), 4: pre and co exposure
affect Vmax and ka, 5: pre and co exposure affect Vmax, ka and faecal deconjugation rate
```

```
X1 <- matrix(NA, nrow = N, ncol = par_var)
colnames(X1) <- colnames
X1 <- as.data.frame(X1)

for(i in 1:par_var){
  X1[,i] <- rep.int(Mean[i], times=N)
}

## experimental % remaining after tobramycin treatment
X1$modf_Vmax_GCA[1] <- 1      #control
X1$modf_Vmax_GCA[2] <- 0.5    #pre exposure, effect on Vmax, no effect on ka
X1$modf_Vmax_GCA[3] <- 1      # co exposure, effect on ka, no effect on Vmax
X1$modf_Vmax_GCA[4] <- 0.5  #pre and co exposure, effect on Vmax and ka
X1$modf_Vmax_GCA[5] <- 0.5  #pre and co exposure, effect on Vmax, ka and faecal deconjugation rate

X1$modf_p_GCA[1] <- 1          #control
X1$modf_p_GCA[2] <- 1          #pre exposure, effect on Vmax, no effect on ka
X1$modf_p_GCA[3] <- 0.9        #co exposure, effect on ka, no effect on Vmax
X1$modf_p_GCA[4] <- 0.9        #pre and co exposure, effect on Vmax and ka
X1$modf_p_GCA[5] <- 0.9        #pre and co exposure, effect on Vmax, ka and faecal deconjugation rate

X1$modf_Vmax_GCDCA[1] <- 1           #control
X1$modf_Vmax_GCDCA[2] <- 0.667       #pre exposure, effect on Vmax, no effect on ka
X1$modf_Vmax_GCDCA[3] <- 1           #co exposure, effect on ka, no effect on Vmax
X1$modf_Vmax_GCDCA[4] <- 0.667       #pre and co exposure, effect on Vmax and ka
X1$modf_Vmax_GCDCA[5] <- 0.667       #pre and co exposure, effect on Vmax, ka and faecal deconjugation
rate

X1$modf_p_GCDCA[1] <- 1        #control
X1$modf_p_GCDCA[2] <- 1        #pre exposure, effect on Vmax, no effect on ka
X1$modf_p_GCDCA[3] <- 0.59     #co exposure, effect on ka, no effect on Vmax
```

```
X1$modf_p_GCDCA[4] <- 0.59    #pre and co exposure, effect on Vmax and ka
X1$modf_p_GCDCA[5] <- 0.59    #pre and co exposure, effect on Vmax, ka and faecal deconjugation rate

X1$modf_Vmax_GDCA[1] <- 1          #control
X1$modf_Vmax_GDCA[2] <- 0.82       #pre exposure, effect on Vmax, no effect on ka
X1$modf_Vmax_GDCA[3] <- 1          #co exposure, effect on ka, no effect on Vmax
X1$modf_Vmax_GDCA[4] <- 0.82       #pre and co exposure, effect on Vmax and ka
X1$modf_Vmax_GDCA[5] <- 0.82       #pre and co exposure, effect on Vmax, ka and faecal deconjugation
rate

X1$modf_p_GDCA[1] <- 1         #control
X1$modf_p_GDCA[2] <- 1         #pre exposure, effect on Vmax, no effect on ka
X1$modf_p_GDCA[3] <- 0.81      #co exposure, effect on ka, no effect on Vmax
X1$modf_p_GDCA[4] <- 0.81      #pre and co exposure, effect on Vmax and ka
X1$modf_p_GDCA[5] <- 0.81      #pre and co exposure, effect on Vmax, ka and faecal deconjugation rate

## DCA is passively transported, so only a ka is included
#pre exposure: ka is multiplied by modf_Vmax_DCA
#co exposure: ka is multiplied by modf_p_DCA
# pre+co exposure: ka is multiplied by both modf_Vmax_DCA and modf_p_DCA
X1$modf_Vmax_DCA[1] <- 1           #control
X1$modf_Vmax_DCA[2] <- 1.138       #pre exposure
X1$modf_Vmax_DCA[3] <- 1           #co exposure
X1$modf_Vmax_DCA[4] <- 1.138       #pre and co exposure
X1$modf_Vmax_DCA[5] <- 1.138       # pre and co exposure

X1$modf_p_DCA[1] <- 1              #control
X1$modf_p_DCA[2] <- 1              #pre exposure
X1$modf_p_DCA[3] <- 0.27           #co exposure
X1$modf_p_DCA[4] <- 0.27           # pre and co exposure
X1$modf_p_DCA[5] <- 0.27           # pre and co exposure
```

```
### deconjugation rate
X1$modf_fdec_GCDCA[1] <- 1          #control, no effect on faecal deconjugation
X1$modf_fdec_GCDCA[2] <- 1          #pre exposure, no effect on faecal deconjugation
X1$modf_fdec_GCDCA[3] <- 1          #co exposure, no effect on faecal deconjugation
X1$modf_fdec_GCDCA[4] <- 1          #pre and co exposure, no effect on faecal deconjugation
X1$modf_fdec_GCDCA[5] <- 0.472      #pre and co exposure, reduced faecal deconjugation

X1$modf_fdec_GCA[1] <- 1            #control, no effect on faecal deconjugation
X1$modf_fdec_GCA[2] <- 1            #pre exposure, no effect on faecal deconjugation
X1$modf_fdec_GCA[3] <- 1            #co exposure, no effect on faecal deconjugation
X1$modf_fdec_GCA[4] <- 1            #pre and co exposure, no effect on faecal deconjugation
X1$modf_fdec_GCA[5] <- 0.281        #pre and co exposure, reduced faecal deconjugation

##Combine parameters
parameters <- setNames(X1, colnames)
Gdose <- parms %>% filter(parm=="Gdose")

#step 2: initial values compartment
inits <- c(dose_GCDCA=Gdose$GCDCA,
        AL_GCDCA=0,
        ALu_GCDCA=0,
        Aup_GCDCA=0,
        Alow_GCDCA=0,
        Acol_GCDCA=0,
        AI_GCDCA=0,
        AF_GCDCA=0,
        AR_GCDCA=0,
        AS_GCDCA=0,
        AB_GCDCA=0,
        dose_GDCA=Gdose$GDCA,
```

```
        AL_GDCA=0,
        ALu_GDCA=0,
        Aup_GDCA=0,
        Alow_GDCA=0,
        Acol_GDCA=0,
        AI_GDCA=0,
        AF_GDCA=0,
        AR_GDCA=0,
        AS_GDCA=0,
        AB_GDCA=0,
        dose_GCA=Gdose$GCA,
        AL_GCA=0,
        ALu_GCA=0,
        AI_GCA=0,
        AF_GCA=0,
        AR_GCA=0,
        AS_GCA=0,
        AB_GCA=0,
        AL_DCA=0,
        Acol_DCA=0,
        AI_DCA=0,
        AF_DCA=0,
        AR_DCA=0,
        AS_DCA=0,
        AB_DCA=0)

#step 3: exposure
qd <- eventTable(amount.units = amount.units, time.units = time.units) %>%
  add.dosing(dose=0.00001, cmt="AL_GCDCA",nbr.doses = 1, do.sampling=FALSE) %>%
  et(seq(from = time.0, to = time.end, by = time.frame))
```

```
#step 4: differential equations

PBK <- RxODE({

 ##Physiological parameters

 #Tissue volumes

 VF = VFc_all*BW_all;    #       {L or Kg}       ; volume of fat tissue (calculated)

 VL = VLc_all*BW_all;    #       {L or Kg}       ; volume of liver tissue (calculated)

 VR = VRc_all*BW_all;    #       {L or Kg}       ; volume of  richly perfused tissue (calculated)

 VS = VSc_all*BW_all;    #       {L or Kg}       ; volume of  slowly perfused tissue (calculated)

 VB = VBc_all*BW_all;    #       {L or Kg}       ; volume of  blood excluding portal vein (calculated)

 VI = VIc_all*BW_all;    #       {L or Kg}       ; volume of intestinal tissue (calculated)

 VG= VGc_all*BW_all;     #       {L or Kg}       ; volume of gall bladder tissue (calculated)

 VLu=VLuc_all*BW_all;    #       {L or Kg}       ; volume of intestinal lumen (calculated)


 #Blood flow rates

 QF = QFc_all*QC_all;    #       {L/hr}          ; blood flow to fat tissue (calculated)

 QL = QLc_all*QC_all;    #       {L/hr}          ; blood flow to liver tissue (calculated)

 QS = QSc_all*QC_all;    #       {L/hr}          ; blood flow to  slowly perfused tissue (calculated)

 QR = QRc_all*QC_all;    #       {L/hr}          ; blood flow to richly perfused tissue (calculated)

 QI = QIc_all*QC_all;    #       {L/hr}          ; blood flow to intestines (calculated)


 ## Scaling of maximal transport rate (Vmax) for ASBT- and BSEP-mediated BA transport

 WL=20*BW_all; #weight of liver, g

 SF_BSEP=aBSEP_all*MWBSEP_all*Hep_all*WL*10^-9; # {mg BSEP/entire lever}; scaling factor

