Microbiome Expert Workshop
Porto, 8-9 July 2019

Workshop Report No. 36

EUROPEAN CENTRE FOR ECOTOXICOLOGY AND TOXICOLOGY OF CHEMICALS

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European Centre for Ecotoxicology and Toxicology of Chemicals
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Gut microbiome associated metabolic capacity and host-microbiome co metabolism

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SUMMARY

In order to assess the progress on the research findings regarding the 'Gut microbiome associated metabolic capacity and host-microbiome co metabolism', an ECETOC'19 – Microbiome experts meeting was organised in Porto, Portugal. (8-9th July, 2019). The Workshop comprised of scientists working on Gut Microbiome research in the fields of host health and disease. Ten scientific presentations from the participants belonging to various research institutes, Universities and companies were held. The second day of the workshop included active discussion between the participants regarding the knowledge gaps in the field of gut microbiome associated metabolism.

One major motive behind the ECETOC'19 Workshop was to bridge the knowledge gaps and produce research ideas for the CEFIC-LRI (ELUMICA) project. The ELUMICA project aims at elucidating the gut microbial metabolic capacity and understanding the metabolism-related gut microbial functionality. In order to successfully fulfil the target and to get as many informative inputs from scientists and research in the field, the expert meeting was organised by Prof. Dr. Bennard van Ravenzwaay and Mr. Oliver de Matos.
The Workshop was divided in two parts, the first part being introduction and insight to the various research conducted by the expert scientists and their respective labs in the field of gut-microbiome associated metabolism.

The various presentations involved research findings on the following topics; gut microbiota and host co-metabolism of xenobiotics, influence of intestinal microbiota on phytochemicals present in natural products, in-vitro based analysis of gut-host co-metabolism, characterisation of human gut microbiome, the use of fecal metabolomics to understand the gut microbiome, in-silico based xenobiotic concentration predictions of gut microbial metabolism and standardisation of tools for detailed microbiome-associated metabolism analysis.
The second part of the Experts Meeting involved cumulative discussion rounds between two groups formed among the participants related to potentially unaddressed and already known research findings in order to bridge the knowledge gaps and address the unknowns. Knowledge gaps related to a broad range of questions such as defining what a good microbiome is, which matrix could be regarded as a functional end-point to understand microbiome-associated metabolic processes, questions related to interindividual variability, the necessity of biomarkers for disease identification and so on were majorly discussed. More specific research questions such as direct microbiome-specific drug reactions, gut microbiome-specific enzyme activity, host-factors determining microbiome-drug interactions, predicting microbial functionality using metagenome-level information, understanding more about microbe-microbe interactions and its role on the metabolism and so on were also discussed during the break-out group round.

Many research findings that have contributed significantly in understanding the host-microbiome co-metabolism, metabolic capacity of intestinal microbiome, metabolome profiling of the microbiome associated process and determining the functionality of the gut microbiome based on the genes/proteins responsible were brought up and discussed intensively about. It was not only informative, but the meeting undeniably provided with different perceptions and aspects in handling the research in the field.

This workshop report provides an overview of the different presentations made an should be seen as a contribution to integrating the metabolic capacity of the gut microbiome as an essential part of its host metabolic activity in the assessment of toxicants. It can be concluded that several metabolic pathways are predominantly mediated by gut microbiome metabolism. Taking into account the profound differences between the gut microbiome communities in different species, it is not unlikely that what is currently considered as "species specific" effect, may in fact not be related to the toxic-dynamic aspects of substances but at least occasionally need to be seen in the light of host (species) specific gut microbiome metabolism. Therefore, further research, to elucidate gut microbiome metabolism in humans and laboratory animals will contribute to a better understanding of translational toxicology.

1. WORKSHOP OVERVIEW

The gut microbiome research has gained immense attention and is under the limelight since recent years. Important research initiatives like the Human Microbiome Project (HMP), European based Metagenomics of Human Intestinal Tract (MetaHIT) and many others paved the way to understanding the (GI) microbiota in depth [1]. The gut microbiota have been known to fulfill important functions in the host, like calories extraction from diet, synthesis of essential amino acids and vitamins, metabolism of lip and bile acids, supporting the immune system of the host, maintenance of homeostasis, prevention of infections and so on [2, 3]. The gut microbiome is known to predominantly consist of bacterial flora that outnumber Eukarya and archaea by 2-3 orders of magnitude [4]. To comprehend the broader view of gut microbial metabolic functions, it is necessary to understand the gut bacterial transformation mechanisms that have immense significance in the fields of Pharmacology and Toxicology.

In order to improve understanding of the influence of intestinal microbiome on host health, integration of knowledge of the gut microbial community composition with its functionality in terms of metabolic process, is necessary. This involves the association of omics approaches to unravel the mechanistic and functional understanding of the host-microbiome interactions. Chemicals/xenobiotics/test compounds induced alterations in the microbial metabolism would promise a greater insight into understanding the microbial functionality in host organisms (such as rodents and humans).

Bridging the knowledge gaps regarding xenobiotic microbial metabolism and microbiome-associated metabolites in both rodents and humans stands necessary. The interspecies differences regarding the microbiome and functionality of microbiome will be address in the LRI research project (ELUMICA). The ELUMICA study plan aims to summarise and compare the microbial capacity in terms of functionality and chemical compound0 induced reactions. The Cefic-LRI research plan of ‘Elucidating the microbial metabolic capacity’ ELUMICA stands as the main motive behind organising the 2019 ECETOC Microbiome Expert Workshop in Porto, Portugal.

2. WORKSHOP STRUCTURE

Around 12 experts in the field of gut microbiome research from various Universities, companies and research institutes took part in the ECETOC Workshop, Porto from 8 to 9 July 2019. The experts presented their research work and the future aspects of their work in the field of Gut Microbiome. The presentations were followed by discussion sessions of two break out groups regarding the unknowns and the knowns in the major areas of microbiome related metabolic capacity. The list of the participants of the Expert Workshop can be found in Appendix A and the detailed program is given in Appendix B.

3. WORKSHOP AIMS AND OBJECTIVES

The Cefic LRI research project along with the ECETOC Workshop aims to explore the current knowledge and to identify the gaps to gain a deeper perspective from the experts in the field of microbiome-related metabolic capacity. The dissemination of knowledge gained from regulatory and scientific perspectives through presentations at the International Conference organised by ECETOC was the main motive.

The Microbiome Expert Workshop was proposed based on an LRI research activity in which the metabolic capacity of the gut microbiome is to be elucidated employing multi-omics approaches and microbiome community analysis to be used in future chemical risk assessment. The two break out groups that were formed in the second day of the Workshop aimed at discussing all the knowns and unknowns in the field from regulatory, scientific and industrial point of views. As a result of the discussion rounds, concrete summarisation was deduced to aid future research.

4. PRESENTATION SUMMARIES

The following chapter focuses on summarising the research work, outcomes and future aspects conducted in the laboratories of various expert scientists who participated in the ECETOC Workshop.

4.1 Presentation 1: ELUMICA – Elucidating Microbial Metabolic Capacity by Dr. Saskia Sperber (BASF, DE)

The gut microbiome consists of bacteria, archaea, fungi, viruses, protists and other species. Bacteria are predominantly present and 2-3 times more in order than the other species. The functions of gut microbiota involve calorie extraction, synthesis of essential vitamins and amino acids, lipid and bile acid metabolism, prevention of infection, support of the immune system and a role in endocrine functioning. Therefore, it makes gut microbiota one of the contributors of health and disease of the host. Gut microbiota plays a major role in (de)toxification of chemicals/xenobiotics, making it a key topic to explore in the fields of pharmacokinetics and toxicology (LRI proposal).

