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University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Department of Anatomy and Neuroscience

Head of Dept. John F. Cryan

Effects of Psychotropic Drugs on the Microbiota-Gut-Liver-Brain Axis

Thesis presented by

Sofia Cussotto, MPharm

Under the supervision of

Prof. John F. Cryan

Prof. Timothy G. Dinan

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Microbiome Ireland
Interfacing Food & Medicine

Department of Anatomy & Neuroscience
University College Cork

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Declaration

This thesis submitted is my own work and has not been submitted for any other degree, either at University College Cork or elsewhere.

Author Contributions

All the work conducted in this thesis was performed independently by the author with the following exceptions:

Chapter 2: Conall Strain performed RNA extraction from caecal samples. Fiona Fouhy and Veronica Peterson carried out the bioinformatic analysis. Ronan Strain carried out the SCFA analysis.

Chapter 3: Alvaro Lopez Gallardo performed bile acid quantification and heatmap construction. Anna Golubeva helped with the Ussing chambers experiment. Conall Strain performed RNA extraction from caecal samples. Fiona Fouhy carried out the bioinformatic analysis.

Chapter 4: Jacinta Walsh performed the HPLC drug detection from blood and caecum samples and faecalase assay. Conall Strain and Fiona Fouhy carried out the bioinformatic analysis.

Chapter 5: Sanzhima Garmaeva and Thomaz Bastiaanssen carried out the bioinformatic analysis.

Signed



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Publications and presentations

Published manuscripts relevant to this thesis

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Cryan JF, O'Riordan, KJ, Cowan CSM, Bastiaanssen TFS, Boehme M, Codagnone MG, **Cussotto S**, Fulling C, Golubeva AV, Guzzetta KE, Long-Smith CM, Lyte JM, Martin J, Moloney GM, Morelli E, Morillas E, O'Connor R, Pereira J, Peterson VL, Rea K, Ritz NL, Sandhu KV, Sherwin E, Spichak S, van de Wouw M, Ventura Silva AP, Wallace-Fitzsimons SE, Hyland N, Clarke G, Dinan TG. The Microbiota-Gut-Brain Axis, *Physiol Rev*. (In Press).

Other published manuscripts

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[#]**Cussotto S**, [#]Walsh J, Strain CR, Fouhy F, Stanton C, Dinan TG, Hyland N, Clarke G, Cryan JF, Griffin BT, Differential Effects of the Gut Microbiota on the Pharmacokinetics of Antipsychotic Drugs. *To be submitted to: ASPET Drug Metabolism and Disposition*

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Conference posters and oral presentations

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Cussotto S, Strain CR, Fouhy F, Stanton C, Dinan TG, Cryan JF. Differential effects of psychotropic drugs on gut microbiota composition, *ECNP Workshop on Neuropsychopharmacology for Early Career Scientists in Europe*, Nice, March 2019. Poster

Cussotto S, Strain CR, Fouhy F, Stanton C, Dinan TG, Cryan JF. Differential effects of psychotropic drugs on intestinal function and gut microbiota composition, *19th Meeting of International Society for Serotonin Research*, Cork, July 2018. Poster

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Cussotto S, *UCC Science for All* competition, "Psychotropics and the Chamber of Secrets… Our gut!", March 2018

Cussotto S, *Researchfest Inspirefest* competition, Psychotropics and the Chamber of Secrets… Our gut!", June 2018 (https://www.youtube.com/watch?v=qasrvjYYqs0)

Abstract

There is a growing recognition of the involvement of the gut microbiota in drug metabolism and *vice versa* the impact of drug intake on the microbiome. In this thesis, we focus our attention on psychotropic medications (from the Greek root *psychè* = mind and *tropòs* = turning). With few isolated studies showing that brain-targeting medications can have antimicrobial activity *in vitro*, we sought to investigate the impact of psychotropics on the microbiome and intestinal physiology *in vivo*. Across a range of psychotropic medications, lithium, valproate, aripiprazole and fluoxetine significantly impacted the microbiome composition and diversity. These effects were not directly linked to changes in intestinal permeability or short-chain fatty acids levels (*Chapter 2*).

The mood stabilisers lithium and valproate significantly impacted bile acid metabolism and targeted a set of bile-metabolising bacteria. Two mechanisms hypothesised as possible players in the bile-targeted effects of lithium and valproate, hepatic inflammation and intestinal permeability, did not seem to play any overt role in the disruption of bile pathways (*Chapter 3*).

We next investigated whether perturbations of the microbiome, through administration of probiotics or antibiotics, could alter the pharmacokinetics of olanzapine and risperidone, two antipsychotic medications. Antibiotics increased the blood levels of olanzapine (AUC, area under the curve) but did not influence the absorption of risperidone. The antibiotics did not have a direct effect on the expression of CYPs involved in the metabolism of antipsychotics. Among the bacterial genera detected by 16S sequencing, the relative abundance of *Alistipes* significantly correlated with the AUC of olanzapine but not risperidone, suggesting that this bacterium might play a role in the pharamacokinetic alterations observed in olanzapine-treated rats *(Chapter 4).*

Lastly, intrigued by the findings of *Chapter 2*, we moved on to look at the microbiome-targeting effects of psychotropic drugs in a human population, the Dutch LifeLines DEEP cohort. Although the small sample size and certain limitations which should be

addressed in future population-based studies, minor effects of drug consumption on the human gut microbiota were detected (*Chapter 5*).

Overall, these results provide novel insight on the role exerted by psychotropic medications on the microbiota-gut-liver-brain axis. Possible implications of this work include optimisation of drug efficacy or toxicity, use of the microbiome as a tool to distinguish responders from non-responders and improvement of personalised medicine.

Chapter 1

General Introduction

1.1 Gut Microbiota

Our gut houses a staggering amount of microorganisms that estimates consider contains 150 times as many genes as our genome (Qin et al., 2010). This population is mainly composed of bacteria belonging to 500-1000 different species (Qin et al., 2010). Fungi, archaea, and viruses are also present in the gut but less is known about their underlying functions. The intestinal microbiota does not remain stable throughout lifespan, in fact the microbiota of newborn infants, acquired at delivery, is characterised by low diversity and a relative dominance of the phyla Proteobacteria and Actinobacteria (Kurokawa et al., 2007). The microbial composition of the neonatal gut is influenced by a number of factors including antibiotic use, diet, mode of delivery, environmental factors and the maternal microbiota (Dominguez-Bello et al., 2010; Faa et al., 2013; Koenig et al., 2011; Marques et al., 2010). Interestingly, the microbiota of formula-fed infants is significantly different from the microbiota of breastfed infants (Bezirtzoglou et al., 2011; Lee et al., 2015; Wang et al., 2015). Moreover, vaginally delivered infants acquire the vaginal microbiota of the mother, whereas infants delivered by caesarean section are colonised by other environmental sources (Dominguez-Bello et al., 2010). In the first months of life, the number of strict anaerobes such as *Clostridium*, *Bacteroides* and bifidobacteria increases gradually and after 1 year of age a complex adult-like microbiota is established. The adult microbiota is more stable over time and more complex than the neonate microbiota (Hamady and Knight, 2009). The gut microbiota is involved in basic human biological processes, including modulating the metabolic phenotype, epithelial development, and innate immunity (Nicholson et al., 2012b; Sommer and Bäckhed, 2013; Willson et al., 2018). Chronic diseases such as obesity, inflammatory bowel disease (IBD), diabetes mellitus, metabolic syndrome, atherosclerosis, alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), cirrhosis, and hepatocellular carcinoma have been associated with the human microbiota (Haghikia et al., 2018; Lavelle and Sokol, 2018; Ponziani et al., 2019; Sircana et al., 2018; Soderborg et al., 2018).

Taxonomic levels used to classify the microbiome include: phylum (i.e. Firmicutes), class (i.e. Bacilli), order (i.e. Lactobacillales), family (i.e. Lactobacillaceae), genus (i.e. *Lactobacillus*) and species (i.e. *Lactobacillus gasseri*).

1.2 Microbiota-Gut-Brain Axis

The microbiota-gut-brain axis is a bidirectional pathway through which the brain regulates the activity of the gut and *vice versa*. This bidirectional axis functions through a series of different routes (Bercik et al., 2012; Dinan et al., 2015; Rhee et al., 2009) and comprises an afferent and an efferent pathway (**Figure 1.1**).

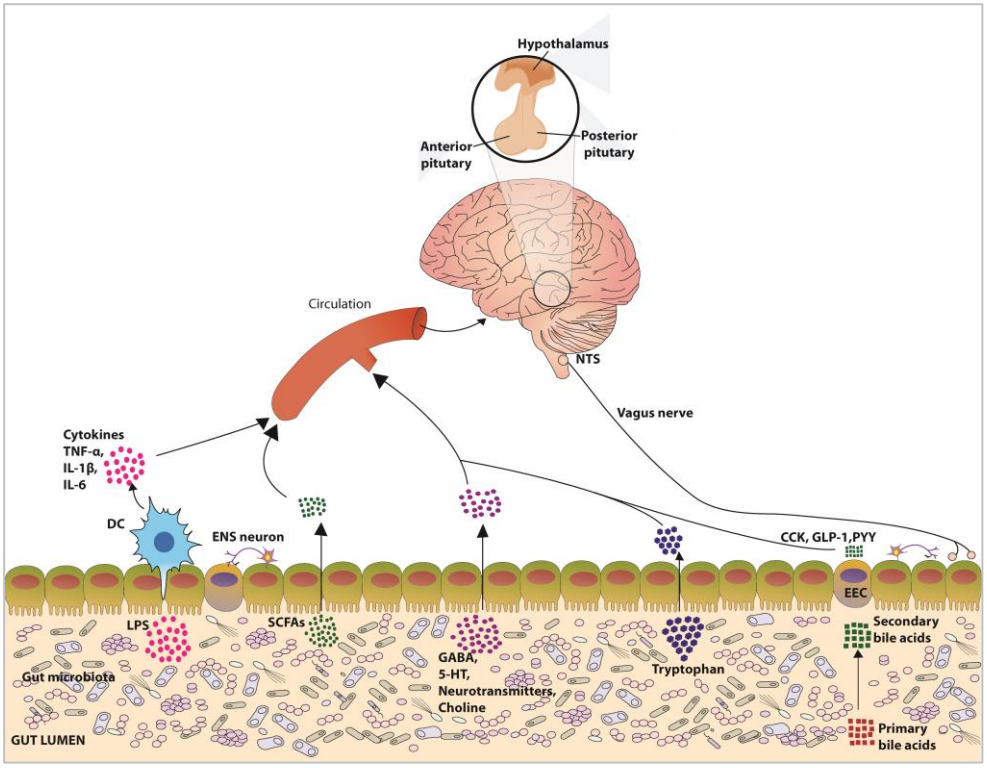


Figure 1.1 The gut microbiota communicates to the brain through several routes that characterise the microbiota-gut-brain axis. *These routes include the vagus nerve, production of SCFAs, immune activation with production of immune mediators, production of neurotransmitters and tryptophan. The gut microbiota is also able to convert primary bile acids into secondary bile acids, which activate receptors on EECs and stimulate the secretion of gut peptides. Neuroactive compounds produced by gut microbiota enter the circulation and reach the brain, subsequently affecting neuroendocrine function. Abbreviations: 5-HT (5-hydroxytryptamine) serotonin, CCK cholecystokinin, DC dendritic cell, EEC enteroendocrine cell, ENS enteric nervous system, GABA ɣ-aminobutyric acid, GLP-1 glucagon-like peptide-1, IL interleukin, LPS lipopolysaccharide, NTS nucleus tractus solitarii, PYY peptide YY, SCFAs short chain fatty acids. Figure adapted from (Cussotto et al., 2018).*

1.2.1 Afferent Signalling

The vagus nerve, the tenth cranial nerve that has both efferent and afferent divisions, is a major modulatory constitutive communication pathway between the bacteria exposed to the gut and the brain (Bercik et al., 2011; Bravo et al., 2011). The immune system provides a further route of communication between gut microbes and the brain, in fact microbiota and probiotics have a direct effect on the immune system (Duerkop et al., 2009; Forsythe and Bienenstock, 2010). Symbiotic bacteria are crucial for the maturation of the immune system in fact, they provide signals for the development of key lymphocyte subsets (Edelman and Kasper, 2008). Moreover, gut bacteria contribute to intestinal epithelial cell maturation and can induce alterations in the circulating levels of pro- and anti-inflammatory cytokines that directly affect brain function, especially areas such as the hypothalamus, where IL-1 and IL-6 provide a potent release of CRF (Duerkop et al., 2009). Gut bacteria contribute to the host metabolism by production of metabolites such as bile acids, choline and short chain fatty acids (SCFAs, namely acetic propionic and butyric acid) that are able to influence a range of physiological and metabolic functions (De Vadder et al., 2014). The free SCFAs are also able to cross the blood-brain barrier (BBB) through monocarboxylate transporters and act in several brain regions (Vijay and Morris, 2014). Although it remains to be established whether the microbiota can produce neuropeptide-like compounds, it is capable of generating a number of neurotransmitters and neuromodulators (Cryan and Dinan, 2012; Nicholson et al., 2012a). Members of the genera *Candida*, *Streptococcus*, *Escherichia* and *Enterococcus* synthesise 5-hydroxytryptamine (5-HT); members of the genera *Escherichia*, *Bacillus* and *Saccharomyces* generate dopamine and/or noradrenaline; members of the genus *Lactobacillus* produce acetylcholine; and members of the genera *Lactobacillus* and *Bifidobacterium* produce gamma-aminobutyric acid (GABA) (Barrett et al., 2012; Cryan and Dinan, 2012; Lyte, 2014; Nicholson et al., 2012a). An example of the connection of the gut microbiome with the host neurophysiological systems is a study showing that the excitability of gut sensory neurons located within the myenteric plexus of the ENS relies on the presence of the normal commensal microbiota for proper functioning (Neufeld et al., 2011). Several studies have suggested that another mechanism involved in the gut-brain communication is tryptophan metabolism.

Tryptophan is an essential amino acid and is a precursor to many biologically active agents, such as serotonin (Ruddick et al., 2006). Most of the tryptophan is metabolised to kynurenine and the disruption of this metabolic pathway has been linked to both gastrointestinal and brain disorders (Fitzgerald et al., 2008). The first rate-limiting step in the kynurenine metabolic cascade is catalysed by some enzymes (specifically indoleamine-2,3-dioxygenase and tryptophan 2,3-dioxygenase) whose activity can be induced by inflammatory mediators and by corticosteroids, respectively (Ruddick et al., 2006). Evidence suggests that a probiotic bacterium, *Bifidobacterium infantis*, can alter concentrations of kynurenine through modulation of the gut microbiota (Desbonnet et al., 2008). Another class of molecules, gut-derived peptides, can reach the systemic circulation and bind receptors on immune cells and vagus nerve terminals thereby enabling indirect gut-brain communication (Lach et al., 2017).

1.2.2 Efferent Signalling

The HPA axis represents the main efferent route from the brain to the gut. When activated, the resulting secretion of cortisol (in humans) or corticosterone (in rodents) affects immune cell activity; both locally in the gut and systemically (Del Rey, 2008). Neuronal efferent activation includes also the efferent branch of the vagus nerve that, when activated induces a release of acetylcholine which, in turn, affect the levels of cytokines (Paton et al., 1971).

1.3 Microbiota-Gut-Liver Axis

The crosstalk between the intestine and liver is increasingly recognised, strengthened by the parallel rise in incidence of liver diseases and gastrointestinal and immune disorders (Hartmann et al., 2015; Schnabl and Brenner, 2014). The gut and the liver communicate via tight bidirectional links through the biliary tract, portal vein and systemic circulation. The liver communicates with the intestine by releasing bile acids and many bioactive mediators into the biliary tract and the systemic circulation. In the intestine, host and microorganisms metabolise endogenous (bile acids and amino acids) as well as exogenous (from diet and environmental exposure) substances, the products of which translocate to the liver through the portal vein thus influencing liver function (Starkel and Schnabl, 2016) (**Figure 1.2**). Drugs and xenobiotics, which are commonly metabolised in the liver, can also translocate from the gut to the liver and *vice versa*, being exposed to the gut microbiota (Zimmermann et al., 2019b). Some key communication routes between the gut and the liver are discussed in this section.

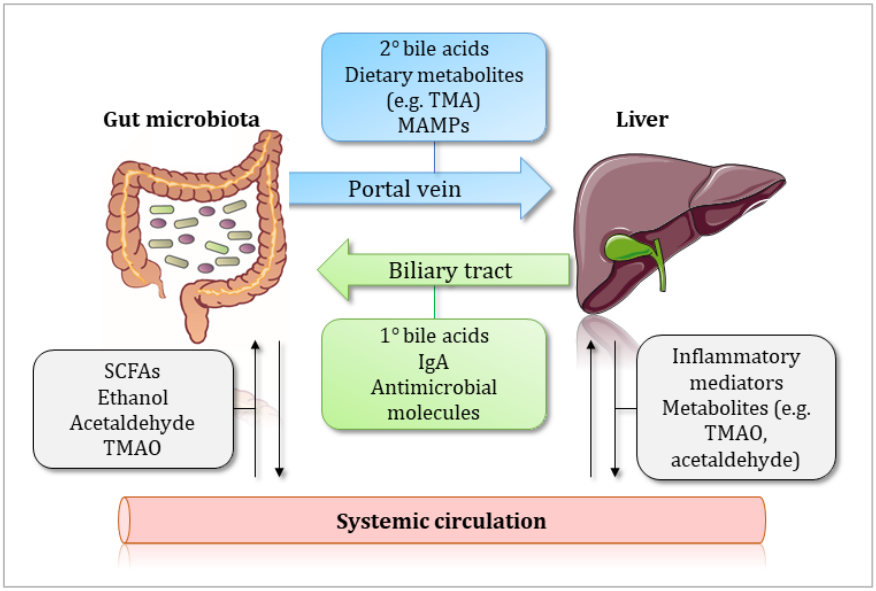


Figure 1.2 Bidirectional communication between gut and liver. *The liver releases primary bile acids and antimicrobial molecules (IgA and angiogenin 1) into the intestinal lumen through the biliary tract. Conversely, gut products such as microbial metabolites and MAMPs translocate to the liver via the portal vein. The systemic circulation also allows the exchange of substances between the gut and the liver. Liver metabolites from dietary, endogenous or xenobiotic substances (e.g. free fatty acids, choline metabolites, ethanol metabolites) are transported to the intestine through the capillary system. Abbreviations: MAMPs microbial-associated molecular patterns; SCFAs short-chain fatty acids; TMA trimethylamine; TMAO trimethylamine N-oxide. Figure adapted from (Tripathi et al., 2018).*

1.3.1 Enterohepatic circulation of bile acids

Bile acids (BAs) are amphipathic molecules synthesised from cholesterol in the liver. Following conjugation to either glycine or taurine, BAs are released in the biliary tract and, together with other biliary components, enable emulsification and absorption of dietary fats, cholesterol, and fat-soluble vitamins (Chiang, 2013). About 95% of the BAs are actively reabsorbed in the distal ileum and carried back to the liver (Chiang, 2013; Wahlstrom et al., 2016). The remaining 5% are deconjugated, dehydrogenated and dehydroxylated by the intestinal microbiota to form secondary bile acids, which reach the liver via passive absorption into the portal circulation. The liver recycles BAs and secretes them back to the biliary tract completing the "enterohepatic circulation" i.e. a system of exchange between the gut and the liver. A carrier-mediated process transports hydrophilic primary bile acids across cell membranes for uptake into intestinal epithelial cells. Regulatory effects of BAs have been mostly studied in regard to farnesoid X receptor (FXR) and Takeda G-protein-coupled receptor 5 (TGR5). BAs bind to FXR in the enterocytes and induce transcription of an enterokine, fibroblast growth factor 19 (FGF19; FGF15 in mouse). FGF19 reaches the liver through the portal vein and down-regulates the *de novo* synthesis of BAs by inhibiting key enzymes involved in the synthesis of new BAs (Zarrinpar and Loomba, 2012). Additionally, BAs bind to TGR5 on the plasma membrane and act on tissues beyond the liver. This binding mediates host energy expenditure (Broeders et al., 2015; Pols et al., 2011) glucose homeostasis (Thomas et al., 2009) and anti-inflammatory immune responses (Perino and Schoonjans, 2015; Schaap et al., 2014). BAs and the gut microbiota closely interact and modulate each other. BAs exert direct control on the intestinal microbiota. By binding to FXR, they induce production of antimicrobial peptides such as angiogenin (ANG1) and RNase A family member 4 (RNASE4), which are directly involved in inhibiting gut microbial overgrowth and, subsequently, gut barrier dysfunction (Inagaki et al., 2006; Parseus et al., 2017). An alteration in the gut microbiota composition can shift the balance between primary and secondary bile acids and their subsequent enterohepatic cycling, the metabolic effects of which are not comprehensively understood. However, because of differences in the affinity of these two classes of BAs for the FXR, these shifts have been associated with increased

hepatic bile acid synthesis and metabolic stress (Arab et al., 2017; Jiang et al., 2015a; Mouzaki et al., 2016; Ridlon et al., 2014).

1.3.2 Intestinal permeability in the microbiota-gut-liver axis

The main components of the intestinal barrier, enterocytes, are tightly attached to adjacent cells by apical junctional proteins that include claudins, occludins, E-cadherins and junctional adhesion molecules (JAMs) (Turner, 2009). This barrier confines the passage of microbes and molecules from the gut lumen, while allowing active transport of nutrients across the junctions. The intestinal barrier is strengthened by several additional lines of defence: Mucins (heavily glycosylated protein aggregates) form a physical barrier between luminal bacteria and the underlying epithelial layer (Turner, 2009); antibacterial lectins, such as regenerating islet-derived protein IIIɣ (REG3G), which are produced by intestinal Paneth cells, target bacteria associated with mucosal lining (Abreu, 2010; Gallo and Hooper, 2012). As additional line of defence, immunoglobulins (specifically secretory immunoglobulin A) produced by plasma cells and transported into the intestinal lumen, can neutralise microbial pathogens by blocking epithelial receptors (Mantis et al., 2011). Finally, commensal bacteria are closely associated with the gut mucosa, and strengthen barrier integrity by stimulating cell-mediated immunity via toll-like receptor mediated signalling (Abreu, 2010; Rakoff-Nahoum et al., 2004) or by producing metabolites that directly strengthen tight junctions (short chain fatty acids) (Wachtershauser and Stein, 2000; Yaku et al., 2012; Ziegler et al., 2016) and inhibit other microbes (Graham et al., 2017; Lobos et al., 2017; Walsh et al., 2017). An important association between the gut microbiota, inflammation and gut barrier integrity is provided by *Akkermansia muciniphila*, a Gram-negative anaerobe that colonises the intestinal mucus layer. Reduced levels of *A. muciniphila* have been associated with thinning of mucus layer (compromising gut barrier integrity) and increased inflammation, which promote both alcoholic and non-alcoholic liver damage (Everard et al., 2013; Grander et al., 2018). *A. muciniphila* has also been shown to induce beneficial effects on metabolism and weight control in diet-induced obesity (Dao et al., 2019; Depommier et al., 2019).

When the gut barrier is compromised, microbes and microbe-derived molecules can translocate to the liver through the portal system, causing inflammation and hepatic injury. Some translocated intestinal products may also directly interact with host factors and contribute to exacerbation of liver disease (Filliol et al., 2017; Lopez-Lazaro, 2016; Scott et al., 2017a; Wu et al., 2017b; Zhu et al., 2013).

1.3.3 Systemic circulation in the microbiota-gut-liver axis

Bacteria and MAMPs

Intestinal dysbiosis (e.g. small intestinal bacterial overgrowth, SIBO) and increased intestinal permeability can lead to translocation of microbial-associated molecular patterns (MAMPs) in the portal circulation. On reaching the liver, MAMPs induce localised inflammation through pattern recognition receptors (PRRs) on Kupffer cells (Seki et al., 2007) and hepatic stellate cells (Gabele et al., 2008; Isayama et al., 2006). Endotoxin-mediated activation of Toll-like Receptor 4 (TLR4) (Gabele et al., 2008; Isayama et al., 2006) along with TLR9 (Hartmann et al., 2012), are the primary drivers of immune response in liver disease. TLR signalling in Kupffer cells activates a downstream proinflammatory cascade, leading to MYD88-mediated activation of NF-kB (Seki and Schnabl, 2012).

Choline metabolites

Choline is a macronutrient important for liver function, brain development, nerve function, muscle movement and metabolism (Zeisel and da Costa, 2009; Zeisel et al., 1991). Importantly, rodents fed a choline-deficient diet have been used to model human non-alcoholic steatohepatitis (Han et al., 2017; Muraki et al., 2017; Rutenburg et al., 1957). Choline is processed by the host into phosphatidylcholine, which assists in excretion of very-low density lipoproteins (VLDL) particles from the liver. This prevents hepatic accumulation of triglycerides and subsequent liver steatosis.

Additionally, intestinal bacteria play a key role in the conversion of choline to trimethylamine (TMA) (Craciun and Balskus, 2012; Rath et al., 2017; Romano et al., 2015). TMA, in turn, can translocate to the liver through the portal circulation where it is converted to trimethylamine *N*-oxide (TMAO) (Canyelles et al., 2018). The importance of methylamines is increasingly being recognised with respect to liver, cardiometabolic and even neurological disorders. Increased systemic circulation of TMAO is concomitant with reduced levels of host-produced phosphatidylcholine, an imbalance characteristically seen in those with intestinal dysbiosis (Tang et al., 2013; Wang et al., 2011b; Yin et al., 2015). Increased TMAO in circulation can also augment the incidence of abdominal aortic aneurysm in mice and lower hippocampal blood flow (Beilfuss et al., 2018; Conrad et al., 2018).

Free fatty acids

Free fatty acids include short-chain fatty acids (SCFAs) and long-chain fatty acids (LCFAs). Butyrate, propionate (produced by bacterial fermentation) and acetate (produced by both host and bacteria) are the dominant SCFAs in the large intestine. Butyrate is an energy source for the enterocytes and facilitates maintenance of intestinal barrier (Wachtershauser and Stein, 2000; Yaku et al., 2012; Ziegler et al., 2016). Alcohol-induced liver injury is characterised by reduced butyrate and propionate (Chen et al., 2015c; Cresci et al., 2017) and increased acetate (possibly produced by ethanol metabolism in the lumen, but predominantly derived from ethanol metabolism in the liver). A reduction in butyrate is linked to weakening of intestinal tight junctions and hence an increase in permeability (Peng et al., 2009; Yan and Ajuwon, 2017). Butyrate supplementation in the form of tributyrin reduced ethanol-induced intestinal permeability and subsequent liver injury in mice on a short-term alcohol diet (Cresci et al., 2017). However, how tributyrin mechanistically protects intestinal barrier function still needs to be clarified. Luminal species of LCFAs include pentadecanoic acid (C15:0), palmitic acid (C16:0), heptadecanoic acid (C17:0) and stearic acid (C18:0). In mice fed alcohol chronically, pentadecanoic acid and heptadecanoic acid, which are only produced by bacterial fermentation, were

significantly reduced when compared to control mice on isocaloric diet (Chen et al., 2015c). In the same study, there was also an overall reduction in total LCFAs which was associated with decreased luminal lactobacilli (known metabolisers of saturated LCFAs). Even though it has not yet been demonstrated that LCFA supplementation can restore *Lactobacillus* spp., dietary supplementation of *Lactobacillus rhamnosus* increased luminal LCFAs (Shi et al., 2015), suggesting that *Lactobacillus*-induced increase in intestinal free fatty acids contribute to its probiotic effects.

Ethanol and acetaldehyde

The mucosa of the GI tract absorbs ethanol by simple diffusion. Gut microbiota and enterocytes express alcohol-metabolizing enzymes such as alcohol dehydrogenase, which co-metabolises ethanol into acetaldehyde and acetate. In germ-free mice, the absence of microbiota was associated with increased hepatic expression of ethanol-metabolizing enzymes, which led to faster ethanol elimination from the blood (Chen et al., 2015a). Acetaldehyde, a metabolite of ethanol, has been implicated in weakening the intestinal tight junctions compromising the gut barrier and allowing translocation of microbial products (Chaudhry et al., 2016; Chen et al., 2015b; Mir et al., 2016; Yan and Schnabl, 2012). Acetaldehyde has also been associated with downregulating the expression of antimicrobial peptides (AMPs) in the intestine (Hartmann et al., 2013; Yan et al., 2011) and eliciting inflammatory and adaptive host immune responses (Mottaran et al., 2002; Park et al., 2016; Wang et al., 2011a).

1.4 Bile Acid Synthesis, Metabolism and Microbiota

Bile acids are the major functional components of bile. They are synthesised from cholesterol in the liver, stored in the gall bladder and subsequently released into the small intestine (Chiang, 2013). The host enzymes and feedback pathways involved in bile acid synthesis are well characterised. A crucial mediator in bile acid metabolism is the gut microbiota, with its unique enzymatic machinery capable of deconjugating

bile acids in the intestine and therefore playing an essential role in bile acid homeostasis (Ramirez-Perez et al., 2017).

1.4.1 Bile acids synthesis

Bile acids are a product of the steroid cholesterol and are generated through either the classical or alternative pathway in hepatocytes. The host enzymes responsible for bile acid synthesis are cytochrome P450 family enzymes, mainly CYP7A1, CYP8B1 and CYP27A1 (Chiang, 2013). Following synthesis of new bile acids, these are conjugated to either glycine or taurine, a process that increases their solubility for secretion into biliary fluid. The process is carried out by bile acid cholyl-CoA synthetase (BAC) activity and amidation at C24 to either glycine or taurine by the enzyme bile acid-CoA:amino acid N-acyltransferase (BAT). As a result, the majority of bile acids excreted from the liver are conjugated (**Figure 1.3**). Bile is secreted into the duodenum via the common bile duct and its main physiological roles in the small intestine are the emulsification of fats, the release of fat-soluble vitamins and the regulation of cholesterol metabolism (Begley et al., 2005a). Feedback regulation of specific enzymes results in alterations to the ratio of individual bile acids in the bile acid pool. Overall regulation of bile acid synthesis is through feedback inhibition of CYP7A1. Direct interaction between bile acids and the farnesoid X receptor FXR downregulates CYP7A1 gene expression (Chiang, 2013). Alternatively, FXR is activated locally in the enterocytes by bile acids and promotes the expression of the mediator Fgf19, which enters circulation and represses CYP7A1 in hepatocytes through interaction with a specific cellular receptor (Fgfr4). The enterohepatic circulation of bile acids is very efficient, with 95% of bile acids actively reabsorbed in the terminal ileum and transported back to the liver via the portal circulation. Approximately 5% of bile salts are lost through the faeces (Chiang, 2013).

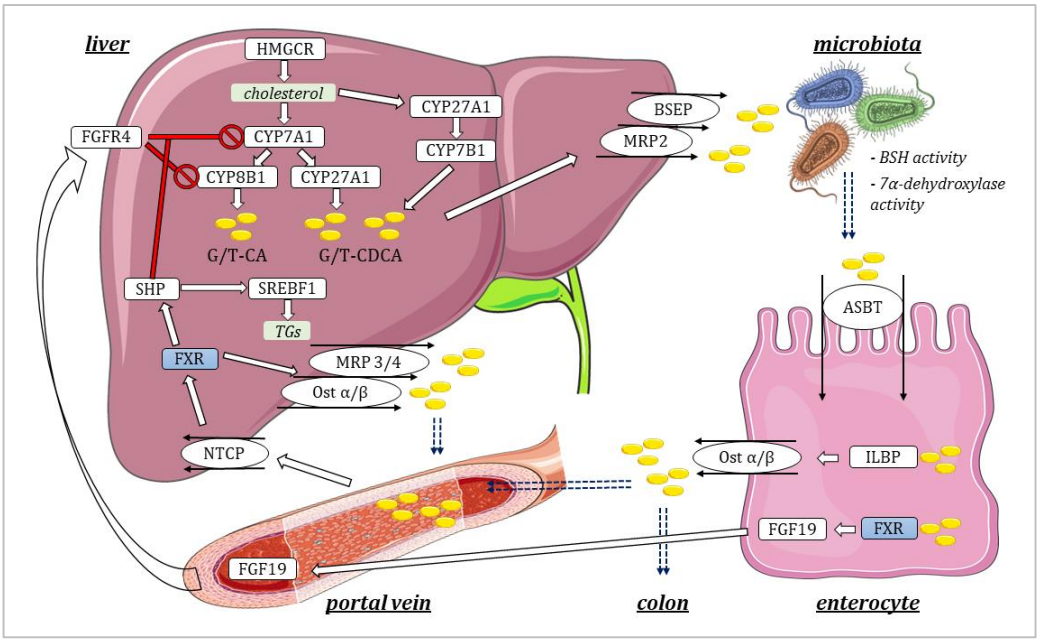


Figure 1.3 Transporters and enzymes involved in the synthesis and enterohepatic circulation of bile acids. *Abbreviations: ASBT apical sodium-bile acid transporter, BSEP bile salt export pump, BSH bile salt hydrolase, CYP cytochrome P450, FGF19 fibroblast growth factor 19, FGFR4 fibroblast growth factor receptor 4, FXR farnesoid X receptor, G/T-CA glyco/tauro-cholic acid, G/T-CDCA glyco/tauro-chenodeoxycholic acid, HMGCR 3-hydroxy-3-methylglutaryl-CoA reductase, ILBP ileal lipid binding protein, MRP multidrug resistance protein, NTCP Na^+-taurocholate cotransporting polypeptide, Ost α/β organic solute transporter α/β, SHP small heterodimer partner, SREBF1 sterol regulatory element binding transcription factor 1, TGs triglycerides.*

1.4.2 Bile acids metabolism: gut microbiota as a key player

The chemical composition of bile acids is strongly influenced by the gut microbes, which have bile-related enzymatic activity (Ridlon et al., 2014) (**Figure 1.4**). Bile salt hydrolases (BSH; EC 3.5.1.24) are microbial enzymes that belong to the Ntn-hydrolase superfamily of proteins. These enzymes cleave the amide bond between the glycine and taurine moiety conjugated to the steroid nucleus of bile salts. This cleavage liberates bile acids and is the crucial first step for further bile acid alterations by the gut microbiota (Begley et al., 2006). BSH activity has been reported in the commensal genera *Lactobacillus* (Chae et al., 2013; Corzo and Gilliland, 1999; Elkins et al., 2001; Jayashree et al., 2014), *Bifidobacterium* (Grill et al., 1995; Kim et al., 2004a; Tanaka et al., 2000), *Enterococcus* (Franz et al., 2001; Wijaya et al., 2004), *Clostridium* spp (Coleman and Hudson, 1995; Gopal-Srivastava and Hylemon, 1988; Rossocha et al.,

2005) and *Bacteroides* spp (Kawamoto et al., 1989; Stellwag and Hylemon, 1976). Interestingly BSH activity is not only confined to gut bacteria, but it has been found in *Xanthomonas maltophilia* isolated from soil (Dean et al., 2002; Pedrini et al., 2006) and thermophilic *Brevibacillus* sp isolated from hot springs (Sridevi and Prabhune, 2009; Sridevi et al., 2009). This indicates that BSH activity might be a widespread feature of bacteria adapted to different environments. There is evidence of horizontal transmission of BSH amongst gut bacteria, suggestive of strong evolutionary selection for this activity (Jones et al., 2008). BSH coding sequences are widely distributed in both Bacteria and Archaea, suggesting a strong host-driven selection (Jones et al., 2008). Moreover, the BSH alleles detected in human microbiota were markedly different in other environments such as the murine gut. The host species-specific selection of microbial BSH activity may indicate host species-specific functional differences in BSH activity (Jones et al., 2008). Interestingly, conjugated bile acids are toxic to bacteria, particularly at low pH, and are proposed to influence the growth of bacteria in different regions of the GI tract (Islam et al., 2011). The presence of BSH can thus confer a protective effect for some bacterial species through bile acid deconjugation. Several studies have also shown that BSH is advantageous for bacterial colonisation (Bateup et al., 1995; Begley et al., 2005b; De Smet et al., 1995; Delpino et al., 2007).

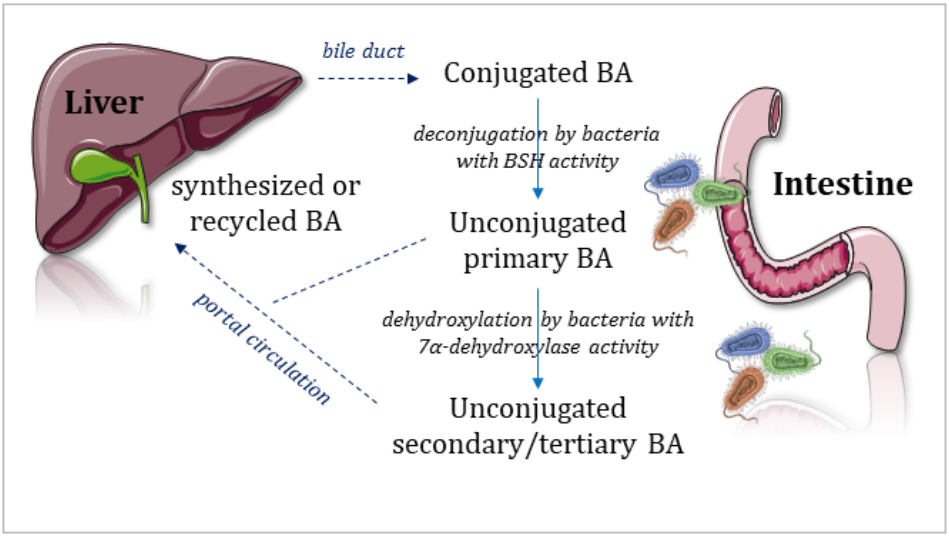


Figure 1.4 The gut microbiota is a key metaboliser of bile acids. *Bacteria with bile salt hydrolase (BSH) and 7α-dehydroxylase activity play a key role in the metabolism of bile acids from conjugated bile acids to primary, secondary and tertiary unconjugated bile acids. The majority of bile acids are then recycled through the portal circulation.*

Once BSH enzymes have liberated bile salts from the taurine or glycine amide they are now accessible to microbial enzymes for further metabolism (**Figure 1.4**). The subsequent bile acid modifications are performed mainly by anaerobes in the lower intestine and they include re-amidation (Ridlon and Hylemon, 2012), oxidation-reduction reactions (Ridlon et al., 2006), esterification and desulfatation reactions. 7α-dehydroxylation, or removal of and hydroxyl (OH) group at the C7 position, is critical for secondary and tertiary bile acid formation and leads to the generation of deoxycholic acid (DCA) from cholic acid (CA), while chenodeoxycholic acid (CDCA) is converted to lithocholic acid (LCA) (Ridlon et al., 2006). In the hepatocyte, secondary bile acids may have different fates; DCA and LCA may be conjugated to glycine or taurine and allowed to circulate with other conjugated primary bile acids, otherwise LCA can be altered by CYP3A4 to generate hyodeoxycholic acid (HDCA) (Xie et al., 2001). Alternatively, LCA may also be converted by *Clostridium* species to the beneficial hydrophilic bile acid ursodeoxycholic acid (UDCA) (Béguet et al., 2004; Hofmann and Hagey, 2008). CA can also be converted to UDCA in a two-step process involving C7α to β epimerization and 12αHSDH oxidation. These activities

are distinct to some members of the *Clostridia*, *Bacteroides* and *E. coli* (Braun et al., 2011).

1.4.3 Bile acids shape the structure of the gut microbiota

Studies on germ-free rodents have demonstrated that microbiota-free animals have more bile acids than conventionally colonised animals (Gustafsson et al., 1957; Sayin et al., 2013; Swann et al., 2011; Wostmann, 1973), suggesting a dynamic interplay between host bile acid composition and gut microbiota. Indeed, the level of bile acids in the intestine can influence the profile of the gut microbiota. Cholic acid (CA) administration in mice promoted the synthesis of antimicrobial bile acids that altered the microbial composition and increased the levels of the more tolerant phylum Firmicutes (Islam et al., 2011). Within this phylum, *Clostridia* and *Erysipelotrichia* were significantly increased along with increased conversion of cholic acid to deoxycholic acid (Islam et al., 2011). This study suggests a potential for bile acids to select bacterial populations with subsequent impact on bile acids signatures (Yokota et al., 2012). Feeding on a diet enriched in saturated milk-derived fats to Il-10 knock-out mice led to enhanced synthesis of tauro-conjugated bile acids which promoted the outgrowth of *Bilophila wadsworthia*, a potential pathobiont associated with gut inflammation. The inflammatory effect was not seen in mice fed a diet rich in vegetable-derived fats (Devkota et al., 2012). Intriguingly, we have recently shown that alterations in the microbial composition in a mouse model of autism were accompanied by reduced bile acid metabolism in the intestine (Golubeva et al., 2017).

1.5 Intestinal Barrier Integrity and the Gut Microbiota

The intestinal tract represents the body's largest interface with the external environment and it has two main functions: as an absorption site, it allows movement of nutrients from the lumen into circulation; as a barrier, it prevents the translocation of harmful entities including microorganisms. Barrier defences include both

immunogenic mechanisms such as immunoglobulins and mucosal lymphocytes as well as non-immunogenic mechanisms such as selective intestinal permeability. Tight junctions, also known as occluding junctions, are multiprotein junctional complexes which prevent the leakage of molecules, transported solutes and water through the intestinal wall. The opening of tight junctions can cause an increase in intestinal permeability which, in turn, acts as a trigger for detrimental effects on distal organs in the host (**Figure 1.5**) (Fasano, 2011; Leonard et al., 2017; Suzuki, 2013). The passage of microbes, microbial products, and antigens into the mucosa can result in activation of the immune system and secretion of inflammatory mediators (Márquez et al., 2016). Increased intestinal permeability has been reported in several diseases including Crohn's disease (Teshima et al., 2012), celiac disease (Heyman et al., 2012), diabetes (Cox et al., 2017), rheumatoid arthritis (Bjarnason et al., 1984) and schizophrenia (Severance et al., 2015), among others.

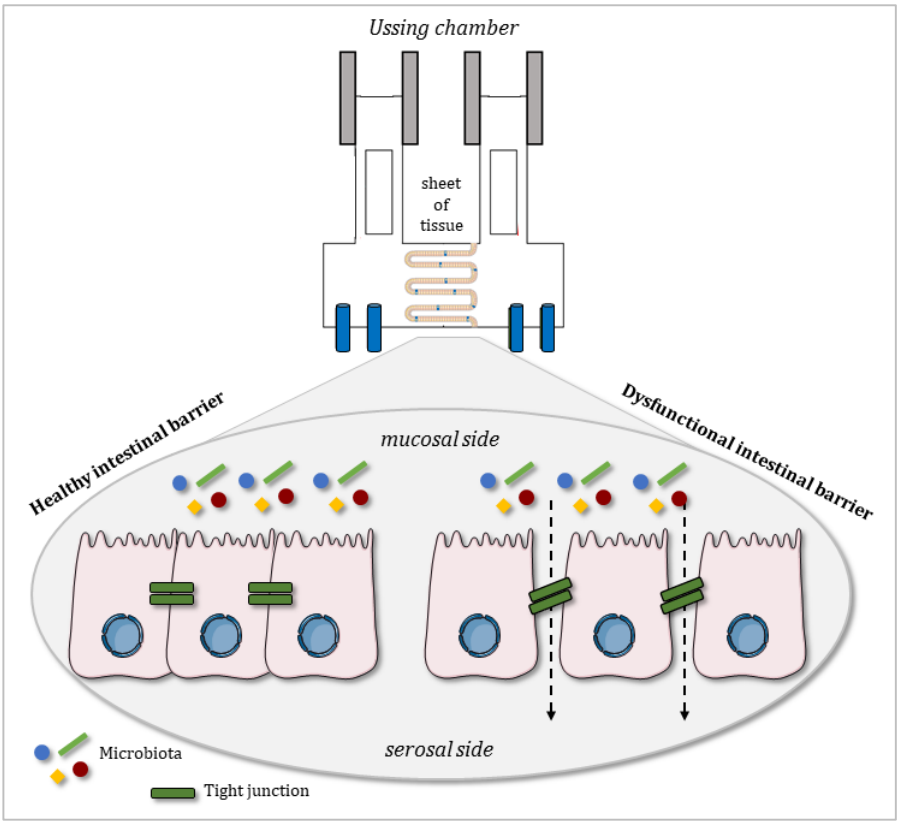


Figure 1.5 Intestinal barrier function. *In physiological conditions, the gastrointestinal epithelium is tightly impermeable. A malfunction in tight junction functionality might increase the leakiness of the barrier. In this condition, bacteria and their products can translocate from the mucosal side to the serosal side, with detrimental consequences for the host. The Ussing chamber apparatus allows to detect changes in intestinal permeability.*

1.5.1 Regulators of intestinal permeability

Intestinal permeability is a highly regulated dynamic process and the main regulators of permeability are:

- Mast cells: substances released from mast cells during degranulation can mediate epithelial and endothelial permeability (Berin et al., 1998; Wershil, 2000). Mediators of mast cells activation include allergens, physical injury, microbial pathogens and various compounds through their associated G-protein coupled receptors (e.g., morphine through opioid receptors) or ligand-gated ion channels (da Silva et al., 2014; Moon et al., 2014).
- Intracellular pathways: nitric oxide (NO) appears to be an important intracellular mediator for regulating the normal physiology of the GI tract. Several *in vivo* and *in vitro* studies have indicated that a low level of NO is important for maintenance of normal mucosal barrier function. Overproduction of NO has been associated to abnormal barrier function (Alican and Kubes, 1996; Salzman et al., 1995).
- Heredity: many hereditary immunological defects might affect intestinal barrier function. Disrupted intestinal permeability was reported in 18% of first-degree relatives and 23% of the spouses of patients with Chron's disease as compared to 3% in controls (Soderholm et al., 1999). The authors of this study suggested that baseline permeability was determined by environmental factors, whereas permeability provoked by acetylsalicylic acid was a function of the genetically-determined state of the mucosal barrier.
- Diet, psychological stress, oxidative stress, exercise, aging and the use of medications: all of these factors have been shown to mediate changes in baseline intestinal permeability (Barau and Dupont, 1990; Deitch et al., 1995; Keshavarzian et al., 1992; Ma et al., 1992; Pals et al., 1997; Saunders et al., 1994; Sigthorsson et al., 1998; Wilson and Baldwin, 1999).
- Gut microbiota: interactions between the gut microbiota and the intestinal barrier might involve changes in permeability. I will discuss this point in the following section.

A growing body of evidence supports the notion that commensal microbes can, directly or indirectly, modulate intestinal permeability. Rats with a stable mixed

aerobic and anaerobic microbiota showed higher intestinal permeability than rats on antibiotics (Garcia-Lafuente et al., 1998) and this was confirmed in a subsequent study in rats where colonisation with *Escherichia coli*, *Klebsiella pneumoniae* and *Streptococcus viridans* significantly increased the lumen to blood clearance of mannitol. On the contrary, colonisation with *Lactobacillus brevis* had the opposite effect and reduced permeability (Garcia-Lafuente et al., 2001). It is still unclear whether these changes in intestinal permeability are the result of direct bacterial action or are mediated by immune mechanisms. In a study using *in vitro* ileal mucosa, the bacterial endotoxin lipopolysaccharide (LPS) caused increased intestinal permeability and bacterial translocation (Go et al., 1995). This effect appeared to be mediated by up-regulation of inducible nitric oxide synthase activity (iNOS) as it was inhibited by iNOS inhibitors (Mishima et al., 1999). As mentioned earlier, immune system activation by luminal bacteria or their products might be a major player in modulating permeability. Release of inflammatory mediators and cytokines can modify intestinal permeability directly or indirectly. For example, in an intestinal monolayer model, interferon directly increased permeability by affecting tight junctions (Madara and Stafford, 1989). Furthermore, neutrophil transmigration induced by the bacterial peptide N-formylmethionyl-leucyl-phenylalanine (FMLP) resulted in a reversible increase in the permeability of an intestinal epithelial monolayer (Nash et al., 1987). More and more evidence supports a role for the microbiota in regulating intestinal permeability.

1.5.2 Measuring intestinal permeability *ex-vivo*

The Ussing chamber technique is a simple and reliable method for investigating intestinal permeability to both macromolecules and ions (Ussing and Zerahn, 1951).
For the assessment of macromolecular diffusion across the intestinal epithelium, a fluorescent tracer flux measurement is performed. This allows measurement of the steady-state rate of transfer of the tracer (e.g. 4 kDa FITC-dextran) across the epithelium from the luminal bath to the basolateral bath. The intestinal section is opened and oriented as a flat sheet to separate the two halves of the chamber. The

intestinal preparation is situated vertically such that the mucosal membrane is facing one chamber half, whereas the serosal membrane the other half-chamber, thus separating the solutions that independently bath each chamber half. The reservoirs above each chamber are water jacketed to enable warming to 37 °C. At each timepoint, samples are collected from the basolateral bath and the amount of FITC-dextran is measured in a spectrophotometer at 485nm excitation / 535nm emission wavelengths. The baseline level of FITC (timepoint zero) is ultimately subtracted from each of the following timepoints (**Figure 1.5**, **Figure 1.6**).

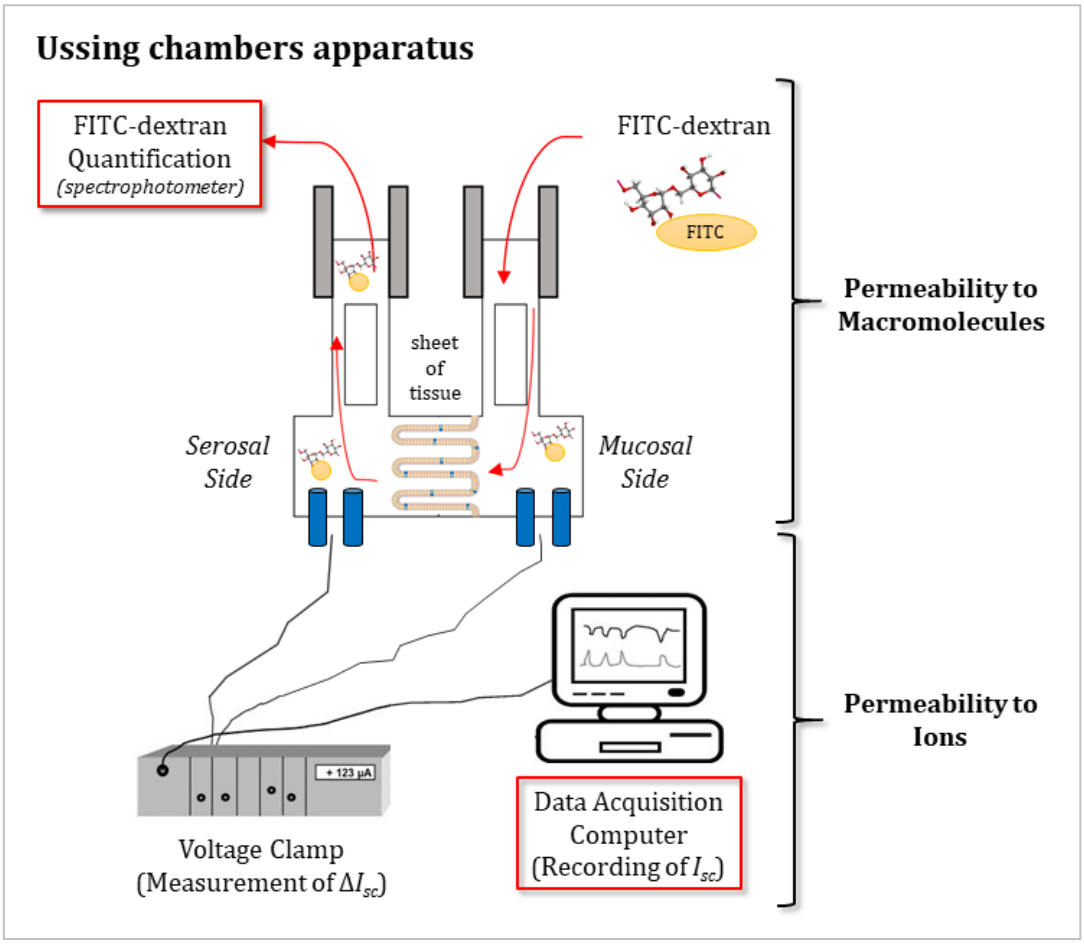


Figure 1.6 Schematic drawing of the Ussing chamber apparatus. *<u>Permeability to macromolecules:</u> A piece of intestine is mounted in the Ussing chamber and FITC-dextran is added to the mucosal compartment. Quantification of FITC in samples collected from the serosal chamber, at different timepoints, provides a readout of permeability to macromolecules. High levels of FITC-dextran in the serosal compartment are associated with loose barrier function. <u>Permeability to ions:</u> In the same apparatus, a 5mV voltage clamp allows for the detection of short-circuit current (I_{sc}). Transepithelial electrical resistance (TEER) is calculated from ΔI_{sc} and voltage, according to Ohm's law. An increase in TEER is associated with tighter barrier function.*

For the assessment of permeability to ions, transepithelial electrical resistance (TEER) is calculated through Ohm's law. In a "leaky" epithelium, the ionic conductance through the paracellular pathway, in contrast to the transcellular pathway, accounts for > 90% of the total transepithelial ionic conductance (Frizzell and Schultz, 1972). Hence, changes in transepithelial ionic conductance can signal untoward effects on the tissue integrity. Changes in the transcellular conductance are usually difficult to detect with the Ussing chambers. Ion conductance through the paracellular pathway of the epithelium is limited by both the tight junctional complex and the relative apposition of the basolateral membranes of adjacent epithelial cells, which determines the volume of the surrounding aqueous column. A constant 5mV voltage is applied and the resulting change in current is measured, an approach that is generally called "voltage clamping". The short-circuit current (I_{sc}) is defined as the charge flow per time when the tissue is short-circuited (**Figure 1.6**).

For the measurement of permeability to both macromolecules and ions, seromuscular stripping of the intestinal preparation might be performed for two main reasons:

1) the seromuscular layers represent a significant diffusion barrier to experimental drugs/isotopes and to nutrients/oxygen, which reduces the viability of the intestinal preparation
2) whole-thickness intestinal preparations undergo rhythmical neuromuscular contractions that produce corresponding changes in the transepithelial voltage potential and thus I_{sc} by physiological means.

The Ussing chamber technique provides a short-term organ culture method that allows precise measurement of electrical and transport parameters of intact, polarised intestinal epithelium. The principal weakness of the Ussing chamber technique lies in the interpretation of a relatively small number of measurements to describe the complex physiological system of the intestinal mucosa. The intestinal mucosa contains many cell types communicating through a variety of systems, which may not be differentiated utilising Ussing chamber measurements. Another minor concern with the Ussing chamber method relates to the limited viability and optimal function of an

ex vivo intestinal preparation, considering that the tissue can be examined for maximum a maximum of three hours, following which it starts degrading.

1.6 Drugs and the Gut Microbiota

The role of the microbiota in health and disease has stretched to all disciplines of medicine and this now includes pharmacology and therapeutics (Walsh et al., 2018).

The field of pharmacomicrobiomics has emerged over the past decade (ElRakaiby et al., 2014; Saad et al., 2012) and has predominantly focused on the impact that the gut microbiota exerts on drug metabolism. A recent investigation *in vitro* assessed the ability of 76 different human gut bacteria to metabolize 271 oral drugs and found that two thirds of the drugs tested were significantly reduced by at least one bacterial strain and that each strain metabolized 11-95 different drugs (Zimmermann et al., 2019a). Also, a growing body of research has demonstrated that several pharmaceutical compounds including paracetamol, digoxin, metformin and cancer drugs among others, influence the human gut microbiota and/or microbial isolated strains. As bacteria can, in turn, modulate drug efficacy and toxicity (Alexander et al., 2017; Currò, 2018; Kelly et al., 2016), the emerging drug-microbe bidirectional interaction might be crucial for future drug development and clinical practice. Moreover, this suggests that drug-related confounding effects should be taken into consideration in future microbiome association studies (**Figure 1.7**).

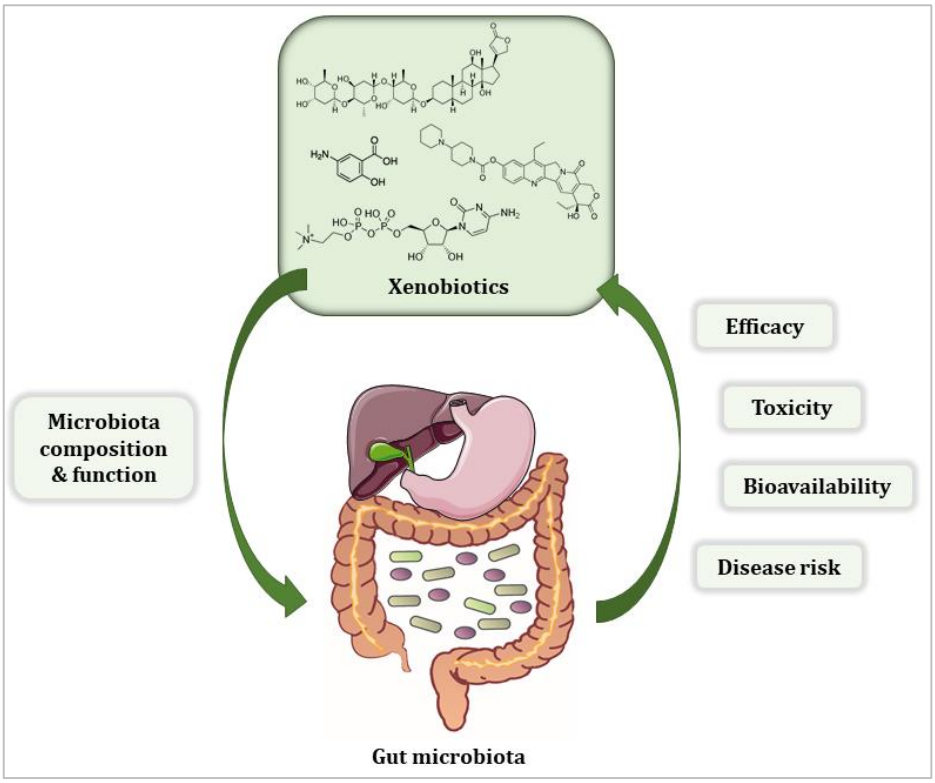


Figure 1.7 The bidirectional link between xenobiotics and gut microbiota. *The gut microbiota alters the chemical structures or the absorption rate of ingested compounds, including dietary components, industrial chemicals, and drugs. These changes affect xenobiotic toxicity, efficacy, bioavailability and disease risk. The microbial enzymes responsible for such transformations are poorly understood. Several xenobiotics alter the composition and function of gut microbiota although the precise mechanisms remain poorly understood. In some instances, xenobiotics display direct antimicrobial activity themselves.*

1.7 Drugs Affect the Gut Microbiota

Antibiotics represent the most direct and effective way of targeting intestinal microbes. Evidence gathered from *in vitro* and *in vivo* studies suggests that a course of short-term antibiotics can substantially change the gut microbiota composition (Jakobsson et al., 2010; Maurice et al., 2013). Several host-targeting non-antibiotic drugs have also been shown to influence the gut microbiota. In a population-based cohort, deep sequencing of gut microbiomes of 1,135 participants showed relations between the microbiota and 19 drug groups (Zhernakova et al., 2016). Other studies have pointed out an association between drug consumption and the microbiome. Analysis of two independent population-level cohorts revealed that, among different

factors, the use of medications was responsible for the largest total variance and interacted with other covariate-microbiota associations (Falony et al., 2016). The composition of the gut microbiota can change in relation to the number and type of medications consumed. Differences in the relative abundance of specific bacteria were detected in individuals taking a single drug, a combination or none. In particular, there were differences in the gut microbiota of individuals taking NSAIDs (nonsteroidal anti-inflammatory drugs) with PPIs (proton-pump inhibitors) versus those taking NSAIDs without PPIs (Rogers and Aronoff, 2016). Regarding polypharmacy, in elderly hospitalised patients there was a significant negative correlation between the number of drugs and microbial alpha-diversity (Chao1 index). Moreover, the number of drugs was associated with the average relative abundance of 15 different taxa, with PPIs, antidepressants and antipsychotics exhibiting the strongest association with single bacteria abundance (Ticinesi et al., 2017).