 SF_ASBT=surface_all*SA_all*10^-6 ; # {cm2/entire ileum} ; scaling factor


 VmaxBSEP_GCDCA=VmaxBSEPc_GCDCA*SF_BSEP*60; # {umol/h/entire liver} maximum speed for
BSEP-mediated GCDCA efflux (calculated)

 VmaxASBT_GCDCA=VmaxASBTc_GCDCA*SF_ASBT*60*modf_Vmax_GCDCA; #{umol/hr/ileum} :
maximum speed for ASBT-mediated GCDCA uptake (calculated)


 VmaxBSEP_GCA=VmaxBSEPc_GCA*SF_BSEP*60; # {umol/h/entire liver} maximum speed for BSEP-
mediated GCA efflux (calculated)
```

VmaxASBT_GCA=VmaxASBTc_GCA *SF_ASBT*60*modf_Vmax_GCA;

#{umol/hr/ileum} : maximum speed for ASBT-mediated GCA uptake (calculated)

VmaxBSEP_GDCA=VmaxBSEPc_GDCA*SF_BSEP*60; # {umol/h/entire liver} maximum speed for BSEP-mediated GDCA efflux (calculated)

VmaxASBT_GDCA=VmaxASBTc_GDCA*SF_ASBT*60*modf_Vmax_GDCA;

#{umol/hr/ileum} : maximum speed for ASBT-mediated GDCA uptake (calculated)

Fast/fed state

#ge=ifelse((ctime>8 & ctime<9.5), ge_all,0); #specifying the duration of gallbladder emptying (1 meal)

ge=ifelse(((ctime >8 & ctime<9.5) | (ctime>12 & ctime<13.5) | (ctime>16 & ctime<17.5)), ge_all,0); # specifying the duration of gallbladder emptying (3 meals)

#(umol/hr/entire liver) calculated

ktj=ifelse(ctime>17.5 | ctime <8, ktj_fasted_all, ktj_fed_all); #specifying different jejunum transit rates for fast/fed state (1/hr)

kti=ifelse(ctime>17.5 | ctime <8, kti_fasted_all, kti_fed_all); #specifying different ileum transit rates for fast/fed state (1/hr)

QGb_all=1-QIb_all; #fraction of bile flow from liver stored in gall bladder, calculated

##Specify modification factors for Vmax of active ileal transport of GCA, GDCA, GCDCA and DCA

modf_Vmax_GCA=modf_Vmax_GCA;

modf_Vmax_GDCA=modf_Vmax_GDCA;

modf_Vmax_GCDCA=modf_Vmax_GCDCA;

modf_Vmax_DCA=modf_Vmax_DCA;

modf_p_GCA=modf_p_GCA;

modf_p_GCDCA=modf_p_GCDCA;

modf_p_GDCA=modf_p_GDCA;

modf_p_DCA=modf_p_DCA;

modf_fdec_GCA=modf_fdec_GCA;

modf_fdec_GCDCA=modf_fdec_GCDCA;

```
## Model calculations

### GCDCA submodel ###

CL_GCDCA=AL_GCDCA/VL;                          # concentration in the liver (umol/L)

CVL_GCDCA=CL_GCDCA/(PL_GCDCA*BP_all);          # concentration leaving the liver(umol/L)

CIl_GCDCA=Alow_GCDCA/VIl_all;                  # concentration leaving the ileum (umol/L)

CI_GCDCA=AI_GCDCA/VI;                  # concentration in the intestinal tissue (umol/L)

CVI_GCDCA=CI_GCDCA/(PG_GCDCA*BP_all);          # concentration leaving the intestinal
tissue(umol/L)

CF_GCDCA=AF_GCDCA/VF;                  # concentration in the fat tissue (umol/L)

CVF_GCDCA=CF_GCDCA/(PF_GCDCA*BP_all);          # concentration leaving the fat tissue (umol/L)

CR_GCDCA=AR_GCDCA/VR;                          # concentration in the rapidly perfused tissue
(umol/L)

CVR_GCDCA=CR_GCDCA/(PR_GCDCA*BP_all);          # concentration leaving the rapidly perfused tissue
(umol/L)

CS_GCDCA=AS_GCDCA/VS;                  # concentration in the slowly perfused tissue (umol/L)

CVS_GCDCA=CS_GCDCA/(PS_GCDCA*BP_all);          # concentration leaving slowly perfused tissue
(umol/L)

CB_GCDCA=AB_GCDCA/VB;                          # concentration in blood excluding portal vein
(umol/L)


d/dt(dose_GCDCA) = -ge*dose_GCDCA+VmaxBSEP_GCDCA*CVL_GCDCA/(KmBSEP_GCDCA +
CVL_GCDCA)*QGb_all ; #change in amount in the gall bladder, umol

d/dt(AL_GCDCA)=QL*(CB_GCDCA -CVL_GCDCA) -VmaxBSEP_GCDCA *CVL_GCDCA
/(KmBSEP_GCDCA + CVL_GCDCA)+ka_GCDCA*modf_p_GCDCA*Aup_GCDCA+VmaxASBT_GCDCA
*CIl_GCDCA /(KmASBT_GCDCA+
CIl_GCDCA)+ka_GCDCA*modf_p_GCDCA*Acol_GCDCA+kam_all*AL_DCA*Gdosel_GCDCA+Acol_DC
A*sens_all*0.05*Gdosel_GCDCA ; # change in amount in liver, umol

d/dt(Aup_GCDCA)= ge*dose_GCDCA +VmaxBSEP_GCDCA *CVL_GCDCA /(KmBSEP_GCDCA +
CVL_GCDCA)*QIb_all-ka_GCDCA*modf_p_GCDCA*Aup_GCDCA-ktj*Aup_GCDCA; # change in amount
in upper intestinal lumen, umol

d/dt(Alow_GCDCA)=ktj*Aup_GCDCA-kti*Alow_GCDCA-VmaxASBT_GCDCA *CIl_GCDCA
/(KmASBT_GCDCA+ CIl_GCDCA); # change in amount in ileum, umol

d/dt(Acol_GCDCA)=kti*Alow_GCDCA-ka_GCDCA*modf_p_GCDCA*Acol_GCDCA-
kdec_all*modf_fdec_GCDCA*Acol_GCDCA;# change in amount in colon, umol