Gut microbiota is highly susceptible to variability as it depends on numerous factors like host's diet, lifestyle, environment, host genetics, age, exposure to xenobiotics and so on. An integrated omics approach allows to gain more knowledge regarding the host-microbial interactions [5]. In the previous research conducted at BASF, significant insight was gained regarding the contribution of gut microbiome metabolism in rats. Therefore, it was necessary to apply an integrative approach linking the functionality and metabolic capacity of both rat and human microbiome. This would not only contribute to understanding the microbiome associated metabolic processes but also interindividual and interspecies variations between rodent and human microbiomes (LRI proposal).

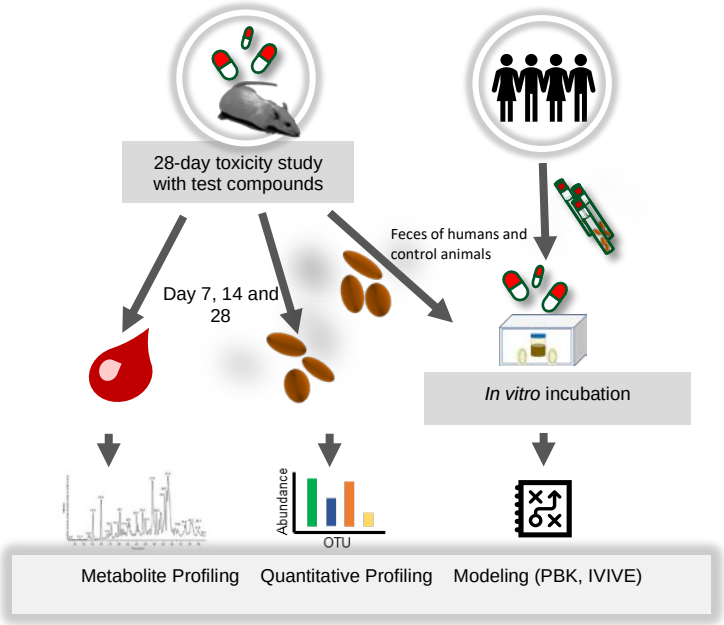


Figure 1: Schematic representation of the study design for metabolite and gut microbial profiling using plasma and fecal matrices respectively

The LRI project ELUMICA aims at unravelling the link between the changes in the gut community induced by drug or test compounds, with the functionality in terms of metabolic processes. The project involves in vivo, in vitro and in silico models to achieve the aim (see figure 1). The in vivo part of the study involves the standard 28-day oral toxicity study of Wistar rats following the (OECD) 407 guidelines. 8-10 test compounds that have low or no systemic availability, low systemic toxicity and having the ability to alter the gut microbial composition (e.g. some antibiotics). Plasma and fecal samples will be collected for the analyses. Plasma and fecal samples will be used for metabolome profiling and fecal samples will be used for quantitative microbial profiling.

Pooled and individual fecal samples from untreated and treated animals from the in vivo will be used for in vitro batch cultivation. Fecal samples of rats from in vivo study and from human donors will be used for in vitro cultivation with test compounds/xenobiotics to study metabolome and microbiome profiling. This will also aid in understanding the interindividual and interspecies differences. Biochemical transformation of bacterial enzymes present in vitro fecal samples from rats and humans will be studied using known chemical probes (or tracers). The probes for the transformation study will be selected based on previously available literature [6, 7].

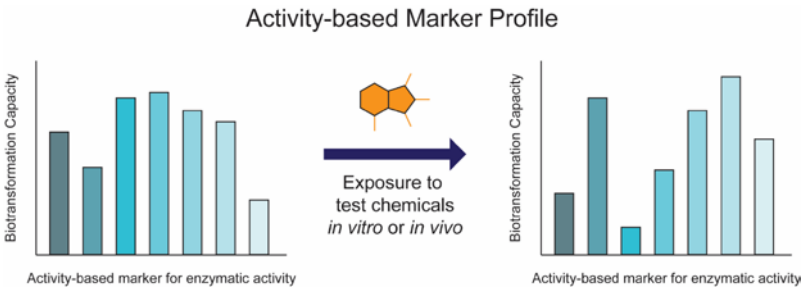


Figure 2: Graphical representation to study bacterial biotransformation using chemical probes to profile the biochemical functionality of gut bacterial enzymes using fecal samples

Finally, computational models will be performed to trace the in vitro concentration-response data to in vivo dose-response effects. The in vitro studies data will be fed into in-silico based physiologically based kinetic models/In vitro to in vivo extrapolation (PBK/IVIVE) models using PBK based reverse dosimetry. IVIVE/PBK models will be established to bridge knowledge about the differences in the microbial functionality and their metabolic capacities of humans and rat and subsequently the interindividual and interspecies differences [8].

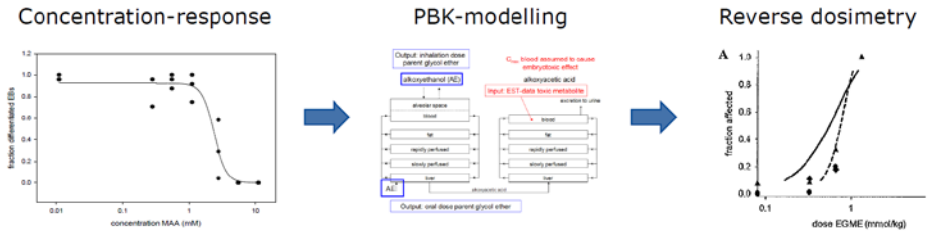


Figure 3: representation of translating in vitro concentration-response curve of toxicity of a specific compound to in vivo dose-response curve using PBK modelling based reverse dosimetry in order to understand adverse effects

Goals of the ELUMICA project are to understand adversity based on changes in key metabolites identified during metabolome profiling, to assess interspecies and interindividual differences based on chemical induced intestinal microbial metabolism. This project also aims at using PBK based reverse dosimetry to predict dose-response changes of key metabolites from in vitro to in vivo conditions, which has huge relevance in risk assessment strategies. Overall, the ELUMICA research project deals will understanding the role of microbiome in chemical-induced toxicity.

4.2 Presentation 2: Influence of the gut microbiome on plasma metabolite patterns by Aishwarya Murali (BASF, DE)

Gut microbiome contributes to the health and disease of the host organism. Quantitatively, the number of microbial cells has been known to be the same range as the cells in a human body. Bacterial species are predominantly present and 2-3 times more in order than the protists, archaea, viruses and other species. The variability of the gut community depends on a lot of factors including the host diet, environment, xenobiotic intake, age of the host and several more. Gut microbiota could be considered as the 'second liver' that plays a role in (de)toxification of chemical compounds. Which is why the gut microbiome research has gained a lot of attention in the fields of pharmacokinetics and toxicological studies [9].

Metabolomics, aids as a powerful tool to understand (changes in) the biochemistry in cells and the entire body of organisms and could be of great value in risk assessment (for example to elucidate mode of action, predictive toxicology and as an aid to improved quality of read across). Metabolomics can be used for identifying relevant biomarkers/metabolite patterns indicating adverse effects/organ toxicity. Other advantageous of metabolomics would include the use of blood/urine/feces as matrix that can be obtained by less invasive methods which enables a more longitudinal study design [10, 11]. A broad range of metabolites can be detected using powerful tools like (HPLC, GC-MS, LC-MS) and other mass spectrometry based analytical methods. The sample materials used for the metabolome profile can be in lesser amounts compared to the sample sizes required for the other omics technologies [9, 12].