1.8 The Gut Microbiota Affects the Pharmacokinetics of Drugs

Pharmacokinetics (from the Greek root *pharmakon* = drug and *kinetikos* = moving, “putting in motion") is a branch of pharmacology dedicated to determining the fate of xenobiotics administered to a living organism. Absorption is one of the four compartments of the pharmacokinetics multi-compartmental model (Arundel, 1997), together with distribution, metabolism and excretion (ADME) (Pacey et al., 2011).
In the next two sections, we provide some of the most compelling evidence on the interaction between gut microbiome and drug absorption/metabolism prior to discussing the relevance to psychotropic compounds.

1.8.1 The gut microbiota affects drug absorption

In pharmacology, absorption is the movement of a substance from the site of administration to the bloodstream (Doogue and Polasek, 2013). Very little is currently known about the role played by the gut microbiota in drug absorption but a few reports on the topic exist. It is interesting to note that all the three studies mentioned in this section use the same experimental approach: manipulation of the gut microbiota through administration of probiotics.

The action of **gliclazide**, a sulfonylurea used to treat diabetes, may be enhanced by administering probiotics. In diabetic rats, the blood levels of gliclazide are higher following a 3-days pre-treatment with probiotics (at the dose of 75 mg/kg) compared to non-treated rats, suggesting that the gut microbiota might mediate the extent of the drug absorption (Al-Salami et al., 2008). In a recent study, a three-day administration of *Lactobacillus reuteri* K8 reduced the absorption of orally administered **acetaminophen** in mice, whereas administration of *Lactobacillus reuteri* K9 did not have an effect (Kim et al., 2018). This effect was probably mediated by probiotic-induced modulation of gut microbial enzyme activity given that the probiotic significantly increased both sulfatase and arylsulfate transferase and significantly decreased β-glucuronidase, which are the bacterial enzymes involved in acetaminophen metabolism. Finally, the antiarrhythmic drug **amiodarone** showed elevated blood levels following administration of probiotics in rats. In detail, the probiotic *E.coli* strain Nissile 1917 was administered to rats for 7 days, followed by a single dose of amiodarone *per os*. The probiotic increased amiodarone plasmatic levels by 43% compared to saline-treated controls, suggesting a microbiota-mediated increase in drug absorption (Matuskova et al., 2014).

1.8.2 The gut microbiota affects drug metabolism

The fate of xenobiotics depends not only by the host but also by the bacteria harbouring our gastrointestinal tract and it has become more investigated, over the past decades, the role of gut microbiome in xenobiotic metabolism. The whole field has

been termed “pharmacomicrobiomics” (Rizkallah et al., 2010). In this paragraph, we offer a glimpse into the known effects of the gut microbiota on drug metabolism.

Digoxin, a cardiac glycoside that has been widely used for hundreds of years to treat heart failure and arrhythmias, is a striking example. This drug is inactivated in the gut by the Actinobacterium *Eggerthella lenta* (Haiser et al., 2013). Moreover, increased consumption of dietary protein in germ-free mice inhibited the reduction of digoxin by *E. lenta* (Haiser et al., 2013). The microbial biotransformation of orally administered **lovastatin**, a drug used for lowering cholesterol levels and reduce the risk of cardiovascular disease, was reduced by concomitant administration of antibiotics in rats (Yoo et al., 2014). This could result in altered systemic concentrations of either the intact drug and/or its metabolites (Yoo et al., 2014). **Amlodipine**, a medication used to treat high blood pressure and coronary artery disease, undergoes clearance when incubated with a faecal suspension, suggesting that the gut microbiota metabolises this drug (Yoo et al., 2016). As a confirmation, a two-days treatment with the antibiotic ampicillin in rats increases the plasma levels of amlodipine, possibly because of the decreased microbial biotransformation in the gastrointestinal tract (Yoo et al., 2016). **Mesalazine**, also known as 5-aminosalicylic acid (5-ASA), is an anti-inflammatory drug used to treat inflammatory bowel disease, including ulcerative colitis or to maintain remission in Crohn's disease (Rachmilewitz, 1989). The faecal microbiota plays a key role in acetylating 5-ASA, with 44% of anaerobic bacteria tested in incubation with the drug exhibiting this property (van Hogezand et al., 1992). The metabolism of **sulfasalazine**, a drug used for the treatment of rheumatoid arthritis, ulcerative colitis and Crohn's disease, is also likely to be mediated by intestinal bacteria. When sulfasalazine was fed to conventional rats, none of the drug was recovered in the urine, faeces or caecum; however if administered in combination with the antibiotic neomycin, the drug was recovered in faeces and caecum (Peppercorn and Goldman, 1972). In addition, when sulfasalazine was given to germ-free rats, recovery of drug in the faeces was over 50% whereas the urine contained an additional 1-2%. In germ-free rats infected with four specific bacteria normally found in the intestinal tract of rodents, sulfasalazine was metabolised as in conventional rats (Peppercorn and Goldman, 1972). Sulfasalazine is metabolised by azoreductases in the gut. The probiotic strains *Lactobacillus acidophilus* L10,

Bifidobacterium lactis B94 and *Streptococcus salivarius* K12 given to rats for three consecutive days increased azoreductase activity in *ex vivo* colon contents with a corresponding increase in sulfasalazine metabolism (Lee et al., 2012). Interestingly however, the same probiotic treatment in rats, followed by an oral 100 mg/kg dose of sulfasalazine, did not alter the pharmacokinetic parameters (Lee et al., 2012). Administration of **diclofenac**, a NSAID, induced enteropathy in mice; however, oral pre-treatment with a bacteria-specific β-glucuronidase inhibitor was able to protect against diclofenac-induced enteropathy (LoGuidice et al., 2012), suggesting that the gut microbiota might play a crucial role in the metabolism of this medication. The antithrombotic effect of **aspirin** seems to be affected by the gut microbiota. In rats, administration of the antibiotic ampicillin significantly prolongs the bleeding time in aspirin-dosed rats (Kim et al., 2016). Moreover, oral administration of ampicillin reduces the aspirin-metabolizing activity of the microbiota by 67% (Kim et al., 2016).

Intestinal microbial azoreductases play a key role in the reduction of **azo dyes** (Chung et al., 1992). A wide variety of anaerobic bacteria isolated from caecal or faecal contents from experimental animals and humans have the ability to cleave the azo linkages to produce aromatic amines (Chung et al., 1992). Moreover, the azoreductase activity in a variety of intestinal preparations is affected by various dietary factors including antibiotics and supplementation with live cultures of lactobacilli (Chung et al., 1992).

Choline and carnitine are dietary amines that have wide-ranging roles in human metabolism (Zeisel and da Costa, 2009) and are precursors of trimethylamine (TMA), a compound that can cause trimethylaminuria when not appropriately metabolised by the host (Mackay et al., 2011). In a recent study, *the* quantification and detailed characterization of the TMA-producing bacteria in human faecal samples have resulted particularly in *Clostridium* XIVa strains and *Eubacterium* sp. strain AB3007 (Rath et al., 2017). In a different study, carnitine metabolism was mediated by Rieske-type oxygenases present in the human microbiota (Zhu et al., 2014).

Chemotherapeutic drugs have also been shown to be metabolised by the gut microbiota (Alexander et al., 2017). Of 30 chemotherapeutic drugs examined *in vitro*,

the efficacy of 10 was found to be significantly inhibited by certain bacteria, while the same bacteria improved the efficacy of six others (Lehouritis et al., 2015). As further corroboration of these findings, the chemoresistance or increased cytotoxicity observed *in vitro* with sample drugs (gemcitabine and CB1954) was replicated in *in vivo* murine subcutaneous tumour models (Lehouritis et al., 2015). The dose-limiting side effect of the common colon cancer chemotherapeutic **irinotecan** is severe diarrhea that arises following reactivation of the drug by symbiotic bacterial β-glucuronidases in the gut (Ma and McLeod, 2003; Mathijssen et al., 2001). Oral administration of a bacterial β-glucuronidase inhibitor protected mice from irinotecan-induced toxicity, suggesting that such inhibitors may be designed to prevent undesirable enzyme activities in the intestine (Wallace et al., 2010). The gut microbiota also plays a crucial role in the metabolism of **5-fluorouracil**, another chemotherapeutic compound (Nakayama et al., 1997). The antineoplastic drug **doxorubicin** is effectively metabolised by *Raoultella planticola in vitro,* as demonstrated by Yan and colleagues (Yan et al., 2018). Specifically, *R. planticola* was shown to deglycosylate doxorubicin into its metabolites 7-deoxydoxorubicinol and 7-deoxydoxorubicinolone via a reductive deglycosylation mechanism. Moreover, doxorubicin was degraded anaerobically by *Klebsiella pneumoniae* and *E.coli* BW25113 *in vitro* (Yan et al., 2018). In a recent study, **5-fluorouracil** (5-FU) and **5-fluoro-2'-deoxyuridine** (FUDR) were found to act through bacterial ribonucleotide metabolism to elicit their cytotoxic effects in *Caenorhabditis elegans* (Garcia-Gonzalez et al., 2017), suggesting that bacteria in the host play an important role in the response to chemotherapeutics. Similar findings were also obtained in a different study (Scott et al., 2017b). Finally, a recent study found that the anticancer immune effects of **cyclophosphamide** are modulated by the gut microbiota. Indeed, the changes induced by this chemotherapeutic on the gut microbiota stimulate the generation of a specific subset of "pathogenic" T helper 17 cells and immune responses typically associated to this medication (Viaud et al., 2013).

Interestingly, two studies have also highlighted a role for the microbiome in patients undergoing anti-programmed cell death 1 protein (PD-1) **immunotherapy** (Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018). The diversity and composition of the microbial community differed between responders and non-

responders, accompanied by functional differences in gut bacteria in responders (including enrichment of anabolic pathways) (Gopalakrishnan et al., 2018). In the same study, immune profiling suggested enhanced systemic and antitumor immunity in responding patients with a favourable gut microbiome as well as in germ-free mice receiving faecal transplants from responding patients (Gopalakrishnan et al., 2018). Resistance to immunotherapy can be attributed to abnormal gut microbiome composition, according to a different study. Antibiotics administration inhibited the clinical benefit of immunotherapy in patients with cancer; moreover, faecal microbiota transplantation (FMT) from cancer patients into germ-free mice ameliorated the antitumor effect only when the donor was a responder, whereas FMT from nonresponding patients failed to do so (Routy et al., 2018).

While a scarce knowledge exists on the link between the microbiome and drug absorption /metabolism, this topic assumes high clinical relevance, considering that changes in absorption and metabolism can correspond to alterations in drug efficacy and toxicity. There are no studies so far exploring the effects of microbial perturbations on psychotropic drug pharmacokinetics and more research is warranted, especially considered that several psychotropics have been shown to alter the gut microbiota composition (see following sections). Overall, the growing evidence underlies a fascinating interaction between intestinal bacteria and drug efficacy (**Figure 1.8**), suggesting that precision medicine strategies should include the intestinal microbiota as a potential treatment modifier (Jobin, 2018).

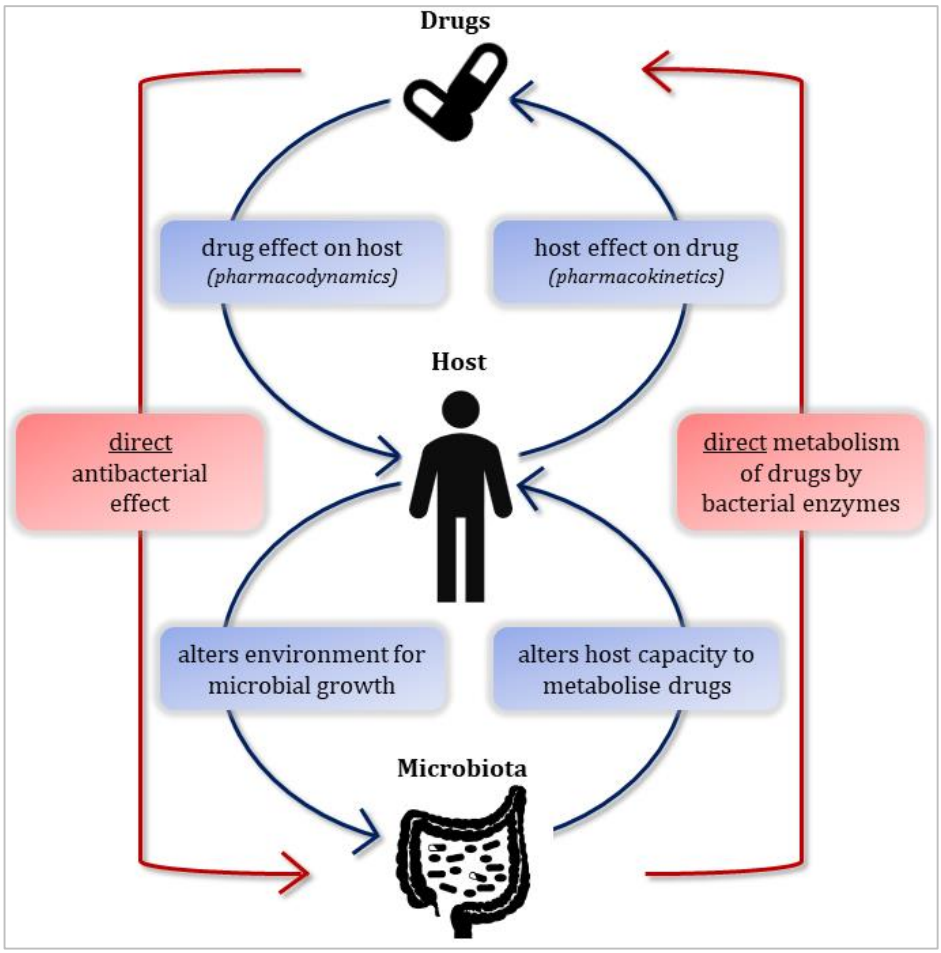


Figure 1.8 The complex interaction between drugs and gut microbiota. *Drugs influence the host through their mechanism of action (pharmacodynamics) and vice versa the host influences the fate of the drugs (pharmacokinetics). In this bidirectional interaction, the gut microbiota plays both a direct (red arrows) and an indirect (blue arrows) role. Drug → Microbiota interactions (left panel): Drugs can directly influence the microbiota through antimicrobial activity (i.e. SSRIs). Drugs can also alter the physiological properties of host organs (i.e. PPI-mediated alterations of pH), which in turn might affect the microbiota composition. Microbiota → Drug interactions (right panel): The microbiota can directly metabolise drugs through bacterial-derived enzymes. The microbiota can also alter the host capacity to metabolise drugs, which in turn might affect the fate of the drug. The precise mechanisms supporting the complex interplay between drugs and gut microbiota are not yet fully elucidated. Figure adapted from (Walsh et al., 2018).*

1.9 Psychotropic Drugs and the Gut Microbiota

In this thesis, I will focus on psychotropic compounds (from the Greek root *psychè* = mind and *tropòs* = turning), which modulate brain and behaviour, and I will explore the scientific evidence on the interaction between psychotropic compounds and the gut microbiome *in vivo* or in isolated strains (*in vitro)* (**Figure 1.9**). For each class of psychotropic compound taken into consideration, sub-sections will be based on the experimental approach used (observations *in vitro*, *in vivo* or in humans). Regarding *in vitro* experiments, some attempts have been made to try and find the best dose translational to the human gut setting. Maier and colleagues have deduced colon concentrations on the basis of drug excretion patterns from published work, and small intestine concentrations on the basis of daily doses of individual drugs. Based on their approximations, a threshold of 20 μM was below the median small intestine and colon concentration of the majority of human-targeted drugs (Maier et al., 2018). It is important to keep this in mind when considering data generated from *in vitro* isolated microbial strains (see **Table 1.1** in *Appendix*).

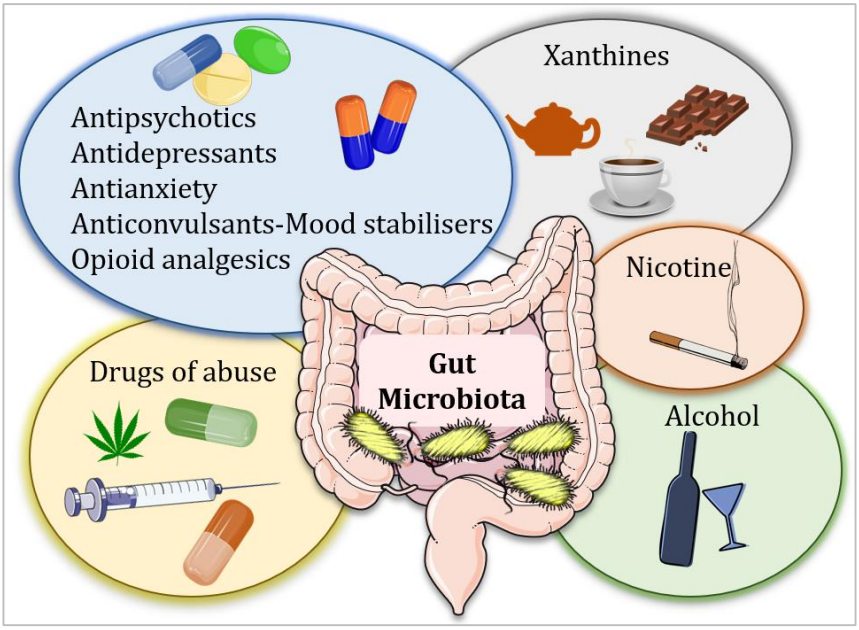


Figure 1.9 Psychotropic compounds affect the gut microbiota composition. *Not only psychotropic medications but also other psychoactive compounds can influence the microbiome either directly or indirectly. Figure from (Cussotto et al., 2019a).*

1.10 Antipsychotics and the Gut Microbiota

Antipsychotics are drugs used for the prophylaxis and acute treatment of psychotic illnesses including schizophrenia and psychosis associated with depression and mania (Gardner et al., 2005). They also have an important role as an alternative or adjunct to benzodiazepines in the management of the acutely disturbed patient, for both tranquillisation and sedation. The common mechanism of action of all antipsychotics is to decrease brain dopamine function by blocking the dopamine D_2 receptors (Laruelle et al., 2005).

Analysis of faecal microbiota from 76 elderly hospitalised patients showed that, among several therapeutic classes, the use of antipsychotics had a strong association with gut microbiota composition (Ticinesi et al., 2017). In a recent study, differences in faecal microbiota between patients with first-episode psychosis and healthy controls, were associated with response after up to 12 months of treatment (Schwarz

et al., 2018), suggesting that the gut microbiota might be involved in treatment response. Specifically, *Lactobacillaceae* and *Bifidobacteria* were highly abundant in patients with first-episode psychosis and correlated positively with severity of psychotic symptoms and negatively with global functioning (Schwarz et al., 2018). In a tour de force *in vitro* screening study of more than 1,000 drugs against 40 representative gut bacterial strains, it was found that 24% of human-targeting drugs inhibited the growth of at least one strain (Maier et al., 2018). Provocatively, nearly all subclasses of the chemically diverse antipsychotics targeted a significantly more similar pattern of species than expected from their chemical similarity, raising the possibility that antimicrobial action may not only manifest as side effect of antipsychotics, but also be part of their mechanism of action (Maier et al., 2018). This hypothesis should be ideally verified by assessing whether microbiome manipulations (i.e. antibiotic administration) have an effect on the efficacy of antipsychotics. Remarkably, the Maier *et al.* study provides an exhaustive justification for the dose used in the *in vitro* screening. The authors argue that, based on drug excretion patterns from published work, the chosen concentration of 20 μM is below the median colon concentration of the human-targeted drugs tested (Maier et al., 2018) and therefore has translational validity. Notably, in their experiment, human-targeted drugs that showed anticommensal activity had lower plasma and estimated small intestinal concentrations than ones with no such activity, suggesting that more human-targeted drugs would inhibit bacterial growth if probed at higher doses, closer to physiological concentrations. In a recent study *in vitro,* the antibacterial activity of antipsychotics against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii was investigated.* Phenothiazines and thioxanthenes showed differential antibacterial activity at concentrations ranging from 64 to 1024 μg/mL, which was independent of antibiotic-resistance patterns (Nehme et al., 2018). How these concentrations translate to those found in the colon following an oral administration of antipsychotics in humans is not clear and is not mentioned in the study, thus these findings might lack translational relevance.

1.10.1 Typical antipsychotics and the gut microbiota

Typical (first-generation) antipsychotics were first developed in the 1950s and the first compounds to come into medical use were the phenothiazines, such as chlorpromazine. Typical antipsychotics are characterised by extrapyramidal adverse effects such as dystonia, Parkinsonian symptoms (bradykinesia, rigidity and tremor), akathisia, tardive dyskinesia, cardiovascular effects such as postural hypotension, prolactin increase and sedation (Leucht et al., 2009).

Evidence from *in vitro* studies

Studies on the interaction between typical antipsychotics and gut bacteria have been only carried out *in vitro*. Other approaches, such as *in vivo* or human observations, are missing from literature, and one reason for this gap might be more and more consideration is directed towards the new class of antipsychotics, the atypical (Skonieczna-Zydecka et al., 2018).

Thioridazine, a phenothiazine antipsychotic, has been shown to possess antimicrobial activity *in vitro* against methicillin-susceptible *S. aureus* (Hahn and Sohnle, 2014; Ordway et al., 2002b), vancomycin-resistant pathogenic strains of *Enterococcus* species (Wainwright et al., 1999), *Mycobacterium tuberculosis* (Amaral et al., 1996; Bettencourt et al., 2000; Ordway et al., 2003; Viveiros and Amaral, 2001), *Pseudomonas aeruginosa* and *Mycobacterium avium* (Viveiros et al., 2005). **Fluphenazine**, another typical antipsychotic, possesses pronounced action against both Gram-positive and Gram-negative bacteria at concentrations of 20-100 μg/mL (Dastidar et al., 1995). Upon investigation of the antimicrobial activity of **trifluoperazine** against 293 strains (from two Gram-positive and eight Gram-negative *genera),* 46 of 55 strains of *S. aureus* were inhibited by doses of 10-50 μg/mL. This drug also inhibited strains of *Shigella* spp., *Vibrio cholerae* and *V. parahaemolyticus* at concentrations of 10–100 μg/mL (Mazumder et al., 2001). *Bacillus* spp. and *Staphylococcus* spp. were strongly inhibited by the antipsychotic **prochlorperazine**; while *E.coli, Salmonella, Klebsiella* and *Pseudomonas* were only moderately sensitive

or resistant to the drug (Rani Basu et al., 2005). **Chlorpromazine**, another typical antipsychotic, had *in vitro* antimycobacterial properties (Kristiansen and Vergmann, 1986; Molnar et al., 1977) and also exerted an inhibitory synergistic effect in combination with certain antibiotics (Amaral et al., 1992). Moreover, this medication has been shown to inhibit significantly the growth of *S. aureus* (Ordway et al., 2002a) and *E.coli* (Amaral and Lorian, 1991; Csiszar and Molnar, 1992). Keeping in mind that these data come from *in vitro* bacterial cultures, it is important to remark that a direct extrapolation and translation into the gut microbiome scenario is not always possible.

1.10.2 Atypical antipsychotics and the gut microbiota

Atypical antipsychotics act on numerous receptors and modulate several interacting transmitter systems. All atypicals (except amisulpride) exhibit greater antagonism of 5-HT_2 receptors than of D_2 receptors, compared with the typical agents. Atypical drugs that do antagonise dopamine D_2 receptors appear to have affinity for those in the mesolimbic system rather than the nigrostriatal system, producing side effects of lesser degree. Clozapine and risperidone exert substantial antagonism of α_2-adrenoceptors, while aripiprazole is a unique drug because it is a partial dopamine D_2-receptor agonist that acts conversely as an antagonist in regions where dopamine is overactive, such as the limbic system (Bennett and Brown, 2008).

Evidence from *in vitro* studies

The effect of **olanzapine** on the growth of two commensal bacterial strains, *E. coli* NC101 and *Enterococcus faecalis* OGIRF was assessed *in vitro* across a range of supraphysiologic concentrations (280 to 560 μg/mL). Olanzapine completely inhibited the growth of *E.coli* at concentrations above 537 μg/mL, while it did not affect the growth of *E. faecalis* (Morgan et al., 2014).

Evidence from *in vivo* studies (rodents)

Most of the studies performed *in vivo* have been focusing on two atypical antipsychotics, olanzapine and risperidone. Administration of **olanzapine** for 3 weeks in rats was able to induce specific alterations of the microbiota profile in both males and females (Davey et al., 2012). Moreover, administration of olanzapine in mice exacerbated the weight gain induced by high-fat diet (Morgan et al., 2014). Interestingly, this effect was absent under germ-free conditions but emerged quickly upon microbial colonization of the gut, suggesting that gut microorganisms might be necessary for the common adverse effect of olanzapine, weight gain (Morgan et al., 2014). As a proof of concept, the impact of antibiotics on olanzapine-induced weight gain was also demonstrated. Coadministration of an antibiotic cocktail in female rats treated with 2 mg/kg of olanzapine for 21 days, attenuated body weight gain, uterine fat deposition, macrophage infiltration of adipose tissue and plasma free fatty acid levels, all of which were increased by olanzapine alone (Davey et al., 2013). More recently, one last experiment has looked at microbiota changes and olanzapine administration. In this case, the prebiotic B-GOS (bimuno galactooligosaccharide) was administered to adult female Sprague-Dawley rats in coadministration with olanzapine (2-week, daily intraperitoneal injection at a dose of 10 mg/kg) and the intake of B-GOS significantly attenuated olanzapine-induced weight gain (Kao et al., 2018). Although B-GOS alone increased *Bifidobacteria* spp., and reduced species within the Firmicutes (*coprococcus*, *oscillibacter*, c. *coccoides, roseburia intestinalis cluster, clostridium XVIII cluster*) and Proteobacteria (*Escherichia/Shigella* spp.) phyla, no effects of olanzapine were observed. This is a discrepancy with other studies, maybe due to the duration and dose of olanzapine administration and/or the method of bacterial analysis. Importantly, additional studies are required to test whether the bacteria affected by B-GOS would proliferate beyond control levels with a longer duration of olanzapine administration at a clinically relevant dose. It is important to note that sex differences might play a key role in response to atypical antipsychotics and thus many studies to date have been performed in females, however more investigations are warranted in male counterparts. The impact of **risperidone** on the gut microbiota has also been investigated *in vivo*. Female mice treated with risperidone at a dose of 80 μg/day over two months exhibited significant excess weight gain, due

to reduced energy expenditure, which correlated with an altered gut microbiota (Bahr et al., 2015b). Interestingly, faecal transplant from risperidone-treated mice into naïve recipients caused a 16% reduction in total resting metabolic rate, attributable to suppression of non-aerobic metabolism (Bahr et al., 2015b). **Aripiprazole**, an atypical antipsychotic with a mode of action that is distinct from most currently available antipsychotic drugs, was able to induce marked changes in microbiota composition in rats following a 4-week treatment at 20 mg/kg/day. The relative abundance of various taxa including *Clostridium*, *Ruminiclostridium*, *Intestinibacter* and *Eubacterium coprostanoligens* was increased by aripiprazole administration (Cussotto et al., 2019b).

Evidence from studies in humans

A recent study has looked at the association between intake of atypical antipsychotics (AAP) and gut microbiota. In a cross-sectional design study, faecal samples of more than 100 bipolar patients were collected and analysed through 16S ribosomal sequencing. Participants were divided into two groups: one group AAP-treated and one group drug-free at the time of faecal sample collection. Atypical antipsychotics included in the AAP cohort were: clozapine, olanzapine, risperidone, quetiapine, asenipine, ziprasodone, lurasidone, aripiprazole, paliperidone, and iloperidone. The microbiota communities of AAP-treated and non-treated patients were significantly separated, with AAP-treated females showing decreased species diversity compared to non-AAP-treated females; while males did not show significant diversity. Three specific genera, *Lachnospiraceae*, *Akkermansia*, and *Sutterella* were differentially abundant in the two groups (Flowers et al., 2017). While this study provides critical insight into the AAP-mediated changes in gut microbiota, the report included no information regarding diet, which is an important environmental factor that drives the composition of gut microbiota. Moreover, the authors observed medication-specific microbiota differences, but it is not known how these translate into functional differences. In a cross-sectional cohort study on psychiatric patients, the effect of AAPs on the gut microbiota was examined. Although no significant differences in

microbiota composition were detected at baseline between AAP users and nonusers, non-AAP users showed an increase in the bacterial genus *Alistipes*. AAP-treated females also had decreased diversity compared with non -treated females (Flowers et al., 2019). One more human study investigated the impact of **risperidone** on gut microbiota composition. In psychiatrically ill children, chronic treatment with risperidone was associated with an increase in body mass index (BMI) and a significantly lower ratio of Bacteroidetes:Firmicutes as compared with antipsychotic-naïve psychiatric controls. Moreover, a longitudinal observation revealed a gradual decrease in the Bacteroidetes:Firmicutes ratio over the ensuing months of treatment with risperidone (Bahr et al., 2015a). Although the small sample size and the fact that polypharmacy was not taken into account, this study offers preliminary evidence that the human gut microbiome is altered in patients treated chronically with risperidone.

1.11 Antidepressants and the Gut Microbiota

Antidepressants are medications used to treat symptoms of depression, social anxiety disorder, seasonal affective disorder and mild chronic depression, as well as other conditions (Delgado, 2004). Antidepressants can be broadly divided into four main classes: tricyclic antidepressants (TCAs), selective serotonin-reuptake inhibitors (SSRIs), monoamine oxidase inhibitors (MAOIs) and novel compounds, some of which are related to TCAs or SSRIs (see SNRIs, serotonin-noradrenaline reuptake inhibitors) (Bennett and Brown, 2008). The mechanism of action is based on the "monoamine hypothesis", which proposes that the main cause of depression is a deficiency of the neurotransmitters noradrenaline (NA) and serotonin (5-HT, 5-hydroxytryptamine) in the brain. However, different classes present different mechanisms of action: SSRIs prevents 5-HT reuptake, TCAs inhibit NA reuptake but effects on 5-HT reuptake vary widely, MAOIs increase the availability of NA and 5-HT by preventing their degradation in the presynaptic terminal (Fiedorowicz and Swartz, 2004).

In a cohort of elderly subjects, intake of antidepressants was strongly associated with changes in gut microbiota composition (Ticinesi et al., 2017). A different population-level analysis of gut microbiome composition found that antidepressants were significantly correlated to microbiome composition (Falony et al., 2016). The field of antidepressants and gut microbiota is in constant expansion, but there is currently not sufficient knowledge on the effect that these drugs exert on the ecology of the gut microbiota. On the other hand, a consistent amount of research has examined the antimicrobial activity that these compounds have against various bacterial strains *in vitro*. In the following sections, evidence related to all subclasses of antidepressant compounds will be taken into consideration.

1.11.1 Tricyclic antidepressants (TCAs) and the gut microbiota

Evidence from *in vitro* studies

TCAs in general inhibit NA reuptake and some compounds can also block 5-HT reuptake to a certain extent (Horn, 1980). All studies to date looking at TCAs and microbiota have been performed *in vitro*.

Clomipramine and imipramine have been shown to possess cytotoxic effects against both human protozoan parasites *Leishmania donovani* and *Leishmania major* (Zilberstein and Dwyer, 1984). Mandal and colleagues have analysed the antimicrobial activity of **Amitriptyline** hydrochloride against 253 bacterial strains (72 Gram-positive and 181 Gram-negative) and 5 fungal strains *in vitro*. Moreover, they carried out a mortality experiment with or without amitriptyline in mice challenged with a virulent strain of *Salmonella typhimurium* (Mandal et al., 2010). Out of 254 bacterial strains, 185 were inhibited at different doses of amitriptyline, with *Staphylococcus* spp., *Bacillus* spp. and *Vibrio cholerae* being the most affected bacteria (Mandal et al., 2010). Regarding fungal strains, amitriptyline inhibited both *Cryptococcus* spp. and *Candida albicans*. Finally, in the *in vivo* experiment, amitriptyline at 25 μg/g and 30 μg/g body weight significantly protected the mice from *Salmonella typhimurium* (Mandal et al., 2010). **Promethazine and imipramine** have

been demonstrated to inhibit the growth of *E. coli* and *Yersinia enterocolitica* through interference with plasmid replication (Csiszar and Molnar, 1992; Molnar, 1988), and imipramine was also able to inhibit the parasite Giardia lamblia (Weinbach et al., 1992). **Desipramine** has been shown to be effective against Plasmodium falciparum (Basco and Le Bras, 1990; Salama and Facer, 1990).

1.11.2 Selective serotonin-reuptake inhibitors (SSRIs) and the gut microbiota

Evidence from *in vitro* studies

SSRIs, as their name indicates, act predominantly by preventing 5-HT reuptake, with little or no effect on NA reuptake (Stahl, 1998).

SSRIs have excellent activity against *Brucellae* (Muñoz-Criado et al., 1996) and they have been shown to be synergistic in combination with antibiotics against some microorganisms such as *Corynebacterium urealyticum* (Garcia-Rodriguez et al., 1991; Munoz-Bellido et al., 1996). Interestingly, SSRIs also affect the normal physiology of some bacteria, for example they inhibit slime production in coagulase-negative staphylococci (Munoz Criado et al., 1997) and inhibit swarming in swarming species in *Proteus* (Muñoz-Criado et al., 1998). Upon analysis of the antimicrobial activity of four SSRIs against *E.coli*, **sertraline** was the most potent antimicrobial compound (Bohnert et al., 2011). Since the discovery of sertraline as a strong antimicrobial, the research has been focused mainly on this compound. Sertraline inhibits the growth of *S. aureus*, *E.coli* and *P. aeruginosa*, and it also has synergy in combination with antibiotics (Ayaz et al., 2015). Moreover, sertraline has potent antifungal activity against Cryptococcus neoformans (Rossato et al., 2016; Trevino-Rangel Rde et al., 2016; Zhai et al., 2012), Coccidioides immitis (Paul et al., 2016) and Candida spp. (Lass-Florl et al., 2003). In a different study it was shown that sertraline was able to kill 97.5% of the promastigotes of *Leishmania donovani* at a dose of 30mg/L while, at the lowest concentration (3 mg/L), it induced significant loss of viability in the promastigotes (61%) (Palit and Ali, 2008). **Fluoxetine** had a strong dose-dependent

antimicrobial activity *in vitro* against *L. rhamnosus* and *E.coli*, while **escitalopram** only exerted a minor antimicrobial effect on *E.coli*, without affecting the growth of *L. rhamnosus* (Cussotto et al., 2019b).

Evidence from *in vivo* studies (rodents)

Evidence from our laboratory has recently shown that 4 weeks of fluoxetine administration in drinking water in rats at a translationally relevant dose of 10 mg/kg/day completely inhibited the growth of *Succinivibrio* and *Prevotella* caecal taxa (Cussotto et al., 2019b). Whether the microbiome changes influence the efficacy and/or toxicity of fluoxetine still needs to be teased apart.

1.11.3 Other antidepressants and the gut microbiota

Evidence from *in vitro* studies

Interestingly, the era of antidepressants started with **isoniazid**, a compound that also has antimicrobial activity against *Mycobacterium tuberculosis* and is currently used to treat tuberculosis (Jena et al., 2014; Lei et al., 2000). **Ketamine** is a non-competitive NMDA (*N*-methyl-d-aspartate) antagonist that acts at the PCP (phencyclidine) binding site in the NMDA receptor and possess a fast onset of action as antidepressant (Bennett and Brown, 2008). Ketamine showed antimicrobial activity *in vitro* against six different strains of bacteria: *S. aureus*, *S. epidermidis*, *E. faecalis*, *S. pyogenes*, *P. aeruginosa* and *E. coli*; with *S. aureus* and *S. pyogenes* being the most sensitive strains (Begec et al., 2013; Gocmen et al., 2008). There is currently little known regarding the effects of ketamine on gut microbiota and other classes of antidepressants, such as MAOIs (monoamine oxidase inhibitors) and SNRIs (serotonin-norepinephrine reuptake inhibitors), have not been investigated. Given the wide range of antimicrobial effects that most antidepressants show against different strains, it is perhaps not

surprising to speculate that SNRIs or MAOIs might exert a microbial effect. This represents a future direction for research.

1.12 Antianxiety Drugs and the Gut Microbiota

Evidence from *in vitro* studies

The literature to date lacks comprehensive studies investigating the effects of antianxiety agents on the gut microbiome, however some studies *in vitro* have been carried out to assess whether these compounds possess antimicrobial activity. **Propranolol** is a beta-receptor blocker that is commonly used to overcome the somatic symptoms of anxiety such as tachycardia and palpitations (Whitlock and Price, 1974). *In vitro* this compound was able to inhibit the growth of *S. aureus* (Kruszewska et al., 2004) and *E.coli* (Hadera et al., 2018). However, the data are divergent, as a different study showed that propranolol did not inhibit the growth of *S. aureus* (Jerwood and Cohen, 2008). More research *in vivo* and in humans is warranted to investigate the microbial effects of antianxiety drugs.

1.13 Anticonvulsants/Mood Stabilisers and the Gut Microbiota

Mood stabilisers are used to treat mood disorders, characterised by intense and sustained mood shifts, typically bipolar disorder, borderline personality disorder and schizoaffective disorder. Many agents described as mood stabilisers are also categorised as anticonvulsants (Rapoport et al., 2009). No population-based studies have been carried out to date looking at the influence of anticonvulsants on the microbiome, but some preclinical data exist and are examined in the following sections.

Evidence from *in vitro* studies

We have recently screened the antimicrobial activity of **lithium and valproate** against *E.coli* and *L. rhamnosus in vitro* and these two medications did not inhibit the growth of the two bacteria (Cussotto et al., 2019b). Interestingly however, valproate has previously been shown to inhibit *Mycobacterium smegmatis* but not to affect *E. coli* (Esiobu and Hoosein, 2003). **Lamotrigine** showed good antibacterial activity against Gram-positive bacteria *B. subtilis*, *S. aureus* and *S. faecalis* (Qian et al., 2009) and inhibition of bacterial ribosome biogenesis (Stokes et al., 2014). Finally, some evidence also showed that gabapentin and topiramate possess differential antimicrobial activity *in vitro* (Kruszewska et al., 2004).

Evidence from *in vivo* studies (rodents)

A 4-weeks administration of **lithium and valproate** in the chow of Sprague-Dawley rats was able to change markedly the caecal microbiome (Cussotto et al., 2019b). Bacterial richness was increased in both treatments compared to vehicle-treated animals; moreover, at the genus level, lithium increased the relative abundance of *Ruminococcaceae* and decreased *Bacteroides*, while valproate decreased the relative abundance of *S24-7 uncultbact* and increased *Ruminococcaceae* (Cussotto et al., 2019b). Valproate, but not lithium, also affected the levels of SCFAs in the caecum. How these microbial changes relate to drug efficacy is not clear. Moreover, it is also not clarified whether these drugs affect directly the gut microbiota (i.e. they reach the caecum) or indirectly (i.e. through gut-brain signalling).

1.14 Opioid Analgesics and the Gut Microbiota

Opioid analgesics act to reduce the intensity and unpleasantness of pain. They produce their effects by activating specific G-protein-coupled receptors in the brain, spinal cord and peripheral nervous system (Trang et al., 2015). Acting as agonists at opioid

receptors, these compounds reduce neuronal excitability and inhibit the release of pain neurotransmitters (Conlon and Bird, 2015).

Evidence from *in vitro* studies

Morphine did not possess antimicrobial activity against any of the 10 microbial strains studied with the agar dilution method (Rosenberg and Renkonen, 1985). Another opioid analgesic, **tramadol**, had strong bactericidal activity *in vitro* against *E.coli* and *S. epidermidis* and weak antimicrobial activity against *S. aureus* and *P. aeruginosa* (Tamanai-Shacoori et al., 2007). **Methadone** exerted antimicrobial activity *in vitro* against *S. aureus*, *P. aeruginosa* and *S. marcescens* (Sheagren et al., 1977).

Evidence from *in vivo* studies (rodents)

In a morphine-dependent murine model, significant shifts in the gut microbiome and metabolome within one day following **morphine** treatment were detected. Morphine was administered through the pellet implantation method, so that plasma levels of morphine were maintained in the 0.6 - 2.0 μg/ml range (range observed in opioid abusers and patients on opioids for moderate to severe pain). Morphine-induced alterations in gut microbial composition were associated to a significant increase in pathogenic bacteria and a decrease in communities associated with stress tolerance (Wang et al., 2018). In a different study in mice, both intermittent and sustained morphine administration influenced the gut microbiome in a way that was causally related to behaviours associated with opioid dependence (Lee et al., 2018). Interestingly, subcutaneous injections of **tramadol** reduce the growth of *S. aureus* through enhancing phagocytes and tissue inflammation, however, it does not eliminate *P. aeruginosa* (Farzam et al., 2018).

Evidence from studies in humans

One study has examined the effect that opioids might have on the gut microbiota in humans. In a cohort of cirrhotic patients, chronic opioid use (hydromorphone N=7, fentanyl N=1, methadone N=1, morphine sulphate N=1, oxycodone N=23, Percocet, N=3, tramadol N=23, and combinations of the drugs N=3) was associated to significant changes in microbiome composition, with lower relative abundance of Bacteroidaceae and Ruminococcaceae (Acharya et al., 2017). This analysis was carried out at drug class level, and it was not possible to discriminate between the effects induced by each single compound.

1.15 Drugs of Abuse, Alcohol, Nicotine and the Gut Microbiota

Considering that accumulating evidence supports the role of the gut microbiota in central nervous system (CNS) function, the interaction between the gut microbiome and drugs of abuse, as well as alcohol and nicotine, represents an expanding field.

Evidence from *in vitro* studies

Ketamine was antimicrobial *in vitro* in a dose-dependent manner against some micro-organisms in propofol, which is a strong growth-promoting factor (Begec et al., 2013). The ketamine MIC (minimal inhibitory concentration) was 19.5 µg/mL for *S. aureus* and 312.5 µg/mL for *E. coli* and *P. aeruginosa*. As ketamine has antidepressant potential, some of its microbial effects have already been described in *Section 5.3*.

Cannabis is obtained from the annual plant *Cannabis sativa* and its varieties *Cannabis indica* and *Cannabis americana*. Psychological reactions to cannabis vary widely, depending on the predisposition of the individual and can include euphoria, memory impairments and time-spatial sense impairments. *In vitro* assays have shown that

cannabis exerts a strong antimicrobial activity against a wide range of micro-organisms (Appendino et al., 2008; M M Ali et al., 2018; Nissen et al., 2010).

Nicotine, one of the main components of tobacco, possesses all the characteristics of a drug of dependence. It modulates dopamine activity in the midbrain, particularly in the mesolimbic system, which promotes the development and maintenance of reward behaviour (Rice and Cragg, 2004). Two studies *in vitro* have evaluated the antimicrobial activity of **nicotine**. The psychotropic compound was active against *E.coli, P. aeruginosa* and *S. faecalis* at a dose of 2 µg/µl (Idrees Zaidi et al., 2012) and against *Listeria monocytogenes* and *Viridans streptococci* at a dose of 10 µg/mL (Pavia et al., 2000).

Evidence from *in vivo* studies (rodents)

The gut microbiota of rats undergoing **methamphetamine**-induced conditioned place preference is different from that of control animals. Moreover, the fecal microbial diversity is slightly higher in metamphetamine-treated rats. The propionate-producing genus *Phascolarctobacterium* is attenuated in metamphetamine-treated rats and the family *Ruminococcaceae* is increased in the same group (Ning et al., 2017). In addition, the SFCA propionate was decreased in the faecal matter of rats that received methamphetamine (Ning et al., 2017). The microbiome might play a role also in **cocaine** addiction: administration of antibiotics in mice induced an enhanced sensitivity to cocaine reward and an enhanced sensitivity to the locomotor-sensitizing effects of repeated cocaine administration (Kiraly et al., 2016). Regarding **cannabis**, recent evidence has shown that modifications in the gut microbiota consequential to diet-induced obesity are prevented in mice treated chronically with **Δ^9 tetrahydrocannabinol** (THC), the major psychoactive constituent of cannabis (Cluny et al., 2015).

Alcohol generally exerts on cells in the CNS a depressant effect that is probably mediated by particular membrane ion channels and receptors (Whitlock and Price, 1974). Alcohol enhances inhibitory $GABA_A$-stimulated flux of chloride through

receptor-gated membrane ion channels, a receptor subtype effect that might be involved in the motor impairment caused by alcohol (Abrahao et al., 2017). Exposure to 4 weeks of chronic intermittent vaporised **ethanol** in mice markedly altered the gut microbiota, increasing the levels of *Alistipes* and decreasing *Clostridium IV, Dorea* and *Coprococcus* (Peterson et al., 2017). In a mouse model of alcoholic liver disease, Bacteroidetes and Verrucomicrobia were increased in mice fed alcohol compared with a relative predominance of Firmicutes in control mice (Yan et al., 2011). Several other studies in rodents have highlighted a correlation between chronic alcohol consumption, leading to liver disease, and microbiome composition (Fouts et al., 2012; Guarner et al., 1997; Yan and Schnabl, 2012). Interestingly, corroborating the idea that the gut microbiome might play a role in alcohol consumption, two dietary means have been used as modulators of the gut microbiome during alcohol consumption. Saturated and unsaturated dietary fats (Kirpich et al., 2016), for example, as well as rhubarb extract (Neyrinck et al., 2017), have been shown to modulate the changes in gut microbiota induced by alcohol intake.

Finally, the psychotropic **nicotine** administered in drinking water influenced the gut microbiota composition in a sex-specific manner in mice. In treated females, *Christensenellaceae*, *Anaeroplasmataceae* and unassigned families in the orders *Bacillales* were significantly reduced. Families such as *Turicibacteraceae* and *Peptococcaceae* were largely increased in male counterparts (Chi et al., 2017).

Evidence from studies in humans

In a human cohort, **cocaine** users displayed a higher relative abundance of *Bacteroidetes* than non-users (Volpe et al., 2014). The composition and diversity of intestinal microbiota in a cohort of 50 patients with substance use disorders (SUD; of which 52% on **heroin** and 30% on **methamphetamine**) was significantly different from those of healthy controls. The relative abundance of *Thauera*, *Paracoccus*, and *Prevotella* was significantly higher in SUD patients compared to healthy participants (Xu et al., 2017). The intestinal microbiota of SUD people would change independently of the type of substance abused, suggesting that the global switch of

lifestyle due to SUD in general could be responsible for the changes in microbiome. Importantly, almost all patients with SUDs are involved in alcohol and tobacco addiction, which may also account for the microbiome effects (Xu et al., 2017). The microbiome of chronic marijuana users displayed a Prevotella:Bacteroides ratio that was 13-fold lower than the one of non-users (Panee et al., 2018). A combination of **THC and cannabidiol (CBD)** has been shown to mitigate experimental autoimmune encephalomyelitis (EAE) by altering the gut microbiome (Al-Ghezi et al., 2017).

Regarding **alcohol**, the mucosa-associated colonic microbiome was altered in alcoholics compared to control participants. Specifically, the alcoholics with dysbiosis had lower median abundances of Bacteroidetes and higher ones of Proteobacteria. Moreover, these alterations were correlated with high levels of serum endotoxin in a subset of the samples (Mutlu et al., 2012). Two similar studies have demonstrated that alcohol-dependent subjects have an increased intestinal permeability which is linked to significant microbiome alterations (de Timary et al., 2015; Keshavarzian et al., 2009; Leclercq et al., 2014). Bacterial overgrowth was found in the jejunum of patients with chronic alcohol abuse (Bode et al., 1984). In cirrhotic patients, the proportion of phylum Bacteroidetes was significantly reduced, whereas Proteobacteria and Fusobacteria were highly enriched compared to healthy controls. Moreover, Enterobacteriaceae, Veillonellaceae and Streptococcaceae were prevalent in patients with cirrhosis at the family level (Chen et al., 2011).

Nicotine consumption and also smoking cessation induced profound changes in the gut microbiome in humans, with an increase of Firmicutes and Actinobacteria and decrease of Bacteroidetes and Proteobacteria at the phylum level. In addition, smoking cessation induced an increase in microbial diversity (Biedermann et al., 2013). The effect of tobacco smoke on the oral and gut microbiome has been recently investigated in a human cohort, where tobacco smokers displayed a higher relative abundance of *Prevotella*, lowered *Bacteroides* and lower Shannon diversity in tobacco smokers compared to controls (Stewart et al., 2018).

1.16 Xanthines and the Gut Microbiota

The three xanthines caffeine, theophylline and theobromine occur naturally in plants. These compounds have complex and incompletely elucidated actions, which include inhibition of phosphodiesterase (the enzyme that breaks down cyclic AMP), effects on intracellular calcium distribution and noradrenergic function (Bennett and Brown, 2008). All xanthines stimulate mental activity to different extents and their effects vary according to the mental state and personality of the subject (Bennett and Brown, 2008).

Evidence from *in vitro* studies

Xanthines were screened against several microbial strain and all compounds displayed antimicrobial activity, with caffeine being the most effective compound (Raj and Dhala, 1965). The morphology of *Aerobacter aerogenes* and *A. cloacae* was affected by **caffeine** (Raj and Dhala, 1965). Coffee also inhibited the growth of *E.coli* and *E. faecalis in vitro* (Tatsuya and Kazunori, 2013). However, this is not the first study showing that caffeine has antimicrobial activity *in vitro*, as a previous experiment had already demonstrated this concept (Daglia et al., 2007).

Evidence from *in vivo* studies (rodents)

A two-weeks administration of cocoa's **theobromine** to healthy adult rats was shown to induce marked changes in gut microbiota composition. Specifically, rats that received a 10% cocoa-containing diet had lower intestinal counts of *E.coli*, whereas rat that received a 0.25% theobromine-containing diet had lower counts of *Bifidobacterium* spp., *Streptococcus* spp. and *Clostridium histolyticum-C. perfingens* group compared to normal-fed rats (Martín-Peláez et al., 2017). Consumption of fermented green tea, containing **theophylline**, was able to restore the changes in gut microbiota composition associated to diet-induced obesity in mice (Seo et al., 2015). In a different study, consumption of 500 μL/day of coffee for three consecutive days

in specific-pathogen-free mice induced *E.coli* and *Clostridium* spp. counts to decrease significantly (Tatsuya and Kazunori, 2013). **Caffeine**-rich Pu-erh tea remodelled the intestinal dysbiosis in mice with metabolic syndrome (Gao et al., 2018). Specifically, *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* were speculated to be the key gut bacterial links between the Pu-erh Tea treatment and metabolic syndrome at the genus and species levels (Gao et al., 2018). Chronic coffee consumption in diet-induced obese rats was accompanied by decreased abundance of *Clostridium Cluster XI* and increased levels of *Enterobacteriaceae*. Moreover, SCFAs (short-chain fatty acids) were largely increased in the coffee-fed rats (Cowan et al., 2014). It is important to note that studies on the effects of caffeine on gut microbiota are not always consistent, for example in a different experiment on rats, 8 weeks of coffee consumption did not alter the gut microbiota composition (Cowan et al., 2013). A 3-weeks regimen with oral administration of 0.7 mg/kg/day in mice decreased *Lactobacillus* ratios compared to controls, but none of the other taxa were affected (Kleber Silveira et al., 2018).

Evidence from studies in humans

Caffeine consumption has received much attention in recent years in relation to microbiome alterations often associated to metabolic disorders. Consumption of 3 cups of coffee daily for 3 weeks in healthy volunteers did not alter faecal profiles of the dominant microbiota, but increased the population of *Bifidobacterium* spp. (Jaquet et al., 2009). Moreover, in some subjects, there was a specific increase in the metabolic activity of *Bifidobacterium* spp. (Jaquet et al., 2009)

1.17 The Gut Microbiota in Psychiatric Disorders

The drugs investigated throughout this thesis are commonly used in the treatment of different psychiatric disorders such as depression and schizophrenia and it is important to highlight that the gut microbiome has been characterised in such psychiatric populations. In this section, I will give a glimpse into the current evidence relating the gut microbiota to psychiatric disorders (**Table 1.2**).

1.17.1 The microbiome in schizophrenia

Schizophrenia is characterised by high rates of co-morbid gastrointestinal problems (Severance et al., 2015). A recent preliminary study of patients with first-episode psychosis identified differences in the microbiota composition, including reduced prevalence of *Lactobacillus* and *Bifidobacteria* species compared to healthy age-matched controls (Schwarz et al., 2018). Interestingly, the differences in microbiota were correlated with severity of negative symptoms and risk for remission at 12-months follow-up but did not correlate with duration of antipsychotic drug treatment. A different study has reported a link between the oropharyngeal microbiome composition and schizophrenia, without indicating the direction of the change (Castro-Nallar et al., 2015). It was recently shown that, compared to healthy controls, schizophrenic patients have significantly lower levels of faecal *Bifidobacterium*, *Escherichia coli* and *Lactobacillus* and higher levels of *Clostridium coccoides*. After 24-weeks of treatment with risperidone, a significant increase in the numbers of faecal *Bifidobacterium* and *E. coli* was observed (Yuan et al., 2018). The authors concluded that drug naïve, first episode patients with schizophrenia showed abnormalities in microbiota composition and that risperidone treatment caused significant changes in faecal bacteria. In a different study, schizophrenic patients showed higher levels of *Proteobacteria, Succinivibrio, Megasphaera, Collinsella, Clostridium, Klebsiella* and *Methanbrevibacter* and lower levels of *Blautia*, *Coprococcus*, *Roseburia* compared to healthy controls (Shen et al., 2018). In addition, 12 significant microbiota biomarkers

were used as diagnostic factors for distinguishing the schizophrenic cohort from the healthy cohort (Shen et al., 2018). Yolken and colleagues employed metagenomic analysis to characterize bacteriophage genomes in the oral pharynx of 41 individuals with schizophrenia and 33 control individuals without a psychiatric disorder. Upon analysis, one bacteriophage genome, *Lactobacillus* phage phiadh, was found to be significantly increased in individuals with schizophrenia, including when controlled for age, gender, race, socioeconomic status and smoking (Yolken et al., 2015). Intriguingly, within the group of individuals with schizophrenia, the level of *Lactobacillus* phage phiadh correlated with the prevalence of immunological disorders as well as with the administration of valproate (Yolken et al., 2015). Several studies have pointed to an association between schizophrenia and the immune response (Kelly et al., 2017b; Lin et al., 1998; Maes et al., 2000) and this provides additional foundation for investigating the microbiome in schizophrenia, given the key role the microbiome plays in maintaining immune function (Belkaid and Hand, 2014; Hooper et al., 2012). The probiotics *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* regulated immune and intestinal epithelial cells which improved control of gastrointestinal leakage in patients with schizophrenia (Tomasik et al., 2015). In a different trial, the two strains *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subs. *lactis* failed to show any effects in attenuating the symptomatology of schizophrenia (Dickerson et al., 2014). The chosen probiotics, however, reduced the risk for severe bowel problems in a small group of outpatients with moderate to severe schizophrenia symptoms. Severance and colleagues conducted a randomized, placebo-controlled, longitudinal pilot study and explored the use of probiotics in the treatment of both yeast gut infection and psychiatric symptoms in schizophrenic patients (Severance et al., 2017). Probiotics decreased Candida albicans antibody levels and gastrointestinal symptoms in male subjects. Trends towards improvement in positive psychiatric symptoms in males treated with probiotics who were seronegative for C. albicans were also observed (Severance et al., 2017).

1.17.2 The microbiome in depression

A growing body of work is currently focused on the microbiome-gut-brain axis in depression (Dash et al., 2015; Foster and McVey Neufeld, 2013). Germ-free mice display reduced depressive-like behaviour compared to control mice (Zheng et al., 2016) and both probiotic and prebiotic treatments have been shown to reduce depressive-like behaviour in rodent models (Bravo et al., 2011; Burokas et al., 2017; Desbonnet et al., 2010). Randomized trials have shown the efficacy of probiotics on mood (Messaoudi et al., 2011; Steenbergen et al., 2015) as well as the ability to reduce responses to stress (Kato-Kataoka et al., 2016). However, other studies have produced controversial results and more trials are needed to demonstrate efficacy and to identify the specific strains that are most beneficial (Bruce-Keller et al., 2018; Doron and Snydman, 2015). Similarly, large, controlled and well-powered studies about the efficacy of prebiotics are warranted (Bruce-Keller et al., 2018). Some difficulties still persist in translating beneficial effects of probiotics in humans (Kelly et al., 2017a). An altered microbial composition has been reported in patients with depression (Jiang et al., 2015b; Kelly et al., 2016; Naseribafrouei et al., 2014; Zheng et al., 2016), with two studies showing a reduction in the relative abundance of *Faecalibacterium*. Another study reported lower levels of *Bifidobacterium* and *Lactobacillus* in depressed patients (Aizawa et al., 2016). Finally, faecal transplantation of microbiota from depressed patients into rodents (microbiota-depleted rats and germ free mice, respectively) was able to transfer anxiety-like and depressive-like behaviours to the recipients (Kelly et al., 2016; Zheng et al., 2016). Interestingly, the rats receiving the FMT, which were treated for 12 weeks (with twice weekly 'top-up' doses), also showed an increase in plasma kynurenine and kynurenine/tryptophan ratio, a change reported in the depressed donor group (although total tryptophan was not significantly altered) as well as an increase in acetate and total short-chain fatty acids, as measured in their faeces (Kelly et al., 2016). In a recent population-based study, a large microbiome population cohort (Flemish Gut Flora Project, n=1,054) was surveyed with validation in independent data sets to study how microbiome features correlated with host quality of life and depression. Butyrate-producing *Faecalibacterium* and *Coprococcus* were consistently associated with higher quality of life indicators. Together with *Dialister*, *Coprococcus* spp. were depleted in depression, even after

correcting for the confounding effects of antidepressants (Valles-Colomer et al., 2019). Gut-brain module analysis of faecal metagenomes identified the microbial synthesis potential of the dopamine metabolite 3,4-dihydroxyphenylacetic acid as correlating positively with mental quality of life and indicated a potential role of microbial γ-aminobutyric acid production in depression (Valles-Colomer et al., 2019).

1.17.3 The microbiome in bipolar disorder

Few studies to date have investigated the link between the microbiome and bipolar disorder. Yolken and colleagues have demonstrated that patients with bipolar mania were approximately twice as likely as other patients to have recently received antibiotics (Yolken et al., 2016). A recent population-based study showed that the microbiome of bipolar patients was different than healthy controls, at least for patients with more severe symptoms (Evans et al., 2017). Specifically, significant differences in two genera of Firmicutes, *Faecalibacterium* and an unclassified member of Ruminococcaceae, were observed (Evans et al., 2017). Among these patients, increased *Faecalibacterium* was associated with improved physical health, reduced depressive symptoms, and better sleep quality. Notably, Ruminococcaceae and *Faecalibacterium* have also been observed to be decreased in patients with depression, and levels of *Faecalibacterium* were negatively associated with depressive symptoms (Jiang et al., 2015b). Atypical antipsychotic treatment was associated with reduced gut biodiversity, particularly in female patients (Flowers et al., 2017). Patients on atypical antipsychotic treatment showed specific taxonomic shifts, with relatively increased levels of Lachnospiraceae, while non-treated individuals had higher levels of *Akkermansia*. Interestingly, probiotic supplementation reduced the rates of rehospitalization in patients recently discharged following hospitalization for mania (Dickerson et al., 2018).