d/dt(AI_GCDCA)=QI*(CB_GCDCA -CVI_GCDCA);       # change in amount in intestinal tissue, umol
```

d/dt(AF_GCDCA)=QF*(CB_GCDCA -CVF_GCDCA) ; # change in amount in fat tissue, umol

d/dt(AR_GCDCA)=QR*(CB_GCDCA -CVR_GCDCA) ; # change in amount in rapidly perfused tissue, umol

d/dt(AS_GCDCA)= QS*(CB_GCDCA -CVS_GCDCA); # change in amount in slowly perfused tissue, umol

d/dt(AB_GCDCA)=(QF*CVF_GCDCA + QL*CVL_GCDCA + QS*CVS_GCDCA + QR *CVR_GCDCA +QI *CVI_GCDCA -(QF+QL+QS+QR+QI) *CB_GCDCA); # change in amount in blood, umol

conc_plasma_GCDCA=(CB_GCDCA/BP_all)+CBfs_GCDCA*sens_all; #concentration in **plasma**, umol

GCA submodel

CL_GCA=AL_GCA/VL; # concentration in the liver (umol/L)

CVL_GCA=CL_GCA/(PL_GCA*BP_all); # concentration leaving the liver(umol/L)

CIl_GCA=Alow_GCA/VIl_all; # concentration leaving the ileum (umol/L)

CI_GCA=AI_GCA/VI; # concentration in the intestinal tissue (umol/L)

CVI_GCA=CI_GCA/(PG_GCA*BP_all); # concentration leaving the intestinal tissue(umol/L)

CF_GCA=AF_GCA/VF; # concentration in the fat tissue (umol/L)

CVF_GCA=CF_GCA/(PF_GCA*BP_all); # concentration leaving the fat tissue (umol/L)

CR_GCA=AR_GCA/VR; # concentration in the rapidly perfused tissue (umol/L)

CVR_GCA=CR_GCA/(PR_GCA*BP_all); # concentration leaving the rapidly perfused tissue (umol/L)

CS_GCA=AS_GCA/VS; # concentration in the slowly perfused tissue (umol/L)

CVS_GCA=CS_GCA/(PS_GCA*BP_all); # concentration leaving slowly perfused tissue (umol/L)

CB_GCA=AB_GCA/VB; # concentration in blood excluding portal vein (umol/L)

d/dt(dose_GCA) = -ge*dose_GCA+VmaxBSEP_GCA*CVL_GCA/(KmBSEP_GCA + CVL_GCA)*QGb_all ; # change in amount in the gall bladder, umol

d/dt(AL_GCA)=QL*(CB_GCA -CVL_GCA) -VmaxBSEP_GCA *CVL_GCA /(KmBSEP_GCA + CVL_GCA) +ka_GCA*modf_p_GCA*Aup_GCA+VmaxASBT_GCA *CIl_GCA /(KmASBT_GCA+ CIl_GCA)+ka_GCA*modf_p_GCDCA*Acol_GCA+kam_all*AL_DCA*Gdosel_GCA+Acol_DCA*sens_all*0.05*Gdosel_GCA ;

change in amount in liver, umol

d/dt(Aup_GCA)= ge*dose_GCA +VmaxBSEP_GCA *CVL_GCA /(KmBSEP_GCA + CVL_GCA)*QIb_all-ka_GCA*modf_p_GCA*Aup_GCA-ktj*Aup_GCA; # amount in upper intestinal lumen, umol

d/dt(Alow_GCA)=ktj*Aup_GCA-kti*Alow_GCA-VmaxASBT_GCA*CIl_GCA /(KmASBT_GCA + CIl_GCA); # amount in ileum, umol

d/dt(Acol_GCA)=kti*Alow_GCA-ka_GCA*modf_p_GCA*Acol_GCA-kdec_all*modf_fdec_GCA*Acol_GCA;# change in amount in colon, umol

d/dt(AI_GCA)=QI*(CB_GCA -CVI_GCA); # change in amount in intestinal tissue, umol

d/dt(AF_GCA)=QF*(CB_GCA -CVF_GCA) ; # change in amount in fat tissue, umol

d/dt(AR_GCA)=QR*(CB_GCA -CVR_GCA) ; # change in amount in rapidly perfused tissue, umol

d/dt(AS_GCA)= QS*(CB_GCA -CVS_GCA); # change in amount in slowly perfused tissue, umol

d/dt(AB_GCA)=(QF*CVF_GCA + QL*CVL_GCA + QS*CVS_GCA + QR *CVR_GCA +QI *CVI_GCA - (QF+QL+QS+QR+QI) *CB_GCA); # change in amount in blood, umol

conc_plasma_GCA=(CB_GCA/BP_all)+CBfs_GCA*sens_all; #concentration in **plasma**, umol

GDCA submodel

CL_GDCA=AL_GDCA/VL;	# concentration in the liver (umol/L)
CVL_GDCA=CL_GDCA/(PL_GDCA*BP_all);	# concentration leaving the liver(umol/L)
CIl_GDCA=Alow_GDCA/VIl_all;	# concentration leaving the ileum (umol/L)
CI_GDCA=AI_GDCA/VI;	# concentration in the intestinal tissue (umol/L)
CVI_GDCA=CI_GDCA/(PG_GDCA*BP_all);	# concentration leaving the intestinal tissue(umol/L)
CF_GDCA=AF_GDCA/VF;	# concentration in the fat tissue (umol/L)
CVF_GDCA=CF_GDCA/(PF_GDCA*BP_all);	# concentration leaving the fat tissue (umol/L)
CR_GDCA=AR_GDCA/VR;	# concentration in the rapidly perfused tissue (umol/L)
CVR_GDCA=CR_GDCA/(PR_GDCA*BP_all);	# concentration leaving the rapidly perfused tissue (umol/L)
CS_GDCA=AS_GDCA/VS;	# concentration in the slowly perfused tissue (umol/L)
CVS_GDCA=CS_GDCA/(PS_GDCA*BP_all);	# concentration leaving slowly perfused tissue (umol/L)
CB_GDCA=AB_GDCA/VB;	# concentration in blood excluding portal vein (umol/L)

d/dt(dose_GDCA) = -ge*dose_GDCA+VmaxBSEP_GDCA*CVL_GDCA/(KmBSEP_GDCA + CVL_GDCA)*QGb_all ; # change in amount in the gall bladder, umol

d/dt(AL_GDCA)=QL*(CB_GDCA -CVL_GDCA) -VmaxBSEP_GDCA *CVL_GDCA /(KmBSEP_GDCA + CVL_GDCA) +ka_GDCA*modf_p_GDCA*Aup_GDCA+VmaxASBT_GDCA *CIl_GDCA /(KmASBT_GDCA/modf_Km_GDCA + CIl_GDCA)+ka_GDCA*modf_p_GDCA*Acol_GDCA+kam_all*AL_DCA*Gdosel_GDCA+Acol_DCA*sens_all*0.05*Gdosel_GDCA; # change in amount in liver, umol

d/dt(Aup_GDCA)= ge*dose_GDCA +VmaxBSEP_GDCA *CVL_GDCA /(KmBSEP_GDCA + CVL_GDCA)*QIb_all-ka_GDCA*modf_p_GDCA*Aup_GDCA-ktj*Aup_GDCA; # change in amount in upper intestinal lumen, umol

d/dt(Alow_GDCA)=ktj*Aup_GDCA-kti*Alow_GDCA-VmaxASBT_GDCA*CIl_GDCA /(KmASBT_GDCA/modf_Km_GDCA + CIl_GDCA); # a change in mount in ileum, umol

d/dt(Acol_GDCA)=kti*Alow_GDCA-ka_GDCA*modf_p_GDCA*Acol_GDCA-kdec_all*Acol_GDCA;# change in amount in colon, umol

d/dt(AI_GDCA)=QI*(CB_GDCA -CVI_GDCA); # amount in intestinal tissue, umol

d/dt(AF_GDCA)=QF*(CB_GDCA -CVF_GDCA) ; #amount in fat tissue, umol

d/dt(AR_GDCA)=QR*(CB_GDCA -CVR_GDCA) ; #amount in rapidly perfused tissue, umol

d/dt(AS_GDCA)= QS*(CB_GDCA -CVS_GDCA); #amount in slowly perfused tissue, umol

d/dt(AB_GDCA)=(QF*CVF_GDCA + QL*CVL_GDCA + QS*CVS_GDCA + QR *CVR_GDCA +QI *CVI_GDCA - (QF+QL+QS+QR+QI) *CB_GDCA); #amount in blood, umol

conc_plasma_GDCA=(CB_GDCA/BP_all)+CBfs_GDCA*sens_all; #concentration in **plasma**, umol

DCA submodel

CL_DCA=AL_DCA/VL; # concentration in the liver (umol/L)

CVL_DCA=CL_DCA/(PL_DCA*BP_all); # concentration leaving the liver (umol/L)

CI_DCA=AI_DCA/VI; # concentration in the intestinal tissue (umol/L)

CVI_DCA=CI_DCA/(PG_DCA*BP_all); # concentration leaving the intestinal tissue(umol/L)

CF_DCA=AF_DCA/VF; # concentration in the fat tissue (umol/L)

CVF_DCA=CF_DCA/(PF_DCA*BP_all); # concentration leaving the fat tissue (umol/L)

CR_DCA=AR_DCA/VR; # concentration in the rapidly perfused tissue (umol/L)

CVR_DCA=CR_DCA/(PR_DCA*BP_all); # concentration leaving the rapidly perfused tissue (umol/L)

CS_DCA=AS_DCA/VS; # concentration in the slowly perfused tissue (umol/L)

CVS_DCA=CS_DCA/(PS_DCA*BP_all); # concentration leaving slowly perfused tissue (umol/L)

CB_DCA=AB_DCA/VB; # concentration in blood excluding portal vein (umol/L)

d/dt(AL_DCA)=QL*(CB_DCA -CVL_DCA) +ka_DCA*modf_Vmax_DCA*modf_p_DCA*Acol_DCA-kam_all*AL_DCA ;

amount in liver, umol

d/dt(Acol_DCA)=kdec_all*modf_fdec_GCDCA*Acol_GCDCA+kdec_all*modf_fdec_GCA*Acol_GCA+kdec

```
_all*Acol_GDCA-ka_DCA*modf_Vmax_DCA*modf_p_DCA*Acol_DCA-0.05*Acol_DCA*sens_all; #
amount in colon, umol

  d/dt(AI_DCA)=QI*(CB_DCA-CVI_DCA);          # change in amount in intestinal tissue, umol

  d/dt(AF_DCA)=QF*(CB_DCA -CVF_DCA) ;        # change in amount in fat tissue, umol

  d/dt(AR_DCA)=QR*(CB_DCA -CVR_DCA) ;        # change in amount in rapidly perfused tissue, umol

  d/dt(AS_DCA)= QS*(CB_DCA -CVS_DCA);        # change in amount in slowly perfused tissue, umol

  d/dt(AB_DCA)=(QF*CVF_DCA +  QL*CVL_DCA  + QS*CVS_DCA + QR *CVR_DCA +QI *CVI_DCA -
(QF+QL+QS+QR+QI) *CB_DCA);                   # change in amount in blood, umol


  conc_plasma_DCA=(CB_DCA/BP_all)+CBfs_DCA*sens_all; #concentration in **plasma**, umol

})


## Solve the model

qd$ctime <- qd$time

print(PBK)


solve.pbk<- as_tibble(solve(PBK, params=parameters, events = qd, inits=inits, cores=4,
covs_interpolation="nocb"))


long <- melt(setDT(solve.pbk), id.vars=1, measure.vars=7:length(solve.pbk), variable.name = "cmp") %>%

  mutate(BA=case_when(str_detect(cmp, "GCDCA") ~ "GCDCA",

                      str_detect(cmp, "GDCA") ~ "GDCA",

                      str_detect(cmp, "GCA") ~ "GCA",

                      str_detect(cmp, "DCA") ~ "DCA"))

long$cmp <- substr(long$cmp, 0,3)