To investigate the effects of antibiotics, the metabolome study employed was a 28-day Wistar rat oral toxicity plan following the OECD 407 guidelines. The test compounds used were antibiotics in two doses that were enough to elicit response but not adverse systemic toxicity. Blood was sampled on days 7, 14 and 28 and feces on day 28 and were used for metabolite profiling using MS-based techniques [9]. About 300 metabolites were analyzed and MetaMap®Tox database was used to predict the modes of action and for visualisation. MetaMap®Tox database consists of the metabolite profiles about thousand compounds, making it a reliable evaluation/visualisation tool to predict the modes of action of new substances that are tested. Metabolite patterns were produced, as in figure 4, from the profiling of plasma and fecal samples from the control and treated animals [12].

Metabolite	Direction	Neomycin sulfate			Gentamicin sulfate			Metabolite class
		7 d	14 d	28 d	7 d	14 d	28 d	
Alanine	↓	0.89	0.81	0.99	0.82	0.76	0.81	Amino acids
Proline	↓	0.96	0.87	0.92	0.83	0.86	0.91	
Tyrosine	↓	0.96	0.87	0.86	0.81	0.78	0.89	
Indole-3-acetic acid	↓	0.55	1.23	0.66	0.59	0.65	0.86	Amino acids related
Ketoleucine	↓	0.94	0.78	0.98	1.04	0.65	0.81	
Indole-3-propionic acid	↓	0.24	0.12	0.30	0.06	0.07	0.42	
Glycerol, lipid fraction	↓	0.64	0.68	0.83	0.85	0.77	0.88	Complex lipids, fatty acids and related
Glycerol, polar fraction	↓	0.94	0.81	0.89	0.91	0.94	0.92	
dihomo-gamma-Linolenic acid (C20:cis[8,11,14]3)	↓	0.72	1.02	0.88	0.92	0.78	0.79	
Sphingomyelin (d18:2,C16:0)	↑	1.04	1.17	1.27	1.17	0.97	1.11	
Sphingomyelin (d18:2,C18:0)	↑	1.08	1.19	1.15	1.01	1.31	0.79	
TAG (C16:0,C18:2)	↓	0.77	0.92	0.91	0.87	0.86	0.83	
TAG (C18:1,C18:2)	↓	0.59	0.71	0.82	0.83	0.87	0.87	
Lysophosphatidylcholine (C20:4)	↓	1.03	0.91	0.92	0.82	0.89	0.90	
TAG (C18:2,C18:3)	↓	0.46	0.62	0.90	0.71	0.65	0.75	
Lactate	↓	0.75	0.83	0.69	1.02	0.71	0.85	Energy metabolism and related
Normetanephrine	↓	0.69	0.66	0.88	0.88	0.65	0.62	Hormones, signal substances and related
3-O-Methyldopamine	↓	0.48	0.23	0.31	0.75	0.70	0.73	
Putrescine	↓	0.87	0.86	0.86	1.00	0.81	0.89	Miscellaneous
Hippuric acid	↓	0.45	0.27	0.20	0.54	0.12	0.17	

Figure 4: Example of a metabolite pattern produced from plasma samples from Neomycin and Gentamycin treated Wistar rats. The generated pattern is a part of MetaMap Tox® database visualisation tool. The blood samples from days 7, 14 and 28 are used in this case, where red boxes indicate significantly increased and blue boxes show significantly decreased metabolite value changes compared to control group

Among the four classes (tetracyclins, lincosamides, aminoglycosides and fluoroquinolones) of antibiotics checked, aminoglycosides showed the most alterations in the plasma metabolite profile. Aminoglycosides are very little/not bioavailable and showed most alterations in metabolites that are involved in lipid, amino acid, bile acid related metabolism processes.

Furthermore, to identify the best matrix for the metabolite profiling, different gut tissues, cecum and feces were analyzed. The metabolome analyses of all the matrices were done to observe antibiotic-induced treatment effects [12]. Three antibiotics; vancomycin, streptomycin and roxithromycin were used in the study. The metabolite profiles of the different gut tissues of the different treatments, did not show significant alterations compared to the cecum and fecal matrices. In feces, the treatment groups showed separation in the clusters with respect to the control, compared to the cecum matrix (see figure 5). Vancomycin and roxithromycin treated metabolites showed clear dose dependent separation in the cecum, whereas vancomycin and streptomycin showed dose dependent separation in fecal samples [12].

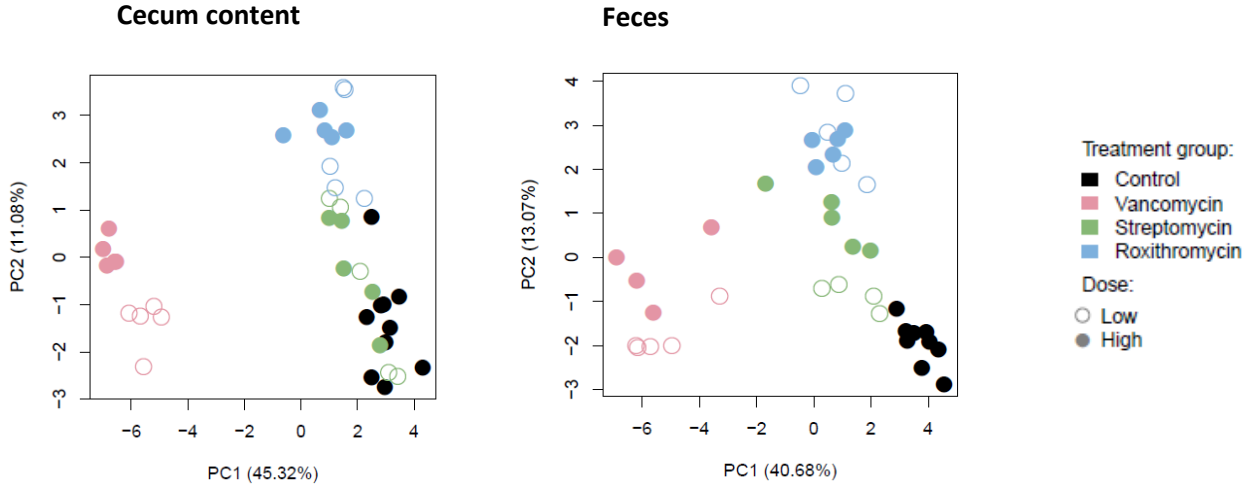


Figure 5: Principle component analysis (PCAs) of metabolite concentration changes in feces and cecum matrices of Vancomycin, Streptomycin and Roxithromycin treated male Wistar rats

Overall, it was concluded that feces could be the best and non-invasive matrix for the analysis of metabolite profiles to study treatment induced effects [12]. This also promotes longitudinal study design and less painful

for the animal models. Another study involved the gut community analysis of fecal samples from lincosamide treatments, Carboxymethyl cellulose or (CMC) (a well-known vehicle to deliver test compounds via gavage) and control diet [13].