Table 1.2 Human studies reporting microbiome alterations in stress-related psychiatric disorders. *Note that obsessive compulsive disorder (OCD), attention-deficit/hyperactivity disorder (ADHD) and panic disorders are not included in the table due to lack of evidence.*

Condition	***Microbiome features***	***References***
Anxiety disorder	Chao 1 richness ↓, *Bacteroidetes* ↓, *Ruminococcus gnavus* ↓, *Fusobacterium* ↓	(Jiang et al., 2018)
Autism	Increased: *Lactobacillus* ↑, *Desulfovibrio* ↑, *Clostridium* ↑, Bacteroides/Firmicutes ratio ↓	(Tomova et al., 2015)
	Sutterella spp. ↑, *Ruminococcus torques* ↑	(Wang et al., 2013)
	Clostridium ↑, *Bacteroides* ↑, *Porphyromonas* ↑, *Prevotella* ↑, *Pseudomonas* ↑, *Aeromonas* ↑, Enterobacteriaceae ↑, *Enterococcus* ↓, *Lactobacillus* ↓, *Streptococcus* ↓, *Lactococcus* ↓, *Staphylococcus* ↓, Bifidobacteria ↓	(De Angelis et al., 2013)
	Lactobacillus ↑, *Prevotella* ↓, *Coprococcus* ↓, Veillonellaceae ↓	(Kang et al., 2013)
	Sutterella ↑, Lachnospiraceae ↑, Ruminococcaceae ↑	(Williams et al., 2011)
	Lactobacillus ↑, *Bifidobacterium* ↓, *Enterococcus* ↓	(Adams et al., 2011)
	Bacteroidetes ↑, *Proteobacterium* ↑, *Actinobacterium* ↓, *Bifidobacterium* ↓	(Finegold et al., 2010)
	Clostridium clusters I and II ↑	(Parracho et al., 2005)
	Collinsella ↑, *Corynebacterium* ↑, *Dorea* ↑, *Lactobacillus* ↑, *Alistipes* ↓, *Bilophila* ↓, *Dialister* ↓, *Parabacteroides* ↓, *Veillonella* ↓	(Strati et al., 2017)

Bipolar disorder	*Faecalibacterium* ↓	(Evans et al., 2017)
Depression	*Bifidobacterium* ↓, *Lactobacillus* ↓	(Aizawa et al., 2016)
	Bacteroidetes ↑, Proteobacteria ↑, Actinobacteria ↑, Firmicutes ↓	(Jiang et al., 2015b)
	Chao 1 richness ↓, *Eggerthella* ↑, *Holdemania* ↑, *Gelria* ↑, *Turicibacter* ↑, *Paraprevotella* ↑, *Anaerofilum* ↑, *Prevotella* ↓, *Dialister* ↓	(Kelly et al., 2016)
	Actinomycineae ↑, Coriobacterineae ↑, Lactobacillaceae ↑, Streptococcaceae ↑, Clostridiales incertae sedis XI ↑, Eubacteriaceae ↑, Lachnospiraceae ↑, Ruminococcaceae ↑, Erysipelotrichaceae incertae sedis ↑	(Zheng et al., 2016)
Post-traumatic stress disorder	Microbial community structure ↔, α- and β-diversity ↔, signature of vulnerability to developing PTSD	(Hemmings et al., 2017)
Schizophrenia	Firmicutes ↑, *Ascomycota* ↑, *Bifidobacterium* ↑ *Lactobacilli* ↑, *Neisseria* ↓, *Haemophilus* ↓, *Capnocytophaga* ↓	(Castro-Nallar et al., 2015)
	Lactobacillus phage phiadh ↑	(Yolken et al., 2015)
	Lactobacillus ↑, *Tropheryma* ↑, *Halothiobacillus* ↑, *Saccharophagus* ↑, *Ochrobactrum* ↑, *Deferribacter* ↑, *Halorubrum* ↑ *Anabaena* ↓, *Nitrosospira* ↓, *Gallionella* ↓ (first episode psychosis)	(Schwarz et al., 2018)

Abbreviations: ↓ indicates a decrease, ↑ indicates an increase, ↔ indicates no changes compared to human healthy controls.

1.18 Aims and Objectives

There is growing recognition of the role played by the gut-brain axis in psychiatric disorders. Despite efforts to characterise how psychotropic medications work at a central level, the influence of these drugs on the gut microbiota and other distal body sites are currently under-investigated. In this thesis we examine the complex relationship between psychotropic medications, the gut microbiome, liver function and intestinal physiology. The investigation of the complex drug-gut interaction carried out in this thesis, has a strong translational potential and could lead to new avenues for clinical practice and personalised medicine. Successful translation of the work here presented, for example, could lead to using the microbiome as a stratification tool and distinguish drug responders from non-responders.

Aim 1: Do psychotropic medications affect the microbiome composition and intestinal permeability?

In recent years it has become evident that many types of drugs can influence the gut microbiota composition (Maier et al., 2018). Moreover, several psychotropic drugs such as antidepressants have shown antimicrobial activity *in vitro* (see *Introduction section 1.9 Psychotropic Drugs and the Gut Microbiota*). With a focus on psychotropic drugs, we wanted to investigate whether these medications could influence microbiome composition both *in vitro* and *in vivo*. The *in vivo* model consisted of a chronic administration (4 weeks) of psychotropic drugs to healthy adult Sprague-Dawley rats. Moreover, we investigated the influence of these medications on intestinal permeability *ex vivo* (*Chapter 2*, **Figure 1.10**).

Aim 2: Do psychotropic medications affect the composition and biotransformation of bile acids?

While analysing the microbiome data of the previous study we noticed that the two psychotropics and mood stabilisers lithium and valproate significantly altered bile acids levels in rats. Thus, we investigated whether the microbiome might have been implicated in the changes observed. We therefore examined pathways involved in the biosynthesis and metabolism of bile acids and hypothesised some mechanistic insights (*Chapter 3,* **Figure 1.10**).

Aim 3: Do modifications of the gut microbiota influence the pharmacokinetics of psychotropic medications?

"Pharmacomicrobiomics" is an expanding field that studies how certain intestinal bacteria can metabolise xenobiotics (Rizkallah et al., 2010). After showing that psychotropic drugs influence the gut microbiota (see *Chapter 2*), we wanted to look at the reverse scenario: would perturbations of the microbiome influence the absorption of the two antipsychotics olanzapine and risperidone? As modulators of microbiome composition, we used two common approaches: antibiotic and probiotic administration. This investigation was carried out in healthy adult rats (*Chapter 4*, **Figure 1.10**).

Aim 4: Do psychotropic medications affect the composition of the human gut microbiota?

Following observations in rats and intrigued by the results from *Chapter 2*, we examined the microbiome-targeted effects of psychotropic medications in a human cohort, the Dutch LifeLines-DEEP population (*Chapter 5,* **Figure 1.10**).

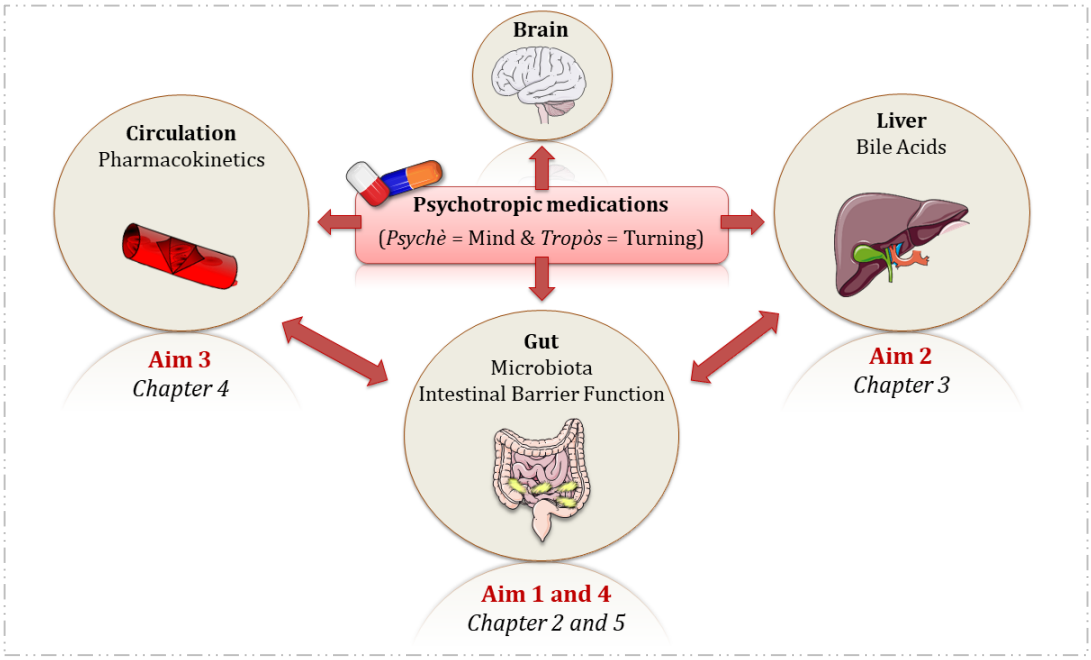


Figure 1.10 Diagram representing the aims of the thesis. *Aim 1 (Chapter 2) investigates the effect of psychotropic medications on the microbiome and intestinal barrier function. Aim 2 (Chapter 3) investigates the effects of the mood stabilisers lithium and valproate on bile acid metabolism and bile-associated microbial taxa. Aim 3 (Chapter 4) focuses on how perturbations of the gut microbiota could impact the absorption of two widely prescribed antipsychotics: olanzapine and risperidone. Aim 4 (Chapter 5) examines the microbiome composition following psychotropic intake in a human population. Aims 2 and 3 are also linked to the investigation of gut microbiota composition.*

Chapter 2

Differential Effects of Psychotropic Drugs on Microbiome Composition and Gastrointestinal Function

Sofia Cussotto[1,2], Conall R. Strain[1,4], Fiona Fouhy[1,4], Ronan G. Strain[1,4], Veronica L. Peterson[1,2], Gerard Clarke[1,3], Catherine Stanton[1,3,4], Timothy G. Dinan[1,3], John F. Cryan[1,2]

[1] APC Microbiome Ireland, University College Cork, Cork, Ireland

[2] Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[3] Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

[4] Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland

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Abstract

Rationale Growing evidence supports a role for the microbiota in regulating gut-brain interactions and thus psychiatric disorders. Despite substantial scientific efforts to delineate the mechanism of action of psychotropic medications at a central nervous system (CNS) level, there remains a critical lack of understanding of how these drugs might affect the microbiota and gut physiology.

Objectives We investigated the antimicrobial activity of psychotropics against two bacterial strains resident in the human gut, *Lactobacillus rhamnosus* and *Escherichia coli*. In addition, we examined the impact of chronic treatment with these drugs on microbiota and intestinal parameters in the rat.

Results *In vitro,* fluoxetine and escitalopram showed differential antimicrobial effects. Lithium, valproate and aripiprazole administration significantly increased microbial species richness and diversity, while the other treatments were not significantly different from controls. At the genus level, several species belonging to *Clostridium*, *Peptoclostridium*, *Intestinibacter* and *Christenellaceae* were increased following treatment with lithium, valproate and aripiprazole when compared to the control group. Animals treated with escitalopram, venlafaxine, fluoxetine and aripiprazole exhibited an increased permeability in the ileum.

Conclusions These data show that psychotropic medications differentially influence the composition of gut microbiota *in vivo* and that fluoxetine and escitalopram have specific antimicrobial activity *in vitro*. Interestingly, drugs that significantly altered gut microbial composition did not increase intestinal permeability, suggesting that the two factors are not causally linked. Overall, unravelling the impact of psychotropics on gastrointestinal and microbiota measures offers the potential to provide critical insight into the mechanism of action and side effects of these medications.

Keywords: *psychotropics, intestinal permeability, gut microbiota, diversity, richness, short-chain fatty acids, antimicrobial*

Introduction

The burden of psychiatric disorders on society continues to grow, with estimates from the World Health Organization (WHO) suggesting that worldwide 322 million and 60 million people are affected by depression and bipolar disorder respectively (World Health Organization, 2017). Treatment options used in the management of psychiatric disorders are often associated with metabolic side effects (Beyazyüz et al., 2013; Correll et al., 2015; Olguner Eker et al., 2017; Reynolds and Kirk, 2010; Tschoner et al., 2007) and high non-response rates (Ackenheil and Weber, 2004; Al-Harbi, 2012; Hatta and Ito, 2014; Nelson, 1998). Over the last decade, a growing body of evidence has highlighted a significant role for the gut microbiota in interactions between the gut and the brain (Bercik et al., 2012; Collins et al., 2012; Cryan and Dinan, 2012; Dinan et al., 2013; Mayer et al., 2014; Mayer et al., 2015). Moreover, alterations in this microbiota-gut-brain axis are associated with several behavioural and psychiatric conditions (Cryan and Dinan, 2012; Foster and McVey Neufeld, 2013; Luna and Foster, 2015). Strikingly, individuals with depression (Jiang et al., 2015b; Kelly et al., 2016; Naseribafrouei et al., 2014; Zheng et al., 2016), bipolar disorder (Evans et al., 2017) and schizophrenia (Dinan et al., 2014; Schwarz et al., 2018) alterations in microbiota composition compared to controls. Moreover, recent data has shown that the transfer of microbiota from humans with depression into microbiota-depleted rats or mice induces a depressive-like phenotype, indicating that the gut microbiota may play a key role in the onset of depressive behaviour (Kelly et al., 2016; Zheng et al., 2016).

Despite substantial scientific efforts to unravel the effects of psychotropic medications on the central nervous system, the impact that these drugs have on gut physiology and microbiota composition is rarely considered. Some isolated studies have shown that antidepressants and antipsychotics possess a microbiota-targeted action. Indeed sertraline, fluoxetine and paroxetine are bactericidal against gram-positive bacteria such as Staphylococcus and Enterococcus (Ayaz et al., 2015; Coban et al., 2009), however, these data are mostly reliant on *in vitro* microbiological studies and are not exhaustive. Regarding antipsychotics, it has been shown that chronic administration of the antipsychotics olanzapine and risperidone affects the gut microbiota

composition in animals and humans (Bahr et al., 2015b; Davey et al., 2012; Kao et al., 2018; Morgan et al., 2014). A recent study has demonstrated that intake of atypical antipsychotics is associated with significant changes in microbiota composition (Flowers et al., 2017), while larger cohort studies have suggested that the use of medications can alter the gut microbiota more generally (Falony et al., 2016; Maier et al., 2018). Behavioural responses to cocaine, another compound belonging to the class of psychotropics, have recently been shown to be affected by gut microbiota shifts in mice (Kiraly et al., 2016).

Importantly, psychotropic medications are often administered orally, thus the gut microbiota represents a plausible target for distal action of these drugs. To this end, we sought to examine the effects that commonly used psychotropics have on microbiota composition and intestinal permeability, which is closely regulated by the gut microbiota (Karl et al., 2017; Ott et al., 2017; Ulluwishewa et al., 2011). Furthermore, we assess the antimicrobial activity of these medications against isolated strains resident in the human gut, L. rhamnosus and E.coli. Unravelling the comparative microbiome and gastrointestinal actions of different psychotropics *in vivo* will provide new insight into the mechanism of these drugs and their side effects and may have a critical impact on future clinical practice and drug discovery efforts.

Methods

Bacterial growth-inhibition assay

Lactobacillus rhamnosus 6118 was grown in anaerobiosis at 37ºC overnight in MRS broth (BD Difco Lactobacilli MRS Broth). *Escherichia coli* APC105 was grown shaking overnight at 37ºC in BHI broth (Oxoid™ Brain Heart Infusion Broth). Overnight cultures were resuspended in broth to an OD reading (optical density at 600nm wavelength) of 0.1, which corresponded to their lag phase. Resuspended cultures were incubated with a range of drugs dissolved in sterile deionised water at different concentrations (100, 400 and 600 μg/mL). The OD of the bacteria was measured every hour for up to seven hours. The vehicle consisted of the dissolution medium only and each curve was produced in triplicates.

Animals

Adult male Sprague Dawley rats (n=8/group; 200-250g on arrival) were obtained from Envigo UK. They were housed 2 per cage and maintained under a 12-h light/dark cycle, provided with chow and water *ad libitum*. Rats in the same cage underwent the same treatment to avoid confounding factors such as coprophagy. Animals were acclimated to housing conditions for one week prior to experimental treatment. Experiments were conducted in accordance with European Directive 2010/63/EU. Approval by the Animal Experimentation Ethics Committee of University College Cork was obtained before commencement of all animal-related experiments.

Drug administration

Each drug was administered for 28 days in drinking water or in the chow and administration continued throughout the behavioural assessment until the animals were culled (**Figure 2.1**, **Table 2.1**).

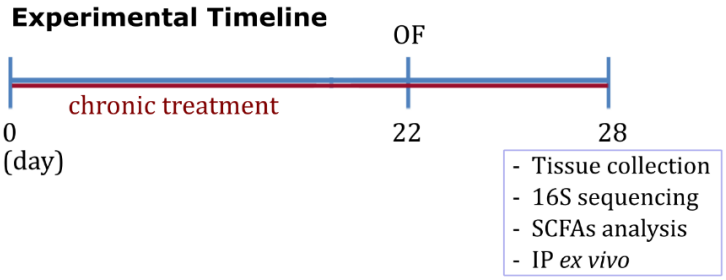
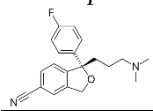
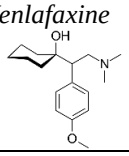
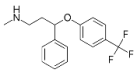
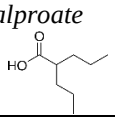
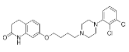


Figure 2.1. Experimental timeline. *Abbreviations: OF* open field, *SCFAs* short-chain fatty acids, *IP* intestinal permeability

Table 2.1 List of psychotropics employed in this study and their mechanism(s) of action. *Abbreviations:* ↑ increase, *SSRI* selective serotonin reuptake inhibitor, *SNRI* serotonin and norepinephrine reuptake inhibitor, *5-HT* serotonin, *NE* norepinephrine/noradrenaline, *NO* nitric oxide, *GABA* ɣ-aminobutyric acid, *D* dopamine

Drug	Class	Main medical uses	Mechanism(s) of action
Antidepressants:			
Escitalopram 	SSRI	Major depressive disorder, generalised anxiety disorder	↑ synaptic levels of 5-HT by blocking the reuptake of the neurotransmitter into the presynaptic neuron
Venlafaxine 	SNRI	Major depressive disorder, generalised anxiety disorder	↑ synaptic levels of 5-HT and NE by blocking the reuptake of the neurotransmitters into the presynaptic neuron
Fluoxetine 	SSRI	Major depressive disorder, generalised anxiety disorder	↑ synaptic levels of 5-HT by blocking the reuptake of the neurotransmitter into the presynaptic neuron
Other Psychotropics:			
Lithium 	Mood Stabiliser	Bipolar disorder, mood-stabiliser, major depressive disorder, schizophrenia	↑ release of 5-HT in the brain, interacts with NO signalling pathway in the brain, modulates glutamate levels, regulates mitochondrial function, lowers levels of inositol triphosphate
Valproate 	Anticonvulsant	Epilepsy, bipolar disorder, schizophrenia	Blocks voltage-gated sodium channels and ↑ brain levels of GABA. It has histone deacetylase-inhibiting effects
Aripiprazole 	Atypical Antipsychotic	Schizophrenia, major depressive disorder, bipolar disorder, obsessive-compulsive disorder	It is silent antagonist of some subpopulations of D_2 receptors but also a high-efficacy partial agonist of other D_2-receptor subpopulations. It has predominantly antagonist activity on postsynaptic D_2 receptors and partial agonist activity on presynaptic D_2 receptors. It is also a partial agonist of the D_3 receptor and the 5-HT_{1A} receptor

The control group received a standard diet (Ssniff, SM Teklad Global 18% Protein Rodent diet, item no. S9912-S710) and drinking water. A second group received 6.38 mg/kg/day of escitalopram oxalate (5 mg/kg/day of free base) in drinking water and standard diet. A third group received 20mg/kg/day of venlafaxine HCl in drinking water and standard diet. A fourth group received 10mg/kg/day of fluoxetine HCl in drinking water and standard diet. A fifth group received 0.2% lithium-supplemented diet, corresponding to approx. 150mg/kg/day, and hypertonic saline water (1.5% NaCl, in order to prevent lithium-induced ionic imbalance). A sixth group received 2% valproate-supplemented diet, corresponding to approx. 1.5g/kg/day, and drinking water. A seventh group received 0.027% aripiprazole-supplemented diet, corresponding to approx. 20mg/kg/day, and drinking water. The concentration of each drug in drinking water and in the chow was determined from the average daily water/food consumption and the average body weight per rat. These dosing regimens have been previously used in chronic behavioural and neurochemical studies in rats (Ariel et al., 2017; Kaminska and Rogoz, 2016; Lyons et al., 2012; Monti et al., 2010; O'Leary et al., 2012; Segnitz et al., 2009; Sogaard et al., 2005; Vidal et al., 2010; Watase et al., 2007). The drinking bottles were protected from light and changed every second day and the chow was stored at 4 ºC during the experiment.

Open Field (OF) Test

To assess possible sedative effects of treatments, the animals were placed in an open arena brightly lit from above and the test was carried out as previously described (O'Mahony et al., 2014). Briefly, 30 minutes before behavioural testing, animals were habituated to the room. The apparatus consisted of a white round arena with a diameter of 90 cm, brightly lit to 1000 lux. At the beginning of the test, animals were placed into the centre of the arena and allowed to explore for 10 min. After testing animals were returned to their home cage. The arena was cleaned with 70% ethanol between trials to ensure that no cue smell remained from the previous trail. Faecal output was manually scored. Total distance travelled was analysed using a tracking software system (Ethovision XT 11.5, Noldus). None of the treatments at the doses tested affected the locomotor activity or the faecal output of the animals (**Figure S.2.1**).

Intestinal permeability

Freshly isolated ileal and colonic tissues were placed in Krebs solution and cut along the mesenteric border. Tissues were then mounted into the Ussing chamber apparatus (Harvard Apparatus, Kent, UK, exposed area of 0.12cm^2) as previously described (Golubeva et al., 2017). 4kDa FITC-dextran was added to the mucosal chamber at a final concentration of 2.5mg/mL; 200µL samples were collected from the serosal chamber after one hour and every 30 minutes for the following 3 hours. FITC was measured at 485nm excitation / 535nm emission wavelengths.

Microbiota composition and Short Chain Fatty Acids analysis in the caecal content

Caecum was harvested and immediately snap-frozen and stored at -80ºC prior to the analysis. DNA was extracted using the Qiagen QIAmp Fast DNA Stool Mini Kit coupled with an initial bead-beating step. The V3-V4 hypervariable region of the 16S rRNA gene was amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library protocol. Samples were sequenced at Teagasc Sequencing Facility (TFRC, Moorepark) on the Illumina MiSeq platform using a 2×250 bp kit. Reads were assembled, processed and analysed following the pipeline, described in *Supplemental Methods*. Short chain fatty acids (SCFAs) were measured by gas chromatography, using a Varian 3500 GC flame-ionization system fitted with a ZB-FFAP column. For construction of the heatmap, Log2 ratios were calculated from group medians of highly abundant bacteria at the genus level using R (version 3.3.2) and R Studio (version 1.0.136).

Statistical analysis

Data are presented as mean + SEM. Intestinal permeability and *in vitro* data were analysed using mixed between-within subjects ANOVA followed by unpaired two-

tailed t-test. Body weight and organ weights were analysed using one-way ANOVA followed by Dunnett's test. 16S rRNA sequencing data was analysed using Kruskal-Wallis non-parametric test followed by Mann-Whitney U test and corrected for multiple comparisons using the Benjamin-Hochberg false discovery rate (FDR) method. Grubbs method was employed to test for any specific outliers (Grubbs, 1950). Threshold for statistical significance was set at $p<0.05$.

Results

Fluoxetine and escitalopram exert specific antimicrobial activity against two bacterial strains resident in the human gut

We tested the effects of psychotropic medications on growth of *Lactobacillus rhamnosus* 6118 and *E.coli* APC105 *in vitro*. These bacterial strains belong to two of the four dominant phyla of the mammalian gut (Firmicutes and Proteobacteria respectively). Each drug was assessed at three different concentrations (100, 400 and 600 µg/mL). Growth curves were performed measuring the optical density (OD) at different time points. Growth of *L. rhamnosus* was completely inhibited by 400 and 600 µg/mL fluoxetine, while growth of *E.coli* was inhibited by 600 µg/mL escitalopram and by all three doses of fluoxetine (**Figure 2.2**). Venlafaxine, lithium and valproate did not exhibit antimicrobial activity *in vitro*. Aripiprazole's antimicrobial activity was not assessed due to its tendency to precipitate with components of the broth, resulting in high risk of false positive data from OD measurements.

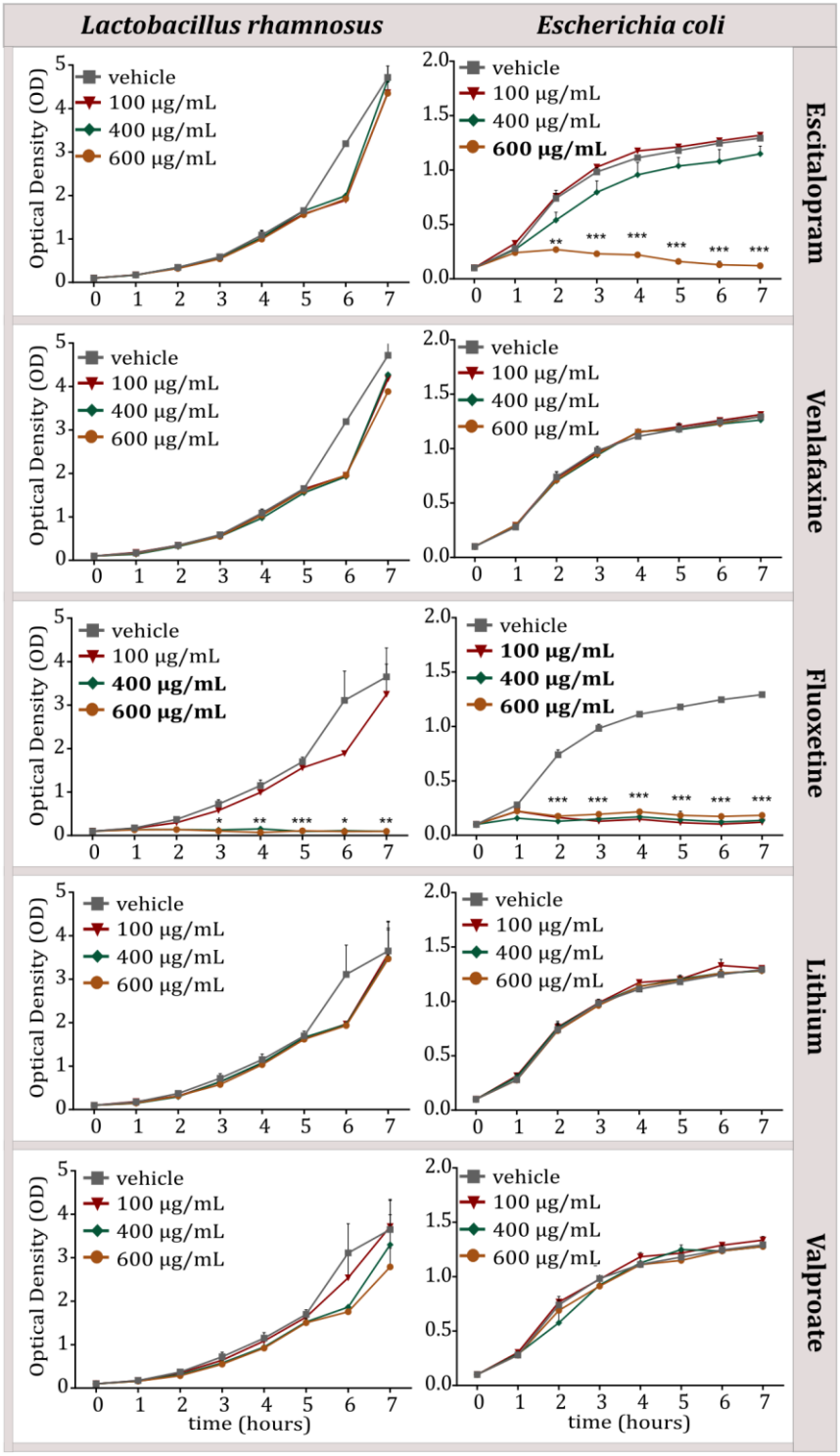


Figure 2.2 Effect of psychotropic drugs on growth of Lactobacillus rhamnosus 6118 and Escherichia coli APC105 *in vitro*. Escitalopram and fluoxetine have differential antimicrobial effects. In bold are bactericidal doses. Statistics: Data are expressed as mean+SEM. *p<0.05, **p<0.01 and ***p<0.001 (n=3/group). Escitalopram effect on E.coli: $F(7;56)=491.682$, $p<0.001$ for the effect of Time, $F(3;8)=82.898$, $p<0.001$ for the effect of Treatment, $F(21;56)=59.87$, $p<0.001$ for the Time×Treatment interaction. Fluoxetine effect on L. rhamnosus: $F(7;56)=42.467$, $p<0.001$ for the effect of Time, $F(3;8)=28.574$, $p<0.001$ for the effect of Treatment, $F(21;56)=15.439$, $p<0.001$ for the Time×Treatment interaction.

Fluoxetine effect on E.coli: $F(7;56)=188.805$, $p<0.001$ for the effect of Time, $F(3;8)=712.570$, $p<0.001$ for the effect of Treatment, $F(21;56)=176.477$, $p<0.001$ for the Time×Treatment interaction. Data were analysed with a mixed between-within subjects ANOVA. F=F-statistic. Mean values in each time point were further compared to the vehicle with unpaired t-test. Statistical outcomes for the t-test are described in Supplemental Material

Administration of fluoxetine, lithium, valproate and aripiprazole significantly alters gut microbiota composition

Our *in vitro* results demonstrated that certain psychotropic drugs differentially modulate the growth of resident gut bacteria. To confirm this in an *in vivo* setting and to assess if any of the treatments induced changes in intestinal microbiota composition, we performed 16S sequencing of bacterial rRNA of the caecum content following chronic administration in rats. The sequencing revealed a significant increase in the bacterial richness and diversity of rats treated with lithium, valproate and aripiprazole as compared to the vehicle-treated group (**Figure 2.3A**). Moreover, separation according to group was further illustrated through principal coordinate analysis (PCoA), with statistical support of the significant separation between escitalopram, venlafaxine, lithium, valproate, aripiprazole and the vehicle ($p<0.05$, **Figure 2.3B**).

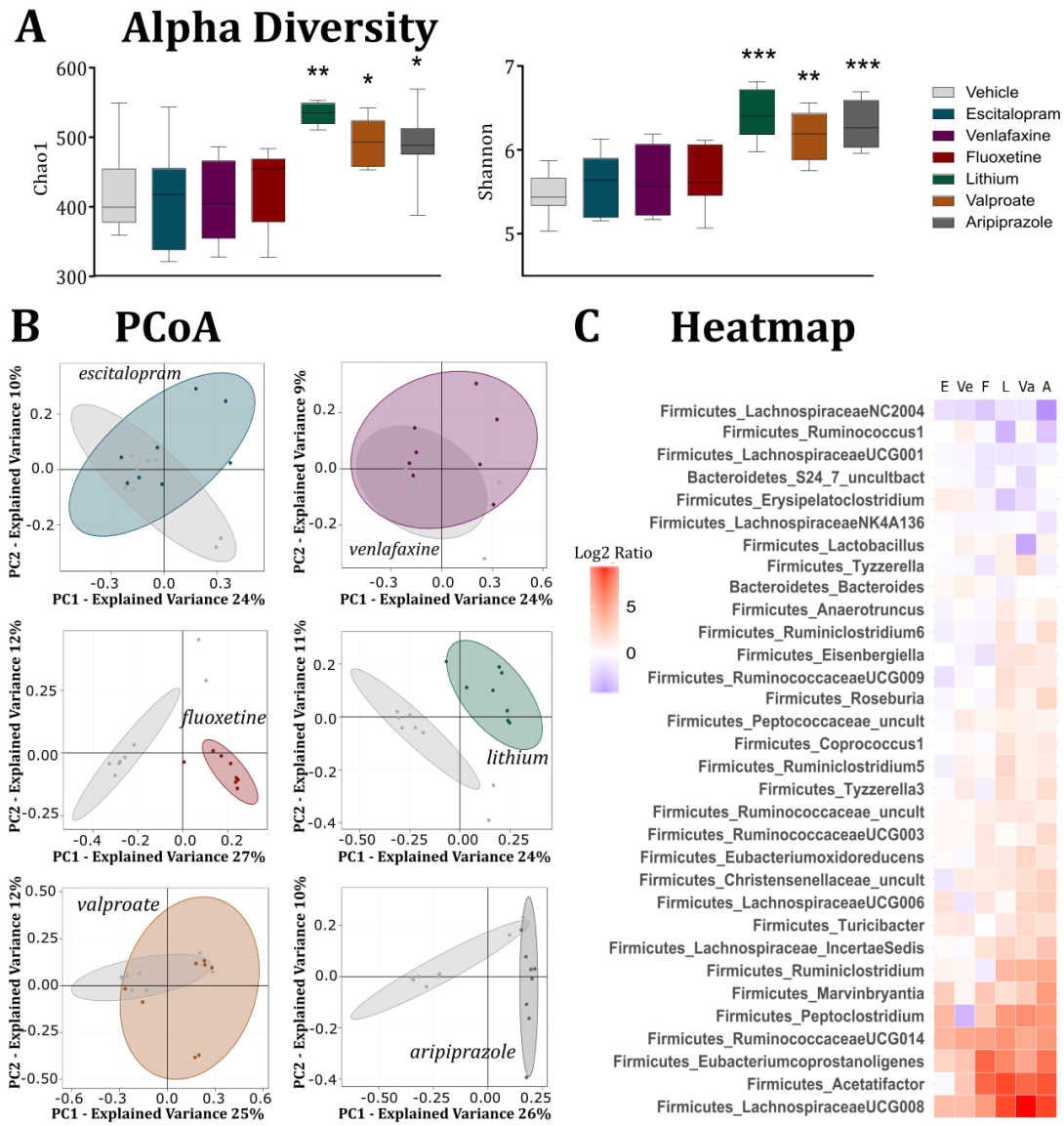


Figure 2.3 Altered microbiota richness and diversity in psychotropic-treated animals as compared to vehicle-treated animals. (A) Alpha diversity. *Statistics:* Kruskal-Wallis test for Chao1 (p=0.000) and Shannon (p=0.000). Mann-Whitney U test for: *Chao1 index:* lithium $U_{(16)}$=6, p=0.006; valproate $U_{(16)}$=10, p=0.021; aripiprazole $U_{(16)}$=12, p=0.036. *Shannon index:* lithium $U_{(16)}$=0, p=0.001; valproate $U_{(16)}$=2, p=0.002; aripiprazole $U_{(16)}$=0, p=0.001. Data are expressed as median and min-to-max values. *p<0.05, **p<0.01 and ***p<0.001 (n=8/group). **(B)** Principal coordinate analysis of Bray-Curtis compiled distance matrix of all microbial relative abundances compared with the vehicle group (light grey ellipse). Escitalopram, venlafaxine, lithium, valproate and aripiprazole show significant variation from the vehicle (Adonis PERMANOVA p<0.05). **(C)** Heatmap of $\log_2$ fold change ratio of medians at the genus level. Red indicates an increase and blue a decrease of taxa in the different treatment groups as compared to vehicle-treated rats. Microbial genera that were significantly different in at least one of the experimental groups compared to the vehicle were selected for the heatmap. E=escitalopram, Ve=venlafaxine, F=fluoxetine, L=lithium, Va=valproate, A=aripiprazole

At the phylum level, lithium induced a significant increase in *Actinobacteria* and a decrease in *Bacteroidetes*; valproate induced an increase in *Actinobacteria, Firmicutes* and a decrease in *Bacteroidetes*; fluoxetine induced a decrease in *Deferribacteres* and aripiprazole induced an increase in *Firmicutes* (**Fig. S.2.2**, **Table S.2.1**). At the family level, lithium, valproate and aripiprazole increased significantly the levels of *Peptostreptococcaceae*, *Clostridiaceae* and *Ruminococcaceae* (**Figure S.2.3**, **Table S.2.2**). Other families were increased by the different treatments, while some of the less abundant families were decreased *(refer to Supplemental Material for details).* At the genus level of the most abundant taxa, lithium increased the relative abundance of *Ruminococcaceae uncultured* and decreased the relative abundance of *Bacteroides* and *Ruminococcus 1*; valproate decreased the relative abundance of *S24-7 uncultbact* and increased the relative abundance of *Ruminococcaceae uncultured*; while aripiprazole decreased the relative abundance of *Ruminococcus 1* (**Figure 2.4**, **Table S.2.3**). None of the treatments significantly affected the genera *Lachnospiraceae uncultured, Akkermansia, S24-7 uncultbact* and *Oscillibacter*.

Interestingly, the relative abundance of minor genera including *Clostridium Sensu Stricto 1*, *Ruminiclostridium 5*, *Intestinibacter*, *Eubacterium coprostanoligens*, *Peptoclostridium*, *Eubacterium oxidoreducens*, *Christensenellaceae uncultured* and *Clostridia Family XIII*, was increased by lithium, valproate and aripiprazole administration (**Figure 2.5**), while antidepressants administration did not influence significantly the abundance of these taxa. Among the antidepressants assessed, fluoxetine induced a marked depletion of the genera *Prevotella 7*, *Prevotella 9* and *Succinivibrio* (**Figure 2.6**).

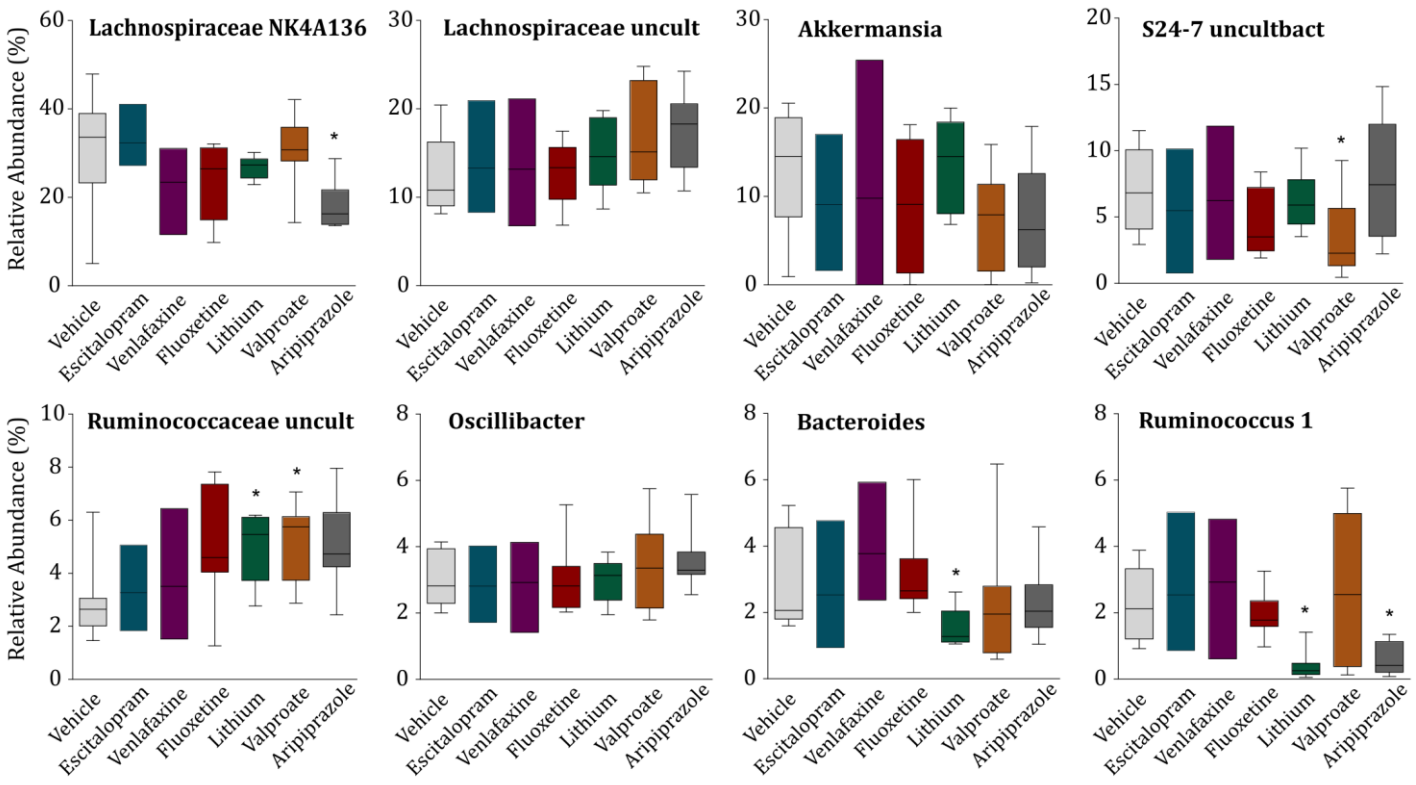


Figure 2.4 Psychotropic drugs differentially affect bacterial composition at genus level of the most abundant taxa. Lithium increased the relative abundance of *Ruminococcaceae uncultured* and decreased the relative abundance of *Bacteroides* and *Ruminococcus 1*. Valproate decreased the relative abundance of *S24-7 uncultbact* and increased the relative abundance of *Ruminococcaceae uncultured*. Aripiprazole decreased the relative abundance of *Ruminococcus 1*. Data are expressed as median and min-to-max values. *p<0.05 (n=8/group). Data was analysed using Kruskal-Wallis non-parametric test followed by Mann-Whitney U test and corrected for multiple comparisons using the Benjamin-Hochberg false discovery rate (pFDR) method *(refer to Supplemental Material for details on statistics*

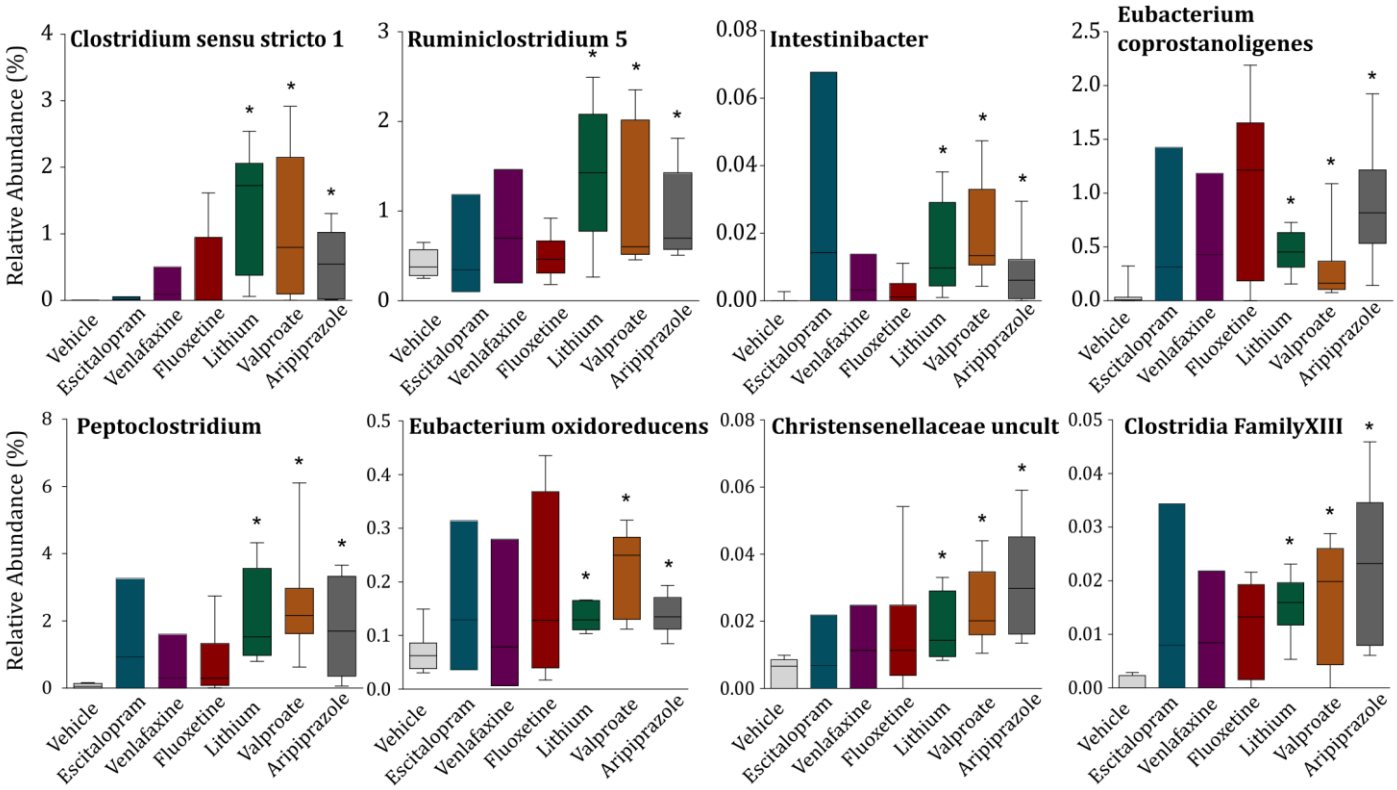


Figure 2.5 Psychotropic drugs differentially affect bacterial composition at genus level of the less abundant taxa. Lithium, valproate and aripiprazole induced an increase in the relative abundance of *Clostridium Sensu Stricto 1*, *Ruminoclostridium 5*, *Intestinibacter*, *Eubacterium coprostanoligens*, *Peptoclostridium*, *Eubacterium oxidoreducens*, *Christensenellaceae* and *Clostridia Family XIII*. Data are expressed as median and min-to-max values. *p<0.05 (n=8/group). Data was analysed using Kruskal-Wallis non-parametric test followed by Mann-Whitney U test and corrected for multiple comparisons using the Benjamin-Hochberg false discovery rate (pFDR) method *(refer to Supplemental Material for details on statistics*

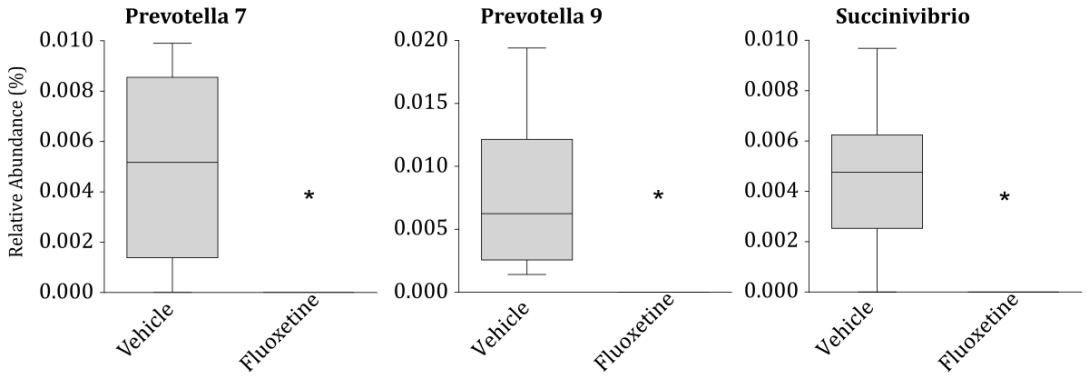


Figure 2.6 Fluoxetine-sensitive genera *in vivo.* The genera *Prevotella 7*, *Prevotella 9* and *Succinivibrio* were all depleted in the caecum of animals treated with fluoxetine. Data are expressed as median and min-to-max values. *$p<0.05$ (n=8/group). Data was analysed using Kruskal-Wallis non-parametric test followed by Mann-Whitney U test and corrected for multiple comparisons using the Benjamin-Hochberg false discovery rate (pFDR) method *(refer to Supplemental Material for details on statistics*)

Valproate and aripiprazole alter the levels of cecal short chain fatty acids (SCFAs)

A key function of gut bacteria is the catabolism of non-digestible dietary fibres resulting in the production of SCFAs, which in turn modulate a number of physiological processes (den Besten et al., 2013; Koh et al., 2016; Morrison and Preston, 2016). We decided to examine the abundance of short-chain fatty acids (SCFAs) in the caecal content in response to psychotropic drugs. Valproate administration induced a significant decrease in the levels of propionate and butyrate while augmenting the levels of isovalerate. Aripiprazole administration induced a significant increase in the levels of acetate and isovalerate (**Figure 2.7**). Two other SCFAs, valerate and isobutyrate, were also quantified and were not affected by any psychotropic treatment (**Figure S.2.4**).

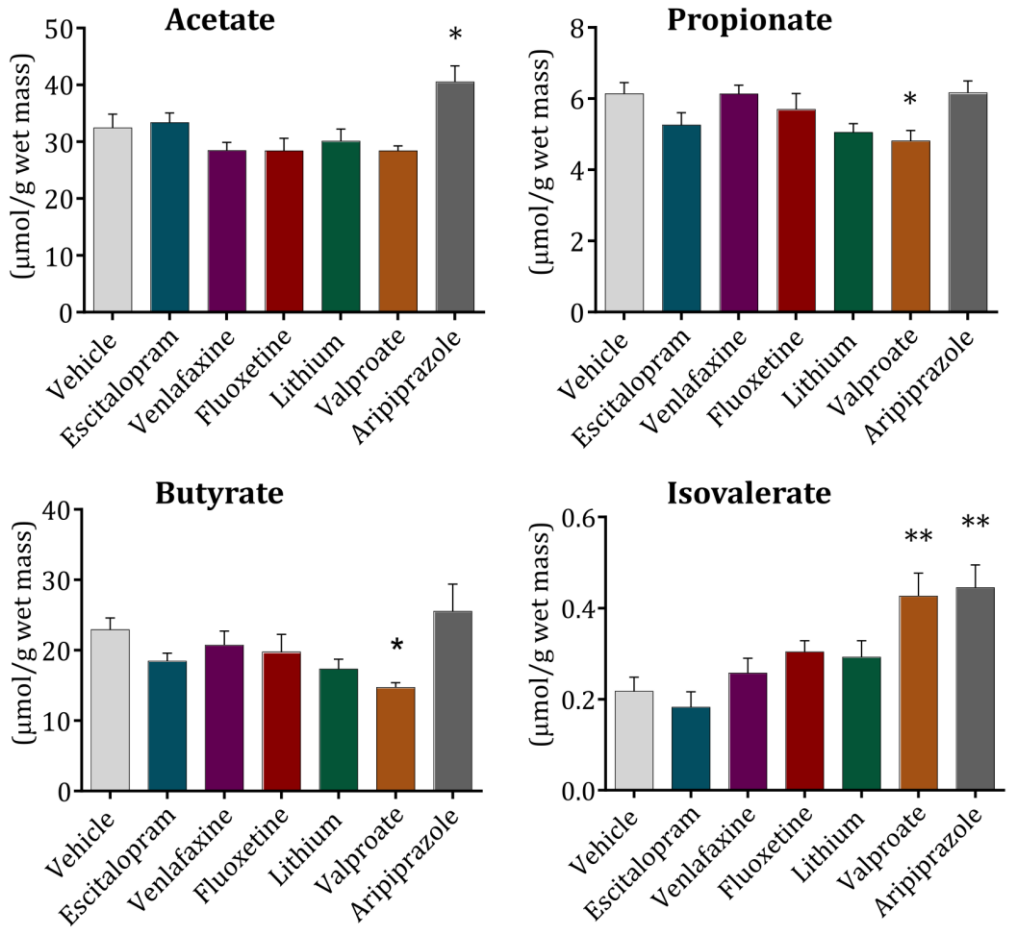


Figure 2.7 Short chain fatty acid (SCFAs) caecal levels. *Statistics:* all SCFAs had $p<0.05$ in one-way ANOVA. Acetate: Dunnett's t-test $p=0.035$ for aripiprazole VS vehicle. Propionate: Dunnett's t-test $p=0.029$ for valproate VS vehicle. Butyrate: Dunnett's t-test $p=0.042$ for valproate VS vehicle. Isovalerate: Dunnett's t-test $p=0.002$ for valproate VS vehicle; $p=0.001$ for aripiprazole VS vehicle. Data are expressed as mean+SEM. *$p<0.05$ and **$p<0.01$ (n=8/group)

Escitalopram, venlafaxine, fluoxetine and aripiprazole administration increases ileal but not colonic permeability

We assessed paracellular intestinal permeability of treated animals in both ileum and colon tissues *ex vivo*. Among the drugs tested, escitalopram, venlafaxine, fluoxetine and aripiprazole administration significantly increased epithelial permeability in the distal ileum as compared to vehicle-treated animals (**Figure 2.8**). None of the drugs at the doses tested affected permeability in the distal colon.

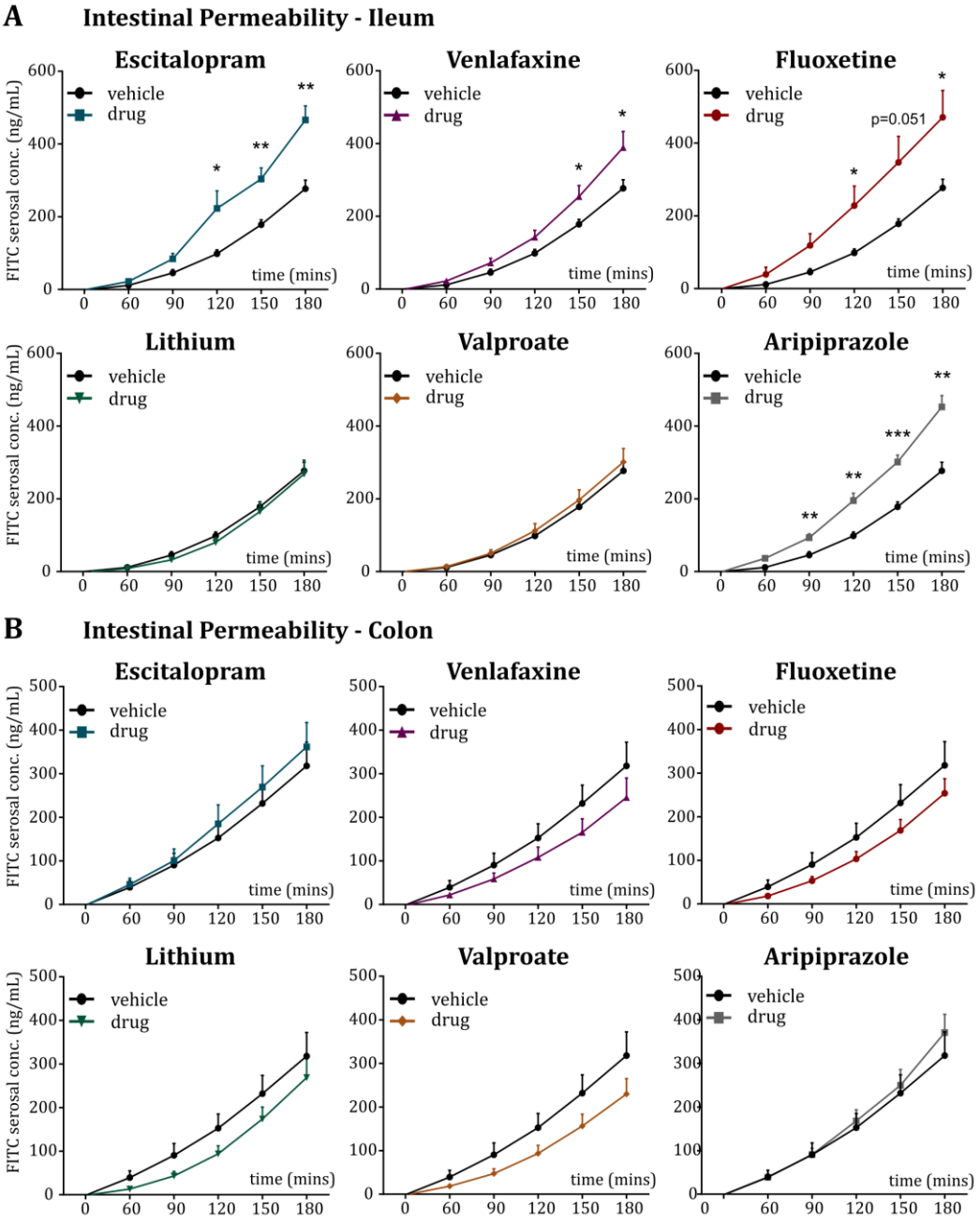


Figure 2.8 Effect of drug treatments on epithelial permeability in small and large intestine. In the different panels, each treatment is compared to the vehicle. (A) In distal ileum, escitalopram-, venlafaxine-, fluoxetine- and aripiprazole-treated rats showed a significant increase in FITC paracellular permeability. (B) In the colon, none of the drugs induced significant changes in intestinal permeability. Data were analysed with a mixed between-within subjects ANOVA. *Statistics:* Distal ileum $F_{(5;235)}$=385.037, p<0.001 for the effect of Time, $F_{(6;47)}$=4.181, p<0.01 for the effect of Treatment, $F_{(30;235)}$=3.490, p<0.01 for the Time×Treatment interaction. Distal colon $F_{(5;235)}$=298.307, p<0.001 for the effect of Time, but no effects for Treatment or Time×Treatment interaction. F=F-statistic. Mean values in each time point were further compared to the vehicle with unpaired t-test. Statistical outcomes for the t-test are described in *Supplemental Material*. Data are expressed as mean+SEM. *p<0.05, **p<0.01 and ***p<0.001 (n=7-8/group)

Discussion

There is an increasing emphasis on the interactions between gut microbiota and drug action across different therapeutic areas including oncology, cardiovascular medicine and even psychiatry. Here we assessed whether orally administered psychotropics can affect microbial and intestinal function in healthy adult rats. Escitalopram and fluoxetine showed differential antimicrobial activity *in vitro*, whereas lithium, valproate and aripiprazole induced significant changes in gut microbiota composition and SCFAs levels *in vivo*. Fluoxetine also induced minor but significant changes in bacterial genera *in vivo*. Escitalopram, venlafaxine, fluoxetine and aripiprazole increased the intestinal permeability in the ileum but not colon.

Limited studies have investigated the effect of psychotropic medications on growth of microbial strains. We found that both SSRIs escitalopram and fluoxetine have diverse antimicrobial activity *in vitro* against *E.coli* and *L. rhamnosus*, two bacteria residing in the human gut (Thursby and Juge, 2017; Walter, 2008). These results are in line with previous work showing that SSRIs possess antimicrobial action *in vitro* especially against gram-positive bacteria such as Staphylococcus and Enterococcus (Ayaz et al., 2015; Coban et al., 2009). Moreover, the antimicrobial activity of some antidepressants has been previously confirmed by the synergistic effects of some SSRIs in combination with antibiotics; as well as their effects against some antibiotic-resistant bacteria (Bohnert et al., 2011; Munoz-Bellido et al., 1996, 2000). Interestingly the two drugs, despite both belonging to the same class of antidepressants, showed different effects, with fluoxetine having strong antimicrobial activity and escitalopram being a weak antimicrobial. A possible mechanism through which fluoxetine and escitalopram might inhibit bacterial growth is through their action as efflux pump inhibitors, which interferes with the normal functioning of the bacteria (Bohnert et al., 2011). Intriguingly, among the drugs assessed *in vitro*, only fluoxetine induced overt shifts in gut microbiota composition *in vivo*. Moreover, differences in drug doses might occur in the two experimental settings, making it challenging to directly compare the experiments. These data highlight that some caution is required in extrapolating the results of *in vitro* assays to predict the effects of drugs on complex gut microbial ecology.

The composition of the gut microbiota was substantially changed *in vivo* in lithium-, valproate-, and aripiprazole- treated animals. Interestingly, the microbial shifts were often consistent across groups, with a significant increase in the relative abundance of minor genera (**Figure 2.5**); while highly abundant genera were generally not affected and were decreased in few instances (**Figure 2.4**; i.e. *Lachnospiraceae NK4A136*, *Ruminococcus 1*, *Bacteroides*). Consistent with changes in bacterial genera, the alpha diversity was also augmented in animals treated with lithium, valproate and aripiprazole, suggesting an increase in microbial richness and diversity. Administration of the SSRI fluoxetine also induced changes in microbiota composition, specifically decreasing the genera *Prevotella 7*, *Prevotella 9* and *Succinivibrio* (**Figure 2.6**).