## Mass balance calculations

solve.pbk %>%
mutate(OUT=dose_GCDCA+AL_GCDCA+Aup_GCDCA+Alow_GCDCA+Acol_GCDCA+AI_GCDCA+AF_
GCDCA+AR_GCDCA+AS_GCDCA+AB_GCDCA+dose_GCA+AL_GCA+Aup_GCA+Alow_GCA
+Acol_GCA+ AI_GCA+AF_GCA +AR_GCA+AS_GCA+AB_GCA+
dose_GDCA+AL_GDCA+Aup_GDCA+Alow_GDCA+
Acol_GDCA+AI_GDCA+AF_GDCA+AR_GDCA+AS_GDCA+AB_GDCA+AL_DCA+Acol_DCA+AI_DCA
+ AF_DCA+AR_DCA+AS_DCA+AB_DCA,
```

```
            MB=Gdose$GCDCA+Gdose$GDCA+Gdose$GCA-OUT) %>%
  ggplot() +
  geom_line(aes(x=time, y=MB))
```

Chapter 6.

General discussion

6.1 Overview of the main findings

The gut microbiota is important for host health and wellbeing. The microbial metabolites including bile acids, short chain fatty acids and amino acids are crucial molecules which help sustain host physiological homeostasis (Herrema and Niess 2020; Lam et al. 2016; Wu et al. 2018). In previous studies, non-systemically available antibiotics which induced targeted changes in microbiome communities were administered in 28 day animal studies to rats in order to study the consequences for host metabolome patterns (Behr et al. 2019; de Bruijn et al. 2020). Taxonomic profiling and functional metabolites were measured in order to understand the composition and function of the gut microbiota including the consequences of microbial metabolism for host metabolic patterns (Behr et al. 2018a; Mancabelli et al. 2017; Murali et al. 2023; Song et al. 2019).

This thesis focused on the role of intestinal microbiota in bile acids homeostasis and the effects of antibiotics on intestinal microbiota and bile acid metabolism and reuptake from the intestinal tract applying new approach methodologies (NAMs). Gut microbiota composition changes induced by the selected antibiotics in *in vitro* anaerobic fecal incubations were characterized by 16S rRNA analysis profiling. Intestinal deconjugation and further metabolism of conjugated bile acids was investigated to study the functional capacity of gut microbiota via LC-MS analysis of the bile acids present in these anaerobic fecal incubation samples. Bile acid reuptake across the intestinal cell layer was studied using an *in vitro* Caco-2 cell transwell model. The results obtained provided insight in the modes of action underlying the effects of the antibiotics on the fecal and blood bile acid patterns in the host (Zhang et al. 2023). Moreover, an *in silico* approach, consisting of physiologically based kinetic (PBK) modeling, was used to predict the effects of the antibiotic tobramycin on *in vivo* plasma bile acid concentrations in human subjects. Altogether, these *in vitro* and *in silico* studies of the current thesis illustrate the feasibility of using new approach methodologies (NAMs) to study the effects of xenobiotics on intestinal microbiota and related bile acid metabolism and on intestinal reuptake and the consequences of such effects for bile acid plasma levels in the host.

In **Chapter 2**, taxonomic profiling of rat untreated fecal samples following *in vitro* incubation with the selected antibiotics (colistin sulfate, tobramycin, meropenem trihydrate and doripenem hydrate) under anaerobic conditions was performed via 16S rRNA analysis. The results obtained showed that of the tested antibiotics especially tobramycin influenced the gut microbiota composition. Also the consequences of the antibiotic treatments for bile acid patterns were characterized in these anaerobic fecal incubations with the antibiotics. The *in vivo* study reported increased levels of fecal conjugated bile acids upon tobramycin treatment while colistin sulfate treatment showed limited effects (Murali et al. 2023). These effects on fecal bile acid levels could be due to an effect of the antibiotic on bile acid metabolism by the intestinal microbiota and/or on the process of bile acid reabsorption. In the *in vitro* anaerobic fecal incubation model the effects of the two antibiotics (colistin sulfate and tobramycin) on

the deconjugation of a series of conjugated bile acids (taurocholic acid, taurochenodeoxycholic acid, glycocholic acid and glycochenodeoxycholic acid) were quantified. At the concentrations tested, calculated to mimic the dose levels used in the reported *in vivo* studies (McConnell et al. 2008) tobramycin was shown to significantly inhibit this deconjugation while colistin sulfate did not have a significant affect. The effects observed *in vitro* were in line with what was reported *in vivo* where tobramycin also had more pronounced effects on bile acid profiles, increasing fecal levels of these conjugated bile acids due to inhibition of bile acid deconjugation. These increases in fecal bile acid levels could however also in part be due to an inhibition of the intestinal bile acid reuptake. To study second mode of action potentially underlying increased levels of fecal bile acids, an *in vitro* Caco-2 transwell cell model was applied to study the impact of tobramycin on the transport of taurocholic acid (TCA), the bile acid most significantly modified upon tobramycin exposure *in vivo* in the 28 day rats studies. The results obtained revealed that tobramycin significantly inhibited the intestinal reuptake of TCA. This inhibition of intestinal reuptake of TCA may provide an additional mode of action underlying the increased fecal levels of this bile acid upon treatment of the rats with tobramycin. Thus, the effects of tobramycin in the *in vitro* model systems provided novel and complementary understanding, explaining the effects of tobramycin on intestinal microbiota and fecal bile acids in the *in vivo* 28 day rat study reported in literature (Murali et al. 2023). In summary, the results of this chapter provided a proof-of-principle on how to use *in vitro* models to elucidate *in vivo* bile acid changes, and understand the mode(s) of action behind the effects of antibiotics, in this case tobramycin, on bile acid profiles.

In **Chapter 3**, it was studied whether the effects on intestinal bile acid deconjugation and transport can be quantified via the *in vitro* model systems by using mixtures of bile acids instead of individual bile acids, which provides a more realistic situation. In order to fulfill this aim, the deconjugation of a mixture of selected bile acids (taurocholic acid, taurochenodeoxycholic acid, glycocholic acid and glycochenodeoxycholic acid) were incubated anaerobically with rat or human fecal samples with or without tobramycin. Additionally, the effect of tobramycin on the transport of the individual bile acids or a mixture of bile acids across a Caco-2 cell layer was studied. The results showed that it is feasible to use a mixture of bile acids to study the impact of tobramycin on the reduction of bile acid deconjugation and of intestinal transport via *in vitro* model systems, thereby eliminating the necessity to study the effects for each individual bile acid in a separate experiment. The isolated and combined bile acid experiments showed subtle differences pointing out mutual competitive interactions between the bile acids and indicating that the use of bile acid mixtures is preferred over use of single bile acid given that also *in vivo* bile acids occur in mixtures.

In **Chapter 4**, the aim was to study the impact of a series of other selected antibiotics (lincomycin, streptomycin, vancomycin), and tobramycin for comparison, on the intestinal

reuptake of conjugated bile acids (TCA, TCDCA, GCA and GCDCA) using a Caco-2 cell layer *in vitro* transwell model system. The results demonstrated that both pre-exposure and co-exposure of the cells to the antibiotics and the bile acids, affected bile acid transport, to an extent that depended on the antibiotic, its concentration and the tested type of conjugated bile acid. Tobramycin at a concentration in line with *in vivo* dose levels at which this antibiotic caused effects on bile acid homeostasis *in vivo*, was able to reduce bile acid transport after pre-exposure of the cells. This observation is in line with its effect on protein synthesis and thus on the expression of bile acid transporters at the ribosome level. In addition, upon co-exposure of the cells to all four antibiotics and the bile acids, a significantly reduction on the bile acid transport was detected, especially on the transport of taurochenodeoxycholic acid (TCDCA) and glycochenodeoxycholic acid (GCDCA) with a potency of the antibiotics that decreased in the order vancomycin > tobramycin = streptomycin > lincomycin. The effects shown in this chapter likely contribute to the effects of these antibiotics on *in vivo* bile acid changes, and show the feasibility of using a new approach methodology (NAM) to study the effects of the antibiotics on intestinal bile acid reuptake.

In **Chapter 5**, a physiologically based kinetic (PBK) model that included microbial deconjugation and intestinal reuptake via the apical sodium dependent bile acid transporter (ASBT) was applied to predict the systemic plasma bile acid concentrations in human upon treatment with the antibiotic tobramycin. The PBK simulation results predicted that exposure to a dose level similar to the high dose level used in the rat *in vivo* studies of 1000 mg/kg bw/d tobramycin would reduce human plasma C_{max} levels of GCA, GCDCA, GDCA and the combined unconjugated BAs (uBAs) by 36.1 %, 18.7 %, 14.5 % and 33.8 % respectively. At this high dose level the effect of tobramycin on intestinal microbial deconjugation appeared not to affect plasma C_{max} levels, which can be explained by the fact that this microbial deconjugation happens mainly in the colon where bile acid reuptake is limited. The predictions for the effect of tobramycin on human plasma bile acid levels were comparable to the actual plasma bile acid levels reported in rats exposed to tobramycin at the same dose level of 1000 mg/kg bw. Altogether, the PBK model appeared to provide a reliable tool to evaluate the effect of oral exposure to tobramycin on host bile acid homeostasis via effects on intestinal bile acid deconjugation and reuptake.

In the following sections, the results of the present thesis and future perspectives are discussed in some more detail.

6.2 General discussion and future perspectives

In this project, our aim was to use new approach methodologies (NAMs) to study the effects of antibiotics on gut microbiota and bile acid metabolism thereby contributing to the 3Rs (reduction, refinement, replacement) of experimental animal studies. In the next sections the following aspects related to the NAMs and results of the present thesis are discussed in more detail:

- Use of anaerobic fecal incubations to mimic intestinal microbiota metabolism.

- Caco-2 cell transwell model to measure *in vitro* bile acid intestinal transport

- *In silico* PBK model to predict (effects on) plasma bile acid concentrations.

- Interspecies and interindividual differences.

- Consequences of (effects on) bile acid homeostasis for human health.

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

6.2.1 Use of anaerobic fecal incubations to mimic intestinal microbiota metabolism.

The gut microbiota and its host have mutual interactions thereby resulting in production of intestinal metabolites that play a key role in sustaining health and wellbeing of the host (Hosokawa et al. 2006; Nicholson et al. 2012). The gut microbiota includes bacteria, viruses, fungi and parasites (Ferranti et al. 2014). Furthermore, the dominant gut microbiota are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobioa*, and the majority of the gut microbiota are obligate or facultative anaerobes (Rinninella et al. 2019; von Martels et al. 2017). The host and its gut microbiota work together to form various kinds of small molecules by metabolism of dietary constituents including also xenobiotics. Many of these intestinal metabolites are important signaling molecules exchanging information between intestine and other organs of the host. These small molecules, including short chain fatty acids (SCFAs), bile acids, choline metabolites, phenolics, vitamins, polyamines, lipids and so on, contribute to changes in cell proliferation and function, can have anti-inflammatory effects, facilitate the absorption of dietary fats and lipid soluble vitamins, modulate lipid metabolism and glucose homeostasis, provide various enzyme functions, can provide dysbiosis biomarkers, and can be a target for the treatment of metabolic diseases (Cani et al. 2007; Halver 2003; Lord and Bralley 2008; Nicholson et al. 2012; Tan et al. 2014; Wang et al. 2011).

To fully define the features of the gut microbiota remains a challenge because many bacteria are adjusted to the anaerobic environment in the gut with many species being unable to survive outside the intestine (Eckburg et al. 2005; Thompson-Chagoyán et al. 2007). In recent years, methods for anaerobic isolation and incubation and new technologies based on the extraction of DNA and amplification of the 16S rRNA have allowed identification and quantification of the components of the gut microbiota directly from collected stools (Mizrahi-Man et al. 2013; Poretsky et al. 2014; Rinninella et al. 2019). For example, the current thesis used 16S rRNA analysis to measure fecal microbiota from rat fecal samples and found that the dominant microbial families include *Verrucomicrobiaceae*, followed by *Porphyromonadaceae* and *Erysipelotrichaceae*, *Lactobacillaceae*, *Ruminococcaceae* and *Lachnospiraceae* (**Chapter 2**). Since the gut microbiota play an important an role in host

metabolism, identification of specific microbiota and illustration of metabolic pathways especially those associated with metabolism of dietary constituents and xenobiotics are helpful to understand the role and influence of the gut microbiota on host metabolism and health. To this end, *in vivo* animal studies are widely used to study composition and function of the gut microbiota via taxonomic profiling from fecal samples, and via metabolomics to characterize specific metabolites such as amino acids, bile acids, lipids and SCFAs based on LC-MS/MS analysis of different matrices (plasma, feces, gut tissues, urine) (Behr et al. 2018b). Although these *in vivo* studies provided insight in the role of the gut microbiota and its metabolites, they are time-consuming, expensive and also bring ethical issues, while it also remains to be elucidated to what extent studies in experimental animals reflect the human situation.

In recent years, a paradigm shift has been made, focusing on developing novel alternative *in vitro* and *in silico* approaches also called new approach methodologies (NAMs) in the field of toxicology and risk and safety assessment. Here such methods were applied also for studying gut microbiota and its metabolites. These approaches included isolation of strains capable of metabolizing dietary components in order to provide insight into the potential microbiota involved in these metabolic conversions *in vivo* (Matthies et al. 2009). This was based on measurement of the activities of specific bacterial strains and/or microbial enzymes isolated from fecal and colonic samples and involved in metabolism of food constituents and xenobiotics (Dabek et al. 2008), or based on investigation of components and functionality of gut microbiota through omics approaches (combination of metagenomics, metatranscriptomics, metaproteomics, metabolomics) (Gosalbes et al. 2011; Roume et al. 2013; Wang et al. 2015; Xiong et al. 2015), and use of mathematical modelling to mimic the gut ecosystem (Manor et al. 2014; Rowland et al. 2018).

In vitro fermentation models are powerful tools to study the human gut microbiota under highly controlled conditions, which allow dynamic observations of different colon regions over time. Generally, the *in vitro* fermentation models include especially two types of models being the *in vitro* batch fermentation model and the *in vitro* continuous fermentation model (Pham and Mohajeri 2018). The *in vitro* batch fermentation model is the simplest and most frequently used model because it is fast, cheap, easy to handle and reproducible. The limitations of this model are also obvious, since because there is no control of pH or nutrient supply it can only perform for relatively shorter periods of time. *In vitro* continuous fermentation models are models such as the Reading model, the PolyFermS model, SHIME models and so on. The ‘Simulator of the Human Intestinal Microbial Ecosystem (SHIME) model can mimic the complete intestinal tract and sustain long term incubations during which it can control pH and add nutrients. The SHIME model has five compartments including the stomach, duodenum, small intestine (jejunum and ileum), ascending, transverse and descending colon (Molly et al. 1994; Van de Wiele et al. 2015). However, the SHIME model is expensive, low-throughput and expert training is needed to handle it. Most *in vitro*

fermentation models incubate fecal samples due to the fact that these samples can be obtained in a non-invasive manner and without causing ethical issues (Pham and Mohajeri 2018). It is also of importance to mention that the selection of the fermentation media has to be considered and might be chosen such as to closely mimic the gastrointestinal conditions. In this thesis, a simple and fast *in vitro* anaerobic fecal incubation model was used to study the intestinal microbiota metabolism and the effects of antibiotics on the gut microbiota and related bile acid profiles (**Chapter 2 and 3**). PBS buffer was selected as incubation medium because it provided lower detection limits for the bile acids while it was considered to also be close to the *in vivo* intestinal situation without supplying additional nutrients. The results obtained upon 24 h anaerobic rat fecal fermentation via 16S rRNA sequencing revealed that, in line with the *in vivo* data, of all antibiotics tested tobramycin affected microbial communities to the largest extent.

It was also reported (Murali et al. 2023) that tobramycin affected the fecal and plasma metabolome substantially resulting especially in an increase in the fecal levels of taurocholic acid (TCA) in the *in vivo* study. Moreover, it is of interest to mention that in fecal samples as such no conjugated bile acids were detected which implied that to study intestinal microbiota mediated deconjugation extra external conjugated bile acids had to be added to the *in vitro* incubations, and were subsequently shown to be efficiently deconjugated with time in the anaerobic fecal incubations (**Chapter 2**). These results corroborated that this deconjugation of conjugated bile acids by the fecal microbiota is highly efficient. It is of interest to note that the total mass recovery decreased from 94.4 % to 56.8 % at the end of the fecal incubations, indicating that CA and CDCA formed upon deconjugation were further degraded into other metabolites which were not quantified (**Chapter 3**). The reason underlying this time dependent decrease in the bile acid recovery may be attributed to bile acid metabolism pathways enabling their use as energy source (Ma and Patti 2014). Such a result is also in line with the increased levels of *Firmicutes* detected in the fecal samples upon tobramycin treatment and the fact that some studies reported that increased *Firmicutes* levels caused increased energy production (Turnbaugh et al. 2006). In **Chapter 3** it was shown that this deconjugation of bile acids can also be adequately detected when using a mixture of bile acids in anaerobic rat or human fecal incubations thus eliminating the need to characterize the effects for each individual bile acid in separate incubations.

In the anaerobic fermentation model, the medium used was PBS without supplemented nutrients which implicated that incubations should be limited to short periods of time of up to 24 h at most. Such anaerobic fecal incubations need less time and space, are easy to handle, and cheaper while many samples can be incubated at the same time. The drawbacks of the model are also obvious; it is a static culture model, which implies that only short-term fermentation studies are possible with limitations in microbiological growth conditions. Such anaerobic fecal batch incubations are particularly useful for studying the metabolite profile of dietary compounds resulting from their conversion by gut microbiota as these models are