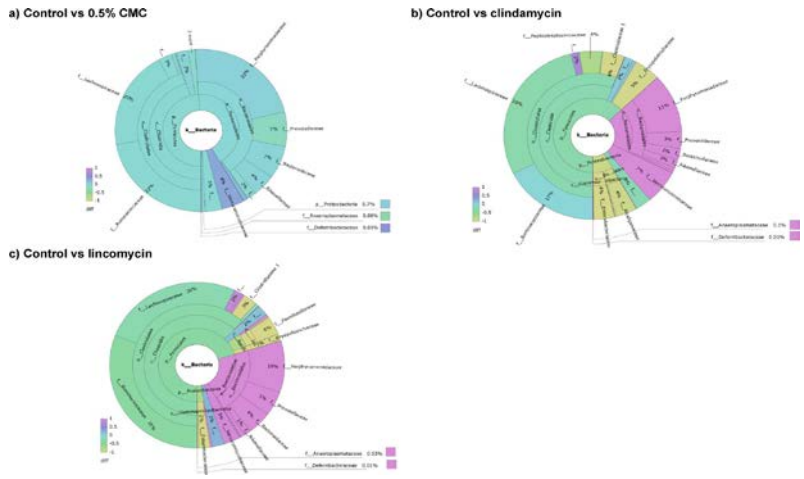


Figure 6: Pairwise Krona plots showing the differences in microbial community composition up to family level after 0.5% CMC, Lincomycin and Clindamycin treatments of Wistar rats. Pink colour indicates a reduction and yellow shows increment of bacterial flor w.r.t. the control flora.

The pairwise Krona plots showed similar alterations in the gut communities in the 0.5% CMC treated and control animals but there were significant variations in the communities of the lincosamide treated animals w.r.t controls (see figure 6). In the lincosamide treated animals, there was a significant reduction in the Bacteriodetes and Verrucomicrobia phyla compared to the controls. A considerable increment in the Firmicutes and Proteobacteria communities occurred in both the lincosamide (clindamycin and lincomycin) treated animals with respect to the control animals. Significant changes in the metabolites of the feces, and to a lesser extent in the plasma of the lincosamide treated animals could be related with the gut community shift produced by the antibiotics [13].

Finally, the bile acid pool was analyzed using the feces and plasma samples from the controls and antibiotic treated animals. 20 bile acid metabolites were analyzed using the two matrices and metabolome profile was observed to check sex dependent and treatment dependent effects in both feces and plasma matrices. Like previously known results, the CMC and controls clustered together in both fecal and plasma matrices [14]. Much clearer separation between the treatments and the control/CMC vehicle could be observed in the fecal samples compared to the plasma, see figure 7.

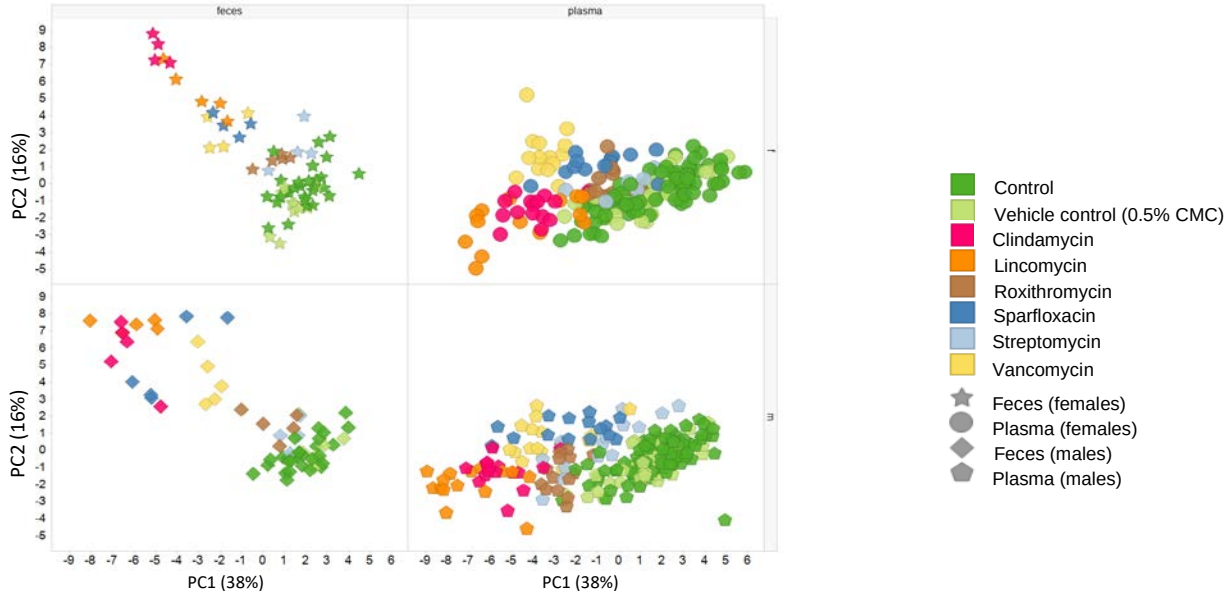


Figure 7: PCA of fecal and plasma metabolite profiles of control and treated male and female Wistar rats.

The bile acid metabolites in the plasma matrix did not contribute much to the PCA separation of the different treatments. Further, very little sex- or treatment-dependent segregation could be observed in the plasma matrix [14]. The research work regarding the role of gut microbial metabolites and determination of plasma metabolite patterns induced by xenobiotic exposure, was solely conducted at the department for experimental toxicology and ecotoxicology of BASF SE Germany. The presentation involved published results from BASF SE regarding the influence of gut microbiome on the plasma metabolite patterns which predominantly included the doctoral thesis of Dr. Christina Behr.

4.3 Presentation 3: Contribution of the gut microbiota to host endogenous and xenobiotic metabolism by Prof. Jonathan Swann (Imperial College London, UK)

The presentation focused on the host-gut microbiome co-metabolism and the respective impacts on the host endogenous metabolism, immune metabolic interactions and xenobiotic metabolism. The host as well as the gut microflora require to carry out metabolic processes in order to survive. Some metabolisms are specific to the host/gut microbiota and some processes are performed together (co-metabolism). To start with, the endogenous metabolism has been observed to be altered based on the host exposure to microbial metabolites. The major altered metabolic pathway was observed to be the bile acid metabolism. Bile acids are the signaling molecules that represent the host-microbial interactions. They can enter the enterohepatic circulation, get re-conjugated into the liver, re-enter the bile and hence, the intestine. Germ-free mice have been observed to lack the secondary bile acids, on conducting the bile acid profiling. There is a study published by the respective investigating the altered bile acid metabolites by the gut microbiota in the host organism [15]. In the study, bile acid metabolite profiling was compared between the germ-free and antibiotic treated

mice. Biosynthesis of steroids and FXR/PXR activation were significantly altered in the germ-free mice due to the lack of secondary bile acids (as in figure 8).

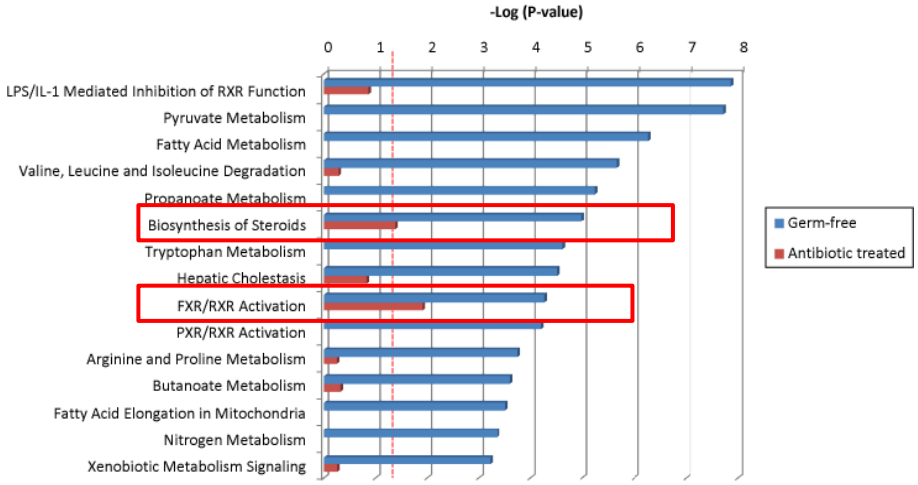


Figure 8: Significantly regulated canonical gene expression pathways in GF (germ-free) rats (blue bars) and pathways common to antibiotic-treated rats (red bars)