Some previous studies have shown that, among psychotropic medications, antipsychotics in particular exert an effect on the gut microbiota. Both atypical antipsychotics olanzapine (Davey et al., 2013; Morgan et al., 2014) and risperidone (Bahr et al., 2015b) induced changes in gut microbiota composition in rodents and children, respectively. Chronic treatment with risperidone was associated with significantly lower ratio of Bacteroidetes:Firmicutes in healthy young males and with an increase in gut microbiota diversity when compared to control participants. Interestingly, we found the same trend in our animals treated with aripiprazole (a compound that belongs to the same therapeutic class of risperidone): at the genus level, 21 of the 26 genera belonging to Firmicutes were significantly increased and 2 genera belonging to Bacteroidetes were decreased. In addition, the microbial alpha-diversity of aripiprazole-treated rats was significantly increased, in line with data on risperidone-treated children. The results, however, are not always consistent. In a bipolar disease cohort, for example, treatment with atypical antipsychotics induced a decrease in microbial diversity, with the effect being present in females but not in males (Flowers et al., 2017). In the same study, the bipolar cohort treated with atypical antipsychotics showed, at the microbiota genus level, a significant increase in *Lachnospiraceae* abundance and a significant decrease in *Akkermansia.* Also, a recent large-scale study *in vitro* looking at the effect of several non-antibiotic drugs on human

gut bacteria *in vitro*, found that *Akkermansia* was significantly more sensitive than all other strains to atypical antipsychotics (Maier et al., 2018). In contrast with these findings, treatment with the atypical antipsychotic aripiprazole did not affect the two aforementioned genera in our rats. The fact that antipsychotics cluster together on microbiome despite different chemical structures and CNS effects, implicates that direct bacterial activity may be part of their mechanism of action or at least their side effects. An important aspect of this study would be to understand the biological and physiological relevance of the bacteria that were altered in response to psychotropics administration. Even though some of the affected *genera* have not been fully characterised, others have been previously associated to diverse conditions and are described in **Table 2.2**.

It is important to note that further work is required to measure the level of these drugs in the caecum, in order to clarify whether these medications reach the caecum at adequate concentrations and are not completely absorbed in the upper gastrointestinal tract. This investigation will further elucidate if the drugs are having a direct microbial effect or are affecting the gut microbiome through indirect mechanisms (such as the gut-brain signalling). Moreover, future studies might want to assess the microbiome-targeted effects of these medications at lower doses, such as those translational to a human setting.

While some knowledge existed of the impact exerted by antipsychotics on the gut microbiota, other classes of psychotropics (see **Table 2.1**) have not previously been investigated *in vivo*. Here we show that chronic administration of the mood stabiliser lithium and the anticonvulsant valproate significantly affected the microbial composition and richness in rats. The increase in richness might be directly due to the effect of the drugs on the microbial stability and the presence of different bacteria competing for the same niche. This effect on richness might also be time and dose dependent. Principal coordinate analysis of Bray-Curtis (beta-diversity) showed that lithium, valproate and aripiprazole had a significant separation from the vehicle group. On the contrary, psychotropics belonging to the class of antidepressants (specifically escitalopram, venlafaxine and fluoxetine) did not markedly affect this aspect of

microbial richness and diversity. Only fluoxetine clustered far from the vehicle group in the principal coordinate analysis of Bray-Curtis (**Figure 2.3B**).

Table 2.2 List of bacterial *genera* that were altered by psychotropics and their associations with physiological/pathological conditions. Bacterial genera were selected from *Figures 4, 5* and *6* of the manuscript according to the following criteria: taxa that were significantly different from the vehicle in at least one treatment group. Among those taxa, *Ruminococcus 1*, *Clostridia Family XIII*, *Ruminiclostridium 5*, *Peptoclostridium*, *Eubacterium oxidoreducens* and *Succinivibrio* were not included in the table due to lack of information on their physiological role. *Abbreviations:* ↑ increase, ↓ decrease, *BMI* body mass index, *CD* coeliac disease, *CDI Clostridium difficile* infection, *DSS* dextran sulphate sodium, *FA* food allergy, *HIV* human immunodeficiency virus, *NAFLD* non-alcoholic fatty liver disease, *SAMP8* senescence-accelerated mouse prone 8, *T2D* type 2 diabetes

Bacteria Genus	**Physiological / Pathological Condition(s)**	**Reference(s)**
Phylum Firmicutes		
Lachnospiraceae NK4A136	Associated with intestinal barrier function in mice with DSS-induced colitis. ↑ in mice fed a meat protein diet. ↓ in SAMP8 mice. Altered in statin therapy in mice. *Lachnospiraceae* strains restrict intestinal inflammation	(Caparrós-Martín et al., 2017; Chen et al., 2017; Li et al., 2017; Xie et al., 2018; Zhan et al., 2018)
Ruminococcaceae uncult	*Ruminococcaceae* ↑ in mice fed a high-fat diet. ↓ in NAFLD patients. ↓ in CDI patients	(Kim et al., 2012; Raman et al., 2013; Schubert et al., 2014)
Clostridium sensu stricto 1	↑ in infants with high genetic risk of developing CD. ↑ in infants with IgE-mediated FA	(Ling et al., 2014; Olivares et al., 2014)
Intestinibacter	↓ in metformin therapy in patients with T2D	(Forslund et al., 2015; Wu et al., 2017a)
Eubacterium coprostanoligenes	Cholesterol-lowering effects	(Freier et al., 1994; Li et al., 1998; Li et al., 1995; Ren et al., 1996)
Christensenellaceae uncult	↑ in longevity. Highly heritable. Associated to low BMI (anti-obesity)	(Goodrich et al., 2016; Kong et al., 2016; López-Contreras et al., 2018)
Phylum Bacteroidetes		
S_24_7 uncultbact	↑ in aging	(Scott et al., 2017a)
Bacteroides	HLA-B27 transgenic rats colonised with *Bacteroides* develop colitis and gastritis. Positively associated to immunoregulation in HIV-1 infection in humans	(Paquin-Proulx et al., 2016; Rath et al., 1996)
Prevotella 7 *Prevotella 9*	*Prevotella* species ↑ in healthy subjects who exhibit improved glucose metabolism following 3-day consumption of fibers	(Kovatcheva-Datchary et al., 2015)

Short chain fatty acids (SCFAs) are produced in the caecum by microbial fermentation (den Besten et al., 2013; Morrison and Preston, 2016) and are key regulators of several host processes such as metabolism (De Vadder et al., 2014), behaviour (Schroeder et al., 2007) and CNS function (Erny et al., 2015; Huuskonen et al., 2004). With marked alterations present at the microbiota level, it was perhaps not surprising that changes in SCFAs occurred. Valproate and aripiprazole influenced SCFAs abundance in the caecum, with acetate and isovalerate being increased by aripiprazole treatment and propionate, butyrate and isovalerate being differentially altered by valproate (**Figure 2.7**). We next investigated whether changes in SCFAs levels were associated with specific microbial taxa that are known producers of SCFAs. *Clostridium spp.*, a known producer of the SCFA acetate (Koh et al., 2016), was found to be increased in aripiprazole-treated animals, while valproate-treated rats showed a decrease in *Bacteroidetes*, which might explain the reduction in propionate in this experimental group. However, in our study the correlations between bacterial taxa and SCFAs were limited and sometimes discordant, for example *Prevotella* which is a producer of acetate (Koh et al., 2016) was decreased by aripiprazole administration. Therefore, the medications might have a different and direct influence on SCFA levels that are not mediated by the intestinal bacteria and still need to be teased apart.

The impact of psychotropic drugs on gut functionality is poorly understood. Thus, we assessed epithelial permeability in the small and large intestine and found that the three antidepressants escitalopram, venlafaxine and fluoxetine, together with the atypical antipsychotic aripiprazole, increased epithelial permeability in the ileum. Escitalopram, venlafaxine and fluoxetine share a common mechanism of action that is the blockade of the serotonin (5-HT) transporter (SERT) leading to increases in intrasynaptic 5-HT levels. SERT is not only present in the brain, it is also widely expressed on epithelial cells of the intestinal mucosa where it removes 5-HT from the interstitial space following release by enterochromaffin cells (Chen et al., 2001; Chen et al., 1998; Coates et al., 2004; Wade et al., 1996). 5-HT is involved in the control of intestinal permeability (Bischoff et al., 2009; Haub et al., 2010; Yamada et al., 2003), thus it is plausible to speculate that the changes observed in ileal permeability might be dependent on direct effects of the three antidepressants on SERT. In addition to the antidepressants, also the atypical antipsychotic aripiprazole increased permeability in

the ileum, however, this effect is not SERT-mediated and may be due to its effects on other 5-HT receptors. On the other hand, aripiprazole induced a concomitant shift in gut microbiota and future studies are needed to determine if there is a causal link between microbiota and permeability in this specific treatment group. Interestingly, the action of psychotropics on intestinal permeability was region-specific, with the colon being largely unaffected.

Interestingly, some studies have demonstrated that the gut microbiota of depressed (Kelly et al., 2016; Naseribafrouei et al., 2014) and bipolar (Evans et al., 2017) patients has an abnormal composition. This suggests that the gut-targeted effects of psychotropics might be part of the mechanism of action of these medications, however, this needs to be further investigated and confirmed. In this vein, future studies examining the impact of these drugs on microbiota composition in animal models of mental disorders and subsequently on human cohorts are warranted.

The functional consequences of drug-induced microbiome changes maybe at multiple levels including drug efficacy, kinetics, side effects and safety. Regarding side effects, weight gain has been the most studied in the context of the microbiome due to the relationship between microbiota composition and obesity (Torres-Fuentes et al., 2017) and appetite regulation (Cani et al., 2009; van de Wouw et al., 2017). Indeed, weight gain induced by antipsychotics (including olanzapine and risperidone) can be modulated by targeting the microbiome with antibiotics and prebiotics (Bahr et al., 2015a; Bahr et al., 2015b; Kao et al., 2018; Morgan et al., 2014). In a recent meta-analysis, all of the antidepressants tested here increased weight gain in a population cohort (Gafoor et al., 2018), suggesting a dissociation between their effects on microbiome and bodyweight *per se*.

In conclusion, the present study demonstrates that psychotropic medications differentially affect gut microbiota composition and intestinal permeability in healthy adult rats. Interestingly, such changes do not parallel with the impact of these drugs on *in vitro* isolated bacterial strains or on intestinal permeability *per se*. Together, these data highlight the importance of investigating the impact of drugs used for the treatment of psychiatric disorders on microbiota-gut-brain axis function.

Supplemental Material

SUPPLEMENTAL TABLES

Table S.2.1 Statistics values for relative abundance of bacterial PHYLA in each treatment group as compared to the vehicle.

PHYLUM	*Escitalopram*		*Venlafaxine*		*Fluoxetine*		*Lithium*		*Valproate*		*Aripiprazole*	
	p	(i/m)Q	p	(i/m)Q	p	(i/m)Q	p	(i/m)Q	p	(i/m)Q	p	(i/m)Q
Firmicutes	0.505	0.042	0.505	0.050	0.721	0.075	0.083	0.025	0.003 *	0.017 *	0.015 *	0.017 *
Bacteroidetes	0.959	0.075	0.234	0.025	0.328	0.042	0.001 *	0.017 *	0.005	0.025	0.195	0.025
Verrucomicrobia	0.328	0.025	0.442	0.042	0.234	0.033	0.959	0.092	0.105	0.033	0.195	0.033
Proteobacteria	1.000	0.083	0.195	0.017	0.130	0.025	0.798	0.075	0.505	0.058	0.328	0.05
Actinobacteria	0.382	0.033	0.050	0.008	0.505	0.050	0.000 *	0.008 *	0.002 *	0.008 *	0.195	0.017
Cyanobacteria	0.574	0.050	0.721	0.075	0.105	0.017	0.105	0.033	0.505	0.050	0.234	0.042
Deferribacteres	0.328	0.017	0.234	0.033	0.007 *	0.008 *	0.959	0.083	0.130	0.042	0.442	0.058
Tenericutes	0.279	0.008	0.959	0.083	0.878	0.083	0.574	0.058	0.574	0.067	0.505	0.067
Noblasthit	0.645	0.058	0.574	0.058	0.645	0.058	0.130	0.042	0.878	0.092	0.878	0.083

*p<0.05, Mann-Whitney U test, Benjamini-Hochberg critical value [$(i/m)Q$] with a Q (false-discovery rate) of 0.2. N=8/group. Red colour indicates an increase; blue colour indicates a decrease in the relative abundance of bacterial taxa as compared to vehicle

Table S.2.2 Statistics values for relative abundance of bacterial FAMILIES in each treatment group as compared to the vehicle.

FAMILY	*Escitalopram*		*Venlafaxine*		*Fluoxetine*		*Lithium*		*Valproate*		*Aripiprazole*	
	p	(i/m)Q	p	(i/m)Q	p	(i/m)Q	p	(i/m)Q	p	(i/m)Q	p	(i/m)Q
Actinobacteria												
Bifidobacteriaceae (Bifidobacteriales)	0.959	0.187	0.505	0.068	0.798	0.162	0.000 *	0.013 *	0.000 *	0.013 *	0.279	0.098
Coriobacteriaceae (Coriobacteriales)	0.279	0.034	0.195	0.080	0.959	0.183	0.000 *	0.017 *	0.721	0.153	0.382	0.110
Nocardiaceae (Actinomycetales)	0.878	0.170	0.959	0.110	0.645	0.140	0.959	0.183	0.038 *	0.051 *	1.000	0.183
Bacteroidetes												
Bacteroidaceae (Bacteroidales)	0.878	0.157	0.130	0.025	0.279	0.076	0.021 *	0.034 *	0.382	0.119	0.382	0.106
S 24_7 (Bacteroidales)	0.442	0.059	0.798	0.021	0.105	0.038	0.574	0.149	0.021 *	0.034 *	0.959	0.170
Deferribacteres												
Deferribacteraceae (Deferribacterales)	0.328	0.047	0.234	0.076	0.007 *	0.013 *	0.959	0.179	0.130	0.085	0.442	0.115
Firmicutes												
Enterococcaceae (Lactobacillales)	0.382	0.055	0.798	0.153	0.195	0.059	0.130	0.098	0.038 *	0.055 *	0.161	0.068
Lactobacillaceae (Lactobacillales)	0.959	0.174	0.083	0.030	0.105	0.042	0.083	0.076	0.000 *	0.004 *	0.328	0.102

Peptostreptococcaceae (Clostridiales)	0.279	0.030	0.959	0.034	0.015 *	0.017 *	0.000 *	0.004 *	0.000 *	0.008 *	0.002 *	0.017 *
Christensenellaceae (Clostridiales)	0.574	0.089	0.083	0.089	0.083	0.029	0.015 *	0.029 *	0.003 *	0.017 *	0.001 *	0.013 *
Clostridiaceae 1 (Clostridiales)	0.083	0.008	0.645	0.051	0.279	0.080	0.000 *	0.008 *	0.007 *	0.021 *	0.000 *	0.004 *
Defluviitaleaceae (Clostridiales)	0.878	0.166	0.505	0.106	0.083	0.034	0.007 *	0.025 *	0.083	0.068	0.003 *	0.025 *
Family XIII (Clostridiales)	0.878	0.162	0.130	0.085	0.645	0.132	0.038 *	0.055 *	0.279	0.106	0.065	0.042
Eubacteriaceae (Clostridiales)	0.798	0.150	0.083	0.119	0.505	0.123	0.028 *	0.051 *	0.234	0.094	0.234	0.089
Peptococcaceae (Clostridiales)	0.721	0.123	0.130	0.047	0.328	0.094	0.021 *	0.038 *	0.028 *	0.047 *	0.161	0.059
Ruminococcaceae (Clostridiales)	0.645	0.098	0.050	0.008	0.161	0.055	0.028 *	0.047 *	0.021 *	0.030 *	0.021 *	0.034 *
Erysipelotrichaceae (Erysipelotrichales)	0.328	0.042	0.195	0.042	0.959	0.179	0.050	0.064	0.021 *	0.038 *	0.234	0.076
Veillonellaceae (Veillonellales)	0.021	0.004	0.279	0.127	0.000 *	0.004 *	0.002 *	0.021 *	0.010 *	0.025 *	0.000 *	0.008 *
Staphylococcaceae (Bacillales)	0.105	0.013	0.234	0.132	0.015 *	0.021 *	0.038 *	0.059 *	0.161	0.089	0.130	0.051
Proteobacteria												
Succinivibrionaceae (Aeromonadales)	0.130	0.025	0.028	0.140	0.002 *	0.008 *	0.021 *	0.042 *	0.021 *	0.042 *	0.002 *	0.021 *
Tenericutes												
Uncult. rumen bacteria (Mollicutes)	0.442	0.068	0.878	0.114	0.798	0.170	0.442	0.128	0.442	0.123	0.003 *	0.030 *
Uncult (Mollicutes)	0.645	0.119	0.505	0.102	0.382	0.102	0.234	0.102	0.105	0.076	0.028 *	0.038 *

*p<0.05, Mann-Whitney U test, Benjamini-Hochberg critical value $[(i/m)Q]$ with a Q (false-discovery rate) of 0.2. N=8/group. Red colour indicates an increase; blue colour indicates a decrease in the relative abundance of bacterial taxa as compared to vehicle. The following families where not significantly different from the vehicle in either treatment group and therefore are not reported in the table: *Lachnospiraceae, Verrucomicrobiaceae, Prevotellaceae, Porphyromonadaceae, ClostridialesvadinBB60, Rhodospirillaceae, Gastranaerophilales uncult, Rikenellaceae, Anaeroplasmataceae, Noblasthit1, Comamonadaceae, ratAN060301C (Bacteroidetes), Aerococcaceae, Acidaminococcaceae*

Table S.2.3 Statistics values for relative abundance of bacterial GENERA in each treatment group as compared to the vehicle. This table can be found in the excel file "Table S3" at the following link: https://link.springer.com/article/10.1007%2Fs00213-018-5006-5#SupplementaryMaterial

SUPPLEMENTAL FIGURES

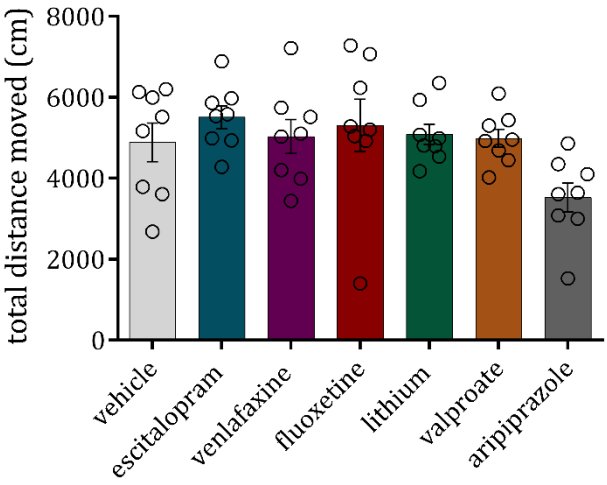


Figure S.2.1 Open field test indicating the total distance moved (locomotor activity). N=8/group. Data were homogeneous and normally distributed so were analysed with a one-way ANOVA followed by Dunnett's test.

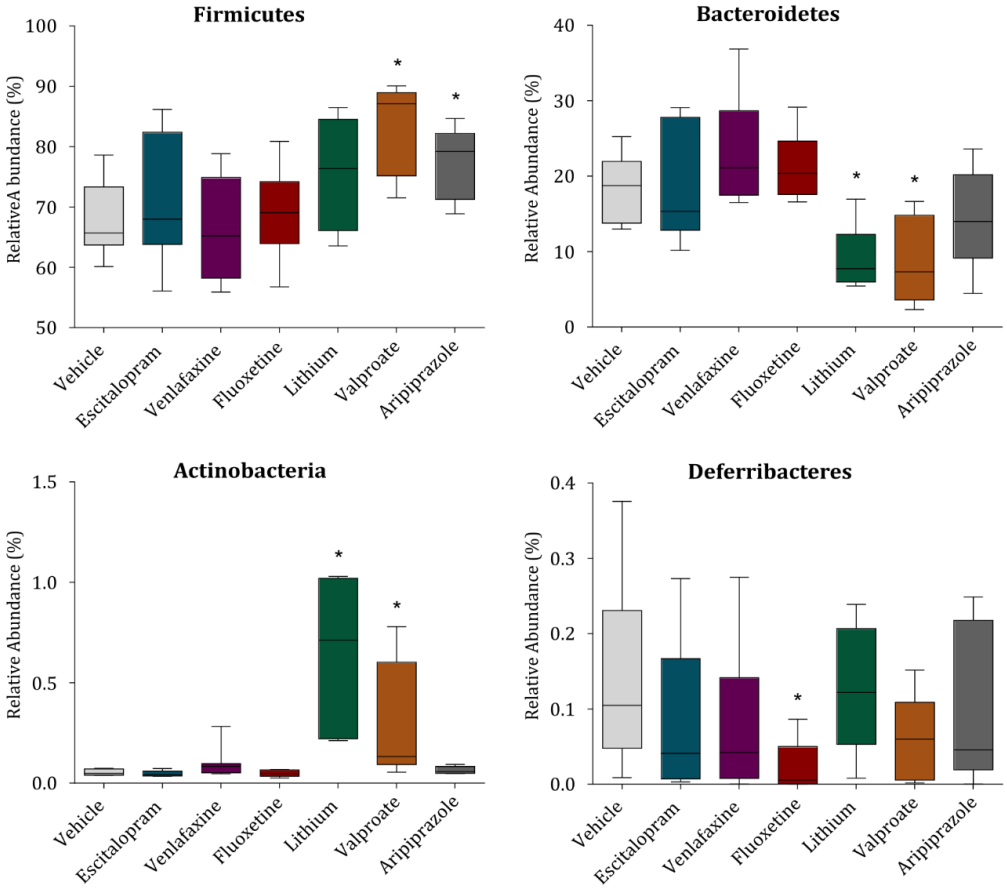


Figure S.2.2 Significant differences at phylum level. *p<0.05, Mann-Whitney U test with a Q (false-discovery rate) of 0.2. N=8/group.

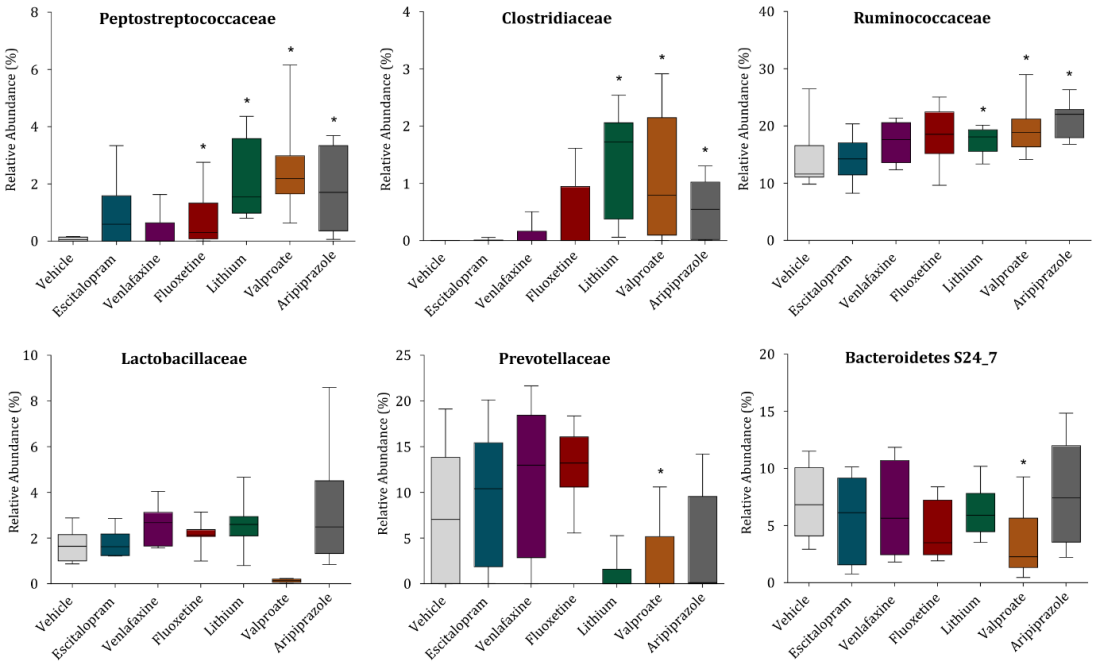


Figure S.2.3 Significant differences at family level. *p<0.05, Mann-Whitney U test with a Q (false-discovery rate) of 0.2. N=8/group.

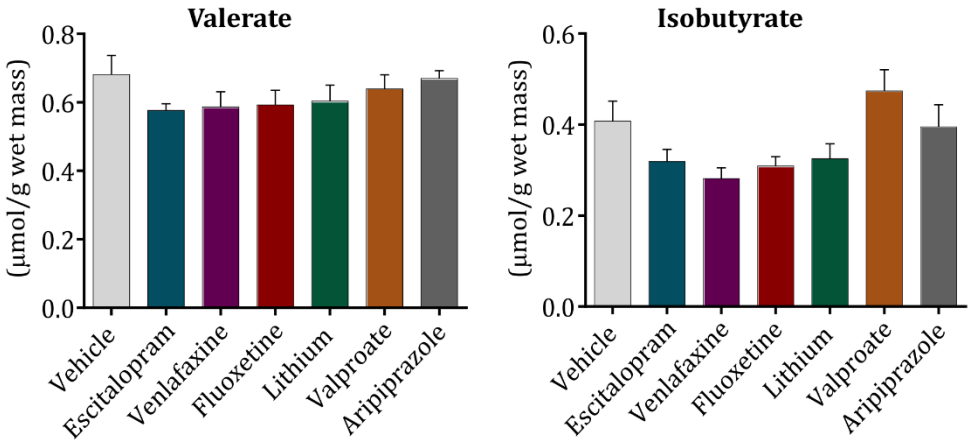


Figure S.2.4 Concentrations of the two SCFAs valerate and isobutyrate in the caecum. Data are expressed as mean + SEM (n=8/group).

SUPPLEMENTAL METHODS

Caecal microbiota composition and short chain fatty acids (SCFAs) analysis
Caecum was harvested, snap frozen and stored at -80ºC prior to the analysis. Caecal content was further split into halves for the 16S sequencing and SCFAs analysis.

- ***Caecal content DNA extraction***

DNA extraction was performed using the QIAmp Fast DNA Stool Mini Kit (Qiagen, Sussex, UK) coupled with an initial bead-beating step. Briefly, 200 mg of each caecal sample were vortex-mixed in a 2 ml screw-cap tubes (Sarstedt, Wexford, Ireland) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.0 mm sterile zirconia beads plus a single 3.5 mm diameter bead (BioSpec Products, Bartlesville, USA) with 1 ml of Qiagen InhibitEX® buffer. Following steps were according to manufacturer's instructions. DNA was quantified using the QubitTM 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) and the Qubit® dsDNA HS Assay Kit (Life Technologies, Oregon, USA). Extracted DNA was kept frozen at -20ºC until further analysis.

- ***16S rRNA Gene Sequence-based microbiota analysis***

The V3-V4 hypervariable region of the 16S rRNA gene were amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol (http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Briefly, first PCR was done using forward primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Each 25 µl PCR reaction contained 5 ng/µl microbial genomic DNA, 1 µM of each primer and 12.5 µl 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK). The PCR conditions follow as: initial denaturation at 95 ºC x 3 min; 25 cycles of 95 ºC x 30 s, 55 ºC x 30 s, 72 ºC x 30 s; and 72 ºC x 5 min for final extension. PCR products were purified with Agencourt AMPure XP system (Beckman Coulter Genomics, Takeley, UK). In the next step, dual indices and Illumina sequencing adapters were attached to PCR products using the Nextera XT Index Kit (Illumina, San Diego, CA). Each 50 µl PCR reaction contained 5 µl purified DNA, 5 µl index primer 1 (N7xx), 5 µl index primer 2 (S5xx), 25 µl 2x Kapa HiFi Hot Start Ready mix and 10 µl PCR grade water. PCR amplification was completed using the previous program but with only 8 amplification cycles instead of 25. Following this, a second clean-up step with the Agencourt AMPure XP system was done. PCR products were quantified, normalised and pooled in an equimolar fashion using the Qubit® dsDNA HS Assay Kit (Life Technologies, Oregon, USA). Next steps in the library preparation were carried out by Teagasc Next Generation DNA Sequencing Facility (Teagasc, Moorepark, Food Research Centre) prior to 2×250 (bp) paired-end sequencing on the Illumina MiSeq platform, using standard Illumina sequencing protocols.

- ***Bioinformatic sequence analysis***

Bioinformatic sequence analysis was performed as previously described (Murphy et al., 2017). Briefly, paired-end sequences were assembled using FLASH (Magoč and Salzberg, 2011) and analysed using QIIME v1.8.0 (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010). Sequences were quality checked and the remaining sequences were clustered into operational taxonomic units using USEARCH (v7-64bit) (Edgar, 2010). Taxonomic ranks were assigned with a BLAST search against the SILVA SSURef database release 123 (Quast et al., 2013). Alpha and beta diversities and Bray-Curt dissimilarities were generated in RStudio (version 1.1.419) using the "vegan" and "car" packages. Principal coordinate analysis (PCoA) plots were visualised with ggplot2 (V 2.2.1) using OTU values normalised with the wisconson function in the vegan package (v. 2.5-1). Ellipses of the PCoA were calculated using the stat_ellipse function in ggplot2 (v 2.2.1) with confidence interval (CI) set to 0.90. Explained percent variance was calculated and plotted for x and y axis. Adonis function (PERMANOVA, permutations=999) in the vegan package (v 2.5-1) was performed on Bray-Curtis matrix on three dimensions. Relative abundance of bacterial taxa was expressed as % of identified sequences.

- ***Heatmap construction***

Log2 ratios were calculated from group medians of highly abundant bacteria at the genus level using R (version 3.3.2) and R Studio (version 1.0.136). Heatmaps were created in R and imported into InkScape to refine the image.

- ***Short chain fatty acids concentration analysis from cecum content***

Analysis of the main SCFA (acetate, propionate and butyrate) and a branched chain fatty acid (BCFA) (iso-butyrate) were carried out in supernatants of homogenised caecal content by gas chromatography (GC), as previously described (Wall et al., 2012). Briefly, at least 100 mg of each caecal sample were vortex-mixed with MilliQ water and incubated at room temperature for 10 min. Following this, the supernatant was obtained by centrifugation (24000 g, 5 min, 4°C), filtered through 0.2µm filters and mixed with 2-Ethylbutyric acid (Sigma Aldrich Ireland Ld, Wicklow, Ireland) as an internal standard. A gas chromatograph Varian 3800 GC flame-ionization system, fitted with a Zebron ZB-FFAP column (30m x 0.32mm x 0.25µm) (Phenomenex, Macclesfield, UK) was used for quantification of SCFA. Chromatographic conditions were as follow: GC oven temperature was held initially at 50 °C for 0.5 min, then raised stepwise, by 10 °C/min, until it reached 140 °C. It was then raised by 20 °C/min up to 240 °C, and held for 5 min. The temperature of the injector and the detector were set at 240°C and 300°C, respectively. Helium was used as the carrier gas at a flow rate of 1.3 ml min^{-1}. A standard curve was built with increasing concentrations of a standard mix containing the SCFAs and BCFA analysed (Sigma Aldrich Ireland Ld, Wicklow, Ireland). Peaks were integrated by using the Varian Star Chromatography Workstation v6.0 software. The concentration of each SCFA was calculated using the linear regression equations ($R2 \geq 0.999$) from the corresponding standard curves. Standards were included in each run to check calibration. Data were presented as µmol/wet caecum weight (g).

SUPPLEMENTAL STATISTICS

Statistical output of the t-test related to Figure 2.2 (Effect of psychotropic drugs on growth of Lactobacillus rhamnosus 6118 and Escherichia coli APC105 in vitro)

Escitalopram effect on E.coli: $t_{(4)}$=8.99, p<0.01 for 600µg/mL at 2h, $t_{(4)}$=16.396, p<0.001 for 600µg/mL at 3h, $t_{(4)}$=45.962, p<0.001 for 600µg/mL at 4h, $t_{(4)}$=40.531, p<0.001 for 600µg/mL at 5h, $t_{(4)}$=32.087, p<0.001 for 600µg/mL at 6h, $t_{(4)}$=37.523, p<0.001 for 600µg/mL at 7h. Fluoxetine effect on L. rhamnosus: $t_{(4)}$=3.487, p<0.05 for 600µg/mL at 2h, $t_{(4)}$=3.7, p<0.05 for 400µg/mL at 2h, $t_{(2.051)}$=5.75, p<0.05 for 600µg/mL at 3h, $t_{(2.099)}$=5.530, p<0.05 for 400µg/mL at 3h, $t_{(4)}$=8.451, p<0.05 for 600µg/mL at 4h, $t_{(4)}$=7.82, p<0.01 for 400µg/mL at 4h, $t_{(4)}$=14.223, p<0.001 for 600µg/mL at 5h, $t_{(4)}$=14.778, p<0.001 for 400µg/mL at 5h, $t_{(4)}$=4.479, p<0.05 for 600µg/mL at 6h, $t_{(4)}$=4.450, p<0.05 for 400µg/mL at 6h, $t_{(4)}$=5.335, p<0.01 for 600µg/mL at 7h, $t_{(4)}$=5.335, p<0.01 for 400µg/mL at 7h. Fluoxetine effect on E.coli: $t_{(2.021)}$=12.261, p<0.01 for 600µg/mL at 2h, $t_{(4)}$=12.908, p<0.001 for 400µg/mL at 2h, $t_{(4)}$=11.144, p<.001 for 100µg/mL at 2h, $t_{(4)}$=19.036, p<0.001 for 600µg/mL at 3h, $t_{(4)}$=21.205, p<0.001 for 400µg/mL at 3h, $t_{(4)}$=21.952, p<0.001 for 100µg/mL at 3h, $t_{(4)}$=49.952, p<0.001 for 600µg/mL at 4h, $t_{(4)}$=40.429, p<0.001 for 400µg/mL at 4h, $t_{(4)}$=64.846, p<0.001 for 100µg/mL at 4h, $t_{(4)}$=22.224, p<0.001 for 600µg/mL at 5h, $t_{(4)}$=26.379, p<0.001 for 400µg/mL at 5h, $t_{(4)}$=40.844, p<0.001 for 100µg/mL at 5h, $t_{(4)}$=48.543, p<0.001 for 600µg/mL at 6h, $t_{(4)}$=43.874, p<0.001 for 400µg/mL at 6h, $t_{(4)}$=47.115, p<0.001 for 100µg/mL at 6h, $t_{(4)}$=33.638, p<0.001 for 600µg/mL at 7h, $t_{(4)}$=35.601, p<0.001 for 400µg/mL at 7h, $t_{(4)}$=32.824, p<0.001 for 100µg/mL at 7h.

Statistical output of the t-test related to Figure 2.8 (Effect of drug treatments on epithelial permeability in small and large intestine)

Escitalopram on ileum: $t_{(13)}$= -2.517, p<0.05 at 120min, $t_{(13)}$= -3.785, p<0.01 at 150min, $t_{(13)}$= -4.196, p<0.01 at 180min. Venlafaxine on ileum: $t_{(13)}$= -2.294, p<0.05 at 150min, $t_{(13)}$= -2.245, p<0.05 at 180min. Fluoxetine on ileum: $t_{(13)}$= -2.36, p<0.05 at 120min, $t_{(13)}$= -2.492, p,0.05 at 180min. Aripiprazole on ileum: $t_{(13)}$= -3.46, p<0.01 at 90min, $t_{(13)}$= -4.233, p<0.001 at 120min, $t_{(13)}$= -5.184, p<0.001 at 150min, $t_{(13)}$= -4.401, p<0.01 at 180min.

Chapter 3

The Mood Stabilisers Lithium and Valproate Increase Bile Acids and Bile-Associated Gut Bacteria

Sofia Cussotto[1,2], Alvaro Lopez Gallardo[1,3#], Anna V. Golubeva[1,2#], Conall R. Strain[1,4], Fiona Fouhy[1,4], Catherine Stanton[1,4,5], Susan A. Joyce[1,3], Timothy G. Dinan[1,5], John F. Cryan[1,2]

[#] Equal contributions

[1] APC Microbiome Ireland, University College Cork, Cork, Ireland

[2] Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[3] School of Biochemistry and Cell Biology, University College Cork, Cork, Ireland

[4] Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland

[5] Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

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Abstract

Background: Lithium (mood stabiliser) and valproate (antiepileptic and mood stabiliser) are two psychotropic medications widely used in clinical practice. There is a growing appreciation of the impact of drugs, especially psychotropic drugs, on the microbiome composition. Given the crucial role played by intestinal microbes in bile acid (BA) biotransformation, we wanted to investigate the effects of lithium and valproate on bile acid composition and bile-metabolising bacteria in the intestine.

Results: Chronic (4 weeks) administration of lithium and valproate in rats increased all types of BAs in circulation, faecal content and liver, except for tauro-conjugated bile acids. These effects were accompanied by an increase in some key bile-metabolising bacteria in the gut, such as *Clostridium*, *Eubacterium* and *Bifidobacterium*, measured through 16S rRNA sequencing. Intestinal and hepatic regulation of BA synthesis and transport was disrupted by both medications. Two possible mediators of such changes, liver damage and disruption of intestinal permeability, did not seem to play any overt role in the observed effects.

Conclusions: These findings reveal that the mood stabilisers lithium and valproate influence the composition of bile and correspondent bile-metabolising bacteria in the intestine. Future studies focused on the possible causality and direction of the interaction between drug-induced changes in microbiota and alterations in bile acids composition, as well as the clinical implications, are now warranted.

Keywords: *Mood stabiliser, Microbiota, FXR, Bile acids, Intestinal permeability*

Introduction

Lithium and valproate are two widely used psychotropic medications (from the Greek root *psychè*=mind and *tropòs*=turning, literally "mind-turning") used as mood stabilisers (both drugs) and antiepileptic (valproate). In the context of the microbiota-gut-brain axis, we have recently shown that many medications and especially these two drugs alter substantially the microbiome composition and diversity in rodents (Cussotto et al., 2019b; Davey et al., 2013; Maier et al., 2018). Given that the liver is a crucial component of drug metabolism and because the gut and the microbiota are connected to the liver through a gut-liver axis (Tripathi et al., 2018), it is possible that such changes may result in lithium and valproate also impacting on hepatic function and bile acid (BA) composition.

BA composition is determined by (1) the biosynthesis of primary BAs from cholesterol in the liver and subsequent conjugation to glycine or taurine; (2) bacterial modifications within the large intestine including deconjugation by bacteria with bile salt hydrolase activity (BSH, EC 3.5.1.24), allowing further microbial modification to secondary and tertiary BAs by 7α-dehydroxylating bacteria (EC 1.17.99.5) (Ramirez-Perez et al., 2017). Under physiological conditions, activation of farnesoid X receptor (FXR) is the key regulator of BA synthesis (Tu et al., 2000). FXR induces target genes in both the liver and the intestine, including hepatic small heterodimer partner (SHP) and intestinal fibroblast growth factor 19 (Fgf19), which in turn inhibit BA synthesis via repression of CYP7A1, CYP8B1 and CYP27A1 gene transcription (Kong et al., 2012). FXR activation is strongly influenced by BA composition with chenodeoxycholic acid (CDCA) being a stronger activator than deoxycholic acid (DCA). DCA, in turn, is stronger than lithocholic acid (LCA) and cholic acid (CA) (Parks et al., 1999). The complex pathways and mediators involved in BA synthesis and transport are described in **Figure 3.1**.

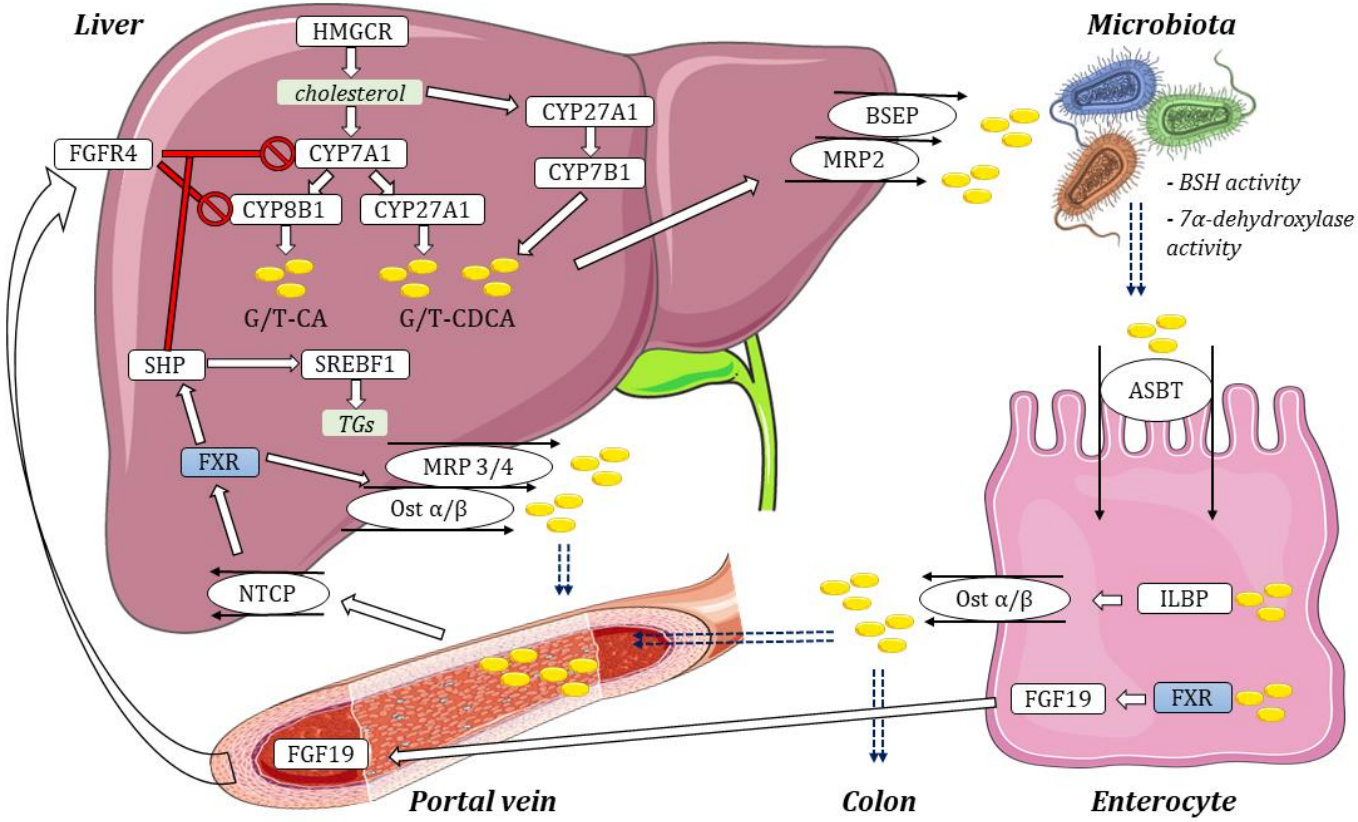


Figure 3.1 Transporters and enzymes involved in the synthesis and enterohepatic circulation of bile acids. The transcript levels of transporters and enzymes are described in Figure 3.6. *Abbreviations: ASBT* apical sodium-bile acid transporter, *BSEP* bile salt export pump, *BSH* bile salt hydrolase, *CYP* cytochrome P450, *FGF19* fibroblast growth factor 19, *FGFR4* fibroblast growth factor receptor 4, *FXR* farnesoid X receptor, *G/T-CA* glyco/tauro-cholic acid, *G/T-CDCA* glyco/tauro-chenodeoxycholic acid, *HMGCR* 3-hydroxy-3-methylglutaryl-CoA reductase, *ILBP* ileal lipid binding protein, *MRP* multidrug resistance protein, *NTCP* Na^+-taurocholate cotransporting polypeptide, *Ost α/β* organic solute transporter α/β, *SHP* small heterodimer partner, *SREBF1* sterol regulatory element binding transcription factor 1, *TGs* triglycerides.

Functionally, altered bile acid levels play a role in intestinal epithelial function (Hegyi et al., 2018), circadian rhythm regulation (Govindarajan et al., 2016; Ma et al., 2009), colon cancer (Ajouz et al., 2014) and glucose metabolism in health and disease (Shapiro et al., 2018). Information regarding the impact of psychotropic medications on microbiome composition is limited; however, in a recent study on rats, we have shown for the first time that certain psychotropic medications including lithium and valproate influence significantly the gut microbiome (Cussotto et al., 2019b). Moreover, in a tour de force *in vitro* screening of more than 1,000 drugs against 40 representative gut bacterial strains, it was found that 24% of human-targeting drugs, including the psychotropic valproate, inhibited the growth of at least one microbial strain (Maier et al., 2018). The authors argued that, based on drug excretion patterns from published work, the chosen concentration of 20 μM was below the median colon concentration of the human-targeted drugs tested and therefore had translational validity (Maier et al., 2018). The microbial changes observed in lithium- and valproate-treated animals might predict the influence of these medications on BA composition and consequently FXR activation. In clinical practice long-term administration of both lithium and valproate can be associated to a certain degree of hepatotoxicity (Hayes et al., 2016), giving further rationale for investigating BA metabolism and liver function. The aims of the current study were: (1) to determine whether lithium and valproate altered BA concentrations, (2) to determine whether such changes were associated with alterations in specific BA-metabolising bacterial taxa, (3) to examine some of the most obvious mechanisms at the hepatic and gastrointestinal level that may underpin such effects. To this end, we assessed whether genes involved in hepatic inflammation or changes in intestinal permeability were major contributors to the BA dysregulation observed following chronic administration of the two drugs.

Methods

Animals

Adult male Sprague Dawley rats (n=8/group; 200-250g on arrival) were obtained from Envigo UK. They were housed 2 per cage and maintained under a 12-h light/dark cycle, provided with chow and water *ad libitum*. Rats in the same cage underwent the same treatment to avoid confounding factors such as coprophagy. Animals were acclimated to housing conditions for one week prior to experimental treatment. Experiments were conducted in accordance with European Directive 2010/63/EU. Approval by the Animal Experimentation Ethics Committee of University College Cork was obtained before commencement of all animal-related experiments.

Drug administration

The drug treatment was administered as previously described (Cussotto et al., 2019b). The control group received a standard diet (Ssniff, SM Teklad Global 18% Protein Rodent diet, item no. S9912-S710) and drinking water. A second group received 0.2% lithium-supplemented diet, corresponding to approx. 150mg/kg/day, and hypertonic saline water (1.5% NaCl, in order to prevent lithium-induced ionic imbalance). A third group received 2% valproate-supplemented diet, corresponding to approx. 1.5g/kg/day. The concentration of each drug in drinking water and in the chow was determined from the average daily water/food consumption and the average body weight per rat. These dosing regimens have been previously used in chronic behavioural and neurochemical studies in rodents (Monti et al., 2010; O'Leary et al., 2012; Watase et al., 2007). The chow was stored at 4 ºC during the experiment.

Bile acid levels in plasma, colon content and liver

Plasma, colon and liver tissues were collected at sacrifice and immediately stored at -80 ºC until further analysis. Bile acids and salts were assessed as previously described

(Joyce et al., 2014). Briefly, they were extracted following addition of deuterated internal standards. Extracted acids were resuspended in 50% methanol, injected in triplicate and assessed in negative electrospray mode through a C18 Acquity column using an LCT Premier mass spectrometer (Waters Corp). Assessment of extraction efficiency was performed using internal standards, and samples were quantified by standard curve construction for individual bile moieties using targetlynx software (Waters) (refer to *Supplemental Methods*).

Microbiota composition analysis in the caecal content

The caecum microbiota was analysed as previously described (Cussotto et al., 2019b). Briefly, caecum was harvested and immediately snap-frozen and stored at -80ºC prior to the analysis. DNA was extracted using the Qiagen QIAmp Fast DNA Stool Mini Kit coupled with an initial bead-beating step. The V3-V4 hypervariable region of the 16S rRNA gene was amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library protocol. Samples were sequenced at Teagasc Sequencing Facility (TFRC, Moorepark) on the Illumina MiSeq platform using a 2×250 bp kit. Reads were assembled, processed and analysed following the pipeline, described in *Supplemental Methods*.

RNA extractions, reverse transcription and quantitative RT-qPCR

The intestinal tissue and frontal lobe of the liver were rapidly dissected from individual animals and stored at -80 °C. Total RNA was extracted with the mirVana^{TM} miRNA isolation kit (Thermo Fisher Scientific/Ambion) following the manufacturer's protocol. A Nanodrop 1000 (Thermo Scientific, UK) was used to determine RNA concentration. RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Thermo Fisher Scientific/Applied Biosystems) in a G-storm thermocycler (G-storm, Surrey, UK). Genes of interest (listed in *Supplemental material*) were amplified using SYBR Green primers. Each transcript value was calculated as the average of triplicate samples across experimental conditions. Values were normalized to β-actin. Data were analysed with the comparative cycle threshold

method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001) and presented as a fold change vs. vehicle group.

Intestinal permeability *ex vivo*

Intestinal permeability was carried out essentially as described previously (Golubeva et al., 2017). Briefly, freshly isolated ileum samples from 10 naïve adult SD rats were placed in Krebs solution and cut along the mesenteric border. Tissues then underwent seromuscular stripping (to remove the serosal and muscular layers) and were mounted into the Ussing chamber apparatus (Harvard Apparatus, Kent, UK, exposed area of 0.12 cm^2). 4kDa FITC-dextran was added to the mucosal chamber at a final concentration of 2.5 mg/mL; 200 µL samples were collected from the serosal chamber every 30 minutes for the following 3 hours. FITC was measured at 485 nm excitation / 535 nm emission wavelengths. Short-circuit current (I_{sc}) was recorded in a zero voltage clamp mode; transepithelial electrical resistance (TEER) was measured by discharging a 2 mV pulse. To minimize the influence of the intrinsic neuromuscular system, seromuscular stripping was performed. The serosa (visceral peritoneum) and the longitudinal/circular muscle layers of the intestinal wall were removed, leaving only the underlying submucosal elements, remnants of muscle and the epithelium. The experimental design of the study can be found in **Figure S.3.1**.

Dose relevance for the *ex vivo* intestinal permeability assessment

For the assessment of intestinal permeability *ex vivo*, we deduced the concentrations of lithium and valproate in the distal ileum (site of reabsorption of bile acids) on the basis of daily dose consumption and specific assumptions described in detail in **Figure S.3.2**. Based on these approximations, 5 mM lithium and 10 mM valproate mimicked the concentrations found in the rat distal ileum. To further examine the dose-response relationship between drug concentration and intestinal permeability, we assessed the same drugs in concentrations 10 times higher (50 mM lithium and 100 mM valproate).

Statistical analysis

Data are presented as mean + SEM. Data that satisfied both homogeneity and normality tests, were analysed using one-way ANOVA followed by Dunnett's test. Data that did not satisfy either homogeneity or normality tests, were analysed using Kruskal-Wallis non-parametric test followed by Mann-Whitney U test and corrected for multiple comparisons using the Benjamin-Hochberg false discovery rate (FDR) method. Grubbs method was employed to test for any outlier (Grubbs, 1950). Threshold for statistical significance was set at $p<0.05$.

Results

3.1 Lithium and valproate increase the proportion of primary and secondary bile acids in circulation, colon content and liver

We assessed changes in the BA composition at three key sites: portal plasma, colonic content, and liver (as unlike humans rats do not have a gallbladder). BA species have been reported to have FXR agonistic ability (CDCA; DCA; LCA), no agonistic potential (ursodeoxycholic acid, UDCA) or unknown potential (hyodeoxycholic acid, HDCA; hyocholic acid, HCA; muricholic acid, MCA) (Pereira-Fantini et al., 2014). Measurement of portal BA composition was performed to determine the BAs being transported into the liver and therefore most likely to impact liver function and production of new bile. The BA composition of portal blood was markedly altered by lithium and valproate (**Figure 3.2A**, **Table S.3.1**). Lithium and valproate induced an increase in the proportion of all circulating BA species except tauro-conjugated BAs, which were not significantly different from control animals (**Figure 3.2A**). Assessment of the BA composition of colonic content samples allowed us to directly assess the impact of drug-induced dysbiosis. The BA composition of the colon content of both lithium- and valproate-treated rats was significantly altered when compared to control animals. Specifically, the proportion of primary, secondary and tertiary BAs was increased, together with glyco-conjugated BAs. Tauro-conjugated BAs were decreased by both treatment groups. UDCA was affected by lithium only and HCA was not affected (**Figure 3.3A**, **Table S.3.2**). Hepatic levels of bile acids reflected the same concentrations found in circulation, with the only difference that hydrophobic tauro-conjugated BAs were significantly decreased by both medications (**Figure 3.3B**, **Table S.3.3**). The BA heatmaps (**Figure 3.4**) show the marked differences between vehicle-, lithium- and valproate-treated rats both in the liver (the key site for BA synthesis) and in the colon content (being the colon the key site for bacteria-mediated BA modifications).

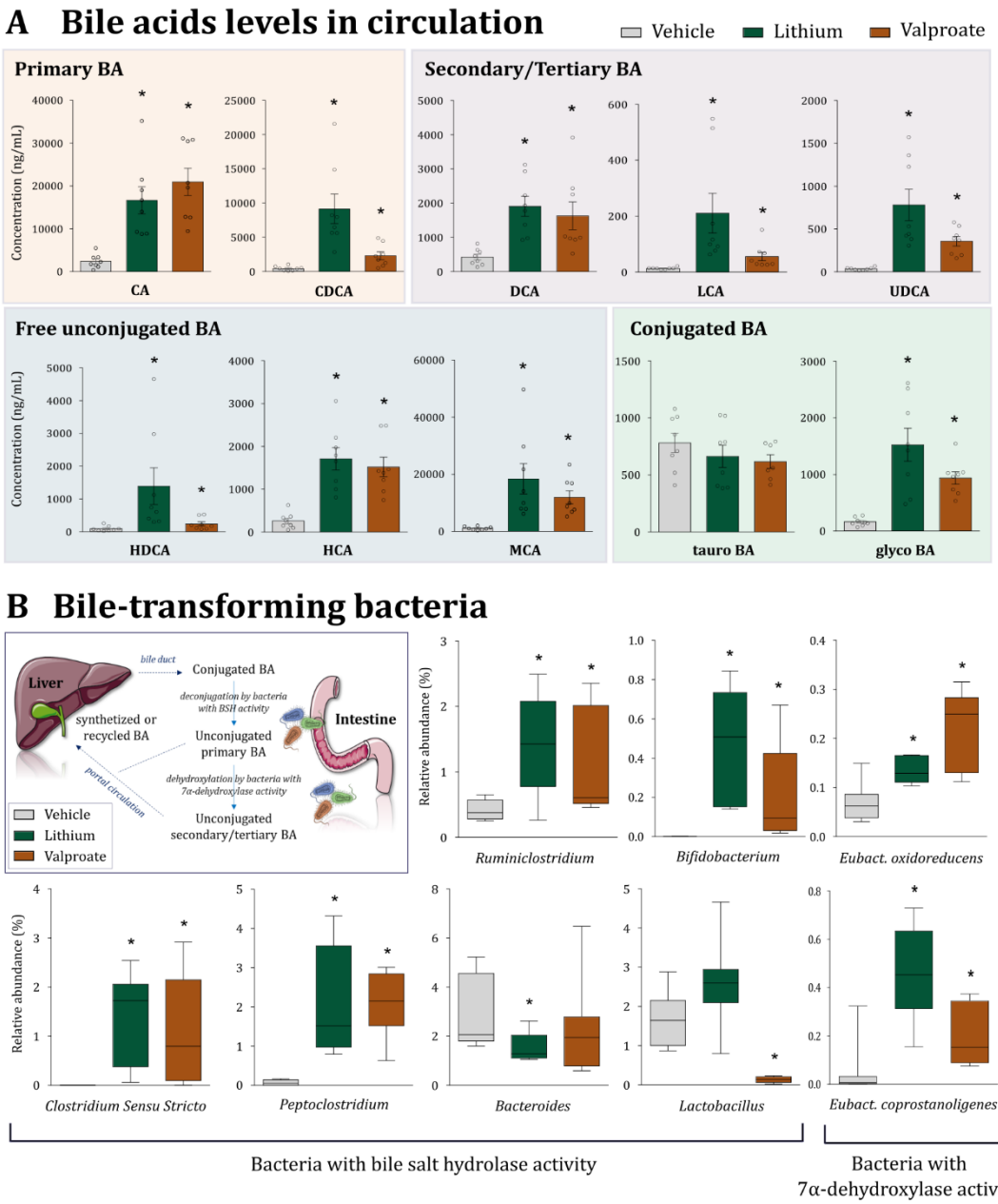


Figure 3.2 Lithium and valproate induce an increase in bile acids and microbial alterations. (A) Alterations in bile acid composition in portal plasma obtained at sacrifice. Except from tauro-conjugated BAs, all other moieties of BAs are increased by lithium and valproate compared to the vehicle group. Data are expressed as mean ± SEM. *p < 0.05 (n = 7-8/group). Details on statistics are described in Table S1. *Abbreviations: CA* cholic acid, *CDCA* chenodeoxycholic acid, *DCA* deoxycholic acid, *HCA* hyocholic acid, *HDCA* hyodeoxycholic acid, *LCA* lithocholic acid, *MCA* muricholic acid, *UDCA* ursodeoxycholic acid. **(B)** Alterations in the relative abundance of bacteria responsible for biotransformation of bile acids in caecum content obtained at sacrifice. The relative abundance of taxa belonging to the genera *Clostridium* and *Eubacterium* is increased by both lithium and valproate administration. Data are expressed as median and min-to-max values. *p < 0.05 (n = 8/group). Data was analysed using Kruskal-Wallis non-parametric test followed by Mann-Whitney U test and corrected for multiple comparisons using the Benjamin-Hochberg false discovery rate (pFDR) method (refer to *Supplemental Material* for details on statistics).

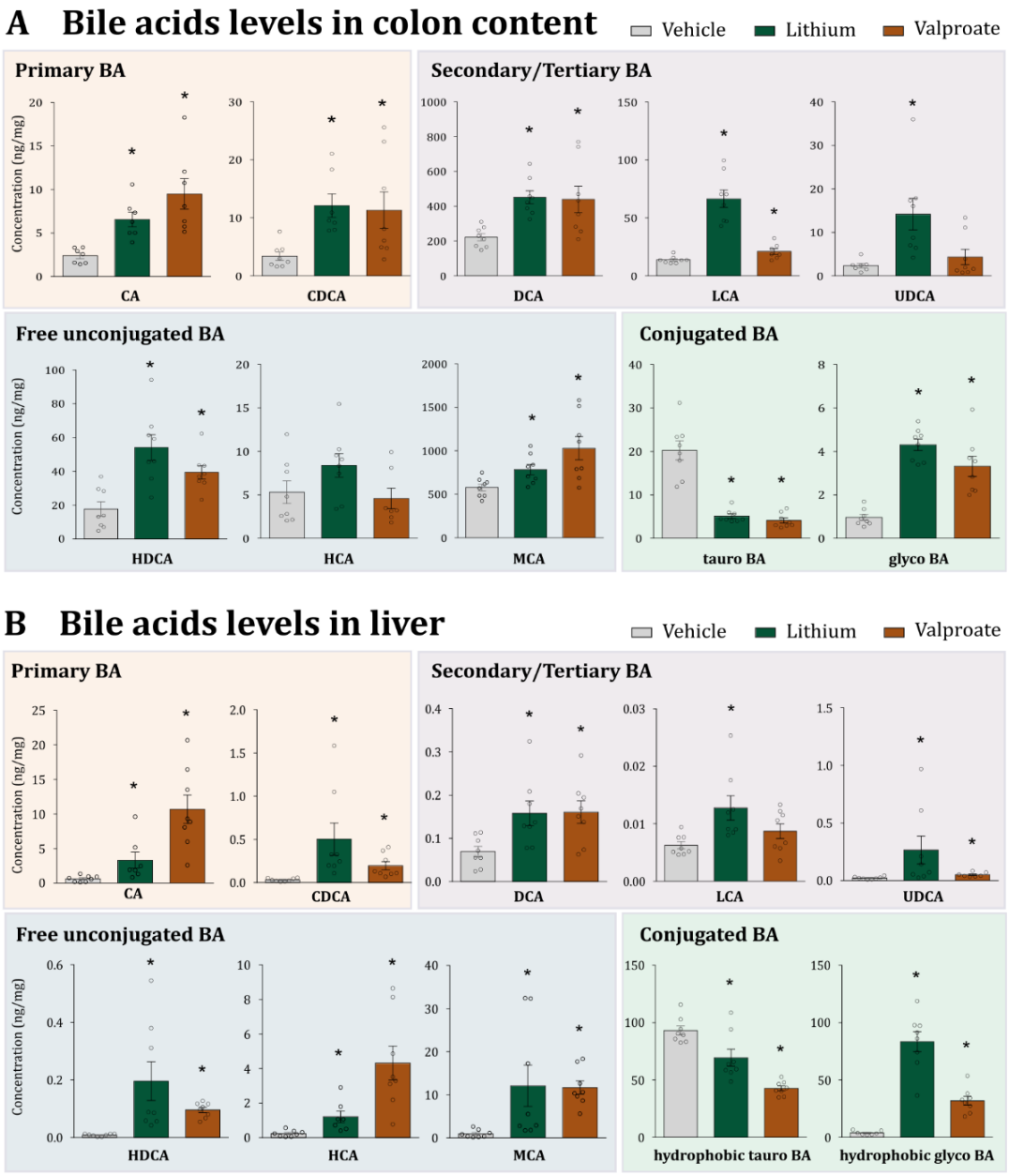


Figure 3.3 Lithium and valproate differentially alter bile acids concentrations. (A) Alterations in bile acid composition in colon content obtained at sacrifice. Data are expressed as mean ± SEM. *p < 0.05 (n = 7-8/group). Details on statistics are described in Table S2. **(B)** Alterations in bile acid composition in the liver obtained at sacrifice. Data are expressed as mean ± SEM. *p < 0.05 (n = 7-8/group). Details on statistics are described in Table S3. *Abbreviations: CA* cholic acid, *CDCA* chenodeoxycholic acid, *DCA* deoxycholic acid, *HCA* hyocholic acid, *HDCA* hyodeoxycholic acid, *LCA* lithocholic acid, *MCA* muricholic acid, *UDCA* ursodeoxycholic acid.