generally convenient and inexpensive, allowing large numbers of substrates or fecal samples to be measured quickly (Gumienna et al. 2011; Macfarlane and Macfarlane 2007). Application of the *in vitro* anaerobic fecal incubations model can also be used to study microbiota mediated conversion of other constituents than bile acids such as for example nutrients, SCFAs, or others. For example, an anaerobic human fecal incubation model was used to study the microbiota mediated degradation of fructoselysine to produce the short chain fatty acid butyrate (Pham and Mohajeri 2018; van Dongen et al. 2021). In another example, polyphenols producing short chain fatty acids by the fecal microbiota anaerobically was investigated (Parkar et al. 2013). In the future *in vitro* anaerobic fecal incubation can be applied to study the conversion or formation of other microbial metabolites. Future *in vitro* fermentation models can also include "diseased" microbiota (Payne et al. 2012). Studying "diseased "microbiota may help investigating the functional role of gut microbiota in inflammatory diseases such as for example Crohn's disease.

6.2.2 Caco-2 cell transwell model to measure *in vitro* bile acid intestinal transport

Enterohepatic circulation of bile acids is a highly efficient pathway with only a minor percentage of the bile acids ending up in feces (Dowling 1972; Hofmann 1977; Hofmann 2009). As a result, a limited effect on the intestinal reabsorption of bile acids can be expected to substantially increase fecal levels of bile acids. In the present thesis the *in vitro* Caco-2 cell transwell model was used to study this bile acid reuptake. The Caco-2 cell line is a cell line derived from human epithelial cells which were taken from a human colon carcinoma. The cells are able to differentiate into a cell monolayer with many properties of the absorptive enterocytes in the small intestine such as for example the presence of brush border enzymes (peptidases, alkaline phosphatase, disaccharidases) (Ferruzza et al. 2012; Lea 2015). The gastrointestinal tract is particularly relevant for the absorption, transport and biotransformation of xenobiotics due to the extensive area of exposure to orally ingested drugs, food additives and contaminants. It has been reported that specific intestinal carriers for sugars, amino acids, di- and tripeptides, vitamins, bile acids, micro-nutrients are also functionally present in Caco-2 cells (Hidalgo and Li 1996). The Caco-2 cells were cultured on permeable filter supports (transwell) that allow free access of ions and nutrients to the two sides of the cell layer which has been reported to improve morphological and functional differentiation, providing a more physiologically relevant model for intestinal transport and toxicity studies (Sambuy et al. 2005a). This system is enterocyte-like, well characterized, easily maintained, and forming adequate cell layers. In addition, transporters and enzymes in Caco-2 cells can be easily modified, resulting in cells with increased levels of for example P-gp (Shirasaka et al. 2006), or CYP3A4 (Thummel et al. 2001). *In vitro* Caco-2 cell transwell models are widely used to study the transport and metabolism of drugs/chemicals across the intestine (Sambuy et al. 2005b; Sun et al. 2008). Bile acid transport depends on the apical sodium- dependent bile acid transporter (ASBT), the ileal bile acid binding protein (IBABP) and the organic solute transporter (ASBT) which are present and active in the Caco-2 cells

(Alrefai and Gill 2007; Wang et al. 2022). The Caco-2 transwell model has also been used before to study intestinal bile acid transport such as the transport of TCA (Hidalgo and Borchardt 1990), and to invesitgate the effect of other compounds on bile acid transport such as the effects of metformin on GCA transport (Carter et al. 2002), and the effects of the mycotoxcin deoxynvalenol (DON) on transport of conjugated bile acids including TCA, TCDCA, GCA and GCDCA (Wang et al. 2022). Also in the present thesis the Caco-2 transwell model was the method of choice to study intestinal bile acid transport and the results of **Chapter 4** show that in the Caco-2 transwell model the conjugated bile acids were transported at 37℃ while transport no longer occurred at 4 ℃, corroborating the role of active transport. Both individual bile acid transport and transport of a mixture of bile acids e were studied in the Caco-2 transwell model, revealing subtle differences between single or combined bile acid transport experiments pointing at mutual competitive interactions and indicating that use of bile acid mixtures may be preferred over single bile acid because *in vivo* bile acids also occur in mixtures (**Chapter 2,3,4 and 5**).

However, Caco-2 cell models only represent enterocytes, and they do not mimic the complex interactions with other cells existing in the human intestinal epithelium (Leonard et al. 2010). Co-cultures of Caco-2 cells with other cell types have been developed to even better represent the intestinal epithelium (Antunes et al. 2013). For example, co-culture of Caco-2 cells with mucus-producing HT29-MTX cells is a more accurate cell model in which production of mucus makes the model more similar to the human intestinal mucosa (Behrens et al. 2001), thus resembling the small intestinal barrier in a better way (Araújo and Sarmento 2013). In addition, 3D cell culture models may prove more dynamic and variable than 2D cell culture models, for example by including specific matrices and/or scaffolds for obtaining "closer to *in vivo*" cultures and mimicking the biochemical microenvironment of the intestine in a better way (Huh et al. 2011; Ravi et al. 2015). Nowadays, *in vitro* 3D intestinal model development focuses also on "organ-on-a- chip", in this case "gut-on-a-chip". The advantage of organ-on-a-chip is that such models may better represent the whole organ physiology thereby providing culture conditions more close to the real *in vivo* situation (Huh et al. 2011). The organ-on-a-chip models can be extended to body-on-a-chip models that integrate various tissues and enable investigation of the crosstalk of different organs enabling determination of pharmaceutical features of chemicals at a higher functional level than previous cell models. Future studies on enterohepatic circulation of bile acids could also consider use of such 3D intestinal body-on-a-chip models, which will reveal whether similar results as now obtained in the Caco-2 transwell models can be obtained. This more advanced intestinal model may prove of value especially for studies on bile acid related intestinal diseases such as intestinal inflammation and intestinal cancer (Zhang and Qiao 2023). In addition, gut-on-a-chip can be used to study kinetics of bile acids, and the effects of drugs or other xenobiotics on these processes, as now studied in the Caco-2 system.

6.2.3 *In silico* PBK model to predict (effects on) the plasma bile acid concentrations

PBK models can be used for the prediction of effects on metabolite concentrations in the whole body in a NAM strategy. Such a PBK model can include all organs of interest for the toxicokinetics and toxicodynamics of a compound or compounds of interest. Inclusion of a gut microbiota compartment in PBK models is feasible and has been implemented in the PBK model for prediction of human plasma bile acid levels in the present thesis (**Chapter 5**). Previously a PBK model that included metabolism by the intestinal microbiota was developed to predict gut microbial metabolism of the isoflavone daidzein to its metabolite S-equol and the consequences of this microbial metabolism for plasma concentrations of daidzein and S-equol (Wang et al. 2020). Other examples are the PBK model to predict systemic concentrations of zearaleone taking its conversion to alpha-zearalenone by intestinal microbiota into account (Mendez-Catala et al. 2021) and models that predict plasma levels of pyrrolizidine alkaloids taking the intestinal microbial reduction of pyrrolizidine N-oxides to the corresponding pyrrolizidine alkaloids into account (Widjaja et al. 2022). In addition, mathematical models of bile acid metabolism contributed to bile acid research. The early models described the enterohepatic circulation of CA, CDCA and DCA and especially the gallbladder contraction and emptying in great detail (Cravetto et al. 1988; Hofmann et al. 1983; Molino et al. 1986). More recent models included the kinetics of LCA, UDCA or the bile acid analog obeticholic acid in a similar design (Edwards et al. 2016; Woodhead et al. 2014; Zuo et al. 2016). Additionally, a PBK model including farnesoid X-mediated autoregulation of bile acid synthesis and selective transport mechanisms were defined (Voronova et al. 2020) and a PBK model describing systemic bile acid metabolism and detailed intestinal transit of bile acids (Sips et al. 2018). In the current thesis, a PBK model was used to predict the effects of tobramycin on the active transporter for intestinal reuptake of bile acids, assumed to be ASBT, thereby affecting bile acid plasma profiles. A major advantage of our PBK model is that the majority of input parameters is derived *using in vitro* or *in silico* methods. This PBK model also provides a first proof-of-principle for the incorporation of active intestinal bile acid uptake in a bile acid PBK model. The PBK model simulations predicted that exposure to tobramycin at the dose level similar to what was used in the reported 28 day rat study (Murali et al. 2023) would reduce human plasma C_{max} levels of GCA, GCDCA, GDCA and uBA by 36.1 %, 18.7 %, 14.5 % and 33.8 %, especially via an effect on ASBT mediated intestinal reuptake with for GDCA and GCDCA the effect being less pronounced when basal bile acid levels are lower (**Chapter 5**). For future studies it remains to be investigated what would happen at lower dose levels of tobramycin including therapeutic dose levels which are at a maximum of 5 mg/kg bw/day substantially lower than the 1000 mg/kg bw used in the animal and human PBK modelling study in **Chapter 5**. This would require studying the effects of lower concentrations of tobramycin in the Caco-2 transport experiments to determine the expected reduction in ASBT mediated intestinal reuptake and can thus be also achieved using new approach methodologies without a need for human testing. Thus, the use of PBK modeling can save time and efforts and avoid

unnecessary animal or human testing not only in drug clinical investigations, but also in studying effects of chemicals on normal physiology.