The variation in bile acid profiles has been known to be very high in individual animals and is also time dependent. This could be majorly dealt with by pooling the samples rather than using single animals. Other than the bile acid metabolism, short chain fatty acid (SCFA) metabolism has also been observed to have a direct effect of intestinal microbiome-host co-metabolism. SCFA have role in energy homeostasis and aid in the modulation of membrane permeability and function as messenger molecules. In another study conducted in the lab, age-dependent urinary metabolite profiles were compared between the Taiwanese and American populations [16]. Cresols are stated to be co-metabolites that were seen to be increased with age. Production of more cresols lead to lesser Tyrosine and Phenylalanine uptake by the host due to microlesions present in the gut, hereby availing more resources for bacterial growth and metabolism. 4-cresol has been known to be a mutagen that increases cell proliferation and induces DNA damage [17].
Furthermore, gut microbiota is known to modulate host metabolism via immune interactions. Indoleamine-2,3-dioxygenase (IDO) is responsible for tryptophan over-usage hereby resulting in lack of tryptophan availability in the GI tract for microbial metabolism. Increased IDO leads to increased production of quinolinic acid, which is neurotoxic [18]. Neuroinflammation due to increased IDO brings behavioural effects in the host. Inhibition of IDO activity has been observed to protect the mice from depression-like behaviours. Lastly, xenobiotic (or drug) metabolism resulting from the host-microbiota co-metabolism was observed. Certain microbiome-host mediated metabolism of xenobiotics could either lead to activation of the drug or could detoxify it. It was observed that the age-associated increase in the 4-cresyl sulfate had a potential impact on the host xenobiotic metabolism [19]. Similarly, toxicity of hydrazine was studied by comparing the metabonomic profiles of germ-free and conventional rats (see figure 9). No apparent difference in hydrazine metabolism was observed between the germ-free and conventional rats [20].

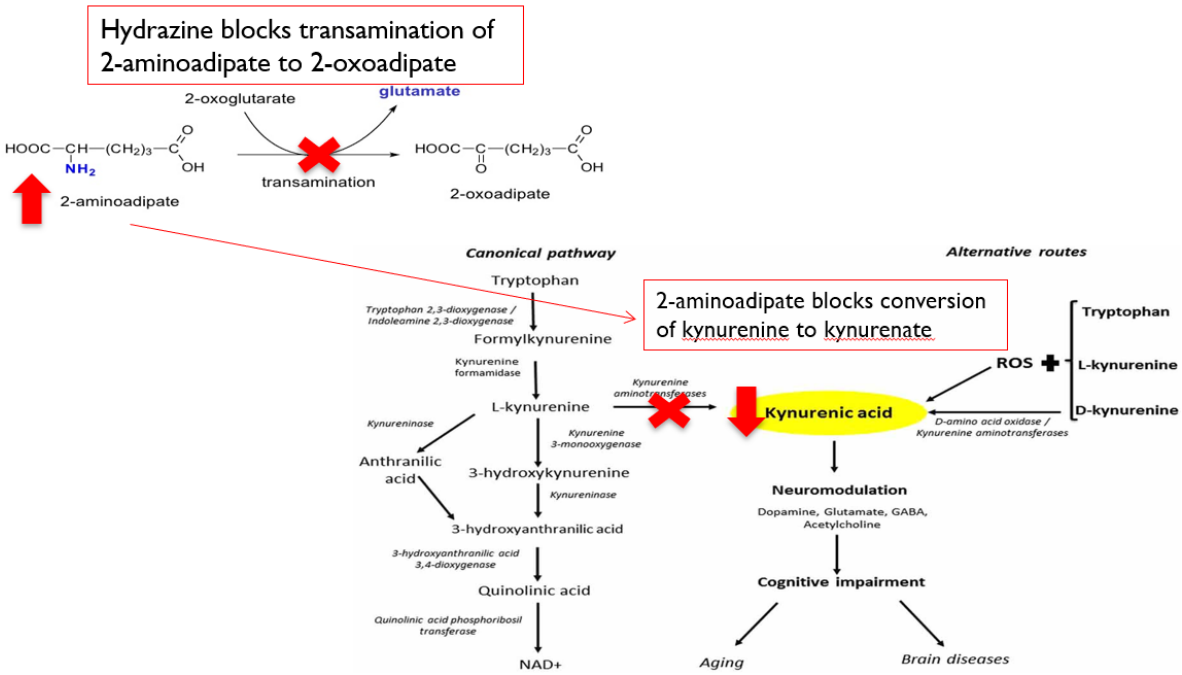


Figure 9: Schematic diagram showing the tryptophan metabolism pathway showing that increased gut bacterial colonisation results in increase in IDO production and hence an increase in kynurenine production, therefore, an increase in toxic metabolites produced.

4.4 Presentation 4: Influence of the microbiota on the phytochemical constituents of natural products and vice versa – metabolomic by Dr. Olaf Kelber (Steigerwald Arzneimittelwerk GmbH, Bayer Consumer Health, DE

The presentation included three topics that address the influence of microbiota on phytochemicals of natural products and vice versa; the anxiolytic activity of flavanols associated with the gut microbiota, effects of a natural product that counteracts gut inflammation and connected changes on GI microflora and finally, phytochemical composition of a natural product and its influence on microbiota [21]. The mice gut microbiota and the anxiolytic properties of kaempferol and quercelin (flavonoids) was studied in gut sterilised and normal animals. Gut sterilisation was conducted by treating the animals with Enrofloxacin. Anxiolytic effect of 1.0 mg kg^{-1} kaempferol was observed and also of 0.1, 0.5, 1.0 and 2.0 mg kg^{-1} quercetin in the non-sterilised mice [21].

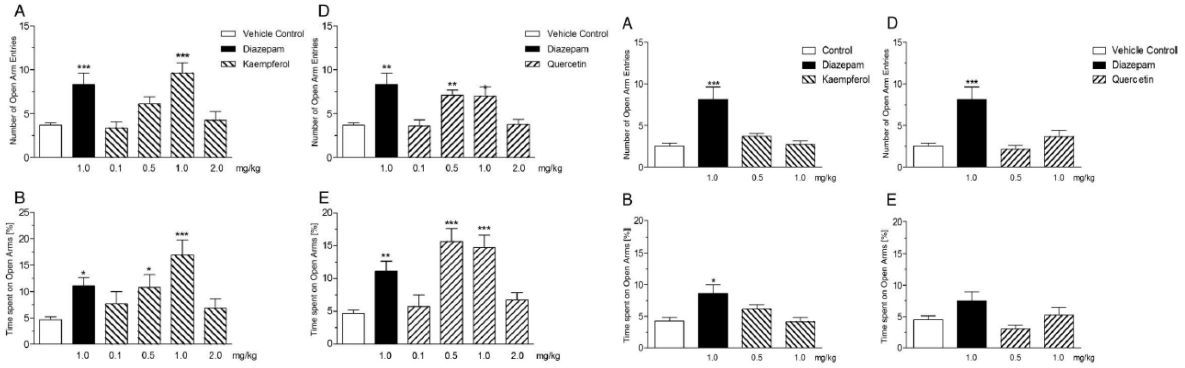


Figure 10: The bar plots show the anxiolytic activity of kaempferol and quercetin by the intestinal microbiota [21]

No anxiolytic effects of kaempferol and quercetin were observed in the Enrofloxacin-based intestinal sterilisation (see figure 10). Hence, it was concluded that the two flavanols are prodrugs that are essential to be activated by the gut microbiota in order to have a beneficial effect on anxiety [21]. Furthermore, a combination natural product, STW 5, was observed to counteract gut inflammation of Wistar rats. Gut inflammation was induced in an animal model of Colitis ulcerosa (UC) by administering DSS in the drinking water during the study [22, 23]. The images observed (see figure 11) reveal that the UC in the rat model, which had been induced by DSS administration, was successfully treated by the natural product STW 5 [22, 24].