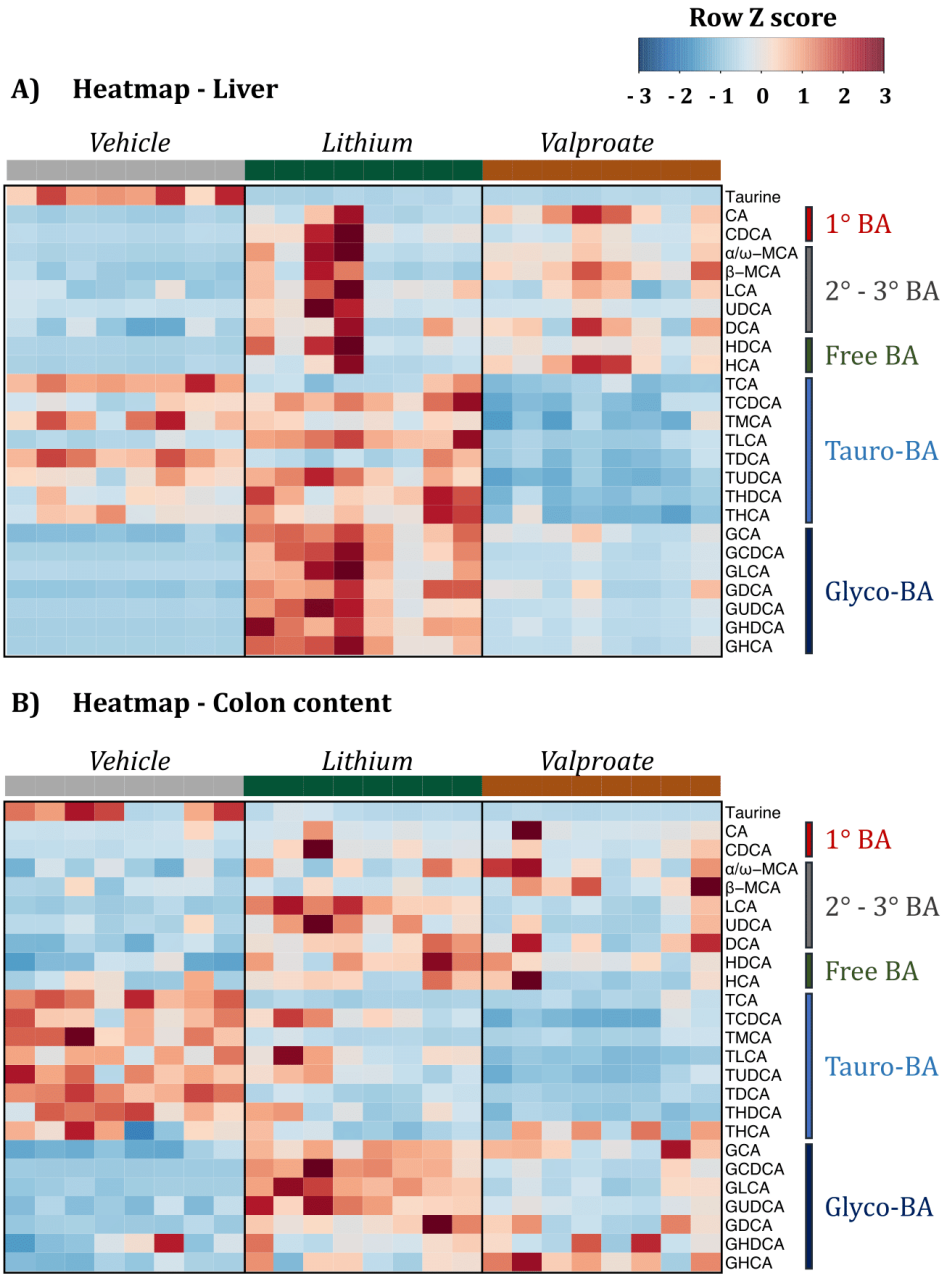


Figure 3.4 Heatmaps of bile acid composition in the liver and colon content. (A) Heatmap of hepatic BA concentrations in vehicle-, lithium- and valproate-treated rats. **(B)** Heatmap of faecal BA concentrations in vehicle-, lithium- and valproate-treated rats. BAs are shown along the y-axis; individual animals are shown along the x-axis. Spearman correlation of absolute quantified bile acid profile was performed. *Abbreviations (in order of appearance)*: *CA* cholic acid; *CDCA* chenodeoxycholic acid; *MCA* muricholic acid; *LCA* lithocholic acid; *UDCA* ursodeoxycholic acid; *DCA* deoxycholic acid; *HDCA* hyodeoxyhcolic acid; *HCA* hyocholic acid; *TCA* taurocholic acid; *TCDCA* taurochenodeoxycholic acid; *TMCA* tauromuricholic acid; *TLCA* taurolitocholic acid; *TDCA* taurodeoxycholic acid; *TUDCA* tauroursodeoxycholic acid; *THDCA* taurohyodeoxycholic acid; *THCA* taurohyocholic acid; *GCA* glycocholic acid; *GCDCA* glycochenodeoxycholic acid; *GLCA* glycolithocholic acid; *GDCA* glycodeoxycholic acid; *GUDCA* glycoursodeoxycholic acid; *GHDCA* glycohyodeoxycholic acid; *GHCA* glycohyocholic acid.

3.2 Lithium and valproate induce significant alterations in bile-transforming bacteria in caecum content

The gut microbiota is essential in shaping the bile acid composition. We examined 16S rRNA sequencing data to determine if administration of lithium and valproate was associated with alterations in the relative abundance of bile acid-biotransforming bacteria. The genera *Clostridium* (Coleman and Hudson, 1995; Gopal-Srivastava and Hylemon, 1988; Rossocha et al., 2005), *Bacteroides* (Kawamoto et al., 1989; Stellwag and Hylemon, 1976; Yoon et al., 2017), *Lactobacillus* (Chae et al., 2013; Corzo and Gilliland, 1999; Elkins et al., 2001; Gu et al., 2014; Jayashree et al., 2014; Lambert et al., 2008; McAuliffe et al., 2005; Ren et al., 2011; Wang et al., 2012) and *Bifidobacterium* (Grill et al., 1995; Kim et al., 2005; Kim et al., 2004a; Kim et al., 2004b; Tanaka et al., 2000; Wang et al., 2012) all carry bile salt hydrolase (BSH) activity; while *Eubacterium* carries 7α-dehydroxylase activity (Coleman et al., 1987; Dawson et al., 1996; Edenharder and Schneider, 1985; Mallonee et al., 1990; Masuda et al., 1984). Following lithium and valproate administration, the proportion of genera *Clostridium*, *Bifidobacterium* and *Eubacterium* was significantly increased (**Figure 3.2B** and **Table S.3.4**). *Bacteroides* and *Lactobacillus* were not increased by either drug. Rather, lithium decreased the levels of *Bacteroides*, and valproate decreased *Lactobacillus* (**Figure 3.2B**).

3.3 Lithium- and valproate-induced reduction in hepatic levels of taurine is accompanied by a decreased expression of taurine transporter TauT

At the hepatic level, lithium and valproate had opposite effects on glyco-conjugated and tauro-conjugated BAs, which were significantly increased and decreased, respectively (**Figure 3.3B**). We hypothesised that the reduction in tauro-conjugated BAs might be due to a low availability of taurine in the liver and our data confirmed this hypothesis, as hepatic taurine was significantly decreased in both treatment groups (**Figure 3.5A**). The two transporters responsible for taurine uptake in the liver are *Gat2* and *TauT (Kubo et al., 2016; Zhou et al., 2012)*, thus we measured the gene expression of these transporters to see whether they were affected by medications. *Gat2* gene

expression was unaffected by either drug, while *Taut2* was significantly decreased in the liver of both lithium- and valproate-treated rats (**Figure 3.5B**). We hypothesise that the increase in glyco-conjugated BAs in the liver (**Figure 3.5B**) might be due to a compensatory mechanism for the lack of tauro-conjugated BAs.

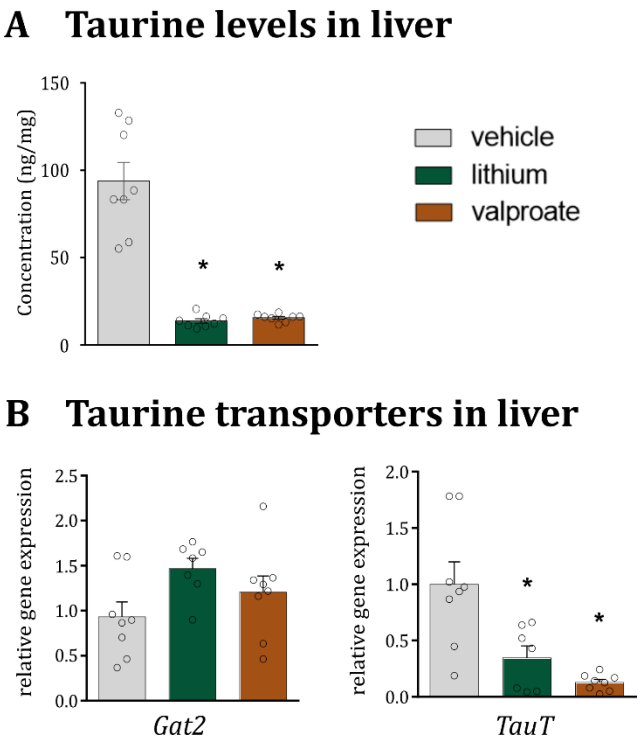


Figure 3.5 Taurine levels in the liver. qRT-PCR analysis of taurine transporters in the liver. (A) Both lithium and valproate decrease the levels of taurine in the liver (KW p=0.000, U=0, p=0.000 for both drugs). **(B)** Both lithium and valproate decrease the gene expression of *Taut* (One-way ANOVA $F_{(2;22)}$=12.01, p<0.001; t-test p<0.01 for lithium, p<0.001 for valproate). *Gat2* is not affected. Data are expressed as mean + SEM. *p<0.05 (n=7-8/group).

3.4 Intestinal and hepatic regulation of bile synthesis and transport is altered by lithium and valproate administration

Given that the levels of circulating bile acids were significantly influenced by both medications, we looked at the gene expression of enzymes and transporters involved in bile acid synthesis and transport in the liver and ileum. The entero-hepatic circulation of BA and the negative-feedback regulation of BA synthesis is a complex system. The relevance of genes analysed in this section is described schematically in

Figure 3.1. Bile acid composition strongly influences FXR activation (Degirolamo et al., 2014; Vaquero et al., 2013) thus we examined the gene expression of key downstream FXR targets. Although the expression of FXR itself was not altered in the liver, two crucial enzymes involved in bile acid synthesis, *CYP7A1* and *CYP27A1* were both increased by lithium and valproate (**Figure 3.6A**). Despite the fact that FXR was unchanged, the expression of the repressor *Shp* was increased by valproate (**Figure 3.6A**). *Hmgcr*, the rate-limiting enzyme responsible to produce cholesterol in the liver, was increased by valproate but not lithium (**Figure 3.6A**). There was no change in the hepatic levels of transporters *Mrp2*, *Bsep* and *Ntcp* (being *Ntcp* the main responsible for the reuptake of BA in the liver); however, the expression of the minor transporters *Mrp3* and *Mrp4* was differentially increased by the two drugs (**Figure 3.6B**). In the ileum, surprisingly, the expression of the repressor *Fgf19* was dramatically depleted in both treatment groups, despite an increase in the expression of FXR (**Figure 3.6C**), suggesting a blunted ability to repress target gene expression. *Ilbp* was decreased in both treatment groups and the transporter *Asbt* was increased following valproate administration (**Figure 3.6D**). In addition, we measured the levels of circulating Fgf19, as this factor is inversely correlated to bile acid synthesis (Gälman et al., 2011; Lundasen et al., 2006; Pattni et al., 2012). Although lithium- and valproate-treated rats showed a decrease in circulating Fgf19, the difference from control animals was not significant (**Fig. S.3.4**).

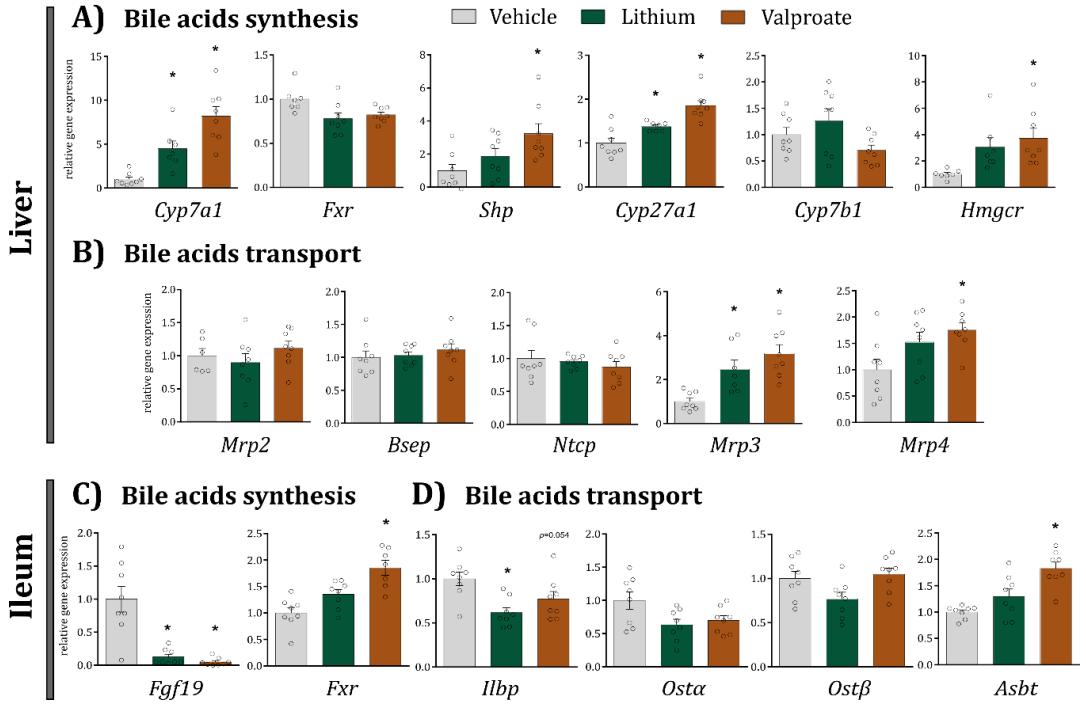


Figure 3.6 qRT-PCR analysis of genes involved in bile acid synthesis and transport pathways in the liver and ileum. (A) Both lithium and valproate increase the gene expression of *CYP7A1* (KW p=0.000, U=1, p=0.002 for lithium; KW p=0.000, U=0, p=0.001 for valproate) and *CYP27A1* (One-way ANOVA $F_{(2;22)}$=19.62, p<0.001; t-test p=0.028 for lithium, p=0.000 for valproate). Valproate increases the gene expression of *Shp* (One-way ANOVA $F_{(2;23)}$=2.35, p<0.05; t-test p=0.013) and *Hmgcr* (One-way ANOVA $F_{(2;21)}$=5.23, p<0.05; t-test p=0.010). **(B)** Both lithium and valproate increase the gene expression of *Mrp3* (One-way ANOVA $F_{(2;22)}$=11.83, p<0.001; t-test p=0.011 for lithium, p=0.000 for valproate). Valproate increases the gene expression of *Mrp4* (One-way ANOVA $F_{(2;23)}$=4.74, p<0.05; t-test p=0.013). **(C)** Both lithium and valproate decrease the gene expression of *Fgf19* (KW p=0.000, U=9, p=0.002 for lithium; KW p=0.000, U=2, p=0.002 for valproate). Valproate increases the gene expression of *Fxr* (One-way ANOVA $F_{(2;22)}$=7.51, p<0.001; t-test p=0.000). **(D)** Lithium decreases the gene expression of *Ilbp* (One-way ANOVA $F_{(2;23)}$=6.62, p<0.001; t-test p=0.000). Valproate decreases the gene expression of *Asbt* (One-way ANOVA $F_{(2;23)}$=14.34, p<0.001; t-test p=0.000). Data are expressed as mean + SEM. *p<0.05 (n=7-8/group).

3.5 Targeted gene expression does not suggest liver or ileum inflammation following lithium and valproate administration

The marked increase in circulating and hepatic levels of bile acids suggested that the liver of lithium- and valproate-treated rats might be damaged or inflamed (Allen et al., 2011). For this reason, we assessed the gene expression of pro-inflammatory and pro-

fibrosis mediators in the liver and in the ileum. However, gene expression of target factors did not suggest inflammation in either liver or ileum level (**Figure 3.7A,C**). *Tnf-α* was significanlty decreased in the ileum of valproate-treated rats. Moreover, liver enzymatic function, measured via circulating levels of alanine aminotransferase (ALT) and aspartate transaminase (AST) was not disrupted in drug-treated rats (**Figure 3.7B**).

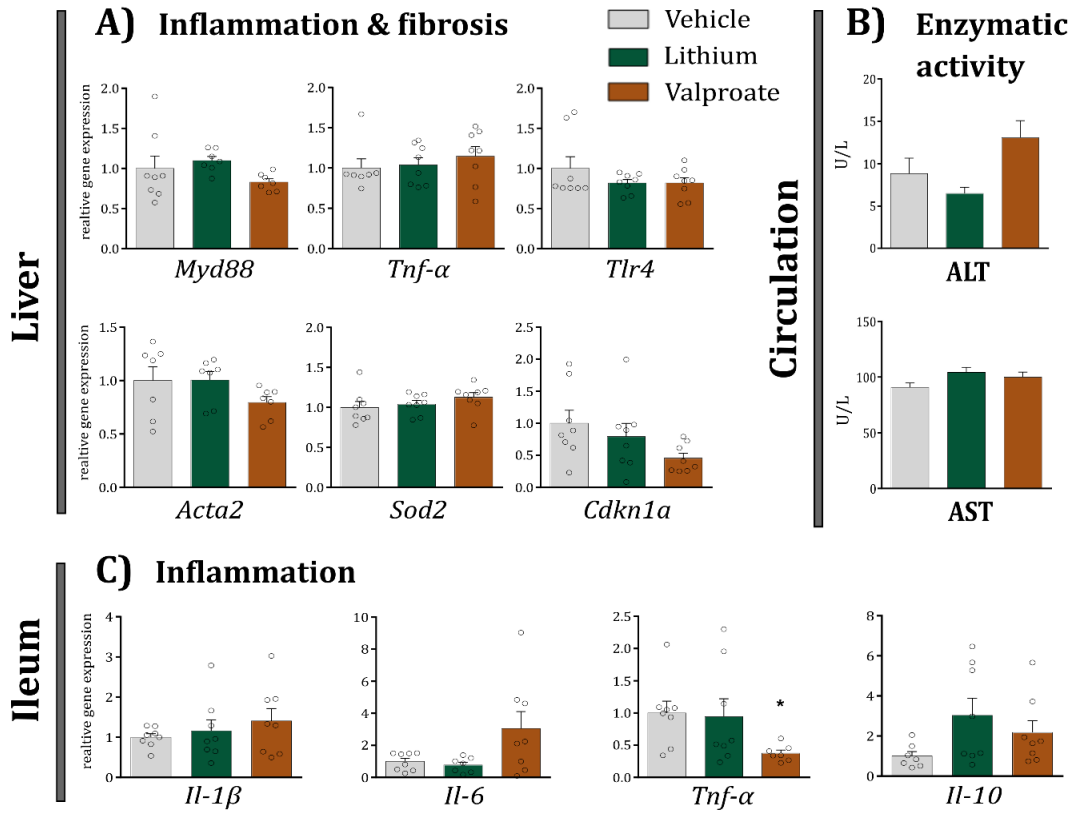


Figure 3.7 qRT-PCR analysis of genes involved in inflammation, fibrosis and hepatic enzymatic function. (A) None of the treatments induce inflammation or fibrosis in the liver as assessed by gene expression. **(B)** None of the treatments alter hepatic enzymatic activity as assessed by AST (aspartate transaminase) and ALT (alanine aminotransferase) plasma levels. **(C)** Valproate decreases the gene expression of *Tnf-α* in the ileum (KW p=0.039, U=5, p=0.008). Data are expressed as mean + SEM. *p<0.05 (n=7-8/group).

3.6 Bile acid dysregulation by lithium and valproate is independent of changes in intestinal permeability

In distal ileum, bile acids are absorbed into the enterocytes through the apical transporter *Asbt* and in turn activate a negative feedback response to the liver (**Figure 3.1**). We hypothesised that abundant levels of BAs in circulation might be the result of functional deficits at the ileum level. Here, as a result of disrupted barrier function, BAs could bypass the enteric wall and be passively reabsorbed in circulation, failing to activate the negative feedback loop to the liver. To characterise epithelial permeability in the distal ileum, we measured the efficacy of macromolecular diffusion across the epithelium *ex vivo* (4 kDa FITC flux). We also assessed changes in ion conductance (transepithelial electrical resistance, TEER). To minimize the influence of the intrinsic neuromuscular system, we performed seromuscular stripping: the serosa (visceral peritoneum) and the longitudinal/circular muscle layers of the intestinal wall were removed, leaving only the underlying submucosal elements, remnants of muscle and the epithelium. At translational concentrations of the two drugs (see **Figure S.3.2** for calculation of these), the intestinal permeability was not disrupted (**Figure 3.8A,B**). Valproate at a concentration of 100 mM significantly increased the permeability to macromolecules (**Figure 3.8A**) and decreased TEER (**Figure 3.8B**), suggesting a disruption in barrier function (less tight). Short-circuit current (I_{sc}), a measure positively correlated with active absorption of Na^+ from the lumen (Tai et al., 1977), was increased by valproate 100 mM and decreased by lithium 50 mM (**Figure 3.8B**).

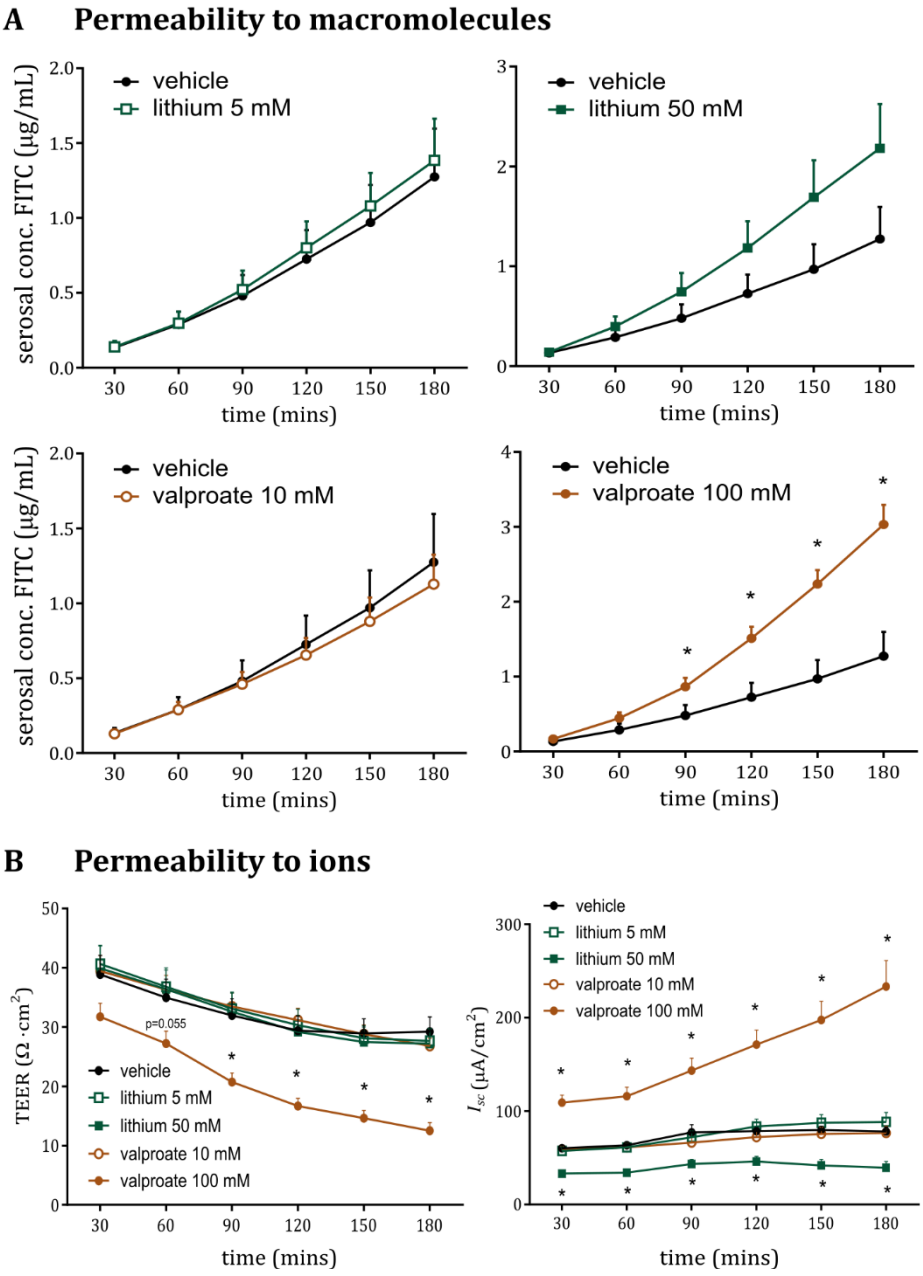


Figure 3.8 Dose-dependent effects of lithium and valproate on permeability in distal ileum. (A) Valproate at a concentration of 100 mM increased FITC paracellular permeability. **(B)** Valproate at a concentration of 100 mM decreased transepithelial resistance (TEER) and increased short-circuit current (I_{sc}). Lithium at a concentration of 50 mM decreased short-circuit current (I_{sc}). Data were analysed with a mixed between-within subjects ANOVA. *Statistics:* Panel A $F_{(5;225)}$=164.88, p<0.001 for the effect of Time, $F_{(4;45)}$=4.18, p<0.01 for the effect of Treatment, $F_{(20;225)}$=7.82, p<0.001 for the Time×Treatment interaction. Panel B-TEER $F_{(5;220)}$=196.75, p<0.001 for the effect of Time, $F_{(4;44)}$=5.14, p<0.01 for the effect of Treatment, $F_{(20;220)}$=3.07, p<0.001 for the Time×Treatment interaction. Panel B-I_{sc} $F_{(5;220)}$=58.67, p<0.001 for the effect of Time, $F_{(4;44)}$=29.31, p<0.001 for the effect of Treatment, $F_{(20;220)}$=16.49, p<0.001 for the Time×Treatment interaction. Mean values in each time point were further compared to the vehicle with unpaired t-test. Statistical outcomes for the t-test are described in *Supplemental Material*. Data are expressed as mean + SEM. *p<0.05 (n=10/group).

Discussion

There is increasing focus on the role of the gut microbiota in drug efficacy and toxicity (Li et al., 2016; Walsh et al., 2018; Wilson and Nicholson, 2009; Zimmermann et al., 2019a). The term pharmacomicrobiomics was recently coined to indicate the effect of microbiome variations on drug disposition, action, and toxicity (ElRakaiby et al., 2014; Rizkallah et al., 2010). In this study we show that the mood stabilisers lithium and valproate increase many BA species in circulation, in the colon content and the liver. These changes were accompanied by an increase in some key intestinal bile acid-metabolising bacteria, mainly belonging to the Firmicutes phylum. Lithium and valproate also altered substantially the gene expression of enzymes and transporters involved in bile acid metabolism. Two possible mediators of such effects, liver damage and disruption of intestinal permeability, did not seem to play a role in these drug-mediated bile effects. Taurine levels in the liver, circulation and colon content were markedly decreased by lithium and valproate administration. While some knowledge already existed on the effects of lithium and valproate on bile acid composition (Kersten and Barth, 1985; Watkins and Klaassen, 1981), here we employ a more comprehensive approach, taking into account the microbiota-gut-liver axis and examining microbial taxa which might play a role in BA metabolism.

We have recently shown that a four weeks administration of psychotropic drugs to adult rats significantly altered the gut microbiota composition (Cussotto et al., 2019b). Here we drill deeper into this dataset to show that specific bile-metabolising taxa (having BSH and 7α-dehydroxylase activity), such as *Clostridium*, *Bifidobacterium* and *Eubacterium* were significantly increased in the circulation of rats undergoing lithium and valproate treatment (**Figure 3.2B**). These microbial changes were accompanied by important changes in BA composition. Independent of BA class (primary, secondary or tertiary), and with the exception of tauro-conjugated BAs, all BAs were significantly increased by lithium and valproate administration (**Figure 3.2A**). FXR is a highly specific BA receptor that is activated by the BAs CDCA, DCA and LCA at physiological concentrations to directly stimulate the transcription of genes that mediate BA transport and synthesis, inhibiting *CYP7A1* and *CYP27A1* transcription (**Figure 3.1**) (Zhang and Chiang, 2001). We therefore measured target

FXR genes involved in BA synthesis and transport in the gut-liver axis. The dominant feedback pathway leading to repression of *CYP7A1* is via FXR-mediated activation of *Fgf19* (**Figure 3.1**). Despite an increase in FXR agonist BAs, we observed a failure in the FXR-*Fgf19* pathway to appropriately feedback to the liver and stop the *de novo* synthesis of BAs, reflected by an increase in both *CYP7A1* and *CYP27A1* gene expression. Strikingly, *Fgf19* gene expression was completely inhibited by both drugs (**Figure 3.6**). The *Fgf19* depletion was unexpected, particularly in the presence of high levels of CDCA, a strong FXR agonist (Soisson et al., 2008), in the circulation and the liver. For compensatory mechanisms, primary and secondary bile acids are often inversely correlated such that an increase in primary would correspond in a decrease in secondary/tertiary and *vice versa*. This was not observed in our study, as all BA species were increased, and no compensatory mechanisms seemed to occur. Regarding conjugated BAs, however, while glyco-conjugated species were increased, tauro-conjugated were often unaltered or decreased, which was reflective of low levels of taurine in all compartments. The decrease in the expression levels of ileal lipid-binding protein *Ilbp* (**Figure 3.6D**) was also reflective of an impaired negative feedback mechanism, being *Ilbp* vital for the maintenance of bile acid homeostasis in the enterohepatic circulation (Praslickova et al., 2012).

Evidence suggests that psychotropic medications can bear a certain degree of hepatotoxicity (Sedky et al., 2012; Selim and Kaplowitz, 1999; Telles-Correia et al., 2017). With such high levels of circulating BAs, we hypothesised that the liver or distal ileum (site of reabsorption of BAs) might be damaged and we assessed the expression of genes involved in inflammation and fibrosis. According to gene expression levels, neither the liver nor the intestine showed signs of inflammatory state (**Figure 3.7**). On the contrary, the expression of *Tnf-α*, a cytokine involved in systemic inflammation, was decreased in the intestine of valproate-treated rats. It was recently shown that UDCA attenuates the release of pro-inflammatory cytokines from colonic epithelial cells *in vitro* and it is protective against the development of colonic inflammation *in vivo* (Ward et al., 2017). The anti-inflammatory activity of UDCA also acts in the liver (Rodrigues et al., 1998). Thus, high levels of UDCA in circulation and colon content might be exerting an anti-inflammatory effect. Whether UDCA is

the main mediator of anti-inflammatory effects and how this counteracts with the increase in other BA species remains unclear.

We then hypothesized a different mechanism, at the ileum level, which might be responsible for the disruption of the negative feedback loop: the intestinal barrier potentially loosened by lithium and valproate might allow BAs to be passively absorbed into circulation and therefore fail to activate *Fgf19*. To assess this, we measured intestinal permeability to macromolecules and ions in an *ex vivo* setting employing Ussing chambers. Following seromuscular stripping, distal ileum tissue from naïve rats was tested in a dose-curve response with exposure to a low and high dose of each drug. Unexpectedly, translationally relevant doses of lithium and valproate (5 mM and 10 mM, respectively) did not exert any effect on permeability (**Figure 3.8**). However, a log fold higher dose of valproate increased permeability to FITC and decreased TEER, implying an increase in intestinal permeability. Short-circuit current (I_{sc}), which is positively correlated to the active absorption of Na^+ from the lumen (Tai et al., 1977), was increased by valproate 100 mM and decreased by lithium 50 mM, both non-translational doses. A possible limitation of the *ex vivo* experiment is that the drugs were tested acutely at a given dose, while the animals that showed drug-induced BA alterations did receive a chronic treatment. However, in our previous study we did assess intestinal permeability on tissue collected after chronic treatment with lithium and valproate (with the difference that there was no seromuscular stripping) and the two drugs did not affect permeability in distal ileum (Cussotto et al., 2019b). These data altogether suggest that the disruption in BA feedback loop is independent of changes in intestinal permeability.

It is worth noting that we observed a decreased body weight in animals receiving lithium and valproate (**Figure S.3.3**). This finding is not in accordance with clinical human observations, as usually these two medications induce body weight gain (Martin et al., 2009; Peselow et al., 1980). Interestingly, BAs might be able to function not only as regulators of BA homeostasis but also as general metabolic integrators. In fact, a recent study has shown that bile acid signalling through TGR5 receptor promoted mitochondrial fission and beige remodelling of white adipose tissue (Velazquez-Villegas et al., 2018). Corroborating these findings, a different study has

shown that administration of BAs to mice increased energy expenditure in brown adipose tissue, preventing obesity and resistance to insulin (Watanabe et al., 2006). Thus, the decrease in bodyweight, epididymal fat and levels of circulating triglycerides observed in lithium- and valproate-treated rats (**Figure S.3.3**) might be the result of massively increased levels of circulating BAs. We hypothesized also the opposite assumption, i.e. the decrease in body weight caused an increase in circulating BAs. This, however, is probably not the case, as studies in humans have proved that body weight gain and levels of total BAs are positively correlated (Alemán et al., 2018; Prinz et al., 2015). It is important to remember that there is an intricate link between bile acids, Fgf19, FXR and body weight. In mice fed a high-fat diet, Fgf19 administration reduced the body weight and improved metabolic function (Fu et al., 2004). Moreover, Fgf19 also plays a role in regulating food intake and appetite (Ryan et al., 2013).

Following *de novo* production of BAs, the final step prior to secretion in the intestine consists of the conjugation of BAs to either glycine or taurine, a process that increases their solubility for secretion into biliary fluid (**Figure 3.1**). Hydrophobic conjugated BAs represent the highest proportion of hepatic BAs. Interestingly, we noticed that lithium and valproate induced a strong decrease in hydrophobic tauro-conjugated BAs in the liver. This effect was probably the result of low availability of hepatic taurine, which was decreased ten-fold in comparison to control animals (**Figure 3.5A**). We hypothesized that lithium and valproate may have depleted the hepatic pool of taurine available for BA conjugation, resulting in an increase in glycine conjugation for compensatory mechanism (**Figure 3.4B**, note that hydrophobic glyco-conjugated BAs are increased). Intrigued by these findings, we measured the gene expression of two crucial transporters responsible for the uptake of taurine in the liver, *Gat2* and *TauT* (**Figure 3.5B**). Lithium and valproate reduced the expression of the taurine transporter *TauT*, explaining the reduction in hepatic taurine levels and subsequent reduction in tauro-conjugated BAs. Taurine is an organic compound widely distributed in animal tissues with many fundamental biological roles, including antioxidation (Jong et al., 2012; Yildirim and Kilic, 2011), osmoregulation (Schaffer et al., 2000), cardiovascular function (Schaffer et al., 2010; Xu et al., 2008) and brain function (Wu and Prentice, 2010). Taurine can cross the blood-brain barrier (Salimäki et al., 2003; Tsuji and

Tamai, 1996; Urquhart et al., 1974) and produces a central anxiolytic effect, probably through activation of the glycine receptor (Kong et al., 2006; Zhang and Kim, 2007). In our animals treated with lithium and valproate, taurine was depleted not only in the liver but also in circulation (**Table S.3.1**), potentially causing a shortage in distal tissues such as the central nervous system. Importantly, the two transporters TauT and Gat2 are also responsible for the uptake of the neurotransmitter GABA. However, TauT is reported to have lower affinity (1.5 mM) for GABA than Gat2 (<80 μM) (Borden, 1996; Tomi et al., 2008), therefore we suppose that the reduced expression of TauT did not impact GABAergic uptake and function. A few studies have looked at the effects of taurine on gut microbiota composition; however, the results to date are conflicting and certainly not conclusive (Sasaki et al., 2017; Yu et al., 2016). Some evidence exists on how the mood stabilisers lithium and valproate can influence brain levels of taurine (Anyanwu and Harding, 1993; Pettegrew et al., 2001) and here we hypothesize a mechanism that is linked to inhibition of the transporter TauT, which is also expressed in the brain (Pow et al., 2002). Future studies investigating the relationship between psychotropic drug action and taurine levels are now warranted.

There are some limitations of the current study worth noting. Changes in body weight have been previously associated to microbiome variations (Duncan et al., 2008; Remely et al., 2015; Santacruz et al., 2009). Although we hypothesise that the decreased bodyweight might be due to the fat-burning and metabolism-regulating properties of BAs, a food intake record of the animals is not provided, therefore we cannot rule out a possible association between weight loss and microbiome composition. Regarding the classification of BA-metabolising bacteria, a limitation of 16S sequencing consists in detecting microorganisms at genus level, without allowing investigation of individual species. Throughout the study, we assume that the abundance of a BA-metabolising genus will be positively correlated to its relevant BA-metabolising species, however a more targeted approach with shotgun sequencing is warranted to confirm our results at species level.

Interestingly, antibiotic administration in mice has been shown to induce BA dysmetabolism and impaired activation of the intestinal FXR/Fgf19 axis with elevated CYP7A1 gene expression (Sayin et al., 2013), thus corroborating the idea that the gut

microbiota plays a role in BA composition. At this stage we cannot, however, define the direction and/or causality of the microbiota-bile interaction. One common property the two drugs share is their HDAC (histone deacetylase) inhibition ability (Krämer et al., 2003; Wu et al., 2013). Thus, it is not odd to suppose that lithium and valproate might block, at epigenetic level, the activation of downstream targets of FXR such as Fgf19 and Ilbp; and more research is warranted to investigate this hypothesis. Provocatively, both psychotropic medications targeted a similar pattern of microbial species, despite their differences in chemical structure and mechanism of action in the brain. This raises the possibility that the microbial action may manifest as side effect or mechanism of action of these drugs. Interestingly, this concept was also hypothesized by Maier and colleagues with regards to antipsychotics, a class of psychotropics that clustered together on the microbiome despite differences in chemical structure (Maier et al., 2018).

In conclusion, in the present study we have demonstrated that the mood stabilisers lithium and valproate induce a bile acid and taurine dysmetabolism which is consistent with changes in key bile acid-metabolising genera in the gut. These data highlight the importance of examining the effects of psychotropic drugs on the microbiome and the microbiota-gut-liver axis, beside the well characterised effects exerted in the central nervous system. Future studies are warranted to examine the causality and direction of the interaction between drug-induced changes in microbiota and changes in bile, as well as the clinical implications.

Supplemental Material

SUPPLEMENTAL TABLES

Table S.3.1 Bile acids levels in the plasma of rats treated with different psychotropic medications (ng/mL)

Bile acid (full name)	Abbreviation	Vehicle Median (IQR)	Lithium Median (IQR) [(i/m)Q]	Valproate Median (IQR) [(i/m)Q]
Unconjugated bile acids				
α/ω-Muricholic acid	α/ω-MCA	513.6 (548.1)	9711 (16567) [0.0138] *	6041 (7952) [0.0086] *
β-Muricholic acid	βMCA	435.8 (557.3)	1987 (2684) [0.0396] *	2995 (3603) [0.0103] *
Chenodeoxycholic acid	CDCA	414.4 (544.4)	7213 (7586) [0.0017] *	1993 (3026.6) [0.0345] *
Cholic acid	CA	2032 (2101)	15342 (11906) [0.0034] *	20198 (18004) [0.0017] *
Deoxycholic acid	DCA	381.2 (402.1)	1857 (1682) [0.0086] *	1012 (1440) [0.0327] *
Hyocholic acid	HCA	215.6 (244.8)	1722 (1096) [0.0121] *	1404 (1208) [0.0069] *
Hyodeoxycholic acid	HDCA	63.24 (119.03)	671.7 (2174.6) [0.0103] *	186.4 (343.48) [0.0431] *
Lithocholic acid	LCA	13.13 (3.684)	108 (349.14) [0.0052] *	35.08 (43.96) [0.0034] *

Ursodeoxycholic acid	UDCA	34.51 (19.01)	486.8 (935.3) [0.0069] *	378.7 (310.8) [0.0052] *
Tauro-conjugated bile acids				
Taurochenodeoxycholic acid	TCDCA	65.99 (33.46)	87.11 (61.78)	41.12 (22.48) [0.0396] *
Taurocholic acid	TCA	233.4 (153.2)	92.6 (156.05)	218.9 (140.1)
Taurodeoxycholic acid	TDCA	84.07 (60.35)	24.5 (21.51) [0.0448] *	19.95 (20.88) [0.0362] *
Taurohyocholic acid	THCA	2.257 (2.755)	1.528 (1.381)	2.013 (3.811)
Taurohyodeoxycholic acid	THDCA	8.422 (4.441)	8.977 (3.583)	6.213 (4.127)
Taurolithocholic acid	TLCA	1.678 (0.644)	5.707 (2.002) [0.0172] *	1.573 (1.63)
Tauromuricholic acid	TMCA	367.2 (231.9)	382.4 (322)	349.4 (149.8)
Tauroursodeoxycholic acid	TUDCA	8.32 (4.067)	7.79 (11.386)	4.97 (2.663)
Glyco-conjugated bile acids				
Glycochenodeoxycholic acid	GCDCA	5.104 (6.969)	275.9 (443.94) [0.0189] *	52.01 (81.08) [0.0396] *
Glycocholic acid	GCA	112.2 (116.81)	819.5 (1092.7) [0.0207] *	748.9 (339.7) [0.0155] *
Glycodeoxycholic acid	GDCA	12.33 (18.624)	246 (116.3) [0.0224] *	92.31 (115.01) [0.0172] *
Glycohyocholic acid	GHCA	5.432 (11.405)	29.33 (37.12) [0.0431] *	8.545 (12.792)
Glycolithocholic acid	GLCA	0.7683 (0.674)	6.587 (7.767) [0.0345] *	1.317 (0.988)

Glycoursodeoxycholic acid	GUDCA	2.53 (6.47)	23.93 (22.62) [0.0362] *	9.652 (10.677) [0.0414] *
Taurine (ng/mL)		1146 (377.6)	856.7 (156.5) [*p*=0.010] #	915.8 (211.7) [*p*=0.042] #

Data that did not satisfy homogeneity of variance and/or normality: * p<0.05, Kruskal-Wallis nonparametric test followed by Mann-Whitney U test. Reported is the Benjamini-Hochberg critical value [(i/m)Q] with a Q (false-discovery rate) of 0.05.
Data that satisfied homogeneity of variance and normality: # p<0.05 one-way ANOVA. Reported is the *p* value of Dunnett's t-test VS vehicle.
N=7-8/group. Red indicates an increase; blue indicates a decrease in plasma bile acids levels compared to vehicle.

Table S.3.2. Bile acids levels in the colon content (faeces) of rats treated with lithium and valproate (ng/mg)

Bile acid (full name)	Abbreviation	Vehicle Median (IQR)	Lithium Median (IQR) [(i/m)Q]	Valproate Median (IQR) [(i/m)Q]
Unconjugated bile acids				
α/ω-Muricholic acid	α/ω-MCA	433.2 (215.9)	637.3 (330.7) [*p*=0.041] #	664.8 (520.9) [*p*=0.035] #
β-Muricholic acid	βMCA	113 (65.21)	127.7 (61.6)	241.5 (293.3) [0.0458] *
Chenodeoxycholic acid	CDCA	2.7 (2.767)	9.543 (10.384) [0.0251] *	7.089 (16.311) [0.0375] *
Cholic acid	CA	2.562 (1.775)	6.28 (2.788) [0.0319] *	8.093 (6.326) [0.0250] *
Deoxycholic acid	DCA	220.7 (107.5)	436.2 (167.2) [0.0028] *	393.2 (411.2) [0.0417] *
Hyocholic acid	HCA	3.797 (6.005)	8.722 (5.454)	3.214 (5.675)
Hyodeoxycholic acid	HDCA	13.53 (21.901)	52.62 (27.09) [0.0347] *	37.79 (9.94) [0.0306] *
Lithocholic acid	LCA	13.66 (4.2)	65.87 (39.97) [0.0014] *	18.61 (10.34) [0.0444] *
Ursodeoxycholic acid	UDCA	2.407 (1.126)	12.4 (11.112) [0.0333] *	1.762 (8.361)
Tauro-conjugated bile acids				
Taurochenodeoxycholic acid	TCDCA	0.7492 (0.3443)	0.5961 (0.4235)	0.1601 (0.2484) [0.0208] *
Taurocholic acid	TCA	4.29 (1.667)	0.6898 (0.0873) [0.0056] *	0.7219 (0.4819) [0.0028] *
Taurodeoxycholic acid	TDCA	2.102 (0.767)	0.4165 (0.3691) [0.0069] *	0.1697 (0.1481) [0.0056] *
Taurohyocholic acid	THCA	0.1386 (0.0728)	0.1012 (0.0285)	0.1479 (0.0847)

Taurohyodeoxycholic acid	THDCA	0.1761 (0.069)	0.1148 (0.0674) [0.0403] *	0.0824 (0.0157) [0.0069] *
Taurolithocholic acid	TLCA	0.1822 (0.0803)	0.1211 (0.0837)	0.0366 (0.0155) [0.0042] *
Tauromuricholic acid	TMCA	12.07 (6.861)	2.392 (1.12) [0.0083] *	1.943 (1.917) [0.0083] *
Tauroursodeoxycholic acid	TUDCA	0.4522 (0.1541)	0.2738 (0.1542)	0.1407 (0.0614) [0.0181] *
Glyco-conjugated bile acids				
Glycochenodeoxycholic acid	GCDCA	0.0477 (0.0153)	0.7781 (0.1506) [0.0278] *	0.2149 (0.2142) [0.0208] *
Glycocholic acid	GCA	0.3905 (0.5762)	2.606 (0.819) [0.0097] *	2.287 (1.276) [0.0222] *
Glycodeoxycholic acid	GDCA	0.0664 (0.035)	0.3664 (0.1206) [0.0292] *	0.2606 (0.426) [0.0278] *
Glycohyocholic acid	GHCA	0.0846 (0.024)	0.1409 (0.0637) [0.0417] *	0.1788 (0.061) [0.0292] *
Glycolithocholic acid	GLCA	0.01995 (0.0058)	0.0910 (0.0438) [0.0111] *	0.0334 (0.0342) [0.0389] *
Glycoursodeoxycholic acid	GUDCA	0.03471 (0.0332)	0.1239 (0.0901) [0.0125] *	0.06932 (0.0191) [0.0264] *
Taurine (ng/mg)		54.31 (60.8)	4.764 (6.567) [0.0444] *	4.557 (2.114) [0.0472] *

Data that did not satisfy homogeneity of variance and/or normality: * $p<0.05$, Kruskal-Wallis nonparametric test followed by Mann-Whitney U test. Reported is the Benjamini-Hochberg critical value [(i/m)Q] with a Q (false-discovery rate) of 0.05. *Data that satisfied homogeneity of variance and normality:* # $p<0.05$ one-way ANOVA. Reported is the *p* value of Dunnett's t-test VS vehicle. N=7-8/group. Red indicates an increase; blue indicates a decrease in plasma bile acids levels compared to vehicle.

Table S.3.3 Bile acids levels in the liver of rats treated with lithium and valproate (ng/mg)

Bile acid (full name)	Abbreviation	Vehicle Median (IQR)	Lithium Median (IQR) [(i/m)Q]	Valproate Median (IQR) [(i/m)Q]
Unconjugated bile acids				
α/ω-Muricholic acid	α/ω-MCA	0.287 (0.3761)	2.651 (16.155) [0.0083] *	5.090 (2.871) [0.0117] *
β-Muricholic acid	βMCA	0.3029 (1.0848)	1.552 (7.5656) [0.0450] *	5.471 (5.31) [0.0133] *
Chenodeoxycholic acid	CDCA	0.02844 (0.0364)	0.2859 (0.674) [0.0033] *	0.1207 (0.2396) [0.0033]
Cholic acid	CA	0.649 (0.7545)	1.906 (3.867) [0.0433] *	9.087 (9.209) [0.0050] *
Deoxycholic acid	DCA	0.06368 (0.0728)	0.1332 (0.1108) [*p*=0.026] #	0.1599 (0.1121) [*p*=0.022] #
Hyocholic acid	HCA	0.2151 (0.2957)	1.060 (1.2973) [0.0350] *	3.405 (4.893) [0.0100] *
Hyodeoxycholic acid	HDCA	0.0091 (0.0089)	0.087 (0.3059) [0.0050] *	0.1012 (0.0464) [0.0067] *
Lithocholic acid	LCA	0.00546 (0.00284)	0.01052 (0.00769) [*p*=0.010] #	0.0086 (0.00618)
Ursodeoxycholic acid	UDCA	0.02014 (0.0158)	0.1070 (0.4678) [0.0383] *	0.04171 (0.0342) [0.0467] *
Tauro-conjugated bile acids				
Taurochenodeoxycholic acid	TCDCA	9.301 (4.719)	16.08 (6.45) [*p*=0.001] #	4.019 (4.184) [*p*=0.014] #
Taurocholic acid	TCA	68.31 (4.07)	41.50 (17.7) [*p*=0.000] #	32.95 (5.18) [*p*=0.000] #
Taurodeoxycholic acid	TDCA	11.17 (5.112)	4.767 (5.161) [*p*=0.000] #	2.761 (2.076) [*p*=0.000] #

Taurohyocholic acid	THCA	0.2239 (0.0808)	0.2686 (0.1989)	0.07879 (0.0242) [0.0283] *
Taurohyodeoxycholic acid	THDCA	0.5304 (0.2369)	0.8630 (0.6109) [0.0467] *	0.2201 (0.122) [0.0183] *
Taurolithocholic acid	TLCA	0.06486 (0.0479)	0.3092 (0.1653) [0.0117] *	0.03216 (0.0503) [0.0500] *
Tauromuricholic acid	TMCA	97.42 (49.19)	76.75 (17.78) [*p*=0.039] #	37.21 (23.48) [*p*=0.000] #
Tauroursodeoxycholic acid	TUDCA	2.12 (0.308)	2.884 (1.179) [*p*=0.028] #	0.5649 (0.4501) [*p*=0.000] #
Glyco-conjugated bile acids				
Glycochenodeoxycholic acid	GCDCA	0.1075 (0.0184)	14.70 (10.432) [0.0150] *	1.795 (2.219) [0.0300] *
Glycocholic acid	GCA	3.046 (3.084)	64.05 (23.26) [0.0167] *	26.15 (10.22) [0.0317] *
Glycodeoxycholic acid	GDCA	0.2062 (0.1313)	8.677 (4.681) [0.0200] *	2.764 (3.624) [0.0350] *
Glycohyocholic acid	GHCA	0.0564 (0.0168)	4.132 (3.11) [0.0233] *	0.5586 (0.6213) [0.0383] *
Glycolithocholic acid	GLCA	0.00161 (0.00145)	0.1066 (0.1591) [0.0133] *	0.01096 (0.0112) [0.0200] *
Glycoursodeoxycholic acid	GUDCA	0.02697 (0.0056)	1.781 (2.139) [0.0183] *	0.2489 (0.252) [0.0333] *
Taurine (ng/mg)		85.94 (61.4)	13.09 (5.02) [0.0017] *	16.11 (3.5) [0.0017] *

Data that did not satisfy homogeneity of variance and/or normality: * p<0.05, Kruskal-Wallis nonparametric test followed by Mann-Whitney U test. Reported is the Benjamini-Hochberg critical value [(i/m)Q] with a Q (false-discovery rate) of 0.05. *Data that satisfied homogeneity of variance and normality:* # p<0.05 one-way ANOVA. Reported is the p value of Dunnett's t-test VS vehicle. N=7-8/group. Red indicates an increase; blue indicates a decrease in plasma bile acids levels compared to vehicle.

Table S.3.4 Statistics values for relative abundance of bile-metabolizing bacterial genera in each treatment group as compared to the vehicle

GENUS (phylum)	***Lithium***		***Valproate***	
	p	(i/m)Q	*p*	(i/m)Q
Clostridium Sensu Stricto (Firmicutes)	0.000 *	0.0027211 *	0.007 *	0.016327 *
Peptoclostridium (Firmicutes)	0.000 *	0.0013605 *	0.000 *	0.002721 *
Ruminiclostridium (Firmicutes)	0.005 *	0.0217687 *	0.028 *	0.031293 *
Bifidobacterium (Actinobacteria)	0.0000 *	0.0040816 *	0.000 *	0.004082 *
Eubacterium oxidoreducens (Firmicutes)	0.003 *	0.0190476 *	0.001 *	0.009524 *
Eubacterium coprostanoligenes (Firmicutes)	0.001 *	0.0122449 *	0.003 *	0.012245 *
Bacteroides (Bacteroidetes)	0.021 *	0.0380952 *	0.382	0.09932
Lactobacillus (Firmicutes)	0.083	0.06531	0.000 *	0.001361 *

*p<<0.05, Mann-Whitney U test, Benjamini-Hochberg critical value [(i/m)Q] with a Q (false-discovery rate) of 0.2. N=8/group. Red indicates an increase; blue indicates a decrease in the relative abundance of bacterial taxa compared to vehicle.

Table S.3.5 List of SybrGreen probes used in the study

Gene	Common gene name	Sequence (5' → 3') left	Sequence (5' → 3') right
Acta2	Alpha-actin-2	CCAGTCGCCATCAGGAAC	TGTGCTGTCTTCCTCTTCACA
Actb	Actin beta	CCCGCGAGTACAACCTTCT	CGTCATCCATGGCGAACT
Asbt	Apical sodium-dependent bile acid transporter	AACGATTGTGATCCCCTACG	CAATGGAAACAGGAATAACAAGC
Bsep	Bile salt export pump	GGGCAGTCACACCCATCTAC	CTTTATCGAGGAGTGAAAAAGTCC
Cdkn1a	Apical sodium-dependent bile acid transporter	GACATCTCAGGGCCGAAA	GGCGCTTGGAGTGATAGAAA
Cyp7a1	Cytochrome P450 family 7 subfamily A member 1	GTGAAGTCCTCCTTAGCTGTG	CAAGTGCAACTGAATGACCTG
Cyp7b1	Cytochrome P450 family 7 subfamily B member 1	CACCTTGCTGGTCCCAGT	CAAGGGAGGTTCACAAGGAC
Cyp27a1	Cytochrome P450 family 27 subfamily A member 1	TTCCAGCTATTTCTACGAGGCTAT	CCGTACTTGGCCTTGTTCA
Fgf19	Fibroblast growth factor 19	TGTAGCCCAAACAGTCCATT	GTTGCTCTGAAGACAATTGCC
Fxr	Farnesoid X receptor	CCACGACCAAGCTATGCAG	TCTCTGTTTGCTGTATGAGTCCA
Hmgcr	3-hydroxy-3-methylglutaryl-CoA reductase	GACCTTTCTAGAGCGAGTGCAT	CGCTATATTCTCCCTTACTTCATCC
Ilbp	Ileal lipid-binding protein	CTCTTGCTTACACGCTCGTAG	CCCAACTATCACCAGACTTCG
Il-1β	Interleukin 1 beta	TGTGATGAAAGACGGCACAC	CTTCTTCTTTGGGTATTGTTTGG
Il-6	Interleukin 6	CCCTTCAGGAACAGCTATGAA	ACAACATCAGTCCCAAGAAGG
Il-10	Interleukin 10	AGTGGAGCAGGTGAAGAATGA	TCATGGCCTTGTAGACACCTT
Mrp2	Multidrug resistance-associated protein 2	TCTCCTCCCAAATACCTCTCC	CTCCATCACCCTCTTCAATATCC
Mrp3	Multidrug resistance-associated protein 3	GTGCTGGCAGGCAAGACT	AAGCACAATGATAAAGTCCGTCT
Mrp4	Multidrug resistance-associated protein 4	GCCAGACCTGGTGAGTTGTT	CGCTCAACAGGGACGACT
Myd88	Myeloid differentiation primary response 88	CCGTGAGGATATACTGTATGAACTG	TTTCTGCTGGTTGCGTATGT
Ntcp	Na^+/taurocholate cotransporting polypeptide	AAGGGGGACATGAACCTCA	CATCATGCCCAAGGCACT
Ost-α	Organic solute transporter alpha subunit	CAATTTCCACTGAGCCCAAT	CCAGGTACACAGCAGATCTTC
Ost-β	Organic solute transporter beta subunit	GCTGCTTCTTTCGATTTCTGTT	ATGCTTTGGTATTTCCGTTCAG
Shp	Small heterodimer partner	TCCAGGACTTCACACAATGC	GTCCCAAGGAGTACGCATAC
Sod2	Superoxide dismutase 2	GGCCATATCAATCACAGCATT	TAGCCTCCAGCAACTCTCCT
Tlr4	Toll-like receptor 4	GGATGATGCCTCTCTTGCAT	TGATCCATGCATTGGTAGGTAA
Tnf-α	Tumor necrosis factor alpha	TGAACTTCGGGGTGATCG	GGGCTTGTCACTCGAGTTTT

SUPPLEMENTAL FIGURES

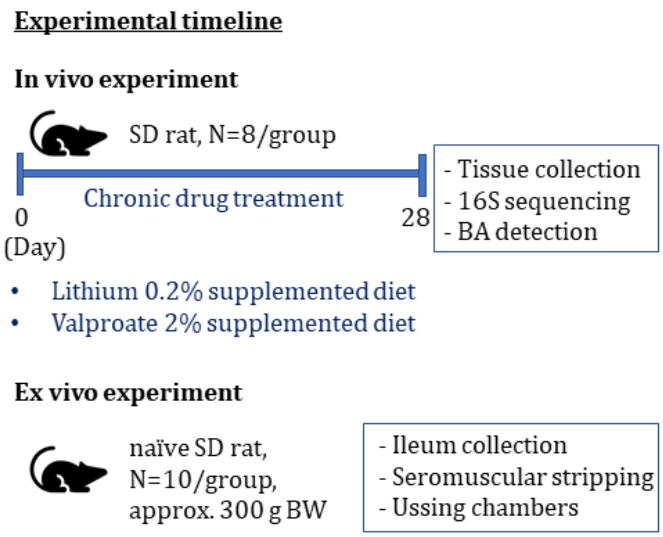


Figure S.3.1 Experimental timeline. *Abbreviations:* BA bile acid, BW body weight.