Dysfunction of bile acid metabolism has been linked with many diseases such as obesity, type 2 diabetes mellitus and bariatric surgery. PBK modeling can help to define critical steps in bile acid dysbiosis and thus direct therapies to counteract this dysbiosis thereby protecting or improving human health. The most important limitation of PBK modeling is that the models require a large amount of data to define all model parameters. This can in part be facilitated by applying quantitative structure activity relationship (QSAR) modelling (Desalegn et al. 2019) and use of suitable *in vitro* experimental data. The limitations of the current PBK model is that it only includes a basic adaptive response, which does not allow for dynamic or adaptive changes due to homeostatic feedback mechanisms such as for example changes in synthesis rate, expression levels of relevant transporters, microbiome composition or intestinal membrane integrity. Future bile acid PBK models can investigate meal dependent changes in plasma bile acids of postprandial responses of bile acid metabolism (Sonne et al. 2016). For example, gallbladder emptying is influenced by meal characteristics such as caloric content, liquid or solid meal, and fat content (Marciani et al. 2013; Sonne et al. 2014). The meal variety for individual patients can be included in the PBK model in future applications, offering patient and/or meal specific modelling of bile acid metabolism.

6.2.4 Interspecies and interindividual differences

It is well known that the human gut microbiota diversity is similar to that of rat at phylum level but not at genus level (Li et al. 2017). Rat gut microbiota studies revealed that the diversity in the gut microbiota community in laboratory rats is larger than that in humans (Manichanh et al. 2010). It is still not well understanded how these interspecies differences affect gut microbial functionality such as for example its metabolic capacity. It is known that *Bacteroides*, *Ruminococcaceae* and *Clostridiales* are dominated in human microbiota while rats have higher abundance of *Prevotella*, and fecal lactate was higher in rats whereas fecal acetate was higher in human, as shown when comparing fecal microbiota composition and the fecal levels of SCFAs and lactate between human and rats (Nagpal et al. 2018). In **Chapter 3** of this thesis, it was shown that there was no species difference in the deconjugation of conjugated bile acids by both human and rat fecal samples which may be ascribed to the fact that a wide range of gut microbiota (eg. *Bacteroides fragilis*, *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Enterococcus*) may be involved in the deconjugation of bile acids by human and rat gut microbiota. The challenge of using rodents to study human gut microbiota is that when translating generated knowledge from rodents to human, the similarities and differences between gut microbiota in the two species have to be considered. Human fecal microbiota transplantation in rodents has facilitated the understanding of human gut microbiota, and its role in metabolism, immunity and disease therapies in recent years. Human fecal microbiota transplantation studies have been applied to treat gastrointestinal disorders like irritable bowel syndrome (IBS) and metabolic disorders such as obesity and type 2 diabetes (Gheorghe et al.

2021; Hartstra et al. 2015; Weingarden and Vaughn 2017). Overall, it is highly important to develop human specific microbiota models to assess the metabolism of gut microbiota. The *in vitro* batch cultures of the present thesis provide a model that will facilitate studies on both species but also on interindividual human differences in intestinal microbiota composition and functionality.

In the present thesis, pooled fecal samples were used to study gut microbiota and its bile acid metabolism. However, gut microbiota composition is variable among individuals due to the influence of factors such as diet, antibiotic treatment, environment, maternal microbiota and genotype (Claesson et al. 2012; Hoskins and Boulding 1976; Lozupone et al. 2012; Mäkivuokko et al. 2012; Martin et al. 2008; Thompson-Chagoyán et al. 2007). It has been demonstrated for example that microbiota abundances and butyrate levels varied significantly between 16 human individuals when studying the degradation of fructoselysine using human fecal incubations (van Dongen et al. 2021). In another study substantial differences existed in microbiota composition and microbial metabolism of epicatechin in human fecal incubations with samples from 24 individuals (Liu et al. 2020). In addition, PBK modeling can also be of use when studying interindividual differences by linking them to Monte Carlo simulations. This approach has been applied for example to assess interindividual differences in the C_{max} of daidzein and its gut microbial metabolite S-equol in human plasma taking into account interindividual differences in gut microbial metabolism and also liver metabolism (Wang et al.). The interindividual variation in the kinetic constants for microbial or liver metabolism needed for this modelling approach could be derived using *in vitro* models with, for the metabolism by the intestinal microbiota use of the anaerobic batch fermentation model also used in the present thesis. To allow conversion of the *in vitro* kinetic data obtained in the batch fermentation model to the *in vivo* situation adequate scaling factors are required. The interindividual differences in defecation mass and the colonic transit time need to be considered when refining the scaling factor when studying the consequences of interindividual variability in intestinal microbial metabolism. In this way, new approach methodologies (NAMs) provide novel tools to study inter-individual differences of intestinal microbial metabolism.

6.2.5 Consequences of (effects on) bile acid homeostasis for human health

Bile acids participate in mediating host's health and disease within various tissues and organs amongst others via their influence on energy homeostasis, glucose and lipid metabolism and endocrine effects by influencing signaling pathways (Vítek and Haluzík 2016). Obesity, diabetes, inflammatory bowel disease (IBD) and other metabolic diseases are diseases widely distributed around the world. It has been suggested that pharmaceutical alteration of bile acid signaling pathways may be a promising treatment for these diseases (Li and Chiang 2012). For example, bile acids can activate the FXR signaling pathway thus regulating enterohepatic circulation of bile acids leading to reduced accumulation in the liver (Thomas et al. 2008). Another example is that bile acids can activate TGR5 signaling pathways in many different

cells thus controlling glucose and energy metabolism (Thomas et al. 2008). Bile acids are cytotoxic at high concentrations because they will induce oxidative stress and membrane damage (Barrasa et al. 2013; Bathena et al. 2015). DCA causes cellular toxicity by increasing formation of reactive oxygen species (ROS) that can cause oxidative stress and DNA damage thereby harming cells and causing diseases (Longpre and Loo 2008). LCA is also cytotoxic, inducing cell apoptosis via a mitochondrial dependent pathway initiated by caspase-8 activity (Katona et al. 2009). CDCA has been reported to induce apoptosis by mitochondrial dysfunction which contributes to the induction of cholestatic liver diseases (Rolo et al. 2004). Therefore, bile acids have dual functions not only beneficial effects but also adverse effects, and it is important to further study these dual effects of bile acids on human health.

In doing so the concept of adverse outcome pathways (AOPs) may provide a useful approach in which also *in vitro* models may prove to be of use. An AOP is a conceptual framework which starts with a molecule initiating event (MIE) and, through a series of intermediate key events, eventually leads to an adverse outcome (Villeneuve et al. 2014). Nowadays, the AOP framework is also used in risk assessment in order to develop predictive methods for human and environmental toxicology in which the mode of action of chemical compounds is better taken into account (Allen et al. 2014). Vinken and colleagues designed an AOP framework starting with inhibition of the bile salt exporting pump (BSEP) leading through key events of bile acid accumulation to the adverse outcome cholestasis (Vinken et al. 2013). This indicates that the Caco-2 transwell *in vitro* model for measuring inhibition on ASBT as used in the present thesis, in addition to other *in vitro* models that enable measuring effects on BSEP mediated bile acid transport, such as primary hepatocyte cultures can be of use to define whether compounds can actually affect this MIE or other steps in the AOP. The results of the present thesis reveal that to detect such effects on bile acid transporters both pre-and co-exposure of the model cells to the test compound and the bile acids are needed to detect different ways by which compounds may influence the transporter mediated bile acid translocation. Pre-exposure studies investigate the effects on intestinal bile acid transport via an indirect mode of action such as for example inhibiting protein synthesis thereby potentially reducing expression levels of bile acid transport proteins, while co-exposure studies investigate the direct inhibiting effect on these transport activities and/ or on passive diffusion (**Chapter 4 and 5**). Therefore, AOPs can satisfy variable purposes and stimulate the development of new *ex vivo* and *in vitro* assays, as well as identifying novel biomarkers in human risk assessment (Vinken et al. 2013).

6.3 Conclusions

Studying the functionality of the gut microbiota helps to define its characterization beyond the structural characteristics derived from 16S RNA analysis. The aim of the present thesis was to use new approach methodologies (NAMs) to study the effects of antibiotics on gut microbiota and bile acid metabolism thereby contributing to the 3Rs (reduction, refinement, replacement) of experimental animal studies. First, combined taxonomic profiling and bile acid profiling

were performed showing that the *in vitro* anaerobic fecal incubation model is a reliable tool to study the effects of xenobiotics on gut microbiota and related metabolism. Given that the enterohepatic circulation is a highly efficient pathway and important in bile acid homeostasis, the *in vitro* Caco-2 cell transwell model was used to study the intestinal reuptake of bile acids. These two *in vitro* models provided alternative testing strategies to illustrate the effects of the antibiotics on gut microbiota and bile acid kinetics. In addition, an *in silico* approach was used to link the *in vitro* data to *in vivo* human plasma bile acid concentrations by PBK modeling simulations. Taking all together the thesis shows that it is feasible to apply new approach methodologies (NAMs) to study the role of the gut microbiota and intestinal transporters in bile acid metabolism and homeostasis in the host.