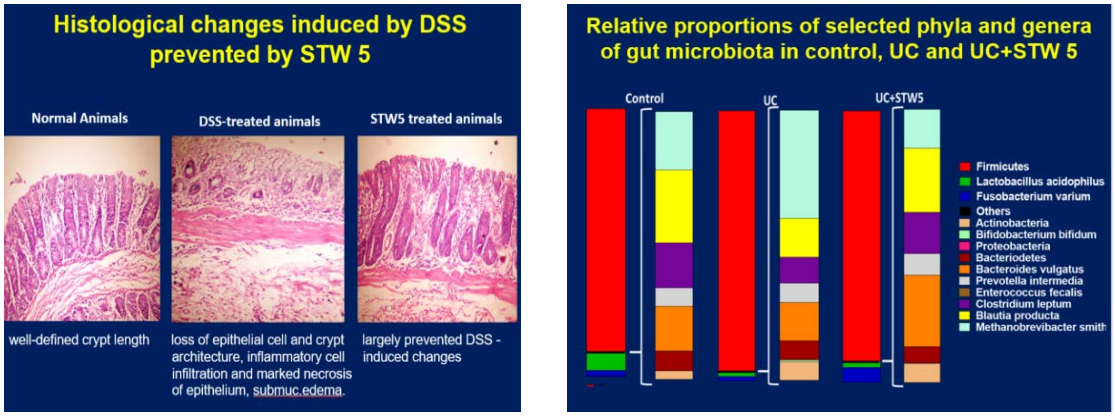


Figure 11: First figure (on the left) shows the changes in the histology observed in normal, DSS-treated and STW 5 combination treated rats. The second figure (on the right) shows relative abundances of gut bacterial population in control animals, UC induced and UC+STW 5 treated rats [24]

Fecal microflora of the study samples was subjected to (qPCR) based bacterial quantitative profiling. The relative abundances of selected bacterial genera and phyla are depicted in figure 11. The variations between the controls, UC induced (by DSS administration) and UC induced + STW 5 treated. In conclusion, the UC induced by DSS administration worked successfully and the treatment of UC by exposure to STW 5 natural product. This indicates the role of STW 5 in the gut inflammation treatment and hence potential influence of the natural product on the metabolic capacity of the intestinal microbiota [24].
Finally, the metabolomics of STW 5 was studied and its effects on the intestinal microbiota and vice versa. The study setup involved incubation of human fecal samples with STW 5 and metabolome profile analysis using (UHPLC-HRMS). The impact of fecal bacteria on STW 5 composition with time can be observed in the figure 12 below [25].

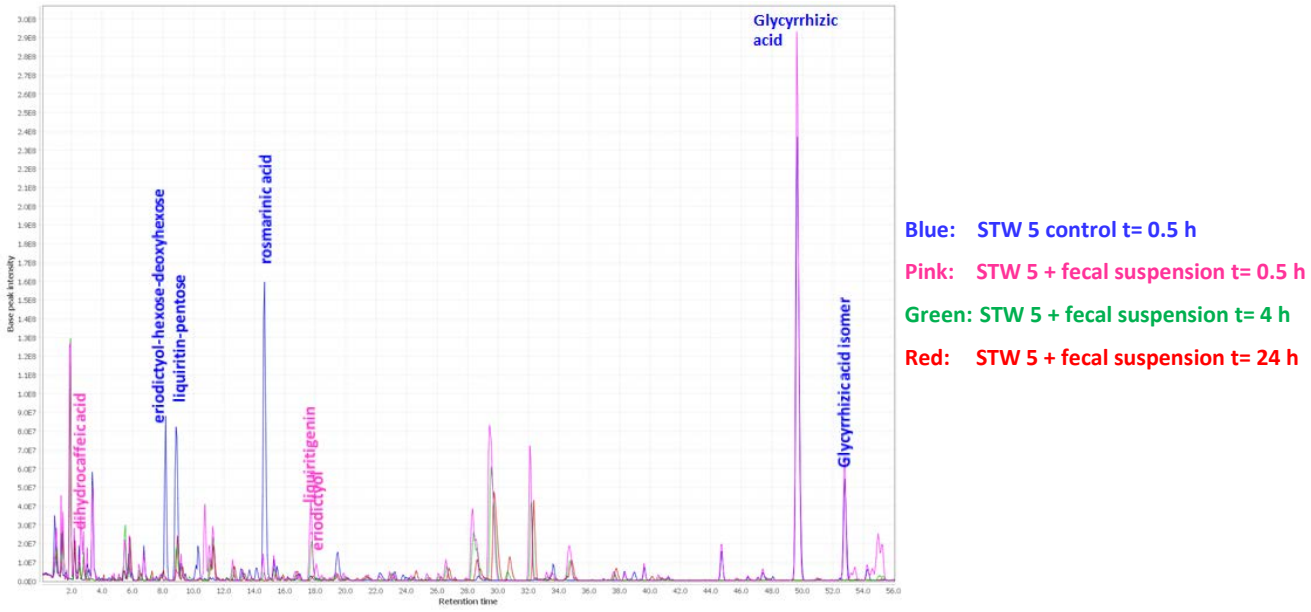


Figure 12: Chromatogram showing peaks from UHPLC-HRMS analysis of STW 5 treated fecal suspensions from different time points vs with controls [25]

Compounds like Liquiritin pentoside and Rosmarinic acid that were present in the natural product STW 5, were observed to undergo microbial transformation to form Linquiritigenin and dihydrocaffeic acid respectively. Additionally, it was observed than the concentration of STW 5 had low impact on the speed of the microbial metabolic reactions. The metabolite levels of liquiritin, glycyrrhizic acid and davidigenin were compared between 10 donors with respect to time (data not shown here).

Bacterial profiling conducted to study the impact of STW 5 on intestinal microbiota revealed time associated clustering (see in Figure 13). The (PCoA) clustering showed clear separation between the controls (PBS vehicle predigested) and the STW 5 treated rat fecal samples. Also, a very apparent time dependent separation was observed in both vehicle and STW 5 treated human fecal samples. This indicated imminent influence of STW 5 natural product on the gut bacterial population, which is also time-dependent.