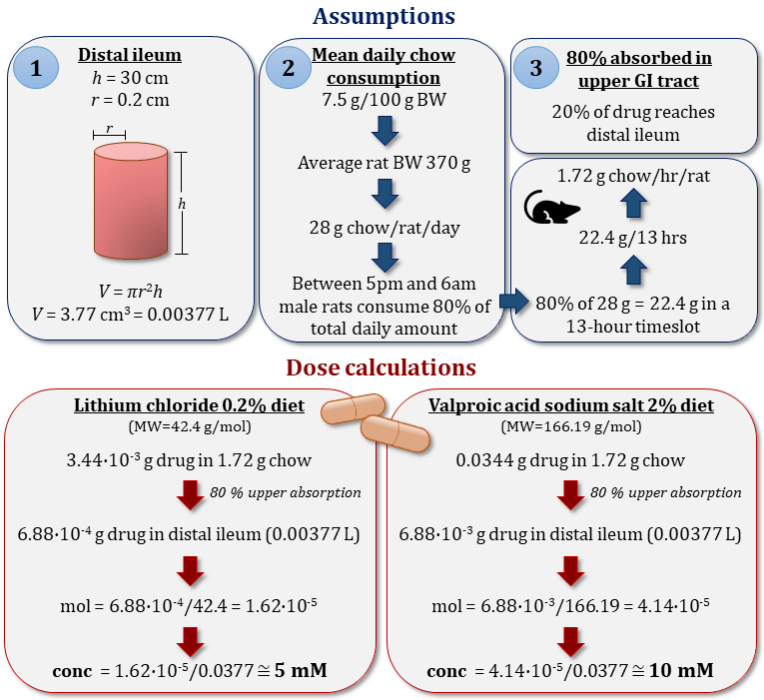


Figure S.3.2 Dose calculation of lithium and valproate for the assessment of intestinal permeability *ex vivo*. *Abbreviations:* BW body weight, MW molecular weight. Reference for assumption 1: (Al-Ansari et al., 2002). Reference for assumption 2: (Sidlo et al., 1995). References for assumption 3: (Baer, 1973) and (Gugler and von Unruh, 1980).

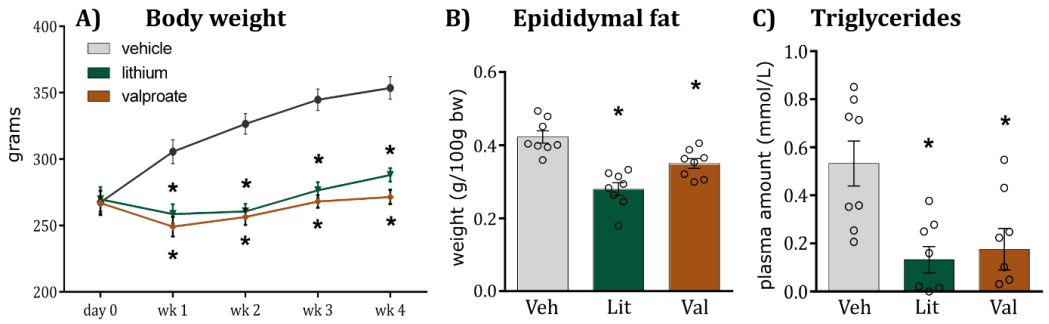


Figure S.3.3 Body weight and fat content. (A) Change in body weight throughout the experiment. Administration of lithium and valproate significantly decreases the body weight. A mixed between-within subjects ANOVA revealed a significant effect for Time [$F_{(4;196)}$=250.06, p<0.001], Treatment [$F_{(6;49)}$=14.15, p<0.001] and a Time × Treatment interaction [$F_{(24;196)}$=12.75, p<0.001]. Lithium and valproate induced a decrease in body weight on weeks 1, 2, 3 and 4 (all p<0.001). **(B)** Epididymal white adipose tissue content expressed as g/100g of body weight. Both lithium and valproate decrease the levels of epididymal fat (One-way ANOVA $F_{(6;55)}$=13.237, p=0.000; t-test p=0.000 for lithium and p=0.020 for valproate). **(C)** Plasmatic levels of triglycerides expressed as mmol/L plasma. Both lithium and valproate decrease circulating levels of triglycerides (One-way ANOVA: $F_{(2;23)}$=7.54, p=0.003; t-test p=0.004 for lithium and p=0.009 for valproate). Data are expressed as mean ± SEM. *p<0.05 (n=8/group).

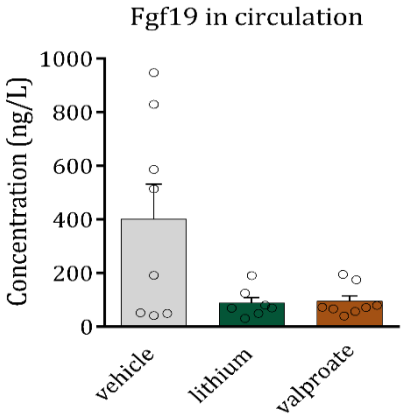


Figure S.3.4 Circulating plasma levels of Fgf19 measured by ELISA kit. Plasma was collected following centrifugation of trunk blood and stored at -80 ºC for further analysis. Triglycerides levels were determined by using the EnzyChromTM Triglyceride Assay kit (ETGA-200; BioAssay Systems).

SUPPLEMENTAL METHODS

Caecal microbiota composition

Caecum was harvested, snap frozen and stored at -80ºC prior to the analysis

- ***Caecal content DNA extraction***

DNA extraction was performed using the QIAmp Fast DNA Stool Mini Kit (Qiagen, Sussex, UK) coupled with an initial bead-beating step. Briefly, 200 mg of each caecal sample were vortex-mixed in a 2 ml screw-cap tubes (Sarstedt, Wexford, Ireland) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.0 mm sterile zirconia beads plus a single 3.5 mm diameter bead (BioSpec Products, Bartlesville, USA) with 1 ml of Qiagen InhibitEX® buffer. Following steps were according to manufacturer's instructions. DNA was quantified using the QubitTM 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) and the Qubit® dsDNA HS Assay Kit (Life Technologies, Oregon, USA). Extracted DNA was kept frozen at -20ºC until further analysis.

- ***16S rRNA Gene Sequence-based microbiota analysis***

The V3-V4 hypervariable region of the 16S rRNA gene were amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol (http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Briefly, first PCR was done using forward primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Each 25 µl PCR reaction contained 5 ng/µl microbial genomic DNA, 1 µM of each primer and 12.5 µl 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK). The PCR conditions follow as: initial denaturation at 95 ºC x 3 min; 25 cycles of 95 ºC x 30 s, 55 ºC x 30 s, 72 ºC x 30 s; and 72 ºC x 5 min for final extension. PCR products were purified with Agencourt AMPure XP system (Beckman Coulter Genomics, Takeley, UK). In the next step, dual indices and Illumina sequencing adapters were attached to PCR products using the Nextera XT Index Kit (Illumina, San Diego, CA). Each 50 µl PCR reaction contained 5 µl purified DNA, 5 µl index primer 1 (N7xx), 5 µl index primer 2 (S5xx), 25 µl 2x Kapa HiFi Hot Start Ready mix and 10 µl PCR grade water. PCR amplification was completed using the previous program but with only 8 amplification cycles instead of 25. Following this, a second clean-up step with the Agencourt AMPure XP system was done. PCR products were quantified, normalized and pooled in an equimolar fashion using the Qubit® dsDNA HS Assay Kit (Life Technologies, Oregon, USA). Next steps in the library preparation were carried out by Teagasc Next Generation DNA Sequencing Facility (Teagasc,

Moorepark, Food Research Centre) prior to 2×250 (bp) paired-end sequencing on the Illumina MiSeq platform, using standard Illumina sequencing protocols.

- ***Bioinformatic sequence analysis***

Bioinformatic sequence analysis was performed as previously described (Murphy et al., 2017). Briefly, paired-end sequences were assembled using FLASH (Magoč and Salzberg, 2011) and analysed using QIIME v1.8.0 (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010). Sequences were quality checked and the remaining sequences were clustered into operational taxonomic units using USEARCH (v7-64bit) (Edgar, 2010). Taxonomic ranks were assigned with a BLAST search against the SILVA SSURef database release 123 (Quast et al., 2013).

Bile acids levels in plasma and faecal material

Bile acids were extracted according to Joyce et al. (Joyce et al., 2014). For the faeces, 80 mg of faecal material was added to dynabeads (Roche) with 300 µl of ice cold 50% methanol containing deuterated internal standards of both Cholic acid and Chenodeoxycholic acid, then subjected to five 30 second rounds of extraction in a dyna-lyser machine (Roche) at 6000 rpm. For plasma extractions, 100 µl was mixed with 300 µl of ice cold 50% methanol also containing deuterated internal standards. Each mixture was vortexed and then centrifuged for 10 mins at 10000 g and the supernatant transferred to a fresh tube. Ice cold acetonitrile (ACN) with formic acid measuring 2 ml was added to each tube, vortexed and agitated at room temperature for 1 hour. Samples were centrifuged again to pellet the debris and the supernatant was added to fresh tubes containing 1 ml of ice cold 100 % ACN. The samples were vortexed and dried under vacuum at +4°C. The dried extracted acids were re-suspended in 150 µl of ice cold 50 % methanol.

- ***Chemicals***

Standard Conjugated bile salts and free bile acids were purchased from Sigma Aldrich and from Steraloids, Inc. (Newport, Rhode Island). HPLC-grade methanol, acetonitrile, water, ammonium acetate, ammonium formate, ammonium hydroxide, formic acid, and acetic acid and water were obtained from Fisher Scientific (Fair Lawn, NJ). Deuterated cholic acid (D-2452) and deuterated chenodeoxycholic acid (D-2772) were purchased from CDN Isotopes Inc. Standards were constructed as 1mg/ml stock solutions of individual sulfated BAs were prepared in water:MeOH (1:1). They were subsequently combined to a final volume of 1.0 ml in water to give a stock concentration of 40 mg/ml for each. Subsequent dilutions were made to which the same volume of deuterated standards was added. Fatty acids were treated similarly

but were resuspended in 100% methanol. These standards were utilized to create standard curves for each analyte examined (Table S4).

- ***Ultra Performance Liquid Chromatography Tandem Mass Spectrometry***

UPLC-MS was performed using a modified method of Joyce et al. (Joyce et al., 2014). Briefly, 5µL from each sample were injected onto a C18 Acquity column (Waters Corp.). Each sample was run in triplicate. Extracts were eluted using a 25-min gradient of 100 % A to 100 % B (A, water, 0.1 % formic acid; B, acetonitrile, 0.1 % formic acid) at a flow rate of 500 µL/min and column temperature of 40°C. Samples were analyzed using an Acquity system (Waters Ltd.) coupled online to an LCT Premier mass spectrometer (Waters MS Technologies, Ltd.) in negative electrospray mode with a scan range of 50–1,000 m/z. Bile acids ionize strongly in negative mode, producing a prominent [M-H]− ion. Capillary voltage was 2.4 Kv, sample cone was 35 V, desolvation temperature was 350°C, source temperature was 120°C, and desolvation gas flow was 900 L/h. Analysis was performed using Waters software Targetlynx for exact quantification against a standard curve for each analyte and Markerlynx for non- biased PCA analysis.

SUPPLEMENTAL STATISTICS

Statistical output of the t-test related to Figure 8 (Dose-dependent effects of lithium and valproate on permeability in distal ileum)

PANEL A (FITC): *Valproate 100 mM* t(18)=-2.113, p<0.05 at 90min, t(18)=-3.183, p<0.01 at 120min, t(18)=-4.038, p<0.01 at 150min, t(18)=-4.232, p<0.01 at 180min.

PANEL B (TEER): *Valproate 100 mM* t(18)=2.053, p=0.055 at 60min, t(18)=3.511, p<0.01 at 90min, t(18)=4.538, p<0.001 at 120min, t(18)=5.118, p<0.001 at 150min, t(18)=5.873, p<0.001 at 180min

PANEL B (I_{sc}): *Lithium 50 mM* t(17)=5.946, p<0.001 at 30min, t(17)=5.752, p<0.001 at 60min, t(17)=3.437, p<0.01 at 90min, t(17)=4.97, p<0.001 at 120min, t(17)=5.2, p<0.001 at 150min, t(17)=4.837, p<0.001 at 180min. *Valproate 100 mM* t(18)=-5.833, p<0.001 at 30min, t(18)=-5.178, p<0.001 at 60min, t(18)=-4.245, p<0.001 at 90min, t(18)=-5.798, p<0.001 at 120min, t(18)=-5.717, p<0.001 at 150min, t(18)=-5.497, p<0.001 at 180min

Chapter 4

Differential Effects of the Gut Microbiota on the Pharmacokinetics of Antipsychotic Drugs

Sofia Cussotto[1,2#], Jacinta Walsh[1,3#], Conall R. Strain[1,6], Fiona Fouhy[1,6], Catherine Stanton[1,4,6], Timothy G. Dinan[1,4], Niall Hyland[1,5], Gerard Clarke[1,4], John F. Cryan[1,2], Brendan T. Griffin[1,3]

[#] Equal contributions

[1] APC Microbiome Ireland, University College Cork, Cork, Ireland

[2] Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[3] School of Pharmacy, University College Cork, Cork, Ireland

[4] Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

[5] Department of Physiology, University College Cork, Cork, Ireland

[6] Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland

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Abstract

The role of the gut microbiome in drug absorption and metabolism has recently come under scrutiny. It remains poorly understood whether the gut microbiome influences the pharmacokinetic parameters of psychotropic drugs and thus potentially alters patient responses. In this study, we investigated the pharmacokinetics of two commonly prescribed antipsychotics, olanzapine and risperidone, in rats whose microbiota had been depleted with an antibiotic cocktail or following administration of the multi-strain probiotic VSL#3.

The bioavailability of olanzapine, but not risperidone, was significantly increased in rats pre-treated with antibiotics. There was no direct effect of the microbiota depletion on the expression of CYP450 enzymes involved in antipsychotic metabolism, as assessed by RT-qPCR and Western blotting. Tight junction transcript level in the duodenum revealed that gut barrier function was not affected by either probiotic or microbiota depletion. The antibiotics, however, significantly altered the metabolic activity of the gut microbiota including faecal β-glucuronidase and β-glucosidase activity. Among the bacterial genera detected by 16S sequencing, the relative abundance of *Alistipes* significantly correlated with the AUC in olanzapine- but not risperidone-treated rats, suggesting that this bacterium might play a role in the pharmacokinetic alterations observed for olanzapine.

These results suggest that the gut microbiome is an important variable determining the bioavailability of orally administered olanzapine but not risperidone. Additional research is warranted to understand the implications and the role of the gut microbiota in the pharmacokinetics of olanzapine in humans, both in terms of the therapeutic and adverse effects of this antipsychotic.

Keywords: Antipsychotic, Microbiome, Pharmacokinetics, Diversity, AUC, CYP

Introduction

The gut microbiome plays a pivotal role in dictating host physiology and health. In recent times, the gut microbiota is coming under increasing scrutiny as a potential determinant of drug pharmacokinetics (Clarke et al., 2019). The traditional role of the gut microbiome on drug pharmacokinetics was thought to be mostly limited to the re-absorption of drug metabolites generated by the host (for example glucuronide conjugates) leading to prolonged half-life of some drugs. Recent studies have highlighted how the microbiome may directly metabolise or degrade drugs (Zimmermann et al., 2019a). In addition, the microbiome can produce metabolites which compete with drugs for drug-metabolizing enzymes or can modulate the latter's gene expression (Carmody and Turnbaugh, 2014; Clarke et al., 2019), albeit few studies to date have demonstrated a clinically significant change in drug pharmacokinetics (estimated to be >20% change in amount of drug reaching the systemic circulation) as a result of altered microbiome (European Medicines, 2010; Vertzoni et al., 2018).

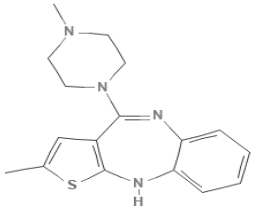
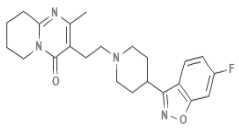
Previous work from our laboratory has linked the gut microbiome to alterations in drug pharmacodynamics; antibiotic-induced microbiota depletion attenuated olanzapine (OLZ)-associated metabolic dysfunction in rats (Davey et al., 2013; Davey et al., 2012). This is consistent with studies in germ-free (GF) mice who do not gain weight following oral delivery of OLZ (Morgan et al., 2014). Although the mechanistic basis for this association is not yet settled, most attention has been placed on the antimicrobial effects of OLZ (Maier et al., 2018; Morgan et al., 2014). A similar role of the gut microbiome in risperidone (RISP)-associated metabolic side effects has also been suggested (Bahr et al., 2015b) and a recent meta-analysis of both animal and human studies linked antipsychotic-induced metabolic dysfunction to the gut microbiome (Skonieczna-Zydecka et al., 2018). The aim of this study was to further extend this research to assess whether perturbations to the gut microbiome could also alter the pharmacokinetic profile of these antipsychotics.

OLZ and RISP are both centrally active, highly lipophilic BCS Class II drugs with comparable half-lives, that are widely used clinically (see **Table 4.1**). Although both

have been associated with microbiome-mediated weight gain *in vivo* (Bahr et al., 2015b; Davey et al., 2013) and antimicrobial effects *in vitro* (Maier et al., 2018; Morgan et al., 2014), they exhibit some differences in pharmacokinetic profiles as well as chemical structure and microbiota exposure. OLZ is well absorbed after oral administration and is cleared primarily via hepatic metabolism, with less than 10% of the drug excreted unchanged in the urine (Korprasertthaworn et al., 2015) and 30% detected in faecal material (FDA, 2019; Sheehan et al., 2010). OLZ is metabolized by the cytochrome CYP system; principally by CYP1A2 and to a lesser extent by CYP2D6. More than 40% of the oral dose of OLZ is subject to pre-systemic metabolism, first-pass effect. OLZ is also subject to glucuronidation via phase 2 enzymes UGT1A4 and to a lesser extent UGT2B10. Knowledge on the phase 2 metabolism of OLZ in rats is currently limited. The metabolism of RISP differs to OLZ. RISP is also well absorbed and its absolute bioavailability is 70%. It is mainly metabolized by CYP2D6 into 9-hydroxyrisperidone (9-OH RISP); CYP3A4 also plays a minor role in the propagation of this active metabolite (Mannens et al., 1993). RISP undergoes little to no phase 2 hepatic metabolism and most of the oral dose, approximately 70%, is recovered unchanged in the urine (Mannens et al., 1993; Markowitz et al., 2002). On this basis, we predicted that OLZ would be more likely to be impacted by the gut microbiome.

It is intriguing to speculate whether perturbations to the gut microbiota could differentially alter the pharmacokinetics of OLZ and RISP and may, in turn, underpin variability in patient response to antipsychotics. In the present study, we depleted the gut microbiota with an antibiotic cocktail to assess whether the microbiota was involved in antipsychotic pharmacokinetics. We also used a more translationally relevant microbiota-directed intervention in the form of a widely used commercially available cocktail of probiotics, VSL#3 (Sarkar et al., 2018).

Table 4.1 Overview of the physiochemical properties of the antipsychotics

Drug name and structure [a]	IUPAC Name	Dosage	Therapeutic Drug concentration	Metabolism	Physiochemical properties Refs			
					MW	Log P	pKa	Solubility
Olanzapine 	2-methyl-4-(4-methylpiperazin-1-yl)-10H-thieno[2,3-b][1,5]benzodiazepine	Target: 10 mg/day	20-80 ng/ml [* & c]	CYP1A2 CYP2D6 UGT1A4 UGT2B10	312 g/mol	2	pKa1=4.01 pKa2=7.2 pKa3=14.2	39.88 mg/ L (H_2O)
Risperidone 	3-[2-[4-(6-fluoro-1,2-benzoxazol-3-yl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydropyrido[1,2-a]pyrimidin-4-one	Range: 2-10 mg/day	N/A	CYP2D6 CYP3A4	410 g/mol	3.49	pKa1=8.76 (2^y amine) pKa2=1.16 (imine)	2.33 mg/ml (H_2O)

Abbreviations : Log P partition co-efficient, *MW* molecular weight, *2^y* secondary, *N/A* not applicable
*(@12hr post dose)
Refs: [a] www.drugbank.ca; [b] www.pubchem.ncbi.nlm.ni.gov; [c] (Hiemke et al., 2011)

Methods

Animals

Adult male Sprague Dawley rats (n=6-7/group; 200-250g on arrival) were obtained from Envigo UK. They were housed 3-4 per cage and maintained under a 12-h light/dark cycle, provided with chow and water *ad libitum*. Rats in the same cage underwent the same treatment to avoid confounding factors such as coprophagy. Animals were acclimated to housing conditions for one week prior to experimental treatment. Experiments were conducted in accordance with European Directive 2010/63/EU. Approval by the Animal Experimentation Ethics Committee of University College Cork (AE19130/P049) was obtained before commencement of all animal-related experiments.

Antibiotic and probiotic treatment

Antibiotics or probiotics were administered for 14 days in water. The antibiotic cocktail consisted of ampicillin 1g/L, vancomycin 500mg/L and imipenem 250mg/L [adapted from (Hoban et al., 2016)] and the solution was provided fresh every second day. The probiotics consisted in VSL#3, a commercially available multi-strain preparation which was administered in a dose of $5 \cdot 10^{10}$ bacteria/kg/day (Rashid et al., 2014) and was provided fresh every day just before the start of the dark cycle (**Figure 4.1A**)

Pharmacokinetic experiments

Rats pretreated with vehicle, probiotic or antibiotic had a 1-day break and subsequently received an acute dosage of either OLZ 20 mg/kg or RISP 15 mg/kg by gavage. Following administration of the antipsychotics, whole blood samples were collected into heparinized tubes from the tip of the tail at different timepoints (30 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h). The plasma was harvested by centrifugation at 10,000G for 10 min and stored at -20°C until further analysis.

High performance liquid chromatography (HPLC) detection of the drugs in plasma and caecal contents

The liquid-liquid extraction (LLE) protocol and HPLC conditions employed for the detection of OLZ and RISP in plasma samples was based on previously published methods with some modifications (Avenoso et al., 2000; Dusci et al., 2002). Detailed information on plasma and caecum preparation as well as drug extraction can be found in the *Supplemental material*. For analyte identification and quantification, calibration standards were prepared by spiking 10 µl or 20 µl of working standard solutions (RISP or OLZ) into 90 µl of blank rat plasma or 200 µl of blank rat caecal contents respectively at final concentrations of 15.6–2000 ng/ml. The calibration standards were treated as described in supplemental material before the analysis. Calibration curves were generated by plotting the peak area ratio (OLZ) or peak area (RISP) of the analyte to internal standard versus the concentration of the analyte using least-square linear regression. The correlation coefficients of the calibration curves were greater than 0.99. The co-efficient of variation (% CV) for the HPLC method, assessed over a 5-day period, was less than 15% for both drugs. The accuracy of the technique was determined by carrying out the extraction procedure on plasma samples spiked with known concentrations of the drug of interest followed by HPLC analysis. Specific information on the HPLC equipment employed can be found in the supplemental material.

Pharmacokinetic and statistical analysis

The maximum plasma concentration (Cmax) and time taken to reach Cmax (Tmax) for OLZ and RISP were estimated directly from the plasma concentration-time profiles. Pharmacokinetic parameters including the area under the plasma drug curve (AUC), clearance (Cl) and mean residual time (MRT) were all calculated using a non-compartmental model. AUC was calculated using the linear trapezoidal method from the first to the last measured plasma concentration i.e. $AUC_{0\text{-}8hr}$. Antipsychotic drug clearance from plasma was estimated by dividing AUC by the administered

drug dose and MRT was calculated by dividing $AUC_{0\text{-}8hr}$ by the area under the first moment curve ($AUMC_{0\text{-}8hr}$). Data are represented as mean ± standard error of the mean (SEM) apart from the plasma pharmacokinetic curve and parameters where data is represented as mean ± standard deviation (S.D.).

Microbiota composition of the caecal content

Caecum was harvested and immediately snap-frozen and stored at -80ºC prior to the analysis. DNA was extracted using the Qiagen QIAmp Fast DNA Stool Mini Kit coupled with an initial bead-beating step. The V3-V4 hypervariable region of the 16S rRNA gene was amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library protocol. Samples were sequenced at Teagasc Sequencing Facility (TFRC, Moorepark) on the Illumina MiSeq platform using a 2×250 bp kit. Reads were assembled, processed and analysed following the pipeline described in *Supplemental Methods*.

RNA extractions, reverse transcription and quantitative RT-qPCR

The duodenum and frontal lobe of the liver were rapidly dissected from individual animals and stored at -80 °C. Total RNA was extracted from the intestinal tissue and the front lobe of the liver with the mirVana^{TM} miRNA isolation kit (Thermo Fisher Scientific/Ambion) following the manufacturer's protocol. A Nanodrop 1000 (Thermo Scientific, UK) was used to determine RNA concentration. RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Thermo Fisher Scientific/Applied Biosystems) in a G-storm thermocycler (G-storm, Surrey, UK). Genes of interest (listed in supplemental material) were amplified using SybrGreen probes. Each transcript value was calculated as the average of triplicate samples across experimental conditions. Values were normalized to β-actin. Data were analysed with the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001) and presented as a fold change vs. vehicle group.

Protein extraction and Western blots

Total proteins from liver samples were extracted through sonication, BCA quantification (Thermo Fisher Scientific) and heat denaturation. Protein levels were detected on SDS-page gels using appropriate primary antibody dilutions against CYP1A2 (1:5000; Abcam ab22717), CYP2D1 (1:1000; Enzo BML-CR3210), CYP3A1 (1:1000; Millipore AB1253), secondary antibody HRP-conjugated to rabbit or mouse (1:10000; Thermo Fisher Scientific). Primary antibody against β-actin (1:1000; Santa Cruz, sc-47778) was used as control for protein loading. Quantification of protein bands was performed using ImageJ software and protein levels were normalized to the β-actin and presented as fold change relative to the vehicle group. Significance was determined by a one-way ANOVA followed by Dunnett's test.

Fecalase assay

Fecalase was prepared from approximately 70 mg fresh-frozen rat faeces collected on day 12 of the 14-day intervention period, according to a modified method previously described (Lee et al., 2002). Briefly, the faecal pellet was suspended in 1 mL phosphate buffer (0.01 M, pH 7.4) and homogenised using a mini BeadBeater machine for 1.5 minutes. The faecal suspension was centrifuged at 2,000 rpm for 5 minutes and the resulting supernatant was centrifuged at 10,000 rpm for 20 minutes. The supernatant from the second centrifugation step (fecalase) was then used for the assay of enzyme activity. For quantification of enzymatic activity, the reaction mixture, containing 50 µl fecalase, 100 µl phosphate buffer (0.01M, pH 7.4) and 100 µl 4-nitrophenyl-β-D-glucopyranoside (1mM, Sigma Aldrich) for β-glucosidase or 100 µl 4-nitrophenyl-β-D-glucuronide (1mM, Sigma Aldrich) for β-glucuronidase, was incubated at 37 °C for 15 minutes. After incubation, 250 µl NaOH (0.5N) was added to stop the reaction and the absorbance was measured at 405 nm (UV-vis spectrophotometer). The Pierce BCA Protein Assay Kit (ThermoFisher Scientific) was used, in accordance with the kit protocol, to measure the total protein concentration in the fecalase samples. Enzyme activity was indicated as the amount required to catalyse the formation of 1 nmole of p-nitrophenol per minute under the standard assay conditions.

Statistical analysis

Data that satisfied both homogeneity and normality tests, were analysed using one-way ANOVA followed by Dunnett's test. Data that did not satisfy either homogeneity or normality tests, were analysed using Kruskal-Wallis non-parametric test followed by Mann-Whitney U test and corrected for multiple comparisons using the Benjamin-Hochberg false discovery rate (FDR) method. Grubbs method was employed to test for any specific outliers (Grubbs, 1950). Threshold for statistical significance was set at $p<0.05$.

Results

Body weight does not change during probiotic and antibiotic administration. Antibiotics increase caecum weight

Rats' body weight was monitored daily. There were no differences across the 14 days period between vehicle-receiving rats and probiotic or antibiotic-treated animals (**Figure 4.1B**). Upon cessation of the study and collection of organs, the caecum weight was significantly increased in antibiotic-treated rats (**Figure 4.1C**).

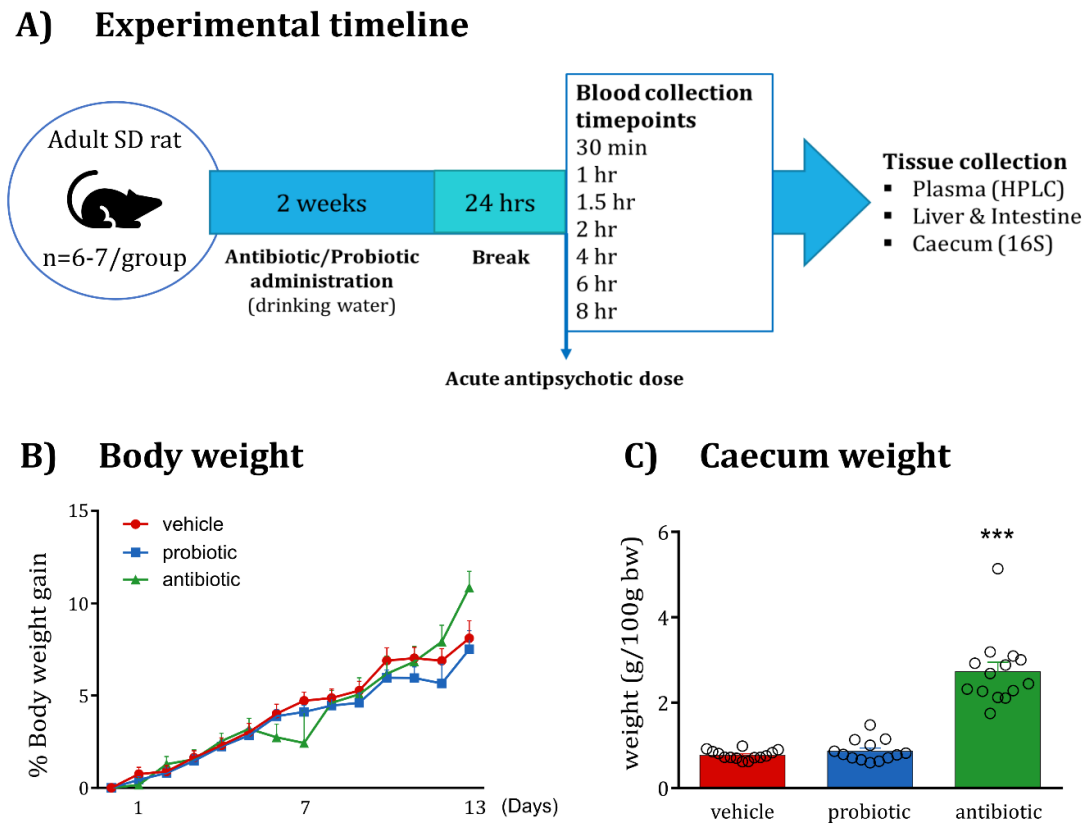


Figure 4.1 Experimental timeline, body weight and caecum weight. (A) Neither probiotic nor antibiotic administration influence the body weight gain. **(B)** Antibiotic administration increases caecum weight (KW p=0.000, U=0, p=0.000). Data are expressed as mean + SEM. *p<0.05 (n=13-14/group).

Antibiotics and probiotics differentially impact the caecal microbiota composition

To confirm the effect of antibiotics on the microbiota composition and to examine whether probiotics also had a microbial effect, we performed 16S sequencing of bacterial rRNA of the caecum content. As expected, the sequencing revealed a significant decrease in the bacterial richness and diversity of rats treated with antibiotics as compared to the vehicle group (**Figure 4.2B**). Moreover, separation according to group was further illustrated through principal coordinate analysis (PCoA), with statistical support of the significant separation between the antibiotic and the vehicle group ($p<0.001$, **Figure 4.2C**). The marked difference between the antibiotic and the vehicle group was also detected at the genus level, with several genera being depleted in the antibiotic group (**Figure 4.2A, Table S.4.3**). The phylum and family microbiome signatures were also significantly disrupted by antibiotics (**Figure S.4.1**). On the contrary, rats that received probiotics for 2 weeks did not show any marked different at the genus level, alpha or beta diversity (**Figure 4.2**).

To assess whether the acute administration of antipsychotics would impact the microbiome *per se*, we checked the alpha diversity in the vehicle group and no significant differences were detected between OLZ- and RISP-treated rats (**Figure 4.2B**).

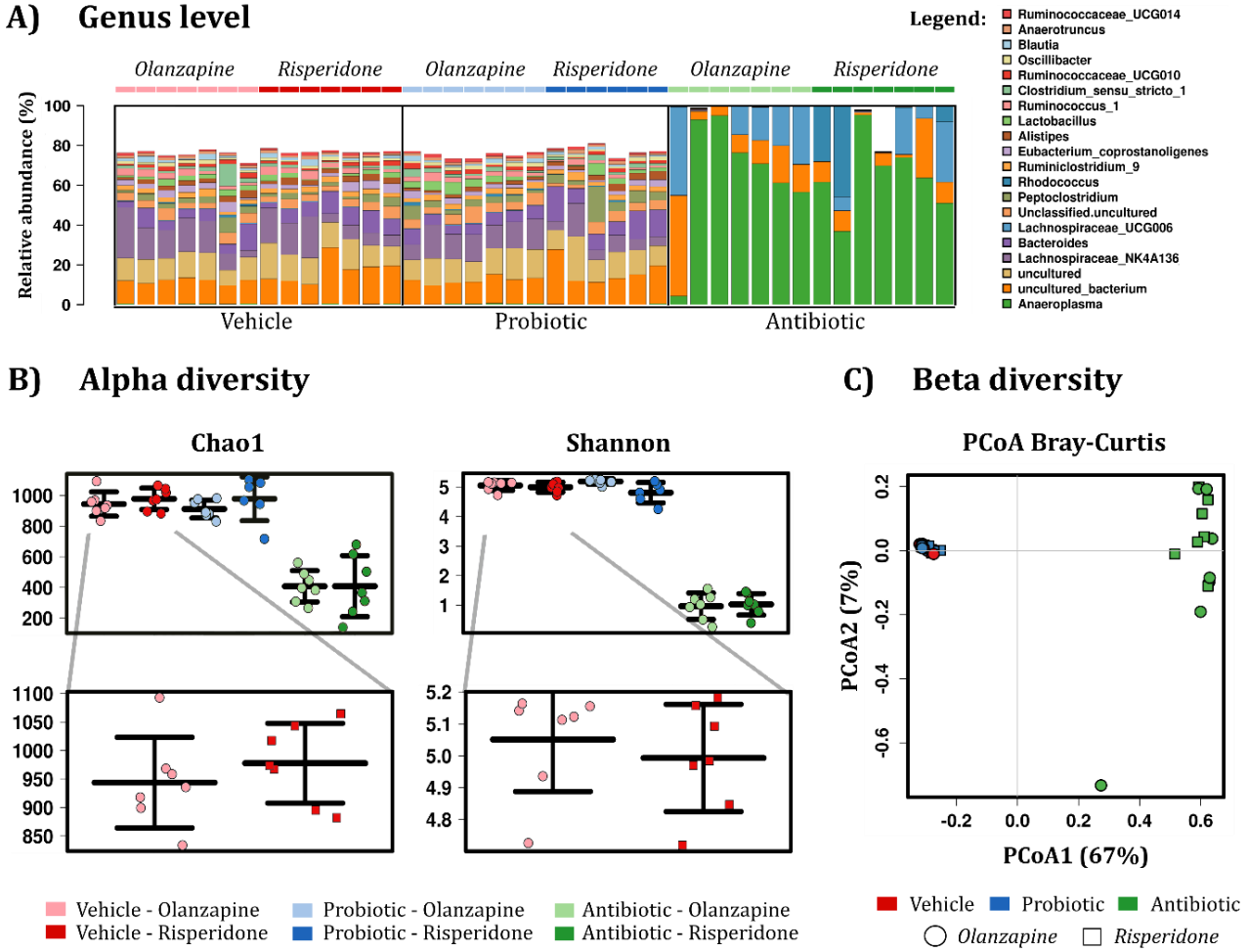


Figure 4.2 Caecum microbiota composition (16S sequencing). (A) Bar charts representing the taxa abundance at the genus level. The 20 most abundant taxa are shown. **(B)** Alpha diversity. Kruskal-Wallis test for Chao1 ($p=0.000$) and Shannon ($p=0.000$). Mann-Whitney U test for: *Chao1* and *Shannon*: antibiotic $p<0.001$ compared to vehicle. Data are expressed as median and min-to-max values. Samples are rarefied to read depth of 32817. **(C)** Beta diversity, principal coordinate analysis of Bray-Curtis compiled distance matrix of all microbial relative abundances compared to the vehicle group. Antibiotic animals show significant variation from the vehicle (Adonis PERMANOVA $p<0.001$, $R^2=0.764$), independent of drug treatment. (n=6-7/group).

At the phylum and family level, several bacteria were altered in the two microbiome-targeted treatments (**Figure S.4.1**, **Tables S.4.1** and **S.4.2**), with antibiotics causing mainly a depletion in several families. At the genus level, antibiotics induced a wide depletion, while the genera *Anaeroplasma* was significantly increased in antibiotic-treated rats and *Lachnospiraceae* UCG006 was increased but not significantly (**Figure 4.3**, **Table S.4.3**). The three bacterial strains present in the VSL#3 probiotic formulation belongs to the genera *Lactobacillus*, *Streptococcus* and *Bifidobacterium*. Among these three taxa, only *Streptococcus* was significantly increased in the caecum of probiotic-treated rats compared to vehicle-treated rats. Other taxa that were significantly increased in probiotic-treated rats included *Erysipelatoclostridium*, *Marvinbryantia* and *Odoribacter* (**Figure 4.3**).

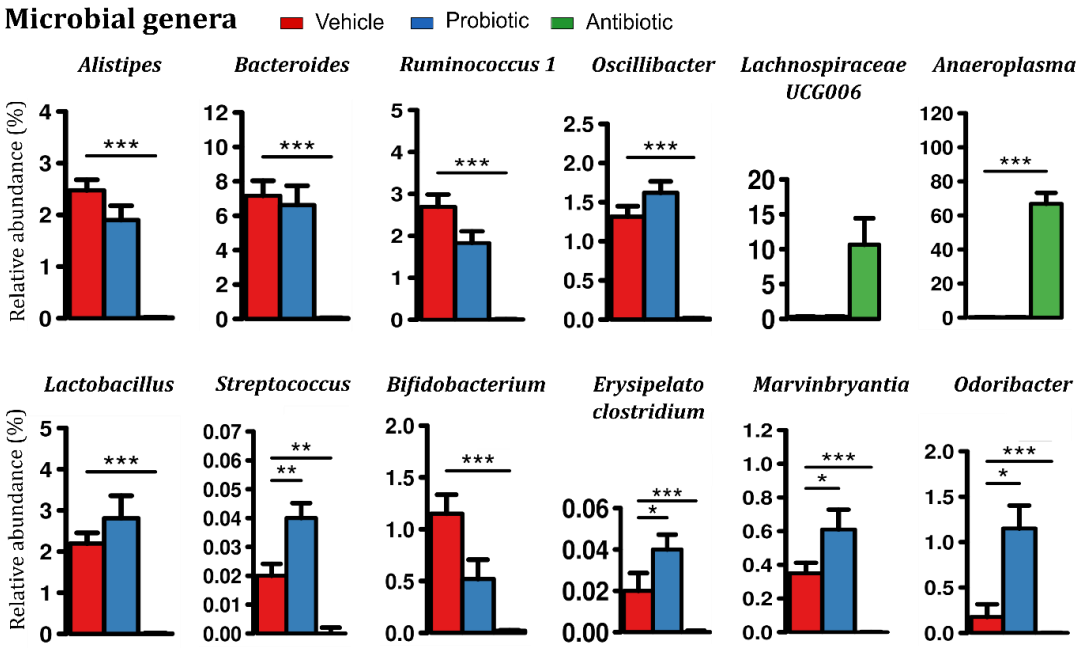
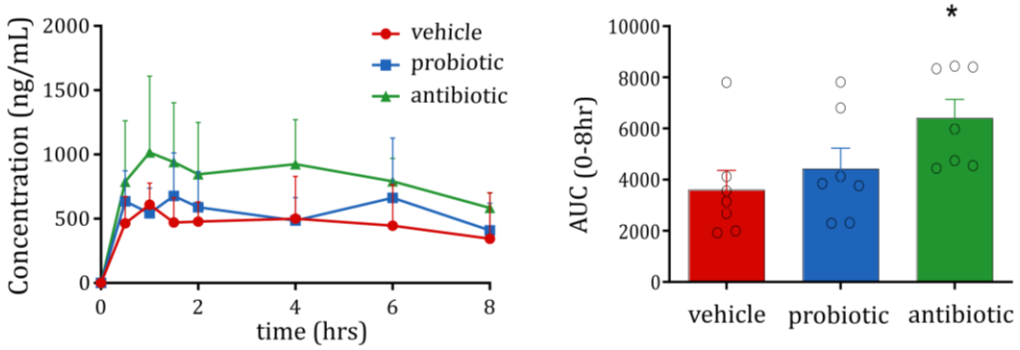


Figure 4.3 Probiotics and antibiotics differentially affect bacterial composition at genus level. Many bacterial genera are completely depleted in antibiotic-treated rats. The genus *Anaeroplasma* is significantly increased in antibiotic-treated rats. *Lachnospiraceae* UCG006 are also increased but not significantly in antibiotic-treated rats. *Streptococcus*, *Erysipelatoclostridium*, *Marvinbryantia* and *Odoribacter* are significantly increased in probiotic-treated rats compared to the vehicle group. Data are expressed as median + SEM. *p<0.05, **p<0.01, ***p<0.001 (n=13-14/group). Data was analysed using Kruskal-Wallis non-parametric test followed by Mann-Whitney U test and corrected for multiple comparisons using the Benjamin-Hochberg false discovery rate (pFDR) method *(refer to Supplemental Material for details on statistics*).

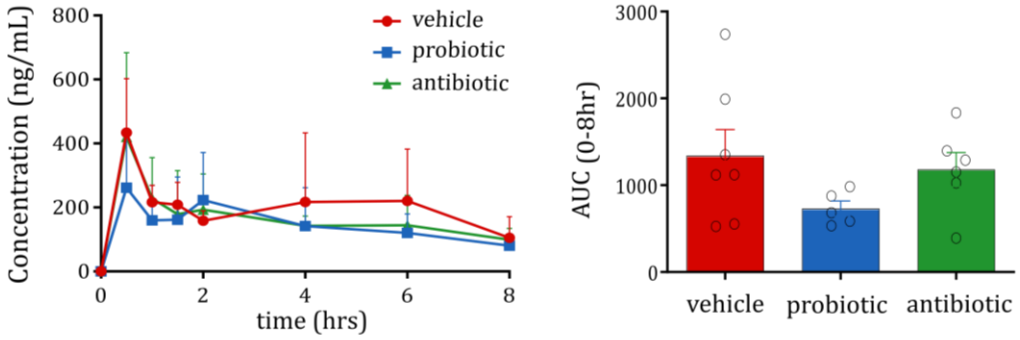
Microbiota-depletion but not probiotic administration influences the absorption of OLZ

The plasma concentration levels of OLZ were determined after oral administration to the vehicle-, probiotic- and antibiotic-treated animals. The mean plasma concentration-time profile of OLZ and AUC (area under the curve) are shown in **Figure 4.4**. The resultant pharmacokinetic parameters are described in **Figure 4.4C**. Antibiotic treatment significantly increased the $AUC_{0\text{-}8hr}$ of OLZ after a single oral dose. Neither of the microbiome manipulations influenced significantly the absorption of RISP although probiotics showed a trend toward a decrease of RISP absorption.

A) Olanzapine - Pharmacokinetics



B) Risperidone - Pharmacokinetics



C) Pharmacokinetics parameters

PK parameter	Olanzapine			Risperidone		
	Vehicle (mean ± SD)	*Probiotic (mean ± SD)*	*Antibiotic (mean ± SD)*	*Vehicle (mean ± SD)*	*Probiotic (mean ± SD)*	*Antibiotic (mean ± SD)*
AUC (0-8 h)	3527 (2066)	4423 (2122)	6416 (1919)	1342 (791)	732 (191)	1182 (476)
MRT (hr)	0.27 (0.04)	0.26 (0.02)	0.26 (0.03)	0.44 (0.21)	0.27 (0.03)	0.31 (0.07)
CL (l/hr/kg)	7.08 (3.20)	5.46 (2.45)	3.37 (1.00)	15 (9.00)	19 (7.7)	11.6 (2.4)
C_{max}(ng/mL)	609.5 (167)	677 (335)	1016 (592)	433 (168)	261 (178)	420 (264)
T_{max} (hr)	1	1.5	1	0.5	0.5	0.5

Figure 4.4 Pharmacokinetic profile of OLZ and RISP after oral administration in rats pre-treated with vehicle, probiotic or antibiotic. (A) Plasma levels and area under the curve (AUC) of OLZ. OLZ (20 mg/kg) was orally administered to rats pre-treated with vehicle, probiotic or antibiotic for 14 days following a 24-hr break (n=7/group, n=3 @1hr timepoint) The AUC (Area Under the Curve) of OLZ is increased by antibiotic administration (One-way ANOVA $F_{(2;20)}$=3.58, $p<0.05$; t-test p=0.033). **(B)** Plasma levels and area under the curve (AUC) of RISP. RISP (15 mg/kg) was orally administered to rats pre-treated with vehicle, probiotic or antibiotic for 14 days following a 24-hr break (n=6-7/group). **(C)** Pharmacokinetic parameters of OLZ and RISP after oral administration in rats pre-treated with vehicle, probiotic or antibiotic. Data are expressed as mean ± SD.

The parameter AUC correlates with the relative abundance of the genera *Alistipes* in OLZ- but not RISP-treated rats

After demonstrating that changes in the microbiome occurred in parallel with changes in the systemic absorption of OLZ (**Figure 4.4A**), we wanted to examine whether the relative abundance of specific taxa was associated to pharmacokinetic parameters [including AUC (area under the curve), Cmax (maximum serum concentration), Tmax (time at which the Cmax is observed) and CL (clearance)] in both antipsychotic-receiving groups. In OLZ-treated rats, the relative abundance of *Alistipes* negatively and significantly correlated with AUC (**Figure 4.5**). This correlation was not observed in RISP-treated rats. All other taxa including *Bifidobacterium* (shown in **Figure 4.5**) did not show significant correlations with pharmacokinetics parameters after adjusting for multiple testing.

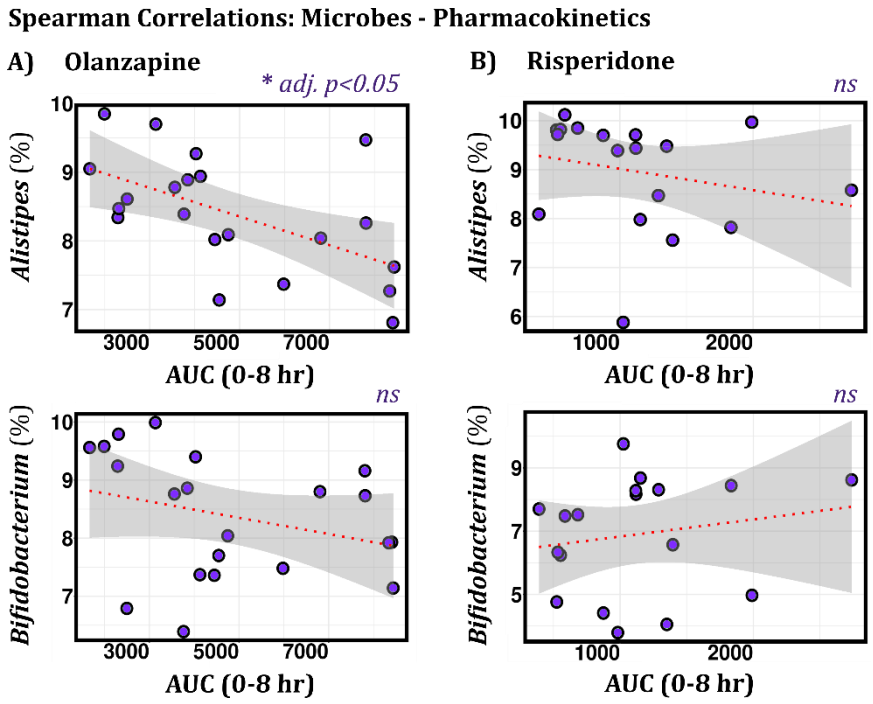


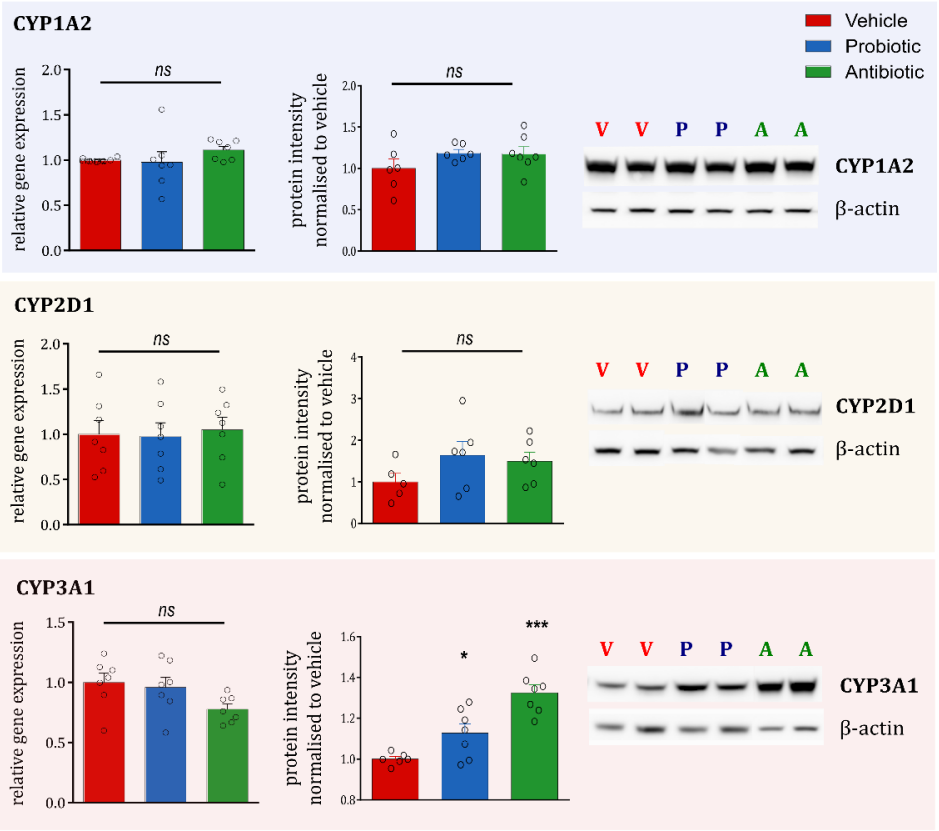
Figure 4.5 Spearman correlations between AUC and the relative abundance of two bacteria in OLZ- and RISP-treated rats. (A) In OLZ-treated rats, AUC significantly correlates with the relative abundance of *Alistipes* (p=0.002245, adj p=0.045, R^2=-0.64). N=21. Bifidobacterium does not correlate with AUC. **(B)** In RISP-treated rats, no significant correlations are observed between AUC and bacterial abundance. N=20. Data are normalized and CLR-transformed. Ns = not significant.

Antibiotics and probiotics do not alter the gene expression of CYPs involved in OLZ and RISP metabolism

To test whether the probiotics or antibiotics had any direct effect on the hepatic expression of cytochromes (CYP) relevant for the metabolism of antipsychotics, RT-qPCR was employed to examine the CYP gene expression at the transcript level. Interspecies comparison of CYPs isoforms present in rats and humans are described in (Martignoni et al., 2006) (**Figure 4.6A**). Neither the probiotic nor antibiotic treatment altered the hepatic mRNA expression of the rat equivalent human isoenzymes implicated in the metabolism of OLZ and RISP (**Figure 4.6A**). To further confirm the transcript findings at protein level, Western blotting analysis was performed for detection of the same CYPs. As shown in **Figure 4.6A**, there was no difference in CYP1A2 or CYP2D1 protein in the livers of probiotic- and antibiotic-treated rats. Conversely, both microbiota-targeted interventions significantly upregulated CYP3A1 protein; the magnitude of the affect being larger in the case of the antibiotic-treated rats ($p<0.001$ vs Probiotic $p<0.05$). As CYP3A1 is, however, only a minor metabolizer of RISP, the significance of this finding is somewhat limited.

A) Liver: CYPs - gene and protein expression

Enzyme (rat)	*Enzyme (human)*	*Relevance*
CYP1A2	CYP1A2	Main metabolizer of olanzapine
CYP2D1	CYP2D6	Main metabolizer of risperidone
CYP3A1	CYP3A4	Minor metabolizer of risperidone



B) Duodenum: tight junctions and MDR - gene expression

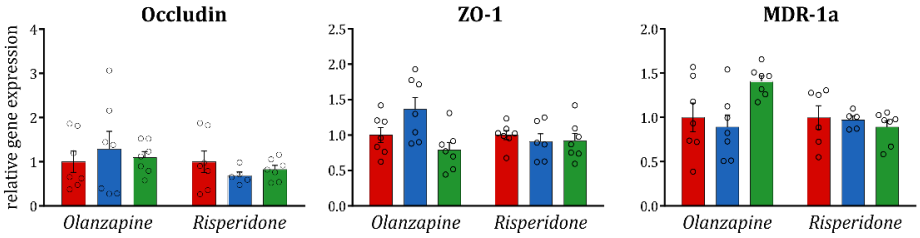


Figure 4.6 (A) Gene expression and protein levels of relevant CYPs in the liver. Probiotic/antibiotic administration does not alter the gene expression and protein levels of CYP1A2 and CYP2D1 compared to the vehicle group. Both probiotics and antibiotics increase the protein levels of CYP3A1 (One-way ANOVA $F_{(2;19)}$=19.25, p<0.001; t-test p=0.049 for probiotic VS vehicle, p=0.000 for antibiotic VS vehicle) without altering gene expression. The protein bands of two representative samples per group are shown. Data are expressed as mean ± SEM. *p<0.05, ***p<0.001, ns=not significant (n=5-7/group). **(B) Gene expression of relevant tight junctions and MDR in the duodenum.** No significant differences are noted across groups. Data are expressed as mean ± SEM (n=5-7/group).

Antibiotics and probiotics do not alter the gene expression of tight junctions and MDR-1a in the duodenum

Previous studies have suggested that antibiotic administration can induce a dysregulation in intestinal barrier function (Spiller, 2018; Tulstrup et al., 2015) and the presence of the gut microbiota is important to maintain normal barrier function. For this reason, we assessed the gene expression of two key tight junctions, occludin and zonula occludens-1 (ZO-1) in the duodenum, main site of absorption of xenobiotics. This was carried out to rule out the possibility that an antibiotic-induced barrier dysfunction in the duodenum might be responsible for the increased absorption of OLZ in circulation; therefore, we analysed the two antipsychotic groups separately. Independently of the acute antipsychotic administered, neither probiotics nor antibiotics induced significant changes in the expression of tight junctions (**Figure 4.6B**). We also assessed the gene expression of multidrug resistance protein 1a (MDR-1a) which is responsible for pumping xenobiotic back in the intestinal lumen potentially affecting drug absorption. The isoform 1a was selected in the duodenum because of its prevalent distribution in this tissue (Cui et al., 2009). Neither probiotics nor antibiotic induced changes in the expression of MDR-1a in the duodenum (**Figure 4.6B**).

Antibiotics deplete the metabolic activity of the gut microbiota

An *ex-vivo* metabolism assay, fecalase, has been previously utilized to elucidate the role of the gut microbiota in the metabolism of lovastatin, aspirin and amlodipine (Kim et al., 2016; Yoo et al., 2014; Yoo et al., 2016). The activity of two microbial-derived enzymes was investigated as a surrogate readout of the metabolic activity of the gut microbiota. The microbiota depletion induced by the antibiotic treatment markedly downregulated the expression of β-glucuronidase faecal enzymatic activity by the end of antibiotic treatment (i.e. day 12 of 14-days intervention period) (**Figure S.4.2B**). Similarly, there was a significant decrease of β-glucosidase activity relative to vehicle-only rats following microbiota depletion. Probiotic treatment significantly decreased β-glucosidase activity (**Figure S.4.2B**).

Discussion

The field of pharmacomicrobiomics studies the effects of microbiome variations on drug disposition, action and toxicity (ElRakaiby et al., 2014; Saad et al., 2012), and has become increasingly investigated in recent years. In this study, we focus our attention on two commonly prescribed antipsychotics with distinct pharmacokinetic profiles, OLZ and RISP, and how their pharmacokinetic properties might be influenced by microbiome manipulations. Our results show that microbiome depletion in adult rats increased the absorption of OLZ following acute administration. On the contrary, RISP's absorption was not influenced by microbiome perturbations.

The main site of absorption of antipsychotics is generally considered to be the stomach and the small intestine, areas of the gastrointestinal tract that are markedly less colonised with bacteria (10^1 and 10^3 bacteria/g respectively) than the bacterial dense large intestine (10^{12} bacteria/g) (O'Hara and Shanahan, 2006; Walsh et al., 2018). The potential effects of the gut microbiota on the absorption or biotransformation of OLZ and RISP would be presumed to be negligible. Our data, however, provides the first evidence that the gut microbiome may play an important role in the pharmacokinetics of OLZ. Lipophilic compounds, including these antipsychotics, have the same or higher permeability coefficients in the distal colon compared with the upper small intestine culminating in greater risk of poor drug absorption from the small intestine.

Antibiotic-associated perturbations of the gut microbiota significantly altered the bioavailability of OLZ; the mean AUC for OLZ in the plasma of the antibiotic group was over 1.5-fold greater than the corresponding value in the vehicle group. These results suggested that the uptake of OLZ from the intestinal tract was increased following microbiota depletion, which may result from the suppression of the metabolic activity of gut microbiota and consequently, reduced pre-systemic metabolism of OLZ. Interestingly, the effect was only present in OLZ, with RISP's absorption not being affected by probiotics or antibiotics.

The observed increase in caecum weight in antibiotic-treated rats has been well characterized previously (Koopman and Kennis, 1980; Savage and Dubos, 1968). In

our study, the antibiotics induced a broad depletion of several genera, allowing *Anaeroplasma* and *Lachnospiraceae* UCG006 to survive and colonise the entire niche. Previous studies have shown that psychotropic medications can impact the gut microbiome composition (Cussotto et al., 2019b; Davey et al., 2012; Maier et al., 2018), thus we assessed whether the microbial diversity (both alpha and beta diversity) was affected by acute administration of either OLZ or RISP. Importantly, our data show that acute antipsychotic-use does not influence the composition of the gut microbiome *per se* (**Figure 4.2B**).

Across 156 genera detected by 16S sequencing, *Alistipes* only correlated with blood levels (area under the curve, AUC) of OLZ- but not RISP-treated rats, suggesting that this specific bacterium might potentially play a role in the pharmacokinetic alterations of OLZ. *Alistipes* has been shown to be increased in mice exposed to chronic intermittent vapourised ethanol (Peterson et al., 2017) in mice receiving faecal microbiota transfer from alcoholic patients with severe hepatitis (Llopis et al., 2016). An increased population of *Alistipe*s has also been observed in depression (Jiang et al., 2015b; Naseribafrouei et al., 2014) and in response to stress (Bangsgaard Bendtsen et al., 2012). Intriguingly, the genus *Alistipes* has been previously linked to patient response to chemotherapy. Specifically, the abundance of *Alistipes* correlated positively with the immunotherapy-induced production of TNF in mice with normal microbiota (Iida et al., 2013). Administration of an antibiotic cocktail (vancomycin, imipenem, and neomycin in drinking water) significantly disrupted the microbiome, impaired immunotherapy efficacy and therefore TNF production (Iida et al., 2013). Furthermore, a recent study investigated the ability of 76 bacterial strains to metabolise over 270 drugs and aimed to identify causal links between microbiota gene content and drug metabolising activities of human gut bacteria. *Alistipes indistinctus* (DSM 22520) was identified as one bacterial strain linked to the chemical modification, or metabolism, of approximately 40 drugs; half of these substrate drugs were greater than 80% metabolised (Zimmermann et al., 2019a). Interestingly, the authors showed greater than 50% of RISP was metabolised after 12hours incubation with this bacterial strain. Our data, however, did not find an association with RISP and *Alistipes*. Although the evidence on the role played by specific taxa on drug response is still

limited, the research by Iida, and Zimmermann, and colleagues' sheds light upon the potential role of *Alistipes* in drug efficacy.