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Chapter 7.
Summary

The gut microbiota plays an important role in human health by contributing to host metabolism via the activity of microbial enzymes, particularly by biotransformation of bile acids and composition. Therefore, gut microbiota is crucial for bile acid metabolism and homeostasis. Several *in vivo* rat studies investigated the interactions between gut microbiota and the fecal and plasma metabolome reporting effects of antibiotics not only on the intestinal bacterial composition but also on the fecal and plasma metabolome showing for example changes in bile acid profiles. Such *in vivo* animal studies, however, are expensive, time consuming, raise ethical issues as well as the question whether effects observed in experimental animals are relevant for humans. Hence, the aim of the present thesis was to investigate the use of new approach methodologies (NAMs) including *in vitro* and *in silico* methods for studies on the effects of antibiotics on bile acid homoeostasis.

In **Chapter 2**, two *in vitro* models were applied to further elucidate the mode of action underlying reported *in vivo* bile acid changes induced by antibiotics (colistin sulfate, tobramycin, meropenem trihydrate and doripenem hydrate). 16S rRNA analysis of rat fecal samples anaerobically incubated with these antibiotics showed that especially tobramycin induced changes in the gut microbiota. Tobramycin was shown to inhibit the microbial deconjugation of taurocholic acid (TCA) and the transport of TCA over an *in vitro* Caco-2 cell layer used as a model to mimic intestinal bile acid reuptake. The effects induced by the antibiotics in the *in vitro* model systems provide novel and complementary insight explaining the effects of the antibiotics on microbiota and fecal bile acid levels upon 28-day *in vivo* treatment of rats. In particular our results elucidate the mode(s) of action underlying the increased levels of TCA in the feces upon tobramycin exposure. All together the results of the this study provide a proof-of-principle on how *in vitro* models can be used to elucidate *in vivo* effects on bile acid homeostasis, and to obtain insight in the mode(s) of action underlying the effect of an antibiotic, in this case tobramycin, on bile acid homeostasis via effects on intestinal bile acid metabolism and reuptake.

In **Chapter 3**, it was investigated if effects on intestinal bile acid deconjugation and transport can be quantified in *in vitro* model systems using mixtures of bile acids instead of studying individual bile acids. To this end deconjugation of mixtures of selected bile acids in anaerobic rat or human fecal incubations and the effect of the antibiotic tobramycin on these reactions was studied. In addition, the effect of tobramycin on the transport of the bile acids in isolation or in a mixture across Caco-2 cell layers was characterized. The results demonstrate that both the reduction of bile acid deconjugation and the inhibition of intestinal bile acid transport by tobramycin can be adequately detected in *in vitro* systems using a mixture of bile acids, thus eliminating the need to characterize the effects for each bile acid in separate experiments. Subtle differences between the experiments with single or combined bile acids point at mutual competitive interactions and indicate that the use of bile acid mixtures is preferred over use of single bile acid given that this is more efficient and that also *in vivo* bile acids occur in mixtures.

In **Chapter 4**, the impact of an additional series of antibiotics (lincomycin, streptomycin, vancomycin and – for comparison to earlier results- tobramycin) on the intestinal reuptake of conjugated bile acids (TCA, TCDCA, GCA and GCDCA) was characterized using a Caco-2 cell layer *in vitro* transwell model system. The results obtained demonstrate that both pre-exposure and co-exposure of the cells to an antibiotic and the bile acids, affected bile acid transport, both to an extent that depended on the antibiotic, its concentration and the type of conjugated bile acid tested. Tobramycin, at concentrations in line with dose levels at which this antibiotic induced effects on bile acid homeostasis in rats *in vivo*, inhibited bile acid transport after pre-exposure of the cells, pointing at an effect on the expression of bile acid transporters via its effects on protein synthesis at the ribosome level. Upon co-exposure of the

Caco-2 cells to an antibiotic and the bile acids, all four antibiotics appeared to significantly reduce the transport of especially the conjugated bile acids TCDCA and GCDCA with a potency that decreased in the order vancomycin > tobramycin = streptomycin > lincomycin. The effects observed likely contribute to the effects reported for these antibiotics on bile acid homeostasis *in vivo*.

In order to further investigate the effects of the antibiotic tobramycin on bile acid homeostasis, in **Chapter 5**, a physiologically based kinetic (PBK) model that included microbial deconjugation and intestinal reuptake via the apical sodium dependent bile acid transporter (ASBT) was applied to predict the systemic plasma bile acid concentrations in human upon oral treatment with the antibiotic tobramycin at levels similar to those shown to affect bile acid homeostasis in the rat experiments. The PBK simulation results predicted that exposure to a dose level similar to the high dose level used in the rat *in vivo* studies of 1000 mg/kg bw tobramycin would reduce human plasma C_{max} levels of GCA, GCDCA, GDCA and the combined unconjugated BAs (uBAs) by 36.1 %, 18.7 %, 14.5 % and 33.8 % respectively. At this high dose level the effect of tobramycin on intestinal microbial deconjugation appeared not to affect plasma C_{max} levels, which can be explained by the fact that this microbial deconjugation happens mainly in the colon where bile acid reuptake is limited. The predictions for the effect of tobramycin on human plasma bile acid levels were comparable to the effects on plasma bile acid levels reported in rats exposed to tobramycin at the same dose level of 1000 mg/kg bw. Altogether, the PBK model appeared to provide a reliable tool to evaluate the effect of oral exposure to tobramycin on host bile acid homeostasis via effects on intestinal bile acid deconjugation and reuptake.

Overall, the current thesis provided a proof-of-principle for the use of new approach methodologies (NAMs) to study the effects of antibiotics on bile acid homeostasis thereby contributing to the 3Rs (reduction, refinement, replacement) of experimental animal studies. Taking all together the thesis shows that it is feasible to apply new approach methodologies (NAMs) to study the role of the gut microbiota and intestinal transporters in bile acid metabolism and homeostasis in the host.

Appendix

Acknowledgement

Biography

List of Publications

Overview of completed training activities

Acknowledgement

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To myself: Be independent, be strong and brave to the future life!

Biography

Nina Zhang was born in Xi'an, China. In 2014, Nina finished her 4 years bachelor degree education in Biotechnology from Southwest University in Chongqing, China. In September of 2016, Nina started her master education in Food quality and safety in Debrecen University in Hungry, where she obtained her master degree in June 2018. In 2019, Nina came to the Netherlands and started her PhD at the Toxicology group of Wageningen University and Research (WUR). Her PhD project was supported by the Long-range Research Initiative (LRI), which is an initiative from the European Chemical Industry Council. The research is extensively reviewed by an External Science Advisory Panel from Cefic-LRI. Her PhD research was related to the use new approach methodologies (NAMs) to study the effects of antibiotics on intestinal microbiota, intestinal cells and related bile acid metabolism. During her PhD, she followed the postgraduate education in toxicology (PET) required for the registration as European Registered Toxicologist (ERT).

List of Publications

Zhang N, Wang J, Bakker W, Zheng W, Baccaro M, Murali A, van Ravenzwaay B, Rietjens IMCM(2022) In vitro models to detect in vivo bile acid changes induced by antibiotics. Archives of Toxicology 96: 3291-3303

Zhang N, Zheng W, Bakker W, van Ravenzwaay B, Rietjens IMCM (2023) In vitro models to measure effects on intestinal deconjugation and transport of mixtures of bile acids. Chemico-Biological Interactions:375: 110445

Zhang N, de Bruijn VMP, Zheng W, Bakker W, van Ravenzwaay B, Rietjens IMCM (2023) Antibiotics reduce intestinal bile acid reuptake in an *in vitro* model system (manuscript in preparation)

Zhang N, Rietjens IMCM, de Bruijn VMP (2023) Application of physiologically based kinetic (PBK) modeling to quantify the effect of the antibiotic tobramycin on bile acid levels in human plasma (manuscript in preparation)

Overview of completed training activities

Discipline specific activities

Cell Toxicology	PET course, online	2020
Pathobiology and Toxicological Pathology	PET course, online	2020
Risk assessment	PET course, online	2020
Organ Toxicology	PET course, online	2021
Toxicogenomics	PET course, online	2021
Epidemiology	PET course, online	2021
Immunotoxicology	PET course, online	2021
Molecular Toxicology	PET course, online	2021
Laboratory Animal Science	PET course, online	2021

Conferences

61st Annual Meeting Society of Toxicology (SOT) and ToxExpo, poster, online, 2022

57th Congress of the European Societies of Toxicology (registered), Ljubljana, 2023

General courses

PhD week	VLAG	2020
The essentials of Scientific writing &Presenting	WGS	2020
Philosophy and Ethics of Food Science and Technology	WGS	2020
Searching and Organizing Literature for PhD	WUR library	2019
Introduction to R	VLAG	2021
Applied statistics	VLAG	2021

Other activities

Preparation of research proposal	Toxicology	2019
General Toxicology	Toxicology	2020
PhD presentation at TOX	Toxicology	2021
Environmental Toxicology	Toxicology	2020

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