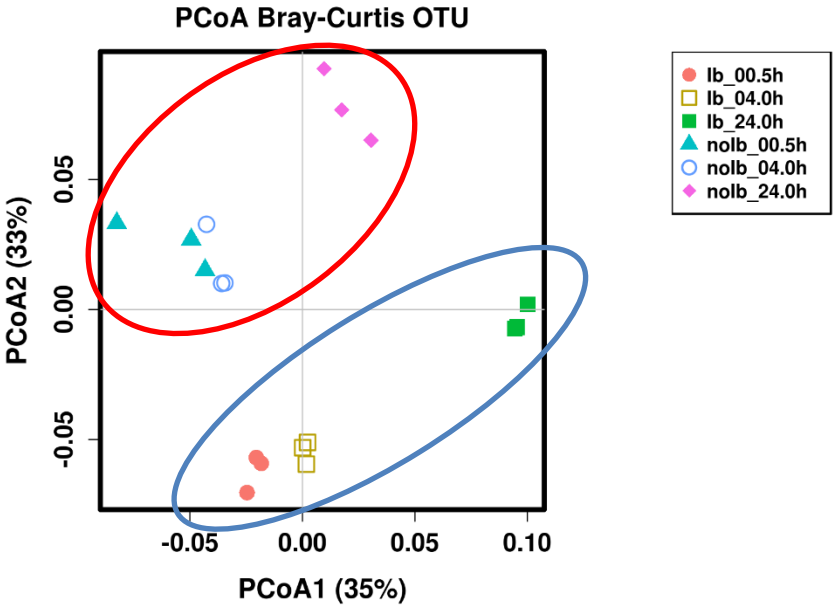


Figure 13: Principle co-ordinate analysis (PCoA) graph depicting the impact of STW 5 over time on the fecal bacterial populations

The effects observed were bidirectionaly as not only the intestinal microbiota had biotransformation properties on the STW 5 composition but also STW 5 has a time dependent effect on the gut bacterial population. Overall, it was concluded that the fecal microbiota could metabolise many phytochemical components of natural products (STW 5) with a strong impact on their structure and activities and with a large inter-individual difference. Natural products could considerable change the intestinal microbiota composition over time [26]

4.5 Presentation 5: Analysis of gut microbial xenobiotic metabolism in vitro by Dr. Silvia W. Gratz (University of Aberdeen, UK)

This presentation involved the impact of gut microbiome on mycotoxins and masked mycotoxins. Mycotoxins are toxic secondary metabolites produced by fungal species whereas the masked mycotoxins are the ones that are (re)activated by the GI microbiome to become toxic [27]. It is not known if the masked mycotoxins produce any direct toxicity or if they elicit indirect toxicity through the release of parent compounds in the gut. To assess this possibility, in vitro models were optimised to understand more about the fate of the masked toxins upon ingestion. The in vitro modeling systems involved 4 different features including the small intestinal hydrolysis activity, intestinal epithelial transport and toxicity mechanisms, colonic microbial metabolism activity and finally, colonic & microbiome toxicity[27].
Small intestinal hydrolysis of masked mycotoxins was analyzed using an in vitro model that comprised of mouth, stomach and Duodenum compartments along with the artificial saliva (pH 6.8), gastric juice (pH 1.3) and duodenal-bile juice (pH 8.1) respectively. The extraction of the mycotoxins and hence Mass Spectrometry based analyses was carried out [28]. The recovered mycotoxins can be observed in the figures below. As shown in figure 14, almost all the mycotoxins could be recovered, that indicate no (or very low) adsorptions of these 'masked' mycotoxins in the gut [28].

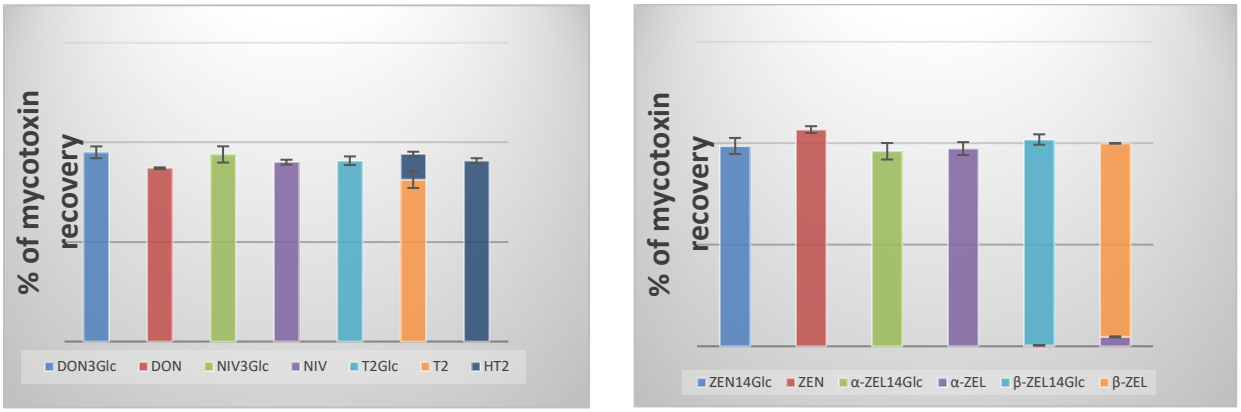


Figure 14: Lack of hydrolysis of masked and free trichothecenes (DON3Glc, ZEN, α-ZEL14Glc, α-ZEL, β-ZEL14Glc, β-ZEL) by artificial digestive juices of the upper gut containing digestive enxymes

Further, to study the intestinal transportation of masked Fusarium mycotoxins in vitro experiments with Caco-2 monolayers were conducted. Caco-2 cells act as the intestinal epithelial monolayer that mimic the in vivo intestinal walls (see figure 15). Fusarium mycotoxins were applied onto the apical/luminal part of the monolayer and hence LC-MS/MS based analysis of the apical and basolateral medium was conducted [28].

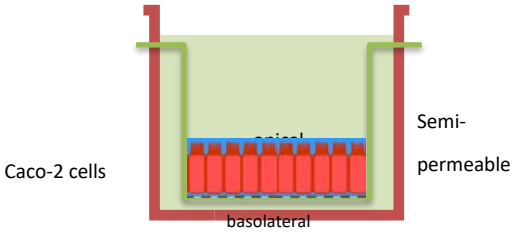


Figure 15: Schematic representation of in vitro model involving Caco-2 cells to study the transportation of Fusarium sp. mycotoxins through the cell monolayers from apical to basolateral compartment

Intestinal absorption was analyzed for various trichothecenes and zearalenone compounds. Some of the results are depicted in the figure 16 below.

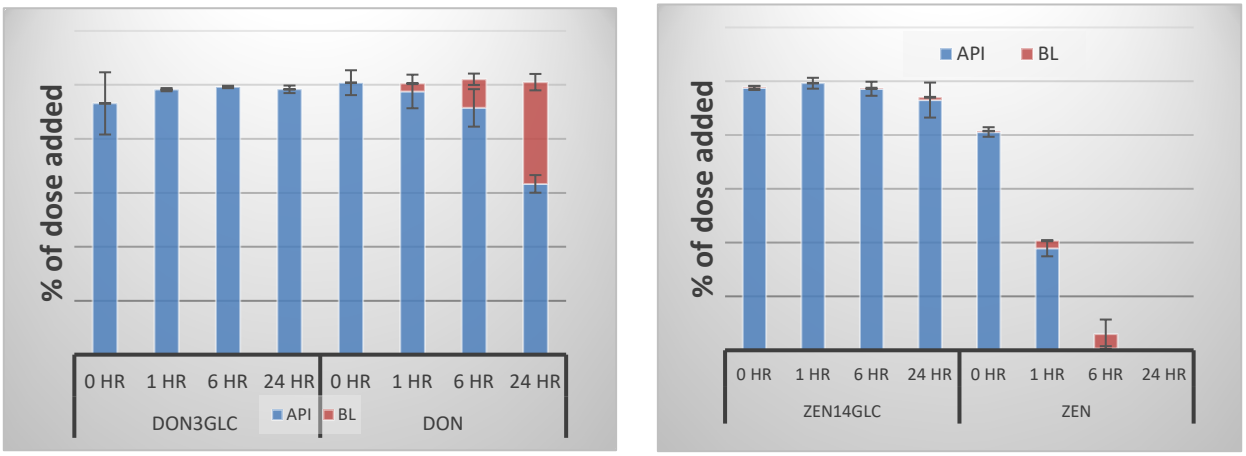


Figure 16: Transport of masked and parent trichothecene mycotoxins (DON3Glc and DON, left figure; ZEN14Glc and ZEN, right figure) through fully differentiated (Caco-2 TC7) monolayers from apical to basolateral compartment

To analyze the colonic microbial hydrolysis, fecal samples from 5 human donors were incubated anaerobically in M2 culture medium and spiked with individual masked mycotoxins. Extraction, clean-up and Mass Spectrometry-based analyses were carried out following the anaerobic incubation for 3 days. Colonic hydrolysis of different mycotoxins including type B trichothecenes, type A trichothecenes and zearalenone compounds were observed [28]. Some of the results have been shown below (see figure 17).