Evidence suggests that antibiotics can induce a barrier dysfunction in the GI tract (Spiller, 2018; Tulstrup et al., 2015). With increased levels of OLZ in the blood of antibiotic-treated rats, we wanted to rule out the possibility that a disruption in intestinal permeability might be the cause of an increased absorption at the duodenum level. According to gene expression of the two tight junctions occluding and ZO-1, antibiotics (and microbiota depletion) did not induce an alteration in duodenal permeability and is unlikely to explain the observations reported here. Finally, the gene expression of multidrug resistance protein 1a (MDR-1a; which is responsible for pumping xenobiotic back in the intestinal lumen) was not disrupted by microbiome manipulations suggesting that MDR1 might not play an overt role in the pharmacokinetic differences seen in OLZ-treated rats.

The increased level of OLZ in the plasma of microbiome-depleted rats was not, however, reflected in the caecum (**Figure S.4.2A**). We hypothesised that the microbial enzyme β-glucuronidase could convert a glucuronide-conjugated metabolite of OLZ (OLZ 10-*N* glucuronide or OLZ 4-*N* glucuronide) into the parent compound; however, our findings did not support this hypothesis.

Antibiotic treatment significantly depleted the enzymatic activity of two microbial enzymes, with the activity of β-glucuronidase falling to levels below the limit of detection (LOD) of the assay (**Figure S.4.2B**). Thus, it seems likely that the increase in plasma OLZ levels might be mediated via an alternative mechanism rather than by antibiotic-mediated interference of enterohepatic recirculation or inhibition of MDR1. The suggested mechanism could be further investigated by comparing the metabolism of OLZ and/or the formation rate of the OLZ metabolite in fecalase from antibiotic-treated rats versus vehicle-only rats.

The antibiotic-induced effects on drug pharmacokinetics appear to be drug specific. Microbiota depletion significantly elevated the plasma levels of OLZ but had no impact on another antipsychotic, RISP. Despite some reports in the literature illustrating a potential role of the gut microbiota in the metabolism of RISP,

perturbations of the gut microbiota in our study did not significantly alter any pharmacokinetic parameter of RISP. Previous work has hinted that the isoxazole ring present in RISP may be liable to cleavage by the gut microbiota (Kim, 2015; Meuldermans et al., 1994). Antibiotic-treatment, such as rifampin, has been previously shown to alter the metabolism of RISP in the liver, albeit via induction of hepatic CYP gene expression (Baciewicz et al., 2013).

Additionally, it is worth noting that, in order to limit pharmacokinetic drug-drug interaction issues, there was a 24-hour intervention-free period to facilitate the excretion of the antibiotics and probiotics from the body prior to acute drug dosing. Moreover, the choice of antibiotics was made to limit their direct effect on host hepatic metabolism. Neither the probiotic or antibiotic cocktail altered the hepatic expression of CYP1A2 and CYP2D1 implicated in the host metabolism of OLZ and RISP both at transcript and protein level (**Figure 4.6**). This finding shows that the pharmacokinetic alteration following antibiotic treatment may be mainly due to a microbiota depletion effect rather than inhibition of hepatic pathways. The protein expression of CYP3A1, which was significantly increased in antibiotic-treated rats, has been previously shown to be induced by macrolide antibiotics in both rat and human hepatocytes (Ledirac et al., 2000). CYP3A1, however, only plays a minor role in the metabolism of antipsychotics.

Little is known regarding the effect of VSL#3 on the expression of various drug-metabolizing enzymes in the liver. Previous research administered VSL#3 probiotics to both conventional and germ-free (GF) mice in the drinking water for 28 days (dose $4.5x10^6$ CFU/ml) and found the probiotic cocktail to have a relatively minor effect on hepatic drug-metabolizing enzyme expression, albeit this finding being in mice (Selwyn et al., 2016). In our study, VSL#3 administration did not impact the pharmacokinetics of antipsychotics, but future studies employing different dosages and time-courses of this multi-strain combination, or examining each probiotic strain individually, are warranted.

To our knowledge, the present study is the first to demonstrate the microbiota-mediated alteration of OLZ pharmacokinetics in a preclinical *in vivo* setting.

Therefore, it is imperative that additional research is carried out in this area to facilitate the critical evaluation of the role the gut microbiota plays in the distribution of antipsychotics in humans. In addition, the mechanisms underpinning the increased OLZ concentration in the systemic circulation of the microbiome-depleted rats remains elusive and need to be clarified. A question arises as to whether the effects of microbiota variations on the absorption of antipsychotic drugs might be dose-dependent or duration-dependent (i.e. acute versus chronic) and future research is now warranted to investigate this important aspect.

Our findings suggest that inter-individual variations in OLZ response might be linked to the gut microbiome composition of the host, with a potential role for the bacterium *Alistipes*. Also, the information here provided might be crucial for clinical settings where antipsychotics and antibiotics are co-administered (Beovic et al., 2016). Unravelling the potential role of antibiotics and prebiotics in modulating the pharmacokinetics of orally administered antipsychotics will provide important new insight into potential drug-drug interactions relevant for clinical practice.

Supplemental Material

SUPPLEMENTAL TABLES

Table S.4.1 Statistics values for relative abundance of bacterial PHYLA in each treatment group (probiotic or antibiotic) as compared to the vehicle.

Phylum	**Probiotic**		**Antibiotic**	
	p value	***(i/m)·Q***	***p value***	***(i/m)·Q***
Firmicutes	ns	ns	0.000	0.0181 *
Verrucomicrobia	ns	ns	0.000	0.0363 *
Bacteroidetes	ns	ns	0.000	0.0545 *
Saccharibacteria	ns	ns	0.000	0.0727 *
Tenericutes	ns	ns	0.000	0.0909 *
Deferribacteres	ns	ns	0.000	0.1090 *
Proteobacteria	ns	ns	0.000	0.1454 *
Lentisphaerae	ns	ns	0.000	0.1636 *
Unclassified	ns	ns	0.000	0.1818 *
Actinobacteria	ns	ns	0.016	0.2

*p<0.05, Mann-Whitney U test, Benjamini-Hochberg critical value [(*i*/*m*)*Q*] with *i*=rank; *m*=total number of tests; *Q* (false-discovery rate)=0.2. N=13-14/group. Red indicates an increase, blue a decrease compared to the vehicle group. ns=not significant

Table S.4.2 Statistics values for relative abundance of bacterial FAMILIES in each treatment group (probiotic or antibiotic) as compared to the vehicle.

Family	**Probiotic**		**Antibiotic**	
	p value	***(i/m)·Q***	***p value***	***(i/m)·Q***
Desulfovibrionaceae	ns	ns	0.000	0.004878 *
Rhodospirillaceae	ns	ns	0.000	0.009756 *
Ruminococcaceae	ns	ns	0.000	0.014634 *
Peptostreptococcaceae	ns	ns	0.000	0.019512 *
Lactobacillaceae	ns	ns	0.000	0.02439 *
Caldicoprobacteraceae	ns	ns	0.000	0.029268 *
Christensenellaceae	0.043	0.019512 *	0.000	0.034146 *
Flavobacteriaceae	ns	ns	0.000	0.039024 *
Clostridiaceae_1	ns	ns	0.000	0.043902 *

Bacteroidales_S24_7_group	ns	ns	0.000	0.04878 *
uncultured_rumen_bacterium	ns	ns	0.000	0.053659 *
Peptococcaceae	ns	ns	0.000	0.058537 *
Streptococcaceae	0.012	0.009756 *	0.000	0.063415 *
Bifidobacteriaceae	ns	ns	0.000	0.068293 *
Victivallaceae	ns	ns	0.000	0.073171*
Prevotellaceae	ns	ns	0.000	0.078049 *
Anaeroplasmataceae	ns	ns	0.000	0.082927 *
Bacteroidaceae	ns	ns	0.000	0.087805 *
Micrococcaceae	ns	ns	0.000	0.092683 *
Porphyromonadaceae	0.008	0.004878	0.000	0.097561 *
Eubacteriaceae	ns	ns	0.000	0.102439 *
Coriobacteriaceae	ns	ns	0.000	0.107317 *
Family_XIII	ns	ns	0.000	0.112195 *
Unknown_Family	ns	ns	0.000	0.117073 *
Deferribacteraceae	ns	ns	0.000	0.121951 *
Erysipelotrichaceae	ns	ns	0.000	0.126829 *
uncultured_organism	ns	ns	0.000	0.131707 *
Alcaligenaceae	ns	ns	0.000	0.136585 *
Verrucomicrobiaceae	ns	ns	0.000	0.141463 *
Rikenellaceae	ns	ns	0.000	0.146341 *
uncultured_bacterium	ns	ns	0.000	0.15122 *
Unclassified	ns	ns	0.000	0.156098 *
Clostridiales_vadinBB60_group	ns	ns	0.001	0.160976 *
Pasteurellaceae	ns	ns	0.001	0.165854 *
Thermoanaerobacteraceae	ns	ns	0.008	0.170732 *
Lachnospiraceae	ns	ns	0.012	0.17561 *
Staphylococcaceae	0.033	0.014634 *	ns	ns

*p<0.05, Mann-Whitney U test, Benjamini-Hochberg critical value [(*i*/*m*)*Q*] with *i*=rank; *m*=total number of tests; *Q* (false-discovery rate)=0.2. N=13-14/group. Red indicates an increase, blue a decrease compared to the vehicle group. ns=not significant

Table S.4.3 Statistics values for relative abundance of bacterial GENERA in each treatment group (probiotic or antibiotic) as compared to the vehicle. p<0.05, Mann-Whitney U test, Benjamini-Hochberg critical value [(*i*/*m*)*Q*] with *i*=rank; *m*=total number of tests; *Q* (false-discovery rate)=0.2. N=13-14/group. Red indicates an increase, blue a decrease compared to the vehicle group. Values in red bold are significantly different from vehicle.

Probiotic			Antibiotic		
genus	*p*	(i/m)·Q	genus	*p*	(i/m)·Q
Streptococcus	0,012	**0,001709**	Lachnospiraceae_NC2004_group	0	**0,001709**
Odoribacter	0,033	**0,003419**	Coriobacteriaceae_UCG_002	0	**0,003419**
Anaerofilum	0,033	**0,005128**	Eubacterium_ventriosum_group	0	**0,005128**
Incertae_Sedis	0,043	**0,006838**	Lachnospiraceae_UCG_005	0	**0,006838**
Marvinbryantia	0,043	**0,008547**	Ruminiclostridium_9	0	**0,008547**
Aerococcus	0,043	**0,010256**	Eubacterium_coprostanoligenes_group	0	**0,010256**
Erysipelatoclostridium	0,043	**0,011966**	Incertae_Sedis	0	**0,011966**
Eisenbergiella	0,048	**0,013675**	Roseburia	0	**0,013675**
Christensenellaceae_R_7_group	0,054	0,015385	Ruminococcus_1	0	**0,015385**
Staphylococcus	0,061	0,017094	Lactobacillus	0	**0,017094**
Lachnospiraceae_UCG_008	0,061	0,018803	Victivallis	0	**0,018803**
Acetitomaculum	0,061	0,020513	Bifidobacterium	0	**0,020513**
Ruminococcaceae_UCG_005	0,085	0,022222	Eubacterium_brachy_group	0	**0,022222**
Eubacterium_coprostanoligenes_group	0,094	0,023932	Marvinbryantia	0	**0,023932**
Ruminiclostridium	0,094	0,025641	Eubacterium_oxidoreducens_group	0	**0,025641**
Peptoclostridium	0,094	0,02735	Ruminococcaceae_UCG_009	0	**0,02735**
Gordonibacter	0,094	0,02906	Enterorhabdus	0	**0,02906**
Coriobacteriaceae_UCG_002	0,105	0,030769	Clostridium_sensu_stricto_1	0	**0,030769**
Ruminiclostridium_6	0,128	0,032479	Unclassified_uncultured_organism	0	**0,032479**
Alloprevotella	0,141	0,034188	Lachnospiraceae_UCG_010	0	**0,034188**
Tyzzerella	0,141	0,035897	Ruminococcus_2	0	**0,035897**
Enterorhabdus	0,155	0,037607	Eubacterium_ruminantium_group	0	**0,037607**
Alistipes	0,155	0,039316	Anaerovorax	0	**0,039316**
Lachnospiraceae_FCS020_group	0,169	0,041026	Ruminococcaceae_UCG_003	0	**0,041026**
Coprococcus_3	0,169	0,042735	Anaerofustis	0	**0,042735**
Jeotgalicoccus	0,169	0,044444	Lachnospiraceae_NK4A136_group	0	**0,044444**
Asaccharobacter	0,169	0,046154	Ruminococcaceae_UCG_010	0	**0,046154**
Roseburia	0,185	0,047863	Parasutterella	0	**0,047863**
Ruminococcus_1	0,22	0,049573	Ruminococcaceae_NK4A214_group	0	**0,049573**
Intestinibacter	0,22	0,051282	Streptococcus	0	**0,051282**
Anaerovorax	0,239	0,052991	Mogibacterium	0	**0,052991**
Lachnoclostridium	0,239	0,054701	Akkermansia	0	**0,054701**
unidentified	0,259	0,05641	Butyrivibrio	0	**0,05641**
Ruminococcaceae_UCG_003	0,28	0,05812	Rothia	0	**0,05812**
Lachnospiraceae_UCG_010	0,302	0,059829	Ruminiclostridium_5	0	**0,059829**
Blautia	0,302	0,061538	Lachnospiraceae_NK4B4_group	0	**0,061538**
Butyricimonas	0,302	0,063248	Eubacterium_nodatum_group	0	**0,063248**
Lachnospiraceae_NK4A136_group	0,325	0,064957	Barnesiella	0	**0,064957**
Ruminococcaceae_NK4A214_group	0,325	0,066667	Anaerotruncus	0	**0,066667**
Mogibacterium	0,325	0,068376	Christensenellaceae_R_7_group	0	**0,068376**
Butyrivibrio	0,325	0,070085	Peptococcus	0	**0,070085**
Enterococcus	0,325	0,071795	Parabacteroides	0	**0,071795**
Ruminococcaceae_UCG_014	0,325	0,073504	Rikenella	0	**0,073504**
Ruminiclostridium_9	0,35	0,075214	Bilophila	0	**0,075214**
Mucispirillum	0,35	0,076923	unidentified	0	**0,076923**
Caldicoprobacter	0,375	0,078632	Lachnospiraceae_UCG_004	0	**0,078632**
Unclassified_uncultured_bacterium	0,402	0,080342	Lachnospiraceae_AC2044_group	0	**0,080342**
Coprococcus_1	0,402	0,082051	Hydrogenoanaerobacterium	0	**0,082051**
Lactobacillus	0,43	0,083761	Paraprevotella	0	**0,083761**
Pasteurella	0,43	0,08547	Lachnospiraceae_FCS020_group	0	**0,08547**
Bilophila	0,43	0,087179	uncultured_rumen_bacterium	0	**0,087179**

Probiotic			Antibiotic		
genus	*p*	(i/m)·Q	genus	*p*	(i/m)·Q
Bifidobacterium	0,458	0,088889	Lachnospiraceae_UCG_008	0	0,088889
Unclassified_uncultured_organism	0,458	0,090598	Alloprevotella	0	0,090598
Allobaculum	0,458	0,092308	Desulfovibrio	0	0,092308
Papillibacter	0,458	0,094017	Natranaerovirga	0	0,094017
Gelria	0,488	0,095726	Ruminococcaceae_UCG_005	0	0,095726
Eubacterium_ruminantium_group	0,488	0,097436	Blautia	0	0,097436
Ruminiclostridium_1	0,488	0,099145	Lachnospiraceae_UCG_001	0	0,099145
Intestinimonas	0,488	0,100855	Eisenbergiella	0	0,100855
Parasutterella	0,519	0,102564	Family_XIII_UCG_001	0	0,102564
Family_eighth_UCG_001	0,519	0,104274	Rikenellaceae_RC9_gut_group	0	0,104274
Candidatus_Arthromitus	0,519	0,105983	Ruminiclostridium_1	0	0,105983
Eubacterium_nodatum_group	0,55	0,107692	Ruminiclostridium	0	0,107692
Lachnospiraceae_UCG_001	0,55	0,109402	Acetitomaculum	0	0,109402
Acetanaerobacterium	0,55	0,111111	Coprococcus_3	0	0,111111
Acetatifactor	0,55	0,112821	Ruminiclostridium_6	0	0,112821
Lactonifactor	0,55	0,11453	Unclassified_uncultured_bacterium	0	0,11453
Lachnospiraceae_UCG_005	0,583	0,116239	Candidatus_Soleaferrea	0	0,116239
Ruminococcus_2	0,583	0,117949	Acetatifactor	0	0,117949
uncultured_rumen_bacterium	0,583	0,119658	Intestinimonas	0	0,119658
Lachnospiraceae_NC2004_group	0,616	0,121368	Anaeroplasma	0	0,121368
Unclassified_unidentified	0,616	0,123077	Bacteroides	0	0,123077
Paraprevotella	0,616	0,124786	Allobaculum	0	0,124786
Bacteroides	0,616	0,126496	Odoribacter	0	0,126496
Turicibacter	0,616	0,128205	Flavonifractor	0	0,128205
Enterobacter	0,65	0,129915	Lachnoclostridium	0	0,129915
Prevotellaceae_UCG_001	0,65	0,131624	Peptoclostridium	0	0,131624
Unclassified	0,65	0,133333	Erysipelatoclostridium	0	0,133333
Akkermansia	0,685	0,135043	Tyzzerella	0	0,135043
Rothia	0,72	0,136752	Anaerofilum	0	0,136752
Desulfovibrio	0,72	0,138462	Ruminococcaceae_UCG_013	0	0,138462
Thalassospira	0,72	0,140171	Family_XIII_AD3011_group	0	0,140171
Ruminococcaceae_UCG_010	0,756	0,14188	Unclassified_uncultured_rumen_bacteriur	0	0,14188
Candidatus_Saccharimonas	0,756	0,14359	Butyricimonas	0	0,14359
Clostridium_sensu_stricto_1	0,793	0,145299	Caldicoprobacter	0	0,145299
Unclassified_uncultured_Mollicutes_bacte	0,793	0,147009	Tyzzerella_3	0	0,147009
Anaerotruncus	0,83	0,148718	Asteroleplasma	0	0,148718
Rikenella	0,83	0,150427	Asaccharobacter	0	0,150427
Hydrogenoanaerobacterium	0,83	0,152137	Alistipes	0	0,152137
Natranaerovirga	0,83	0,153846	Ruminococcaceae_UCG_014	0	0,153846
Oscillibacter	0,83	0,155556	uncultured	0	0,155556
Eubacterium_oxidoreducens_group	0,867	0,157265	Senegalimassilia	0	0,157265
Ruminococcaceae_UCG_009	0,867	0,158974	Shuttleworthia	0	0,158974
Lachnospiraceae_NK4B4_group	0,867	0,160684	Papillibacter	0	0,160684
Barnesiella	0,867	0,162393	Turicibacter	0	0,162393
Flavonifractor	0,867	0,164103	Gordonibacter	0	0,164103
Family_eighth_AD3011_group	0,867	0,165812	Candidatus_Saccharimonas	0	0,165812
Unclassified_uncultured_rumen_bacteriur	0,867	0,167521	Prevotellaceae_UCG_001	0	0,167521
Eubacterium_ventriosum_group	0,905	0,169231	Oscillibacter	0	0,169231
Candidatus_Soleaferrea	0,905	0,17094	Thalassospira	0	0,17094
Asteroleplasma	0,905	0,17265	Coprococcus_1	0	0,17265
Victivallis	0,943	0,174359	Mucispirillum	0	0,174359
Eubacterium_brachy_group	0,943	0,176068	Unclassified	0	0,176068
Ruminiclostridium_5	0,943	0,177778	Pasteurella	0,001	0,177778
Parabacteroides	0,943	0,179487	Sporobacter	0,001	0,179487
Ruminococcaceae_UCG_013	0,943	0,181197	Acetanaerobacterium	0,003	0,181197
Anaerofustis	0,981	0,182906	Candidatus_Arthromitus	0,003	0,182906
Lachnospiraceae_UCG_004	0,981	0,184615	Lactonifactor	0,003	0,184615
Lachnospiraceae_AC2044_group	0,981	0,186325	Gelria	0,008	0,186325
uncultured	0,981	0,188034	Unclassified_uncultured_Mollicutes_bacte	0,056	0,188034

Table S.4.4 List of SybrGreen probes used in the study. Provider company: Eurofins Genomics.

Gene	**Common gene name**	**Sequence (5' → 3')** **left**	**Sequence (5' → 3')** **right**
Actin β	Actin beta	CCCGCGAGTACAACCTTCT	CGTCATCCATGGCGAACT
Occludin	Occludin	GCTATGAAACCGACTACACGACA	ACTCTCCAGCAACCAGCATCT
ZO-1	Zonula occludens-1	AGGCTATTTCCAGCGTTTTGA	AATCCTGGTGGTGGTACTTGC
MDR-1a	Multidrug resistance protein 1a	GAAAGGAATTTACTTCAAACTTGTCA	CACAAGCTTCATTTCCTAATTCAA
CYP1A2	Cytochrome P450 Family 1 Subfamily A Member 2	AATGACATCTTTGGAGCTGGAT	GGGCTCTGTCACAAGTAGCA
CYP3A1	Cytochrome P450 Family 3 Subfamily A Member 1	CATGTCTGAGGATGAAGAATGG	TGTCTCATGAGGGGGAACAT
CYP2D1	Cytochrome P450 Family 2 Subfamily D Member 1	GAGTGTTGGCCAGTGGTCTT	CAGCAGCTCCATGTCTGC

SUPPLEMENTAL FIGURES

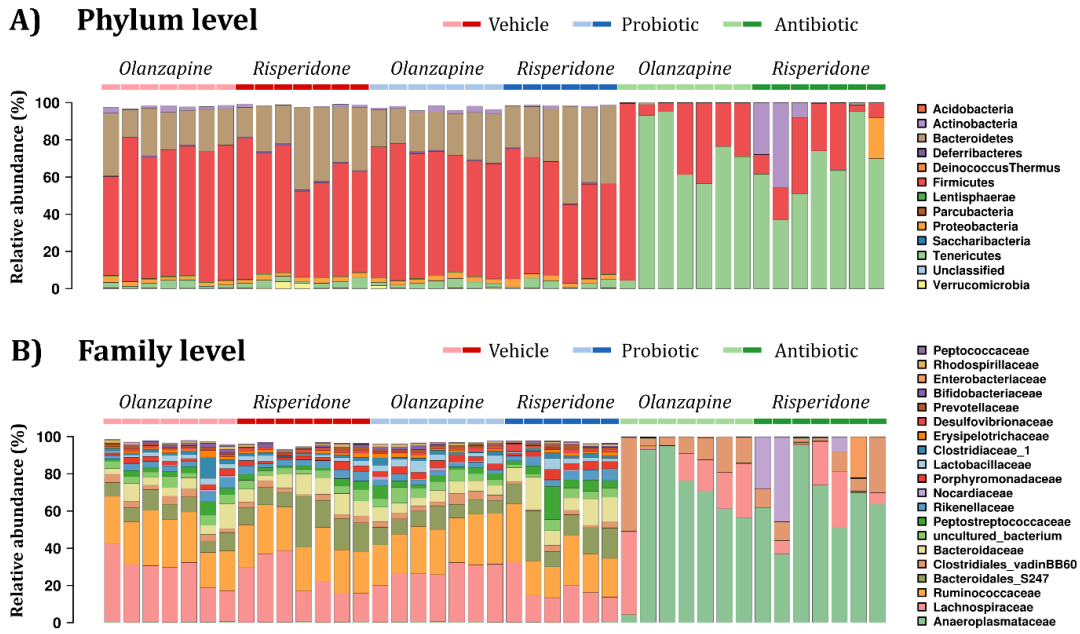


Figure S.4.1 Bar charts representing the taxa abundance at the phylum (A) and family (B) levels. The 20 most abundant taxa are shown.

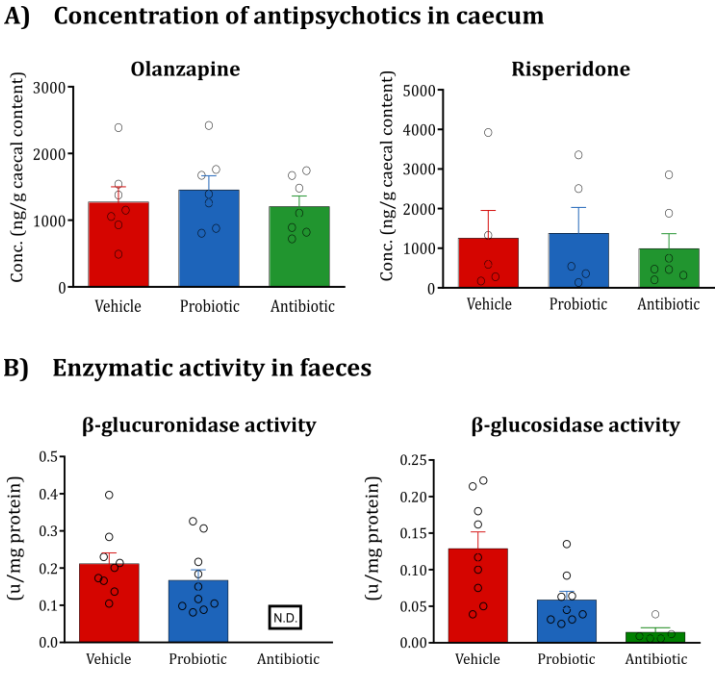


Figure S.4.2 (A) Antipsychotic concentration in the caecum after oral administration in rats pre-treated with vehicle, probiotic or antibiotic. Neither antibiotic or probiotic treatment significantly altered the concentration of OLZ (n=7) or RISP (n=5-7) in the caecum. Data are expressed as mean + SEM. **(B) Impact of microbiota-targeted interventions on the metabolising enzyme activity of rat faecal samples (faecal extract, fecalase)**. β-glucuronidase and β-glucosidase activity in rat faecal samples from vehicle, probiotic and antibiotic-treated rats. Data are expressed as mean + SEM. *p<0.05 ***p<0.001 (n=9-10).

SUPPLEMENTAL METHODS

Caecal microbiota composition

Caecum was harvested, snap frozen and stored at -80ºC prior to the analysis.

- ***Caecal content DNA extraction***

DNA extraction was performed using the QIAmp Fast DNA Stool Mini Kit (Qiagen, Sussex, UK) coupled with an initial bead-beating step. Briefly, 200 mg of each caecal sample were vortex-mixed in a 2 ml screw-cap tubes (Sarstedt, Wexford, Ireland) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.0 mm sterile zirconia beads plus a single 3.5 mm diameter bead (BioSpec Products, Bartlesville, USA) with 1 ml of Qiagen InhibitEX® buffer. Following steps were according to manufacturer's instructions. DNA was quantified using the QubitTM 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) and the Qubit® dsDNA HS Assay Kit (Life Technologies, Oregon, USA). Extracted DNA was kept frozen at -20ºC until further analysis.

- ***16S rRNA Gene Sequence-based microbiota analysis***

The V3-V4 hypervariable region of the 16S rRNA gene were amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol (http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Briefly, first PCR was done using forward primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Each 25 µl PCR reaction contained 5 ng/µl microbial genomic DNA, 1 µM of each primer and 12.5 µl 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK). The PCR conditions follow as: initial denaturation at 95 ºC x 3 min; 25 cycles of 95 ºC x 30 s, 55 ºC x 30 s, 72 ºC x 30 s; and 72 ºC x 5 min for final extension. PCR products were purified with Agencourt AMPure XP system (Beckman Coulter Genomics, Takeley, UK). In the next step, dual indices and Illumina sequencing adapters were attached to PCR products using the Nextera XT Index Kit (Illumina, San Diego, CA). Each 50 µl PCR reaction contained 5 µl purified DNA, 5 µl index primer 1 (N7xx), 5 µl index primer 2 (S5xx), 25 µl 2x Kapa HiFi Hot Start Ready mix and 10 µl PCR grade water. PCR amplification was completed using the previous program but with only 8 amplification cycles instead of 25. Following this, a second clean-up step with the Agencourt AMPure XP system was done. PCR products were quantified, normalized and pooled in an equimolar fashion using the Qubit® dsDNA HS Assay Kit (Life Technologies, Oregon, USA). Next steps in the library preparation were carried out by Teagasc Next Generation DNA Sequencing Facility (Teagasc, Moorepark, Food Research Centre) prior to 2×250 (bp) paired-end sequencing on the Illumina MiSeq platform, using standard Illumina sequencing protocols.

- ***Bioinformatic sequence analysis***

Bioinformatic sequence analysis was performed as previously described (Murphy et al., 2017). Briefly, paired-end sequences were assembled using FLASH (Magoč and Salzberg, 2011) and analysed using QIIME v1.8.0 (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010). Sequences were quality checked and the remaining sequences were clustered into operational taxonomic units using USEARCH (v7-64bit) (Edgar, 2010). Taxonomic ranks were assigned with a BLAST search against the SILVA SSURef database release 123 (Quast et al., 2013). Alpha and beta diversities and Bray-Curt dissimilarities were generated in Calypso (version 8.84) Principal coordinate analysis (PCoA) plots were visualized with ggplot2 (V 2.2.1) using OTU values normalized with the wisconson function in the vegan package (v. 2.5-1). Adonis function (PERMANOVA, permutations=999) in the vegan package (v 2.5-1) was performed on Bray-Curtis matrix on three dimensions. Relative abundance of bacterial taxa was expressed as % of identified sequences.

High performance liquid chromatography (HPLC) detection of the drugs in plasma and caecal contents

1. Plasma Sample Preparation and HPLC Conditions

Olanzapine: The liquid-liquid extraction (LLE) protocol and HPLC conditions employed for the detection of olanzapine in plasma samples was based on (Dusci et al., 2002), with some modifications. 20 µl of the internal standard (I.S.), clozapine, was added to 50 µl of plasma sample (to yield final clozapine concentration of 400 ng/ml) (Discovery Fine Chemicals, UK). After sample alkalinisation with 100 µl of Na_2CO_3 (2M), 750 µl of hexane: dichloromethane: (85:15, v/v) was added as an extraction solvent. Tubes were mixed on an eppendorf shaker at maximum speed (1400 rpm) for 5minutes at 4 °C, followed by sonication for 4 minutes (Bransonic Ultrasonic Cleaner 5510EDTH, Sigma). The clear supernatant was isolated in a new eppendorf. A further 750 µl of the extraction solvent was added to the cloudy pellet. Samples were mixed vigorously for 30 minutes and centrifuged at maximum speed for 10 minutes. The supernatant was again isolated. The tubes were dried under a stream of nitrogen (approx.15-20 minutes) to evaporate the extraction solvent and reconstituted with 100 µl of mobile phase. Compounds were eluted isocratically over a 16 min runtime at a flow rate of 1 ml/min. The mobile phase consists of 14% acetonitrile in water (containing 0.25% H_3PO_4 and 0.05% triethylamine). The limit of quantitation for risperidone was 15.6 ng/ml.

Risperidone: The liquid-liquid extraction (LLE) protocol and HPLC conditions employed for the detection of risperidone in plasma samples was based on (Avenoso et al., 2000), with some modifications. Briefly, 100 µl of NaOH (2M) was added to 50 µl plasma samples spiked with 20 µl of I.S. (to yield final clozapine concentration of 2000 ng/ml). Tubes were vortex-mixed for 30s and 1ml diisopropyl ether-isoamyl alcohol (99:1, v/v) was added as extraction solvent. Following 10 minutes of vigorous mixing, samples are centrifuged at 4000rpm for 10 minutes. The organic phase was back extracted with 100 µl of potassium phosphate (0.1M, pH 2.2) and 30 µl of the acid solution was injected onto the HPLC column. The mobile phase consisted of

acetonitrile: potassium dihydrogenphosphate [0.05M pH 3.7, pH adjusted with 25% phosphoric acid] (30:70) and was filtered through Millipore 0.22 µm Durapore filters (Millipore, Ireland). Compounds were eluted isocratically over a 12 min runtime at a flow rate of 1 ml/min. The column was maintained at room temperature and samples/standards were kept at 8 °C in the cooled autoinjector prior to analysis. The limit of quantitation for risperidone was 62.5 ng/ml.

2. Sample Preparation and Drug Extraction from Caecal Contents

To quantify the amount of risperidone and olanzapine present in the caecum of the rats, 200mg of caecal contents from each animal was isolated and suspended in 1 ml of HPLC grade water and homogenised using a bead-beater for 3·1minute intervals. The same LLE procedure was used to process the caecal samples as that detailed for the plasma samples; the only difference involving the volume of starting material, 100 µl of the homogenised caecal content was used instead of 50 µl plasma.

3. HPLC Equipment

The HPLC with ultraviolet detection (HPLC-UV) system consisted of Agilient 1260 Infinity Binary LC (Agilient Technologies). System components were used in conjunction with EZChrom Elite software (Mason Technology). The detector used was the 1260 Infinity II Diode Array. All samples for both drugs were injected onto a reversed phase Synergi RP 4 µm MAX-RP HPLC Column 4.60 X 250 mm column (Phenomenex), which was protected by a SecurityGuard HPLC (Phenomenex).

Chapter 5

Effect of Psychotropic Drugs on the Human Gut Microbiota: Analysis of the LifeLines-DEEP Cohort

Sofia Cussotto[1,2], Sanzhima Garmaeva[3], Thomaz Bastiaanssen[1,2], Timothy G. Dinan[1,4], Alexandra Zhernakova[3], John F. Cryan[1,2]

[1] APC Microbiome Ireland, University College Cork, Cork, Ireland

[2] Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[3] Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

[4] Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

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Abstract

Increasing evidence suggests that medications used for the treatment of psychiatric disorders can influence the gut microbiome composition. The aim of this study was to investigate relations between intake of psychotropic drugs and microbiome composition in a human cohort. Whole genome shotgun sequencing of the gut microbiomes of 1,126 participants from a Dutch population-based cohort shows minor but significant relations between the microbiome and intake of psychotropic medications, particularly antidepressants. Interestingly, alterations in the genus *Roseburia*, which has previously been shown to be altered in rat studies was found to be affected by psychotropic intake in this study. However, many previously identified psychotropic-induced changes in microbiota composition did not persist in a human cohort. Possible reasons for not detecting other overlapping common bacterial taxa might be the different methods used for microbiome sequencing (whole genome shotgun versus 16S) and the presence of species-specific bacteria associated to human or rat communities.

Keywords: shotgun sequencing, SSRIs, TCAs, diversity, Roseburia

Introduction

Increasing evidence suggests that different host-targeting drugs can affect the gut microbiota *in vitro* and *in vivo* (Falony et al., 2016; Maier et al., 2018). Moreover, we have recently shown that psychotropic medications such as antidepressants and antipsychotics can induce microbiome modifications in a preclinical rodent model (Cussotto et al., 2019b). Other medications that target human cells have been associated with changes in microbiome composition, including antidiabetics (Forslund et al., 2015), proton pump inhibitors (PPIs) (Imhann et al., 2016; Jackson et al., 2016), nonsteroidal anti-inflammatory drugs (NSAIDs) (Rogers and Aronoff, 2016) and atypical antipsychotics (Flowers et al., 2017). The role played by the gut microbiota in both beneficial and undesirable effects of medications is rarely considered. The aim of our study was to assess, in a large cohort of 1,126 individuals, associations between intake of psychotropic medications (antiepileptics, benzodiazepines and antidepressants) and gut microbiota composition. This study represents a follow-up analysis of previously published work which demonstrated that 126 exogenous and intrinsic host factors, including drug intake, collectively explained 18.7% of the variation seen in the inter-individual microbial composition (Zhernakova et al., 2016). Based on these previous findings (Zhernakova et al., 2016), we narrowed down the investigation to focus on psychotropic drugs which were classified according to class, chemical structure (i.e. SSRIs versus TCAs) and individual compound. In addition, we excluded individuals who consumed more than one psychotropic drug. We then discuss the results of our analysis comparing them with previously published work in rodents.

Methods

Population cohort

This study includes stool samples from 1,126 LifeLines-DEEP participants from the general population of the northern part of the Netherlands (58% females, mean age 45 years, range 18-81 years). The demographic information of this population is described in detail in the previously published metagenomics study from Zhernakova and colleagues (Zhernakova et al., 2016). The phenotyping and processing of LifeLines-DEEP has been described previously (Zhernakova et al., 2016). The 1,126 LifeLines-DEEP participants collected stool samples at home. These were immediately stored in the freezer, then collected on dry ice within a few days and transferred to a -80°C facility. All samples were collected over a short period of 3 to 4 months. Sample collection, DNA preparation, processing, and sequence and data analysis were all performed in a standardized manner using laboratory space and equipment described previously (Zhernakova et al., 2016).

We initially divided 1,135 participants in 5 groups based on the class of drugs consumed:

- Antiepileptics (N=5)
- Benzodiazepines, BDZs (N=28)
- Other antidepressants (N=10)
- Selective serotonin reuptake inhibitors, SSRIs (N=29)
- Tricyclic antidepressants, TCAs (N=11)
- Non-users (N=1,052)

Participants who were administered a combination of psychotropic drugs (N=9) were excluded from the analysis resulting in 1,126 individuals, distributed as follows:

- Antiepileptics (N=5)
- Benzodiazepines, BDZs (N=22)
- Other antidepressants (N=7)
- Selective serotonin reuptake inhibitors, SSRIs (N=22)

- Tricyclic antidepressants, TCAs (N=9)
- Non-users (N=1,061)

Inclusion criteria consisted in the intake of at least one, but not multiple, psychotropic drug recorded for the cohort. There were no exclusion criteria for the participants, with the exception of poly-intake of psychotropic drugs.

Table 5.1 Record of psychotropic medications consumption in the LifeLines-DEEP cohort.

Group name	ATC CODE	Drug name	No. of people on drug	Total
Antiepileptics	N03AX09	Lamotrigine	1	**5**
	N03AF01	Carbamazepine	2	
	N03AG01	Valproic acid	2	
	N03AX11	Topiramate	0	
Benzodiazepines	N03AE01	Clonazepam	1	**28**
	N05BA05	Clorazepate	1	
	N05BA06	Lorazepam	1	
	N05CD06	Lormetazepam	1	
	N05CD09	Brotizolam	1	
	N05BA04	Oxazepam	7	
	N05CD07	Temazepam	7	
	N05BA01	Diazepam	9	
Other antidepressants	N06AX11	Mirtazapine	5	**10**
	N06AX16	Venlafaxine	5	
	N06AX05	Trazodone	0	
	N06AX21	Duloxetine	0	
Selective serotonin reuptake inhibitors	N06AB06	Sertraline	2	**29**
	N06AB03	Fluoxetine	3	
	N06AB04	Citalopram	3	
	N06AB10	Escitalopram	3	
	N06AB08	Fluvoxamine	4	
	N06AB05	Paroxetine	14	
Tricyclic antidepressants	N06AA04	Clomipramine	2	**11**
	N06AA09	Amitriptyline	9	

DNA extraction and next generation sequencing

Following storage of the faecal samples at -80°C, DNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen; cat. #80204). The gut microbiome was analysed using paired-end metagenomic shotgun sequencing (MGS) on a HiSeq2000, generating an average of 3.0 Gb of data (about 32.3 million reads) per sample. Samples with read

counts lower than 15 million were removed. Human contamination was further removed by mapping the reads against the human reference genome (build 37) using bowtie2 (version 2.1.0) (Langmead and Salzberg, 2012).

Bioinformatics

Microbial profiling of samples has been performed by mapping read sequences to approximately 1 million clade-specific marker genes using MetaPhlAn 2.2 (Segata et al., 2012), as described in (Zhernakova et al., 2016).

Statistical analysis

Diversity of microbial communities was calculated for every individual using the *diversity* function in the R package '*vegan*' (version 2.5-3) at the genus level (Oksanen et al., 2018). To assess the difference in alpha-diversity between medication groups, one-way ANOVA test has been performed. To assess the heterogeneity of microbial communities between individuals, Bray-Curtis and Aitchison distances were calculated using the *vegdist* function from the same package and the ALDEx2 package (Fernandes et al., 2013), respectively. The approach of Aitchison distance calculation also recruited the absolute counts imputation from the relative abundance obtained with MetaPhLan2.2 and read depth information. To check the homogeneity of the dispersions within medication groups the function *betadisper* with the 'centroid' option was applied to matrices of distance. The significance of differences of dispersions was estimated by *permutest* function using 1000 permutations. To test how much of the inter-individual microbial variation at species level could be explained by antipsychotic medications consumption, we then performed a PERMANOVA (permutational multivariate analysis of variance) corrected for sex, age, read depth, and depression using the *adonis* function from the same package. The *p-value* was determined by 1,000 and 10,000 permutations, and differences were considered significant at $p<0.05$.

To associate the available metadata with microbial relative abundances at species and genus level the function *Maaslin* relying on general linear models from the package 'MaAsLin2' (version 0.2.3) was used (Mallick et al., 2019). Here we were considering only those species and genus that were present in at least 5% of the participants. Factors that have been shown to have influence on the gut microbiome composition earlier (age, sex, read depth) were considered in a multivariate model assessing the relation antipsychotic medications and relative microbial abundances. In each analysis, the default settings of *Maaslin* were applied (Total Sum Scaling (TSS) normalization, log transformation of microbial abundance, Benjamini-Hochberg (BH) correction method for computing the q-value).

Results

We examined the effect of psychotropics medications on alpha-diversity of the microbial community (Shannon index on genus level present in at least 5% of individuals). Psychotropic drugs did not show any significant effect on the alpha-diversity of bacteria compared to non-users (**Figure 5.1**).

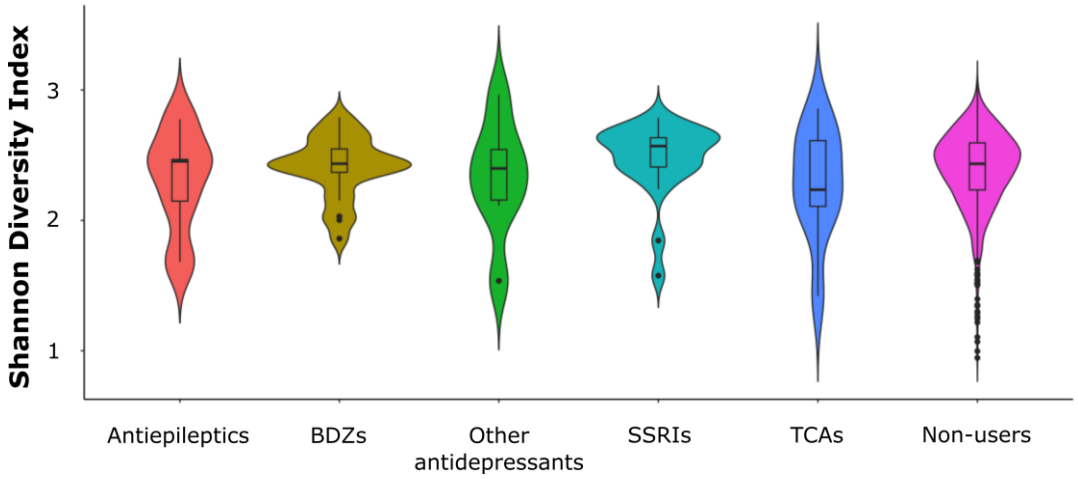


Figure 5.1 Alpha-diversity plots in psychotropic users versus non-users. *Abbreviations*: *BDZs* benzodiazepines, *SSRIs* selective serotonin reuptake inhibitors, *TCAs* tricyclic antidepressants.

Then we assessed whether psychotropic drugs affected the beta-diversity of the microbiome. Here, two different methods have been applied at the species level of bacteria: measurements of Bray-Curtis dissimilarity and Aitchison dissimilarity. Regarding Bray-Curtis dissimilarity, dispersions within medication groups were different ($p<0.05$). After correction of read depth, age and gender, SSRIs and TCAs were shown to influence microbiome composition (Adonis PERMANOVA p=0.0029 and 0.005699 respectively on 10,000 permutations). Thus, usage of SSRIs explained 0.2% of microbial variation as well as TCAs (**Figure 5.2A**). Altogether, psychotropic drugs explained 0.66% of microbial variation (Adonis PERMANOVA p =0.0036; 10,000 permutations). This suggests that a small effect is induced by psychotropic drugs on the microbiota, and some of the effect could be lost due to the small sample

size. Associations remained significant also after correction for depression (SSRIs: R^2=0.00212, p=0.004995; TCAs: R^2=0.00197, p=0.011988; 1,000 permutations). Regarding Aitchison dissimilarity, no significant effects of medications on microbiome were detected (**Figure 5.2B**).

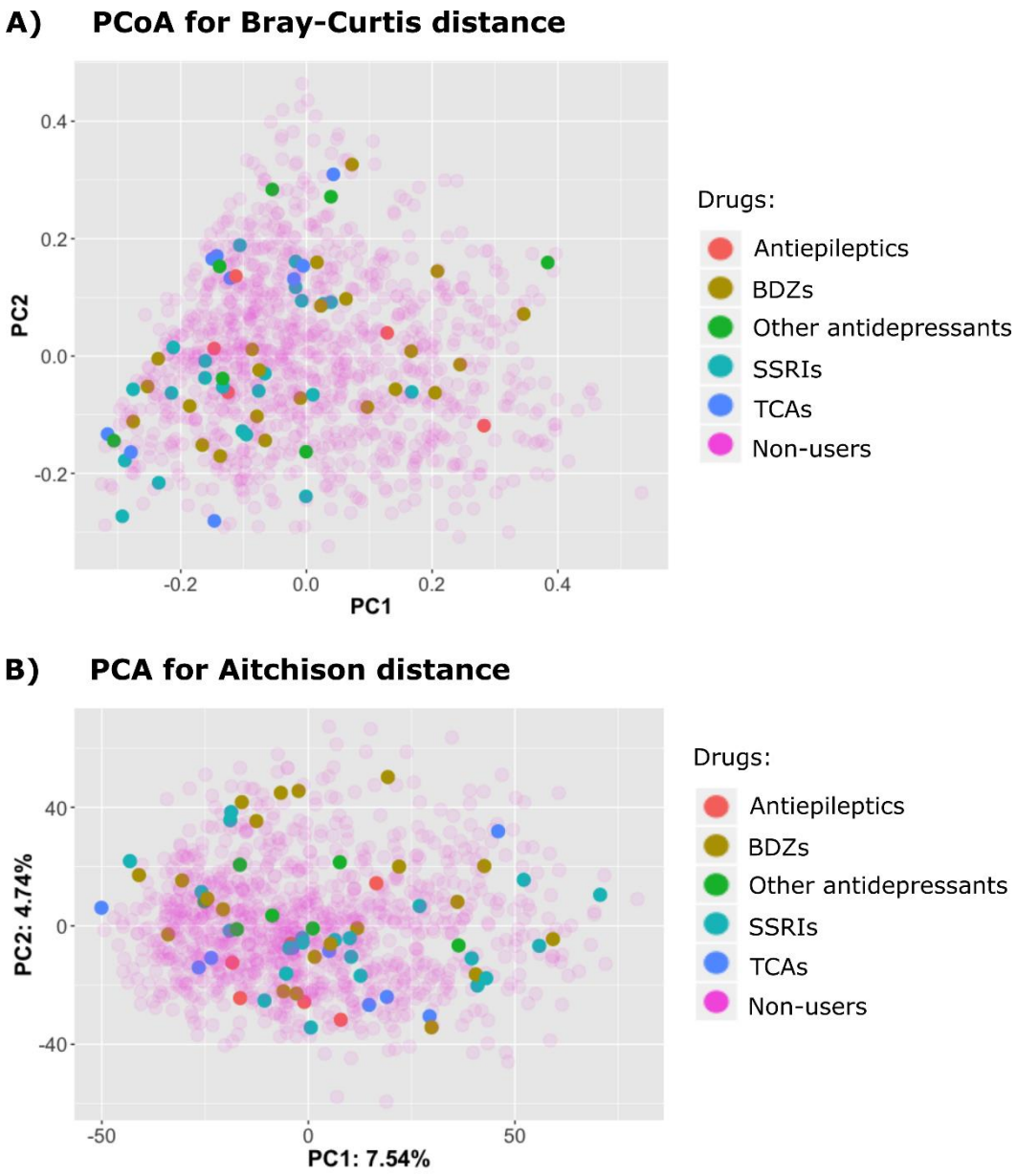


Figure 5.2 Beta-diversity plots in psychotropic users versus non-users. (A) PCoA of Bray-Curtis dissimilarity matrix. **(B)** PCA of Atchison dissimilarity matrix. *Abbreviations*: *BDZs* benzodiazepines, *SSRIs* selective serotonin reuptake inhibitors, *TCAs* tricyclic antidepressants.

Our next aim was to investigate further the influence of single psychotropic pharmaceutical compounds on the microbiome diversity. For this analysis, we selected compounds that were present in at least more than 5 individuals (specifically: oxazepam [N05BA04, N=7], temazepam [N05CD07, N=7], diazepam [N05BA01, N=9], paroxetine [N06AB05, N=14], amitriptyline [N06AA09, N=9]). Due to the small sample size, no significant effects of individual compounds on beta-diversity was detected, after correction for read depth, age, gender and depression.

A major advantage of shotgun sequencing compared to 16S sequencing is the opportunity to recover accurate species-level taxonomic and functional profiles of the microbiome (Ranjan et al., 2016). When analysing associations between species and drug consumed, due to the evident difference in sample size between drug users and non-users, we selected those species whose dispersion and SEM (standard error of the mean) where comparable in the user and non-user groups. Across several species, we found significant associations for SSRIs, TCAs, BDZs, paroxetine and diazepam. The specific associations are listed in **Table 5.2**.

Table 5.2 Associations between psychotropic classes/drugs and bacterial species. Only species whose dispersion and SEM (standard error of the mean) where comparable in users and non-users were selected. *Abbreviations*: *BDZs* benzodiazepines, *SSRIs* selective serotonin reuptake inhibitors, *TCAs* tricyclic antidepressants. The cut-off for significance of *q* value is 0.05.

Psychotropic class/drug	**Bacteria (species)**	**Coefficient**	***p* value**	***q* value**
SSRIs	*Roseburia intestinalis*	0.002421	$1.114 \cdot 10^{-9}$	$1.608 \cdot 10^{-7}$
TCAs	*Methanobrevibacter smithii*	0.011485	$1.286 \cdot 10^{-6}$	$9.277 \cdot 10^{-5}$
BDZs	*Anaerostipes hadrus*	0.001369	$7.479 \cdot 10^{-6}$	$4.623 \cdot 10^{-4}$
	Clostridium leptum	0.000371	$2.170 \cdot 10^{-3}$	$5.050 \cdot 10^{-2}$
Paroxetine	*Roseburia intestinalis*	-0.00187	$1.730 \cdot 10^{-6}$	$1.218 \cdot 10^{-4}$
Diazepam	*Actinomyces viscosus*	$-1.38 \cdot 10^{-5}$	$1.028 \cdot 10^{-3}$	$2.824 \cdot 10^{-2}$
	Methanobrevibacter smithii	-0.01058	$8.602 \cdot 10^{-6}$	$5.172 \cdot 10^{-4}$

Significant associations were also detected between type of drug consumed and bacterial genera (represented in the heatmap, **Figure 5.3**). The genera mostly associated to BDZ consumption were *Corynebacterium*, *Alloscardovia* and *Gemella.* The genera strongly associated to SSRIs were *Roseburia* and *Eggerthella*, while the ones strongly associated to TCAs were *Bartonella* and *Methanobrevibacter*.

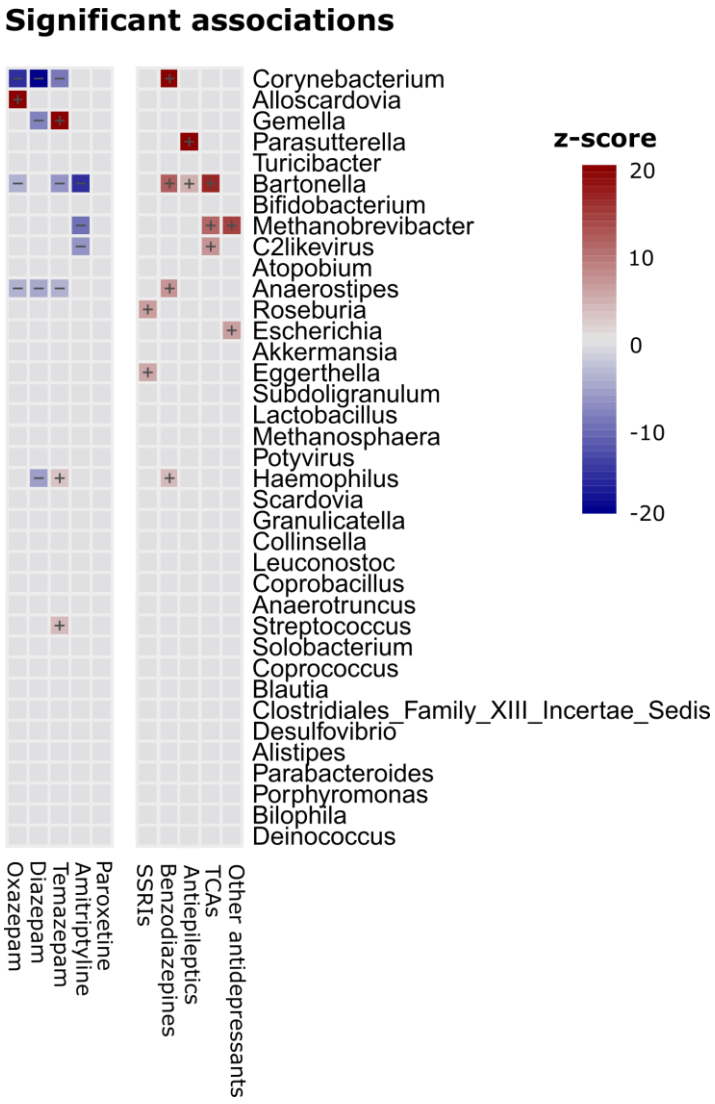


Figure 5.3 Associations between psychotropic classes/drugs and bacterial genera. Only significant associations are shown (*q* value<0.05, BH). Z-scores of associations are centred and normalized, colours represent the direction of an association.

When comparing the human data with our preclinical findings in rats, we found that the genus *Roseburia* was associated to psychotropic consumption in both studies. Specifically, *Roseburia* was positively correlated to SSRI consumption in the LifeLines-DEEP cohort (**Table 5.2**, **Figure 5.3**) and we found it to be increased by the psychotropics lithium (mood stabiliser) and aripiprazole (antipsychotic) in rats (Cussotto et al., 2019b). A possible reason for the detection of only one shared microbial genus might be the different sequencing methods used in the two studies: the analyses in rats were done via 16S sequencing which makes it challenging to compare them to data obtained through shotgun sequencing. Moreover, some species-specific microorganism can occur only in rats or humans. Differences in DNA isolation methods could also explain the discrepancies in identification of bacteria, particularly rare ones.

Discussion

In light of previous studies from our and other laboratories showing that psychotropic medications influence the gut microbiota in *in vitro* and in animal models, we wanted to investigate whether consumption of psychotropics would impact the microbiome in a human cohort. Our analysis shows that there are minor but significant effects of certain psychotropic classes and compounds on microbiome composition.

Despite no differences were detected in alpha-diversity across groups, SSRIs and TCAs influenced significantly the microbiome composition according to the Bray-Curtis dissimilarity measurements (**Figure 5.2A**).

At the species level, the antidepressants SSRIs and TCAs were weakly but positively associated with *Roseburia intestinalis* and *Methanobrevibacter smithii* (**Table 5.2**). Although the functional relevance of such taxa has not been extensively characterised, changes these bacterial species have been reported in different physiological and pathological conditions. *Roseburia intestinalis* is a butyrate-producing bacterium (Duncan et al., 2002) and has been previously shown to protect the colonic mucosa against inflammation (Zhu et al., 2018). *Methanobrevibacter smithii* exerts xylanolytic activity of anaerobic ruminal fungi (Joblin et al., 1990) and was depleted in human obesity (Million et al., 2011). BDZ intake was positively correlated with *Anaerostipes hadrus* and *Clostridium leptum*. *Anaerostipes hadrus* is a butyrate-producing species; *Clostridium leptum* was shown to attenuate inflammation induced by airway allergy (Li et al., 2012) and was significantly altered in patients with ulcerative colitis (Zhang et al., 2007).

More research is warranted to investigate the individual contribution of these bacterial species in drug efficacy or toxicity. Interestingly, bacterial species that were positively correlated to a certain drug class were sometimes negatively correlated to a compound belonging to the same class (or *vice versa*; i.e. *Roseburia intestinalis* is positively correlated with SSRIs but negatively correlated with paroxetine, an SSRI), highlighting the importance of investigating the individual contributions of each drug.

In our study, the investigation at compound-level was limited by the small sample size but future research should address this point.

At the genus level, in our preclinical study we found that fluoxetine completely depleted the growth of *Prevotella* and *Succinivibrio* (Cussotto et al., 2019b) and we wanted to examine whether SSRIs induced similar changes in the LifeLines-DEEP cohort. We were not able to detect *Succinivibrio* in the human cohort, possibly due to the different method used for sequencing, while *Prevotella* was detected but did not show significant associations. Interestingly, the genus *Roseburia* was altered by psychotropics in our preclinical study and showed significant associations in the LifeLines-DEEP cohort. *Roseburia,* that we found increased by lithium (mood stabiliser) and aripiprazole (antipsychotic) in rats, was positively correlated with SSRIs in the LifeLines-DEEP cohort. It is important to note that *Roseburia* was positively associated with SSRIs both at genus and species level (**Figure 5.3**, **Table 5.2**). Intriguingly *Roseburia* was previously shown to be increased in mice receiving fluoxetine, an SSRI (Lyte et al., 2019) and decreased in rats receiving a faecal transplantation from depressed individuals (Kelly et al., 2016). Our findings in the LifeLines-DEEP cohort support therefore an association between *Roseburia* and SSRI intake, although fluoxetine was not tested *per se*.

In a recent study in mice, antidepressants administration reduced richness and increased beta-diversity of gut bacteria compared to controls (Lukić et al., 2019). Moreover, at the genus level, antidepressants reduced the abundance of *Ruminococcus* and *Adlercreutzia.* Strikingly, the antidepressive effect of duloxetine was attenuated by simultaneous administration of *Ruminococcus flavefaciens*. In the present human population, however, we could not find significant changes in *Ruminococcus* or *Adlercreutzia*.

Intriguingly all three benzodiazepines investigated at compound level (oxazepam, diazepam and temazepam) showed a significant negative association with *Corynebacterium* (**Figure 5.3**) while a previous study has shown that psychotropics such as SSRIs and antipsychotics inhibited the growth of *Corynebacterium urealyticum in vitro* (Munoz-Bellido et al., 1996). These data suggest that more

research should investigate the role of the genus *Corynebacterium* and its species in psychotropic intake.

This study does not lack limitations. Firstly, the difference in sample size between psychotropic users and non-users and the lower sample size among users would affect the power of the analysis. Although we corrected the analyses for read depth, age, gender and depression, we acknowledge that other factors including diet, GI symptomatology, smoke, alcohol consumption and comorbidities represent considerable confounder factors. Future studies with bigger sample sizes should take these cofounders into account and try to replicate the findings of the LifeLines-DEEP cohort. Finally, growing research in the field emphasises the effects of antipsychotic drugs on the gut microbiome (Flowers et al., 2017; Maier et al., 2018), with data showing that the metabolic side effects of olanzapine are mediated by microbial states (Davey et al., 2013; Kao et al., 2018; Morgan et al., 2014). Due to the low sample size, antipsychotics were not included in our analysis and a broader analysis including such drugs and others is warranted.

In conclusion, our data show that minor but significant changes in microbiome composition are associated with the intake of psychotropic medications, especially antidepressants, in the Dutch LifeLines-DEEP cohort. The findings are in accordance with the increasing evidence suggesting a microbiota-targeted effect of psychotropic medications.

Chapter 6

General Discussion

6.1 Overview and Summary

In this thesis, I have explored the complex and bidirectional relationship between psychotropic medications (drugs commonly used to treat different psychiatric disorders) and the microbiota-gut-liver-brain axis. The findings here reported have the potential to be translated into clinical practice and represent a cornerstone piece of research for the development of microbiota-based personalised medicine.

Our data show that chronic administration of psychotropic medications influences microbial and intestinal function in healthy adult rats (*Chapter 2*). Specifically, the SSRIs escitalopram and fluoxetine showed differential antimicrobial activity *in vitro*, with fluoxetine having stronger antimicrobial activity. Intriguingly fluoxetine was the only medication that impacted microbial activity both *in vitro* and *in vivo*. Lithium, valproate and aripiprazole administration induced a significant increase in the relative abundance of minor genera, while highly abundant genera were mostly not affected and were decreased in few instances (i.e. *Lachnospiraceae* NK4A136, *Ruminococcus* 1, *Bacteroides*). Microbial diversity and richness were increased by lithium, valproate and aripiprazole. With marked alterations present at the microbiota level, it was perhaps not surprising that changes in SCFAs occurred. Valproate and aripiprazole influenced SCFA abundance in the caecum, with acetate and isovalerate being increased by aripiprazole treatment and propionate, butyrate and isovalerate being differentially altered by valproate. The alterations in SCFA levels seemed independent of changes in specific SCFA-producing taxa. The impact of psychotropic drugs on gut functionality is poorly understood and it is well known that the gut microbiota plays a role in shaping intestinal permeability (Karl et al., 2017; Ott et al., 2017; Ulluwishewa et al., 2011). Thus, we assessed epithelial permeability in the small and large intestine and found that the antidepressants escitalopram, venlafaxine and fluoxetine, together with the atypical antipsychotic aripiprazole, increased epithelial permeability in the ileum. Interestingly, the action of psychotropics on intestinal permeability was region-specific, with the colon being largely unaffected. Together, these data highlight the importance of investigating the impact of drugs used for the treatment of psychiatric disorders on microbiota-gut-brain axis function.

In *Chapter 3*, we drilled deeper into the dataset to show that, across a range of psychotropic medications, the mood stabilisers lithium and valproate increase many BA species in circulation, in the colon content and the liver. Independent of BA class (primary, secondary or tertiary), and with the exception of tauro-conjugated BAs, all BAs were significantly increased by lithium and valproate administration. These changes were accompanied by an increase in some key intestinal bile acid-metabolizing bacteria, mainly belonging to the Firmicutes phylum. Lithium and valproate also substantially altered the gene expression of enzymes and transporters involved in bile acid metabolism. For example, we observed a failure in the FXR-*Fgf19* pathway to appropriately feedback to the liver and stop the *de novo* synthesis of BAs, reflected as an increase in both *CYP7A1* and *CYP27A1* gene expression. With such high levels of circulating BAs, we hypothesised that the liver or distal ileum (site of BA reabsorption) might be damaged and we assessed the expression of genes involved in inflammation and fibrosis. According to gene expression levels, neither the liver nor the intestine showed signs of an inflammatory state. An alternate mechanism which might be responsible for BA dysregulation, a change in intestinal barrier function, did not play any overt role as assessed by Ussing chambers. Moreover, the decrease in bodyweight, epididymal fat and levels of circulating triglycerides observed in lithium- and valproate-treated rats might have been the result of massively increased levels of circulating BAs. With a particular focus on taurine, lithium and valproate decreased the levels of hepatic taurine-uptake transporter *TauT*, which was accompanied by a reduction in tauro-conjugated bile acids. The depletion in circulating taurine has the potential to impact distal sites in which this molecule is crucial, including the central nervous system. These data highlight the importance of examining the effects of psychotropic drugs on the microbiota-gut-liver axis, apart from the effects exerted in the central nervous system.

Having shown that certain psychotropic drugs can influence the microbiome composition and intrigued by the potential clinical relevance of the drug-microbiota interactions, we decided to look at whether perturbations of the gut microbiota could alter the pharmacokinetics of psychotropic drugs, specifically the antipsychotics

olanzapine and risperidone (*Chapter 4*). Administration of antibiotics for two weeks to adult rats was accompanied by increased levels of olanzapine in circulation. This effect was drug-specific, as risperidone did not show the same trend. Antibiotics did not seem to induce barrier function dysregulation, maybe due to the short time-course. Interestingly, the pharmacokinetics of olanzapine correlated with the relative abundance of the bacteria *Alistipes*, a taxon previously associated with response to chemotherapy. These data give new insight into the potential role of the microbiome in drug absorption which, in turn, might have implications for drug efficacy and toxicity.

The work presented in *Chapter 5* is a collaborative project with the University of Groningen, where we examined the effects of psychotropic drugs intake on the human microbiome composition in the LifeLines-DEEP cohort. Here we show that minor but significant changes in the microbiome composition can occur as a result of psychotropic drugs' intake.

Taken together these data support the bidirectional interaction between psychotropic medications and the microbiota-gut-liver-brain axis (**Figure 6.1**). The potential clinical implications of these findings will be discussed in this chapter.

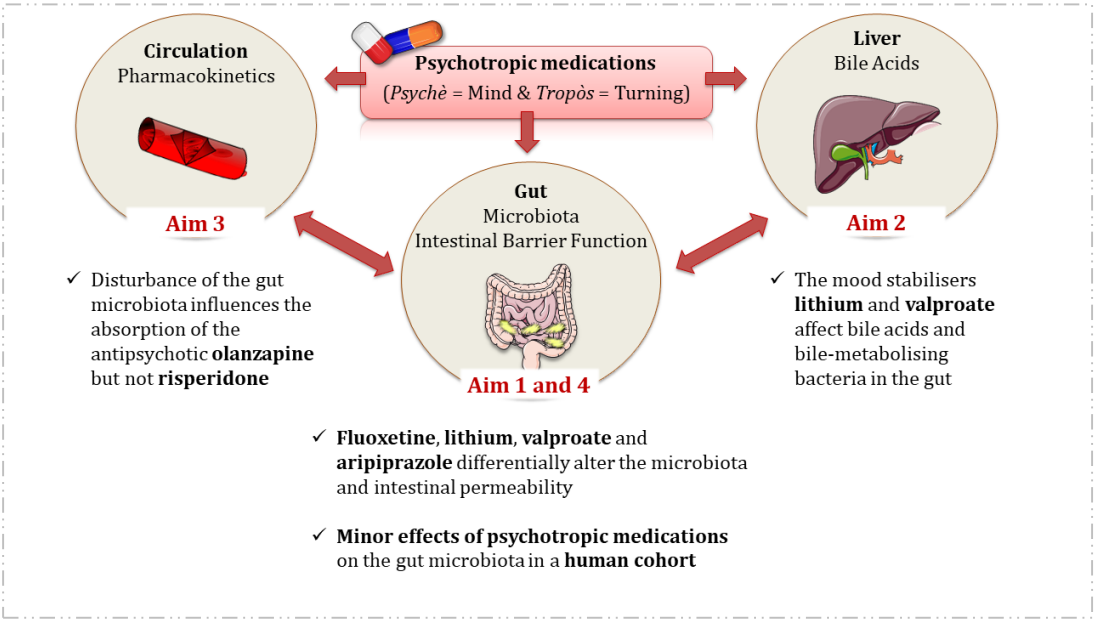


Figure 6.1 Summary of the findings of this thesis.

6.2 Interplay between Psychotropic Drugs and the Gut Microbiota: Unravelling the Implications

In this thesis, we show that the psychotropics fluoxetine (antidepressant), lithium (mood stabiliser), valproate (antiepileptic) and aripiprazole (antipsychotic) significantly alter the microbiome composition and richness. Moreover, the blood absorption of the antipsychotic olanzapine was increased following disturbance of the gut microbiome through administration of an antibiotic cocktail. The emerging drug-microbiome network could guide future clinical practice and drug development. Some of the new avenues for translational applications include improving drug efficacy, mitigating drug side effects, repurposing of human-targeted drugs and more. The implications of our findings will be discussed in this section (**Figure 6.2**).

6.2.1 Mechanism of action and efficacy of psychotropic drugs

The antidiabetic drug metformin represents a clear example of a compound whose beneficial activity on the host depends on intestinal bacteria (Cabreiro et al., 2013; Forslund et al., 2015; Pryor and Cabreiro, 2015). Recent studies using faecal transplantation on germ-free mice and delayed-release formulations confirm that the microbiome is crucial for the mechanism of action of metformin (Buse et al., 2016; DeFronzo et al., 2016; Wu et al., 2017a). The absorption of the levodopa, a drug used in the treatment of Parkinson's disease, is decreased by the presence of the bacterium *Helicobacter pylori* (Hamlet et al., 1999; Hashim et al., 2014; Miyaji et al., 1999). Recent studies have shown that levodopa conversion by bacterial tyrosine decarboxylase in the small intestine was a significant explanatory factor for the increased levodopa/carbidopa dosage regimen required in a subset of Parkinson's patients (Clarke et al., 2019; Maini Rekdal et al., 2019; van Kessel et al., 2019). Our data suggest that microbiome modulation may contribute to the therapeutic effect of certain psychotropics, as we found that drugs with different chemical structures (i.e. lithium, valproate and aripiprazole) impacted the growth of a similar niche of gut microbes. Interestingly, a similar cluster of microbial effects was also reported by

Maier and colleagues regarding antipsychotic drugs (Maier et al., 2018). In that study, nearly all subclasses of the chemically diverse antipsychotics targeted a significantly more similar pattern of species than expected from their chemical similarity, raising the possibility that antimicrobial action may not only manifest as side effect of antipsychotics, but also be part of their mechanism of action.

As discussed earlier, studies have shown that psychiatric populations (i.e. depressed or schizophrenic patients) can have altered microbiota composition and richness, therefore supporting the notion that psychotropics might work on intestinal microbes as part of their mechanism of action. Until now the effects of antipsychotics and other psychotropics on the microbiome have been mainly considered in the context of side effects (e.g. weight gain). Studying whether and how the microbiome might contribute to the mechanism of action of psychotropic drugs will substantially impact clinical practice and shed new light on the efficacy of existing drugs. Antidepressants, for example, bear considerable inter-individual variation in drug response and lack of efficacy, being only 20-30% more effective than placebo (Arroll et al., 2005), therefore continued metabolomic and metagenomic analyses of the microbiome have the potential to increase the individual response to antidepressants.

Further studies are needed to evaluate whether the microbiome-targeting effects of psychotropic drugs play a role in the mechanism of action of such drugs, both in animals and humans. An example of a first attempt to clarify this point comes from Lukić and colleagues who have shown that the antidepressive effect of duloxetine was attenuated by simultaneous administration of *Ruminococcus flavefaciens* in mice (Lukić et al., 2019). Some of the approaches that can and should be employed to prove that microbiome modifications are causative and not epiphenomenological, are described in *Section 6.4*.

6.2.2 Side effects and toxicity of psychotropic drugs

The gut microbiome can also play a critical role in the side effects and toxicity of psychotropic drugs; an important point to remember especially in the context of

medications with narrow therapeutic window. Antibiotic treatment in rats attenuated both the microbial metabolism and the teratogenicity-associated adverse effects of the benzodiazepine nitrazepam (Elmer and Remmel, 1984). Antipsychotic-induced metabolic dysfunction has been linked to shifts in the gut microbiota composition. Olanzapine-induced weight gain is strictly dependent on intestinal microbes, as demonstrated by experiments on germ-free mice (Morgan et al., 2014). As a confirmation of this finding, antibiotic administration in olanzapine-treated rats attenuated the metabolic side effects of olanzapine (Davey et al., 2013). Treatment with the prebiotic B-GOS significantly attenuated the olanzapine-induced weight gain without altering the antagonism of central serotoninergic receptors necessary for drug efficacy (Kao et al., 2018). Strikingly, both olanzapine-induced and risperidone-induced weight gain have been associated with an altered composition of the gut microbiota (Bahr et al., 2015b; Davey et al., 2012). Suppressed energy expenditure, induced by the gut microbiota, was found to cause the observed weight gain in risperidone-treated mice. Furthermore, faecal microbiota transplantation (FMT) from risperidone-treated mice to naive mice induced weight gain and suppressed energy expenditure in the FMT-recipient mice (Bahr et al., 2015b). In addition to the microbiota-targeted effects of psychotropics *in vivo*, in this thesis we have shown that fluoxetine and escitalopram possess differential antimicrobial activity *in vitro*. The microbial effects reported in our work might be responsible for the side effects associated with antidepressants. Possible approaches to mitigating the collateral (anti)microbial effects and related side effects include optimizing drug choice, combining drugs with resistant probiotics or using selectively suppressive drug combinations (Brochado et al., 2018). In *Chapter 3* we have shown that the mood stabilisers lithium and valproate can impair bile acid function and associated bile-metabolising bacteria, thus suggesting that the microbiome might play a role in the hepato-toxicity often related with these medications. In *Chapter 4* we have shown that a microbiome depletion induced a significant increase in plasmatic levels of the antipsychotic olanzapine, but not risperidone. The discrepancy between the two drugs might be due to the different chemical structure (despite belonging to the same therapeutic class). Increased levels of olanzapine might in turn translate into alterations in drug toxicity.

While most of the research to date has been focused on the metabolic side effects of olanzapine and risperidone, further studies are needed to prove the causal role of the microbiome in mediating the side effects of other psychotropic compounds.

6.2.3 Repurposing new antibiotics

When discussing the overlap between psychotropic drugs and antibiotics, iproniazid stands out as a striking example. Isoniazid (a structural analogue of iproniazid) was originally designed to treat tuberculosis and it was accidentally found to cause euphoria in tuberculosis patients. This led to the launch of iproniazid as the first antidepressant on the market (Butler et al., 2019). Moreover, in the context of microbes-mood interaction, exposure to antibiotics has been associated with an increased risk for depression and anxiety (Lurie et al., 2015). On the other hand, minocycline, a tetracycline antibiotic, has been shown to possess antidepressant properties (Pae et al., 2008; Reis et al., 2019; Rosenblat and McIntyre, 2018). Although this has largely been attributed to its ability to affect microglia function in the brain (Dai et al., 2019; Han et al., 2019), we cannot rule out an antimicrobial-mediated effect on mood and depression. Interestingly, microglia function has been reported to be altered in several psychiatric disorders including depression (Mondelli et al., 2017; Perry, 2018; Yirmiya et al., 2015). In addition, microbiota alterations can lead to modifications in microglia function (Erny et al., 2015; Mosher and Wyss-Coray, 2015; Thion et al., 2018), which in turn could impact brain function and behaviour. These examples remind us how sometimes antimicrobial and psychotropic effects can overlap. In this light, an intriguing implication of our results consists in the long-term opportunity for drug repurposing with the aim to develop new antibiotics. Psychotropic drugs with antimicrobial activity could provide a basis for the development of narrow-spectrum, species-specific antibiotics, urgently needed in the era of antibiotic resistance (Brown and Wright, 2016).

6.2.4 Antibiotic resistance: Could psychotropics be contributing?

The use of psychotropic medications might play a role in the acquisition of antibiotic resistance. The antidepressant fluoxetine was recently shown to induce multi-antibiotic resistance through a mechanism involving ROS-mediated mutagenesis (Jin et al., 2018). Specifically, exposure of *Escherichia coli* to fluoxetine at 5-100 mg/L for 30 days promoted its mutation frequency resulting in increased resistance against the antibiotics chloramphenicol, amoxicillin and tetracycline. Fluoxetine led to chromosomal mutations that up-regulated the expression of efflux pumps, further enhancing the antibiotic efflux (Jin et al., 2018). This is a very important aspect to consider, especially given the alarming rise of antibiotic resistance. Efficient control strategies are needed to minimise the emergence of antibiotic resistance due to human-targeting drugs. Strikingly, the antidepressant fluoxetine is among the more prevalent categories of pharmaceuticals detected in the marine environment and therefore in fish (Brodin et al., 2014; Gaw et al., 2014; Kreke and Dietrich, 2008; Schultz et al., 2010; Vasskog et al., 2008), a factor that potentially contributes to the spread of antibiotic resistance. Further work is warranted to investigate the effects of fluoxetine and other psychotropic drugs on the dissemination of antibiotic resistance under environmentally relevant concentrations.

6.2.5 Towards personalised medicine

An interesting direction which will possibly influence future clinical practice is represented by manipulations of the individuals' microbiome aimed at improving therapeutic outcome. Patient populations are not homogenous, and as previously discussed, in addition to the genetics and age of a patient, the host microbiome can influence the therapeutic outcomes. Not only genetic analysis but also the human microbiome could represent a challenge to optimal drug delivery and response (Grice and Segre, 2012). One option for precision medicine would be to alter the route of drug administration to avoid microbiota-drug interactions at the site of action, or tailoring a drug to act specifically within the colon, which could also provide a solution

to unwanted drug-related toxicities in other tissues (Kashyap et al., 2017). Excipients are pharmacologically inert substances that are added to formulations to stabilise the active substance or enhance the function of the dosage form. Recent research supports the possibility that excipients themselves might mediate microbiome changes. Polyethylene glycol (PEG), a polymer used in drug delivery, is linked to changes in the gut microbiota (Kashyap et al., 2013). Specifically, humanised mice fed a standard diet supplemented with 15% PEG 3350 for 10 days had significantly reduced abundance of the families Peptococcaceae and Eubacteriaceae. Further research into excipient-microbiota interactions is an aspect that needs to be considered for future drug formulation and delivery.

A different precision medicine approach consists of altering the patient microbiome to enhance therapeutic efficacy, as an adjunctive therapy of drug response. Means to target the microbiome include faecal transplantation, administration of probiotics, prebiotics, postbiotics or antibiotics. Any of these approaches would require more randomised control trials in order to demonstrate the link between therapeutic efficacy and microbiome manipulations. It is important to remember that there is no such thing as a “normal microbiome” and that many factors can influence the stability of the microbiome including aging, hormones, diet and the external environment. In elderly hospitalised patients, dysbiosis of the microbiome was significantly associated with polypharmacy and mortality (Ticinesi et al., 2017). During the aging process, microbiome changes could impact the efficacy and toxicity of psychotropic medications. Therefore, a personalised approach to drug prescription and delivery, which takes into account the complex drug-microbiota interactions throughout the lifespan, is important if precision medicine is to achieve its potential.

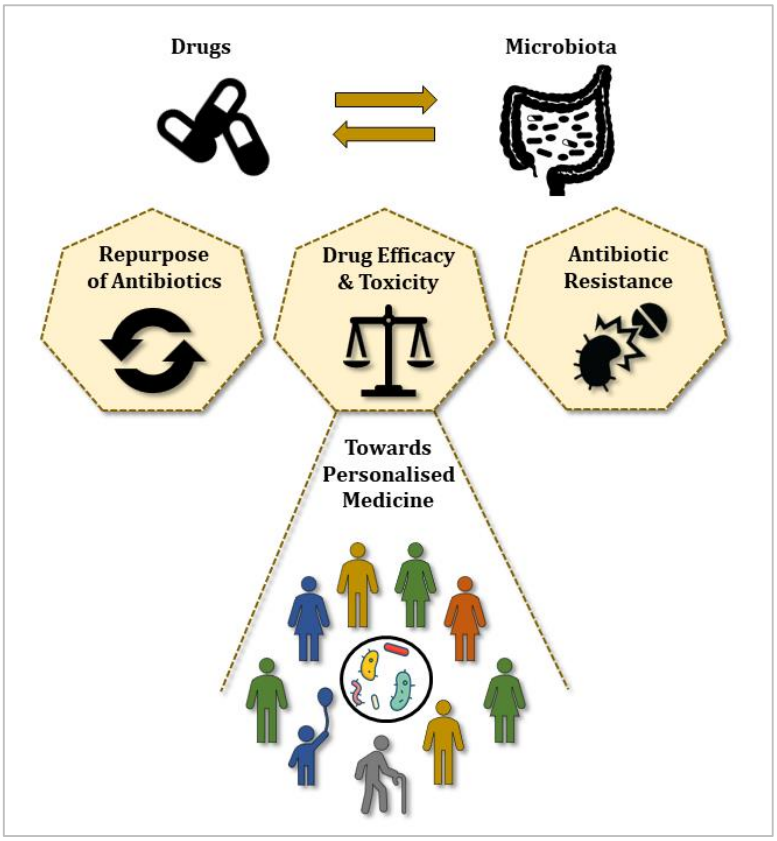


Figure 6.2 Possible implications for the interaction between drugs and the microbiome. *The effect of the microbiome on drug efficacy/toxicity provides a rationale for improving personalised medicine approaches.*

6.3 Psychiatric Disorders, Psychotropic Drugs and the Microbiome: A Complex Trinity

This thesis is focused on the interactions between psychotropic drugs and the microbiota-gut-liver-brain axis. Although our preclinical experiments are carried out in healthy rats, we have previously highlighted that the composition of the gut microbiome can be substantially altered in psychiatric populations (see *Chapter 1*, **Table 1.1** in *Appendix*). Psychiatric disorders are heterogeneous and bear a complex pathophysiology. In addition to genetic predisposition, environmental factors and alterations of biological systems (such as the neuroendocrine and immune systems), the microbiome could also play a pivotal role in the onset/development of such disorders. An intriguing speculative hypothesis that emerges from our research is that

a decreased microbial richness observed in a specific psychiatric population (Kelly et al., 2016) might be counterbalanced through administration of psychotropics which increase the richness itself. We have shown in *Chapter 2* that some psychotropic drugs indeed increase the microbial richness and diversity in healthy rats. Of course, this hypothesis should be tested and shown effective in human clinical cohorts. The microbiota is therefore at the interface between drugs and disease, the result of which will have implications for treatment outcome and efficacy (**Figure 6.3**). Moreover, psychotropic compounds other than those tested in this thesis should be screened for potential microbiota-targeting effects and their functional relevance. Successful translation of our work could lead to using the microbiome as a stratification tool which in turn could identify subgroups of patients that may be more likely to benefit from psychotropic medications.

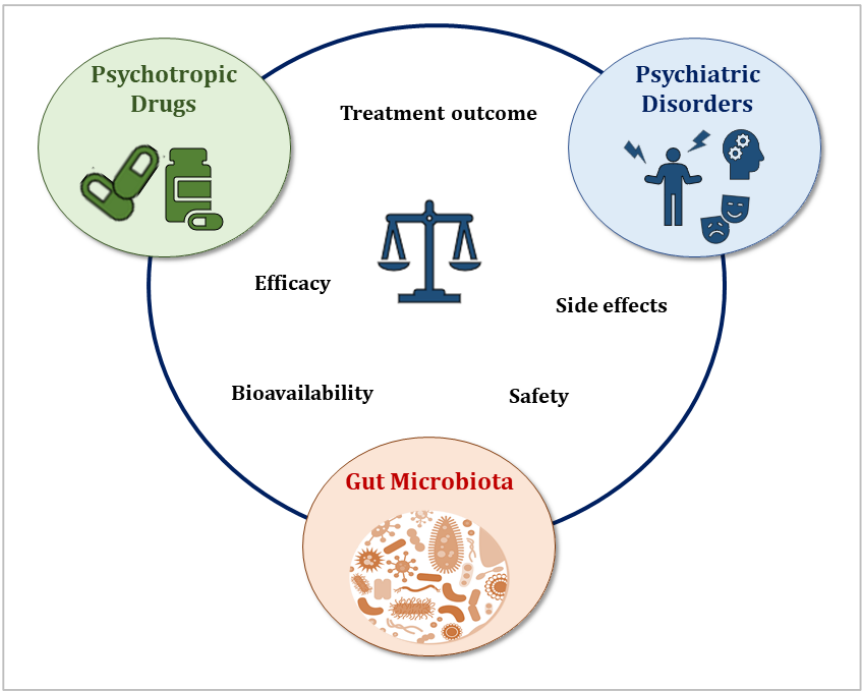


Figure 6.3 Complex interaction between disease state, drugs and the microbiome. *In addition to the bidirectional link between psychotropic drugs and the microbiome, psychiatric disorders can also influence the microbiome composition. This three-compartmental model will, in turn, impact treatment outcome, drug efficacy and toxicity.*

6.4 Drug-Bug Interactions: What Future Studies Are Needed?

The investigation of the role of the gut microbiota in the pharmacodynamics and pharmacokinetics of psychotropic drugs is in its infancy and only a few cross-sectional clinical studies have been conducted thus far. While the field is expanding rapidly and provides encouraging evidence that the microbiome might be a target to improve the response to psychotropic medications, more mechanistic insights are required in order to move beyond associations and examine causal relationships between microbiome composition and drug response/toxicity. A fine example of an attempt to investigate specific bacteria involved in the response to antidepressants is represented by (Lukić et al., 2019). In the study, the authors have characterised the differences in microbiome induced by antidepressants and have subsequently supplemented chosen bacterial species with vehicle- and antidepressant-treated mice. Interestingly, supplementation with the bacteria *Ruminococcus flavefaciens* diminished duloxetine-induced antidepressant activity, while *Adlercreutzia equolifaciens* had no such effect (Lukić et al., 2019); suggesting a mechanism for microbial regulation of antidepressant treatment efficiency.

Different types of experimental models can be used to investigate the microbiome and its interaction with the host, with the aim of gaining mechanistic insight. Here, some of the more common models will be discussed (**Figure 6.4**)

6.4.1 Probiotics, prebiotics and synbiotics

Probiotics refer to candidate species of live bacteria that, when ingested in adequate amounts, confer beneficial health effects upon the host (Butel, 2014). Through interacting with the host microbiota and intestinal epithelium, probiotics exert a wide range of effects on the host health, with various strains improving metabolism, immunity, endocrine function, and slowing aging in preclinical studies (El Aidy et al., 2015; Patterson et al., 2016). Intriguingly, probiotics can exert beneficial effects on

brain physiology and behaviour. *Faecalibacterium prausnitzii* (ATCC 27766) may function as a promising psychobiotic, as it recently demonstrated an anxiolytic and antidepressant-like phenotype in rats, probably via increasing caecal short-chain fatty acids and plasma IL-10 while reducing corticosterone and IL-6 (Hao et al., 2019). Certain bacterial strains or cocktails of bacteria have demonstrated efficacy in improving behavioural symptoms of various disorders, including depression, anxiety and autism (Allen et al., 2016; Bravo et al., 2011; Buffington et al., 2016; Hsiao et al., 2013; Kang et al., 2017; Savignac et al., 2014). These findings have led to the concept of psychobiotics for the treatment of various neurological and psychiatric disorders through targeting of the gut microbiota (Dinan et al., 2013). Psychobiotics are currently defined as microbiota-targeted interventions such as "beneficial bacteria (probiotics) or support for such bacteria (e.g. prebiotics) that influence bacteria-brain relationships" (Sarkar et al., 2016).

Prebiotics are "substrates that are selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). One of the main classes of prebiotics is dietary fibre, often defined as "carbohydrates with a degree of polymerization greater than 2, which fail to be hydrolysed or absorbed in the small intestine" (Stephen et al., 2017). These include inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), resistant starch and other soluble dietary fibres, amongst others. Importantly, prebiotics do not always influence the composition and activity of the gut microbiota in a selective and predictable manner (Bindels et al., 2015). Nonetheless, prebiotic supplementation has been demonstrated to reduce stress-responsiveness, anxiety- and depressive-like behaviour, as well as facilitate changes in hippocampal synaptic efficacy, including increased hippocampal brain-derived neurotrophic factor (BDNF) expression, general hypothalamic neuronal activity, and enhanced cognition and learning. Most studies thus far have been descriptive and are limited to demonstrating prebiotic influence on brain physiology and behaviour (Azpiroz et al., 2017; Burokas et al., 2017; Grimaldi et al., 2018; Jia et al., 2016; Savignac et al., 2013; Vazquez et al., 2015) and studies should aim to understand the mechanisms involved. A growing body of work is also focused on combining prebiotics and probiotics to develop synbiotics (Ford et al., 2014).

The use of specific probiotics, prebiotics or cocktails of synbiotics as adjunctive therapy to improve the response to psychotropic medications (or reduce their side effects) represents a fascinating and powerful venue of direction for research. These investigations should ideally be carried out both in preclinical and clinical settings.

6.4.2 Microbiota depletion: Germ-free models and antibiotics

Germ-free (GF) animals (Williams, 2014) have been invaluable tools for understanding microbe-host relationships. Lacking exposure to microorganisms since birth, GF animals provide insights into how the microbiota is fundamental in shaping behaviour, physiology and neurobiology in the host (Weger et al., 2019). GF rodents are generated via aseptic caesarean section (C-section) and kept free of microbes throughout the lifespan. Animals lacking a microbiome have different development and physiology than animals with commensal bacteria, for example they are smaller in body weight and have impaired intestinal function (Aluwihare, 1971; Jeppsson et al., 1979; Savage et al., 1981), lower concentrations of most gastrointestinal luminal amino acids (Yamamoto et al., 2018), and live longer (Gordon et al., 1966; Luczynski et al., 2016; Tazume et al., 1991). Due to the lack of commensal microbes, GF animals have impaired immune systems, dysregulated hormone signalling, altered metabolism, and differences in neurotransmission from conventional counterparts (Kawase et al., 2017; Neufeld et al., 2011; Pan et al., 2018; Sudo et al., 2004; Weger et al., 2019). Alternatively, colonization of GF mice with specific strains of bacteria has also shown to be a useful approach to interrogate microbiota-host interactions (Gordon and Pesti, 1971). From these gnotobiotic animals, it is possible to investigate mechanisms of communication between specific members of the microbiota and host physiology.

In addition to the GF model, antibiotics are also a useful tool for investigating the impact of microbiota perturbations on brain and behaviour. They offer much greater temporal flexibility and specificity compared to the GF model as they can be delivered acutely or chronically at any stage across an animal's lifespan (Desbonnet et al., 2015; Leclercq et al., 2017; O'Mahony et al., 2014). Additionally, the ability to adjust the

dose of antibiotics allows to control the extent of microbiota depletion, from minor perturbations to substantial ablations of the entire microbiota. A crucial consideration in the use of antibiotics to investigate the microbiota-gut-brain axis is their absorption from the GI tract. Non-absorbable antibiotics (i.e. vancomycin, neomycin, and bacitracin) knockdown the microbiota without entering the circulation and avoiding potential central nervous system (CNS) effects. Other antibiotics such as metronidazole and minocycline can potentially enter the CNS and can have direct action on brain and behaviour (e.g., microglial inhibition with minocycline; (Riazi et al., 2015)); therefore, studies using such antibiotics should be interpreted with caution. Antibiotic administration to laboratory animals has been shown to influence behaviours such as sociability and anxiety (Bercik et al., 2011; Degroote et al., 2016; Desbonnet et al., 2015; Frohlich et al., 2016; Guida et al., 2018; Hoban et al., 2017).

Microbiota-depleted animal models are useful tools for investigating the bidirectional relationship between the gut microbiota and administration of psychotropic drugs. In these models, single species of bacteria can also be administered, and their potential as an adjunctive therapy response can be studied.

6.4.3 Faecal Microbiota Transplantation (FMT)

FMT is a procedure that involves the transfer of intestinal microbiota from one individual to another and it is performed via oral administration of faecal material in rodents. This technique establishes a donor-like microbiome in the GI tract of the recipient, allowing stronger assumptions regarding the causal relationships between gut microbiota and host outcomes to be made. The FMT procedure has become best known for its remarkable success rate in the treatment of refractory *C. difficile* infection (Han et al., 2016; Sekirov et al., 2010; van Beurden et al., 2017; van Nood et al., 2013). FMT has opened up possibilities for more mechanistic investigations of the microbiota's role in various clinical conditions via "humanisation" of the rodent microbiota (FMT from human to rodents) (Kelly et al., 2016; Staley et al., 2017). Intriguingly, various behavioural phenotypes can be transferred by FMT, including

anxiety-like behaviour and aspects of depressive symptomatology, suggesting that the microbiota is a key regulator of anxiety and depression (Bruce-Keller et al., 2015; Kelly et al., 2016; Zheng et al., 2016).

From the drug-microbiota perspective, FMT could be employed to examine whether microbial changes induced by psychotropic medications causally mediate the efficacy of such drugs. One way to do this consists of transplanting faecal material from animals pre-treated with psychotropics into a microbiota-depleted recipient, followed by behavioural assessment. Clearly, a quantification of the drug (and its metabolites) levels that are potentially present in the donor faeces must be performed prior to the transplant itself, to confirm that there is no therapeutic dose of drug in the transplanted material.

6.4.4 Metabolomic approaches: It's not just what's there but what they are doing

In recent years, faecal metabolomics has increasingly gained attention and has shown promising results in characterising microbial metabolic functions (Matysik et al., 2016). The major metabolites produced by gut bacteria include SCFAs, branched chain fatty acids (BCFAs), branched chain amino acids (BCAAs), bile acids, biogenic amines and gases (e.g. CO_2, CH_4) (Nicholson et al., 2012a). Intriguingly, metabolomics analyses have allowed a better understanding on the role of the microbiome in neurological and neurodevelopmental disorders. A recent study revealed that microbial metabolites impact susceptibility to epileptic seizure in mice, linking the anti-seizure effect of ketogenic diet to metabolic mechanisms (Olson et al., 2018). Similar associations between metabolic neuroactive by-products of bacteria and behavioural outcomes have also been described in autism (Hsiao et al., 2013; Sharon et al., 2019).

The shotgun sequencing and the bioinformatic software PICRUSt allow for inferring metabolic potential of the gut microbiota. A fine example of this approach is a recent study where the authors performed a functional analysis of the microbiota neuroactive

metabolic potential in the Belgian Flemish Gut Flora Project population cohort (n=1,054), which was validated further in the Dutch LifeLines DEEP cohort (Valles-Colomer et al., 2019). In this study, specific microbial metabolites were found to be positively correlated to quality of life and depression scores (Valles-Colomer et al., 2019).

In conclusion, faecal metabolomics provides a functional readout of microbial metabolism as well as its interaction with host and environmental factors. Faecal metabolomics can be combined to brain metabolomics specifically to prove causality in the gut-to-brain communication. These types of metabolomics approaches are now warranted to gain a better mechanistic insight on the link between psychotropic drugs and the microbiome.

6.4.5 Human observations and targeted interventions

The relationship between consumption of psychotropic drugs and gut microbiome composition is currently unexplored in humans, therefore observations and interventions are warranted to translate preclinical findings into clinically relevant conditions. Metabolomic approaches, already mentioned in *Section 6.4.4*, should be employed not only in animal models but also in humans to gain a deeper insight on the role played by microbial metabolites. Translational limitations occur because of the clear discrepancy between the rodent and the human microbiota composition, in addition to a variety of cofounding factors (such as diet, comorbidities, smoke, etc.) that can be controlled in rodents but are more difficult to limit in human populations. Large-scale observations in humans should aim to compare the microbiome composition before and after a course of psychotropic drugs and to examine whether it associates with treatment outcome. Moreover, the field of drug-microbe interactions requires longitudinal studies in large-scale cohorts. A recent analysis based on daily sampling has revealed personalized diet-microbiome associations in humans (Johnson et al., 2019). A similar approach is warranted in the drug-microbiome field, with the aim to monitor changes in human gut microbiome composition in response to daily

drug intake. This will allow to test whether each subject's daily drug-microbiome interaction is unique to that individual and will deepen the understanding of personalised drug-microbiome relationships.

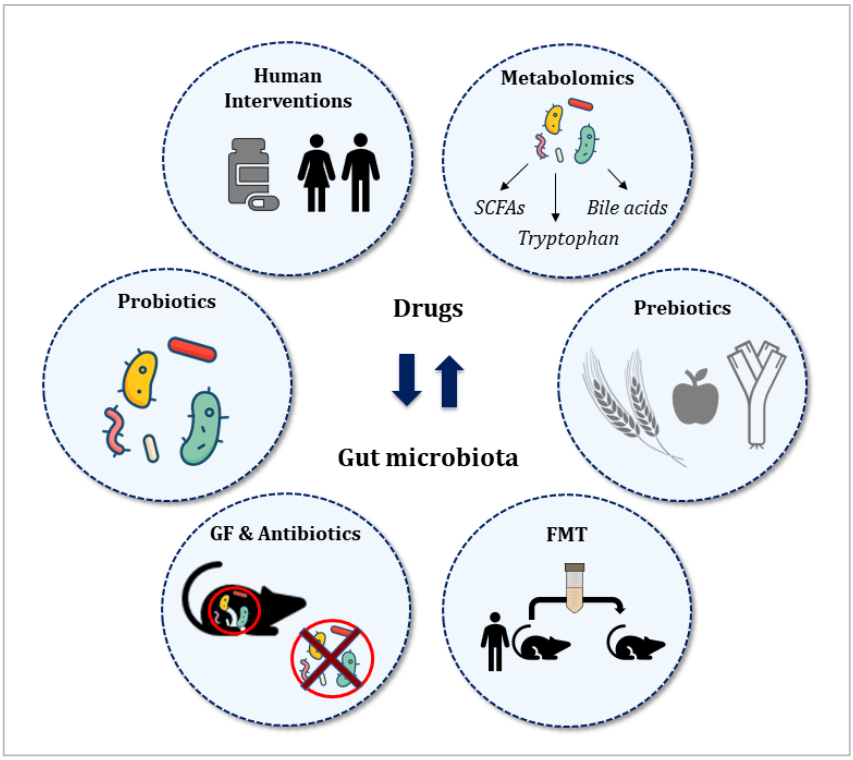


Figure 6.4 Approaches used to manipulate the microbiome for studying mechanisms and causality in the drug-microbiome field. *The methods include probiotics, prebiotics, synbiotics, microbiota-depleted animal models and faecal microbiota transplantation. Metabolomics approaches and large-scale longitudinal human studies are also warranted.*

6.5 Overall Conclusions

This thesis presents data showing that brain-targeting (psychotropic) drugs modulate the gut microbiome composition and intestinal permeability in a preclinical model. Moreover, we demonstrate that the mood stabilisers lithium and valproate increase a population of bile-metabolising bacteria and alter bile signalling potentially through the gut-microbiota-liver route. In this thesis we also show that a disturbance of the microbiome through administration of an antibiotic cocktail significantly impacted the absorption of the antipsychotic olanzapine with a specific bacterium, *Alistipes*, being significantly correlated with the blood levels (AUC) of olanzapine. Minor but significant associations between psychotropic intake (especially antidepressants) and microbiome composition were also observed in the human LifeLines-DEEP cohort. This thesis, together with existing data, provides convincing evidence for the bidirectional link between the microbiome and psychotropic drugs. The findings presented here might inform future clinical psychiatric practice, suggesting that the microbiome should be taken into account when psychotropic medications are administered. Exploration of the role of the microbiome in the efficacy or toxicity of other psychotropic medications is now warranted. An open question that remains is whether subgroups of psychiatric patients would respond better or worse to specific medications or their side effects based on the microbiome composition. To answer this, more translational research is needed.

This thesis represents an appropriate starting point for a deeper investigation of the relative contributions of psychotropic drugs on specific microbial taxa and more research on the implications for clinical practice is now warranted.

"Every day we live and every meal we eat we influence the great microbial organ inside us - for better or for worse."

Giulia Enders, Gut: The Inside Story of Our Body's Most Underrated Organ

Appendix

Table 1.1 Correlations between psychotropic compounds and microbes, divided by experimental design.

Psychotropic class	*Psychotropic compound*	*Experimental approach*	*Details*	*Reference(s)*
Antipsychotics	Aripiprazole	*In vivo*	4-wks administration in rats increase the relative abundances of *Clostridium*, *Ruminiclostridium*, *Intestinibacter* and *Eubacterium coprostanoligens*	Cussotto et al., 2019b
		In humans	The microbiota communities of AAP-treated (including aripiprazole) and non-treated patients are significantly separated. The genera *Lachnospiraceae*, *Akkermansia*, and *Sutterella* are differentially abundant in the two groups	Flowers et al., 2017
	Chlorpromazine	*In vitro*	Antimycobacterial properties	Kristiansen and Vergmann, 1986; Molnar et al., 1977
			Synergistic effect in combination with certain antibiotics	Amaral et al., 1992
			Inhibits significantly the growth of *S. aureus* and *E.coli*	Ordway et al., 2002a; Amaral and Lorian, 1991; Csiszar and Molnar, 1992
	Fluphenazine	*In vitro*	Pronounced action against both Gram-positive and Gram-negative bacteria at concentrations of 20-100 μg/mL	Dastidar et al., 1995

			Against 293 strains from two Gram-positive and eight Gram-negative genera, 46 of 55 strains of *S. aureus* are inhibited by doses of 10-50 μg/mL. Shigella spp., Vibrio cholerae and *V. parahaemolyticus* are also inhibited at concentrations of 10–100 μg/mL	Mazumder et al., 2001
	Olanzapine	*In vitro*	Completely inhibits the growth of *E.coli* NC101	Morgan et al., 2014
		In vivo	3-wks administration in rats alters the microbiota profile in both males and females	Davey et al., 2012
			4-wks administration in mice accelerates weight gain resulting from high-fat diet. The effect is absent under GF conditions but emerges quickly upon microbial colonization of the gut	Morgan et al., 2014
			Coadministration with an antibiotic cocktail in female rats attenuates body weight gain, uterine fat deposition, macrophage infiltration of adipose tissue, plasma free fatty acid levels, all of which are increased by olanzapine alone	Davey et al., 2013
			Coadministration with the prebiotic B-GOS in female rats attenuates olanzapine-induced weight gain	Kao et al., 2018
		In humans	The microbiota communities of AAP-treated (including olanzapine) and non-treated patients are significantly separated. The genera *Lachnospiraceae*, *Akkermansia*, and *Sutterella* are differentially abundant in the two groups	Flowers et al., 2017
			Cross-sectional study on psychiatric patients. No significant differences in microbiota composition at baseline between AAP users and nonusers. Non-AAP users have increase in *Alistipes*. AAP-treated females have decreased diversity compared with non-treated females	Flowers et al., 2019
	Prochlorperazine	*In vitro*	Strongly inhibits Bacillus spp. and Staphylococcus spp.	Rani Basu et al., 2005
	Risperidone	*In vivo*	80μg/day in female mice induces weight gain which correlates with an altered gut microbiota. Faecal transplant from risperidone-treated mice causes a 16% reduction in total resting metabolic rate in naïve recipients, attributable to suppression of non-aerobic metabolism	Bahr et al., 2015b
		In humans	The microbiota communities of AAP-treated (including risperidone) and non-treated patients are significantly separated. The genera *Lachnospiraceae*, *Akkermansia*, and *Sutterella* are differentially abundant in the two groups	Flowers et al., 2017

			Chronic treatment in psychiatrically ill children increases the BMI and reduces the ratio of Bacteroidetes:Firmicutes. There is a gradual decrease in the Bacteroidetes:Firmicutes ratio over the ensuing months of treatment	Bahr et al., 2015a
	Thioridazine	*In vitro*	Antimicrobial activity against methicillin-susceptible *S. aureus*, vancomycin-resistant pathogenic strains of Enterococcus species, Mycobacterium tuberculosis, *Pseudomonas aeruginosa* and Mycobacterium avium	Hahn and Sohnle, 2014; Ordway et al., 2002b; Wainwright et al., 1999; Amaral et al., 1996; Bettencourt et al., 2000; Ordway et al., 2003; Viveiros and Amaral, 2001; Viveiros et al., 2005
	Trifluoperazine	*In vitro*	Antimicrobial activity against 46 of 55 strains of *S. aureus* at doses of 10-50 μg/mL. Antimicrobial against *Shigella* spp., *Vibrio cholerae* and *V. parahaemolyticus* at concentrations of 10–100 μg/mL	Mazumder et al., 2011
Antidepressants	Amitriptyline	*In vitro*	Out of 254 bacterial strains, 185 are inhibited at different doses, with Staphylococcus spp., Bacillus spp. and *Vibrio cholerae* being the most affected bacteria. Amitriptyline also inhibits both Cryptococcus spp. and Candida albicans	Mandal et al., 2010
		In vivo	At doses of 25μg/g and 30μg/g significantly protects mice from Salmonella typhimurium	Mandal et al., 2010
	Clomipramine	*In vitro*	Cytotoxic effects against both human protozoan parasites Leishmania donovani and Leishmania major	Zilberstein and Dwyer, 1984
	Desipramine	*In vitro*	Effective against Plasmodium falciparum	Basco and Le Bras, 1990;

				Salama and Facer, 1990
	Escitalopram	*In vitro*	Antimicrobial effect on *E.coli*, but no effect on *L. rhamnosus*	Cussotto et al., 2019b
	Fluoxetine	*In vitro*	Strong dose-dependent antimicrobial activity against *L. rhamnosus* and *E.coli*	Cussotto et al., 2019b
		In vivo	4-wks administration in rats completely inhibits the growth of *Succinivibrio* and *Prevotella* caecal taxa	Cussotto et al., 2019b
	Imipramine	*In vitro*	Cytotoxic effects against both human protozoan parasites Leishmania donovani and Leishmania major	Zilberstein and Dwyer, 1984
			Inhibits the growth of *E. coli* and Yersinia enterocolitica through interference with plasmid replication. It also inhibits the parasite Giardia lamblia	Csiszar and Molnar, 1992; Molnar, 1988; Weinbach et al., 1992
	Ketamine	*In vitro*	Antimicrobial activity against: *S. aureus*, *S. epidermidis*, *E. faecalis*, *S. pyogenes*, *P. aeruginosa* and *E. coli*; with *S. aureus* and *S. pyogenes* being the most sensitive strains	Begec et al., 2013; Gocmen et al., 2008
			Sustained antimicrobial activity in a dose-dependent manner against micro-organisms in propofol, which is a strong growth-promoting factor	Begec et al., 2013
	Promethazine	*In vitro*	Inhibits the growth of *E. coli* and Yersinia enterocolitica through interference with plasmid replication	Csiszar and Molnar, 1992; Molnar, 1988
	Sertraline	*In vitro*	Potent antimicrobial against *E.coli*	Bohnert et al., 2011
			Inhibits the growth of *S. aureus*, *E.coli* and *P. aeruginosa*, also shows synergy in combination with antibiotics	Ayaz et al., 2015
			Potent antifungal activity against Cryptococcus neoformans, Coccidioides immitis and Candida spp.	Rossato et al., 2016; Trevino-Rangel Rde et al.,

				2016; Zhai et al., 2012; Paul et al., 2016, Lass-Florl et al., 2003
			Kills 97.5% of the promastigotes of Leishmania donovani at a dose of 30mg/L. At the lowest concentration (3mg/L), it induces significant loss of viability in the promastigotes (61%)	Palit and Ali, 2008
Antianxiety drugs	Propranolol	*In vitro*	Inhibits the growth of *S. aureus* and *E.coli*	Kruszewska et al., 2004; Hadera et al., 2018
			Does not inhibit the growth of *S. aureus*	Jerwood and Cohen, 2008
Anticonvulsants/Mood stabilisers	Lamotrigine	*In vitro*	Good antibacterial activity against Gram-positive bacteria *B. subtilis*, *S. aureus* and *S. faecalis.* Inhibition of bacterial ribosome biogenesis	Qian et al., 2009; Stokes et al., 2014
	Lithium	*In vivo*	4-wks administration in rats changes the caecal microbiome, with many genera being affected	Cussotto et al., 2019b
	Valproate	*In vitro*	Inhibits Mycobacterium smegmatis	Esiobu and Hoosein, 2003
		In vivo	4-wks administration in rats changes the caecal microbiome, with many genera being affected.	Cussotto et al., 2019b
Opioid analgesics	Methadone	*In vitro*	Antimicrobial activity against *S. aureus, P. aeruginosa* and *S. marcescens*	Sheagren et al., 1977
		In humans	Chronic opioid use (methadone N=1) in cirrhotic patients induces changes in microbiome composition, with lower relative abundance of Bacteroidaceae	Acharya et al., 2017
	Morphine	*In vitro*	Does not possess antimicrobial activity against any of the 10 microbial strains studied with the agar dilution method	Rosenberg and Renkonen, 1985
		In vivo	Induces dysbiosis in a morphine-dependent murine model. The dysbiosis is associated to an increase in pathogenic bacteria and a decrease in communities associated with stress	Wang et al., 2018
			Intermittent or sustained opioid regimen in mice influences the gut microbiome and this is causally related to behaviours associated with opioid dependence	Lee et al., 2018

		In humans	Chronic opioid use (morphine sulphate N=1) in cirrhotic patients induces changes in microbiome composition, with lower relative abundance of Bacteroidaceae	Acharya et al., 2017
	Tramadol	*In vitro*	Strong bactericidal activity against *E.coli* and *S. epidermidis.* Weak antimicrobial activity against *S. aureus* and *P. aeruginosa*	Tamanai-Shacoori et al., 2007
		In vivo	Subcutaneous injection in BALB/c-sensitive mice reduces the growth of *S. aureus* through enhancing phagocytes and tissue inflammation. It does not help eliminate *P. aeruginosa*	Farzam et al., 2018
		In humans	Chronic opioid use (tramadol N=23) in cirrhotic patients induces changes in microbiome composition, with lower relative abundance of Bacteroidaceae	Acharya et al., 2017
Drugs of abuse	Cannabis	*In vitro*	Strong antimicrobial activity against a wide range of micro-organisms	Appendino et al., 2008; M M Ali et al., 2018; Nissen et al., 2010
		In vivo	Modifications in the gut microbiota consequential to diet-induced obesity are prevented in mice treated chronically with THC	Cluny et al., 2015
		In humans	The microbiome of chronic marijuana users displays a Prevotella:Bacteroides ratio that is 13-fold lower than non-users	Panee et al., 2018
			A combination of THC and CBD mitigates experimental autoimmune encephalomyelitis by altering the gut microbiome	Al-Ghezi et al., 2017
	Cocaine	*In vivo*	Administration of antibiotics in mice induces an enhanced sensitivity to cocaine reward and an enhanced sensitivity to the locomotor-sensitizing effects of repeated cocaine administration	Kiraly et al., 2016
		In humans	Cocaine users display a higher relative abundance of Bacteroidetes than non-users	Volpe et al., 2014
	Heroin	In humans	The composition and diversity of intestinal microbiota in a cohort of 50 patients with SUD (of which 52% on heroin) is significantly different from those of healthy controls. The relative abundance of *Thauera*, *Paracoccus* and *Prevotella* is significantly higher in SUDs compared to healthy participants	Xu et al., 2017
	Metamphetamine	*In vivo*	The gut microbiota of methamphetamine-treated rats differs from that of control rats. The fecal microbial diversity is higher in metamphetamine-treated rats. The genus	Ning et al., 2017

			Phascolarctobacterium is reduced and the family *Ruminococcaceae* is increased in metamphetamine-treated rats	
		In humans	The composition and diversity of intestinal microbiota in a cohort of 50 patients with SUD (of which 30% on metamphetamine) is significantly different from those of healthy controls. The relative abundance of *Thauera*, *Paracoccus* and *Prevotella* is significantly higher in SUDs compared to healthy participants	Xu et al., 2017
Alcohol	*NA*	*In vivo*	4-wks intermittent vaporised ethanol in mice alters the gut microbiota, increasing the levels of *Alistipes* and decreasing *Clostridium IV*, *Dorea* and *Coprococcus*	Peterson et al., 2017
			In a mouse model of alcoholic liver disease, *Bacteroidetes* and *Verrucomicrobia* are increased in mice fed alcohol	Yan et al., 2011
		In humans	Human alcoholics with dysbiosis have lower abundances of Bacteroidetes and higher ones of Proteobacteria	Mutlu et al., 2012
			Alcohol-dependent subjects have an increased intestinal permeability which is linked to significant microbiome alterations	de Timary et al., 2015; Keshavarzian et al., 2009; Leclercq et al., 2014
			In cirrhotic patients, the proportion of phylum Bacteroidetes is significantly reduced, whereas Proteobacteria and Fusobacteria are highly enriched compared to healthy controls. Enterobacteriaceae, Veillonellaceae and Streptococcaceae are prevalent in patients with cirrhosis at the family level	Chen et al., 2011
Nicotine	*NA*	*In vitro*	Active against *E.coli*, *P. aeruginosa* and *S. faecalis* at a dose of 2µg/µl; and against Listeria monocytogenes and Viridans streptococci at a dose of 10µg/mL	Idrees Zaidi et al., 2012; Pavia et al., 2000
		In vivo	Influences the gut microbiota composition in a sex-specific manner in mice	Chi et al., 2017
		In humans	Induces profound changes in the gut microbiome, with an increase of Firmicutes and Actinobacteria and a decrease of Bacteroidetes and Proteobacteria at the phylum level. Smoking cessation induces an increase in microbial diversity	Biedermann et al., 2013

			Tobacco smokers display a higher relative abundance of *Prevotella*, lowered *Bacteroides* and lower Shannon diversity compared to controls	Stewart et al., 2018
Xanthines	Caffeine	*In vitro*	Inhibits the growth of *E.coli* and *E. faecalis*	Tatsuya and Kazunori, 2013; Daglia et al., 2007
		In vivo	Consumption of 500µL/day of coffee for three consecutive days in specific-pathogen-free mice decreases the levels of *E.coli* and *Clostridium* spp.	Tatsuya and Kazunori, 2013
			Caffeine-rich Pu-erh tea remodels the intestinal dysbiosis in mice with metabolic syndrome. *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* are the key gut bacterial links between the Pu-erh Tea treatment and metabolic syndrome	Gao et al., 2018
			Chronic coffee consumption in diet-induced obese rats decreases the abundance of *Clostridium Cluster XI* and increases Enterobacteriaceae. SCFAs are largely increased in the coffee-fed rats	Cowan et al., 2014
			8 weeks of coffee consumption in rats does not alter the gut microbiota composition	Cowan et al., 2013
			Oral administration of 0.7 mg/kg/day caffeine for 21 days in mice decreases *Lactobacillus*	Kleber et al., 2018
		In humans	Consumption of 3 cups of coffee daily for 3 wks in healthy volunteers increases the population of Bifidobacterium spp. In some subjects, there is a specific increase in the metabolic activity of Bifidobacterium spp.	Jaquet et al., 2009
	Theobromine	*In vivo*	2-wks administration of cocoa's theobromine in rats induces marked changes in gut microbiota. Rats that received a 10% cocoa-containing diet have lower counts of *E.coli*. Rats that received a 0.25% theobromine-containing diet have lower counts of Bifidobacterium spp., Streptococcus spp. and Clostridium histolyticum - C. perfingens group	Martín-Peláez et al., 2017
	Theophylline	*In vivo*	Consumption of fermented green tea, containing theophylline, is able to restore the changes in gut microbiota composition associated to diet-induced obesity in mice	Seo et al., 2015

Glossary of microbiome-associated terms

Term	*Definition*
16S rRNA gene/transcript sequencing	Bioinformatics technique where highly conserved regions of the 16S rRNA gene (DNA) or transcript (cDNA) are used to identify present or metabolically active microbes in a sample, respectively
Alpha diversity, beta diversity	Statistical terms used in ecology to describe variability of a dataset. Alpha diversity describes within-sample variability, while beta-diversity describes variability between samples. Many different formulas are available that define diversity differently, putting different weights on aspects like the number of species, how rare/abundant the species are, binary presence/abundance, and even taxonomic distance between species
Faecal microbiota transplantation (FMT)	Treatment where subjects are colonized with processed faecal matter (usually from a healthy donor in clinical cases or from a specific clinical population of interest in experimental studies). To ensure grafting of the donor microbiome, antibiotics (or germ-free animals) are generally used to deplete the recipient microbiome prior to FMT
Germ-free (GF)	A host without a microbiome. Generally, refers to mice and rats that were born and reared in a sterile environment to keep them from developing a microbiome, for the purpose of experimentation
GreenGenes, SILVA, RDP	Sequence databases and tools used to identify which microbes are present in a sample and their taxonomic relationships
Host	The organism (e.g., human, rodent etc.) that houses a given microbiome population
Microbiome	A term often used synonymously with "microbiota" but more precisely used to refer to the collective genome of a given microbiota
Microbiota	The collection of microorganisms found in/on a particular environment or living host
PICRUSt, HUMAnN2, LEfSe, GraPhlAn, MetaPhlAn	Parts of the bioBakery set, software tools developed by the Huttenhower lab, used to analyse microbiome data (https://bitbucket.org/biobakery/biobakery/wiki/Home)
Prebiotics	Nondigestible foods (such as fibres) that have a beneficial effect on the microbiome for the host
Principal coordinate analysis (PCoA) and principal component analysis (PCA)	Statistical methods used for datasets with many numerical values per sample, like microbiota data. The complex data are algorithmically converted to simpler set of values, called principal coordinates or components, with the aim of explaining variation in the data. Useful for visualizing differences between microbiome samples. If a principal coordinate or component is large, this is an indication it is determining a large proportion of the observed variance in the data
Probiotics	Live microbes that have a positive effect on host health when ingested in adequate quantities
Psychobiotics	Targeted interventions of the microbiome to support mental or brain health
QIIME, QIIME2	Quantitative Insights Into Microbial Ecology: Software tools used to analyse microbiome data
Short-chain fatty acids	Metabolic products of dietary fibres produced by commensal gut bacteria (e.g. acetate, propionate, butyrate)

Synbiotics	Synergistic combination of prebiotics and probiotics. The aim is to optimize treatment effects by providing both the beneficial microbes and the nutrients they need to survive and colonize
Whole genome shotgun sequencing	Bioinformatics technique where all DNA in a sample is sequenced to identify which microbes are present in a sample and their functional (metagenomic) potential. More expensive than 16S rRNA sequencing, but gives more reliable functional predictions

List of abbreviations

5-ASA	5-aminosalicylic acid
5-FU	5-fluorouracil
5-HT	5-hydroxytryptamine
AAP	atypical antipsychotic
ADME	absorption, distribution, metabolism and excretion
ALD	alcoholic liver disease
ALT	alanine aminotransferase
AMP	antimicrobial peptides
ANG1	angiogenin
AST	aspartate transaminase
AUC	area under the curve
BA	bile acids
BAC	bile acid cholyl-CoA synthetase
BAT	bile acid-CoA:amino acid N-acyltransferase
BBB	blood-brain barrier
BCAA	branched chain amino acid
BCFA	branched chain fatty acid
BDZ	Benzodiazepine
BHI	Brain Heart Infusion
BMI	body mass index
BSH	Bile salt hydrolases
CA	cholic acid
CDCA	chenodeoxycholic acid
CL	clearance
Cmax	maximum serum concentration
CNS	central nervous system
CRF	corticotropin-releasing factor
CYP	cytochrome P450
DCA	deoxycholic acid
ENS	enteric nervous system
FGF	fibroblast growth factor
FMLP	N-formylmethionyl-leucyl-phenylalanine
FMT	faecal microbiota transplantation
FOS	fructooligosaccharides
FUDR	5-fluoro-2'-deoxyuridine
FXR	farnesoid X receptor
GABA	gamma-aminobutyric acid

GF	Germ-free
GI	gastrointestinal
GOS	galactooligosaccharides
HCA	hyocholic acid
HDCA	hyodeoxycholic acid
HPA	hypothalamus-pituitary-adrenal
IBD	inflammatory bowel disease
IL	interleukin
iNOS	inducible nitric oxide synthase activity
I_{sc}	short-circuit current
JAM	junctional adhesion molecules
LCA	lithocholic acid
LCFA	long-chain fatty acids
LPS	lipopolysaccharide
MAMP	microbial-associated molecular patterns
MAOI	monoamine oxidase inhibitor
MCA	muricholic acid
MIC	minimal inhibitory concentration
NA	noradrenaline
NAFLD	nonalcoholic fatty liver disease
NO	nitric oxide
NSAID	nonsteroidal anti-inflammatory drug
OD	optical density
OF	open field
OLZ	olanzapine
PCoA	principal coordinate analysis
PD-1	anti-programmed cell death 1 protein
PEG	Polyethylene glycol
PPI	proton-pump inhibitor
PRR	pattern recognition receptor
RISP	risperidone
RNASE4	RNase A family member 4
ROS	reactive oxygen species
SCFA	short-chain fatty acid
SERT	serotonin transporter
SSRI	selective serotonin-reuptake inhibitor
SUD	substance use disorder
TCA	tricyclic antidepressant
TEER	transepithelial electrical resistance
TGR5	Takeda G-protein-coupled receptor 5
THC	tetrahydrocannabinol
TLR	Toll-like Receptor
TMA	trimethylamine
TMAO	trimethylamine *N*-oxide
UDCA	ursodeoxycholic acid
UGT	UDP-glucuronosyltransferase
VLDL	very-low density lipoproteins

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