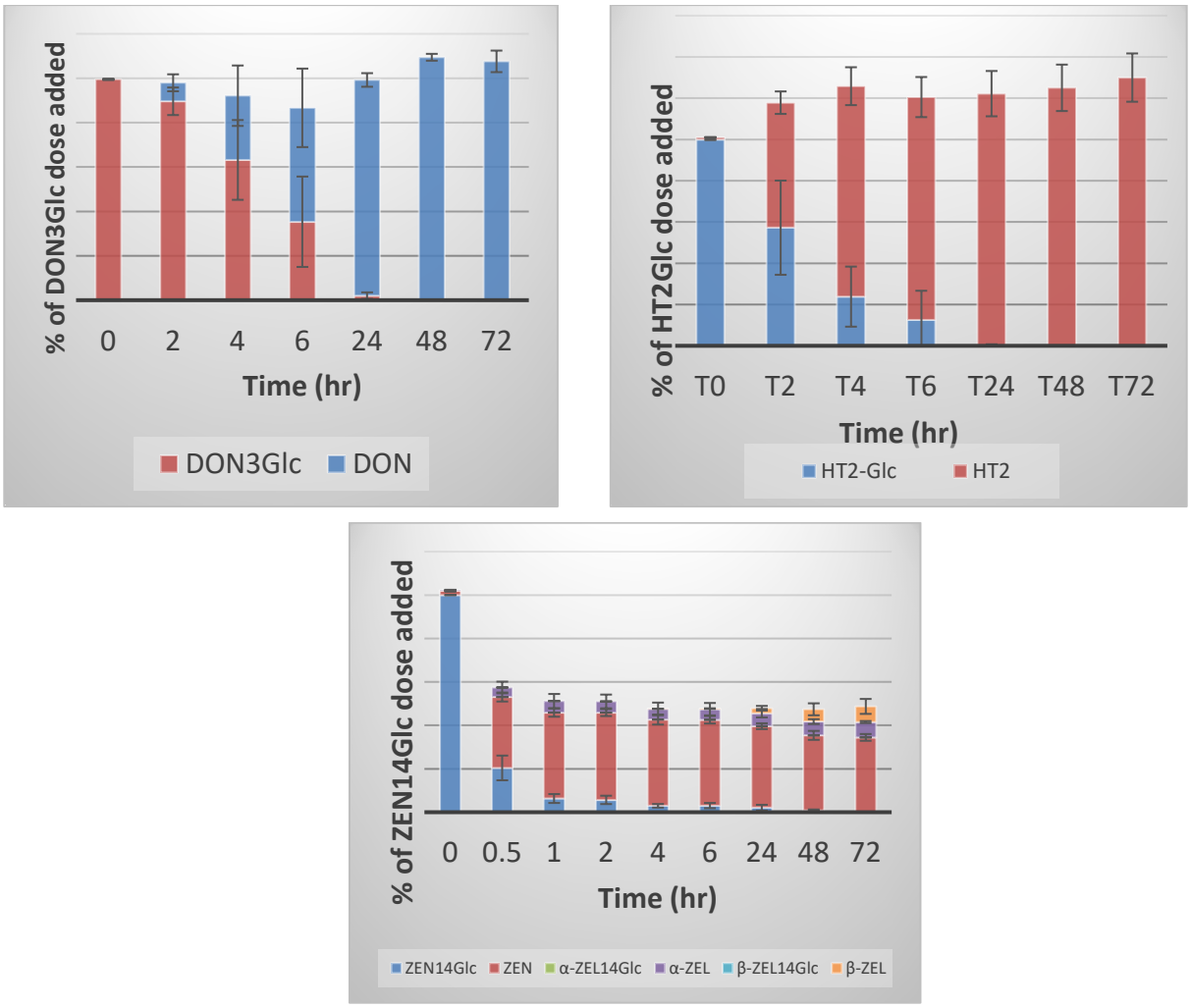


Figure 17: Hydrolysis of masked trichothecenes (DON3Glc, top left figure; HT2Glc, top right figure) and release of free parent trichothecenes (DON, HT2) by human fecal microbiota from 5 donors (0-72 h). Hydrolysis of ZEN14Glc (bottom figure) and release of free parent compounds (ZEN, α-ZEL and β-ZEL) by human fecal microbiota from 5 donors (0-72 h)

Detection of deoxynivalenol (DON) and diepoxy-deoxynivalenol (DOM-1) was performed in urine samples from human donors and metabolite profiling of the two mycotoxins were carried out using the human fecal samples. DON and DOM-1 are prominent mycotoxins produced by Fusarium sp. Microbial hydrolysis of masked mycotoxin (DON3Glc) in different gut sections was also observed [29]. The study plan involved anaerobic batch incubations of gut samples spiked with the masked mycotoxin, followed by extraction, clean-up and LC-MS/MS analysis (see figure 18). Batch cultures of fecal samples are known to mimic the hydrolysis reactions in the large intestine in vivo [29].

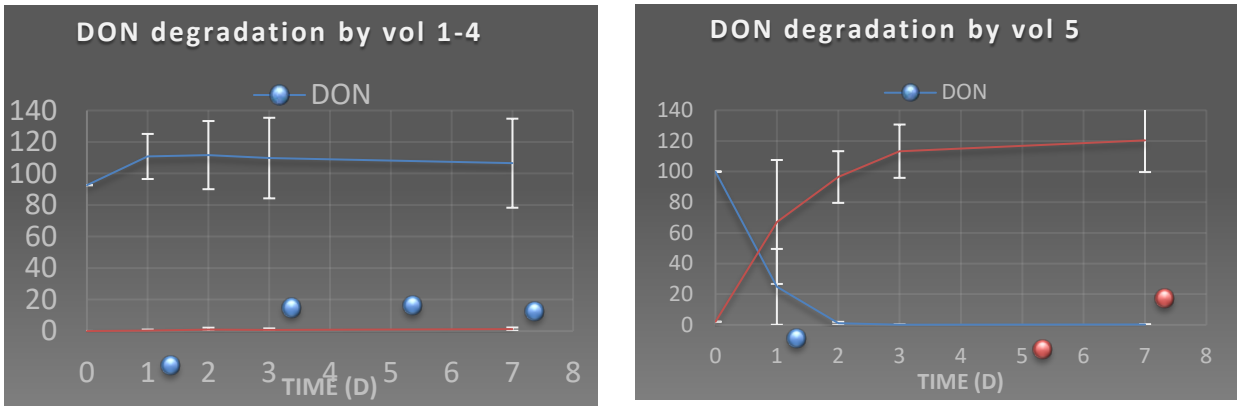


Figure 18: Kinetics of metabolism of DON to DOM-1 by the fecal microbiota of donors 1-4 (left figure) and donor 5 (right figure)

See in figure 19, the hydrolysis of DON3Glc and release of free DON by porcine intestinal microbiota from different regions of the small and large intestines were observed over 0 to 72 h [30]. Furthermore, fecal batch cultures were made in order to mimic the hydrolysis in other regions of the large intestine and was observed that hydrolysis process already starts in small intestine.

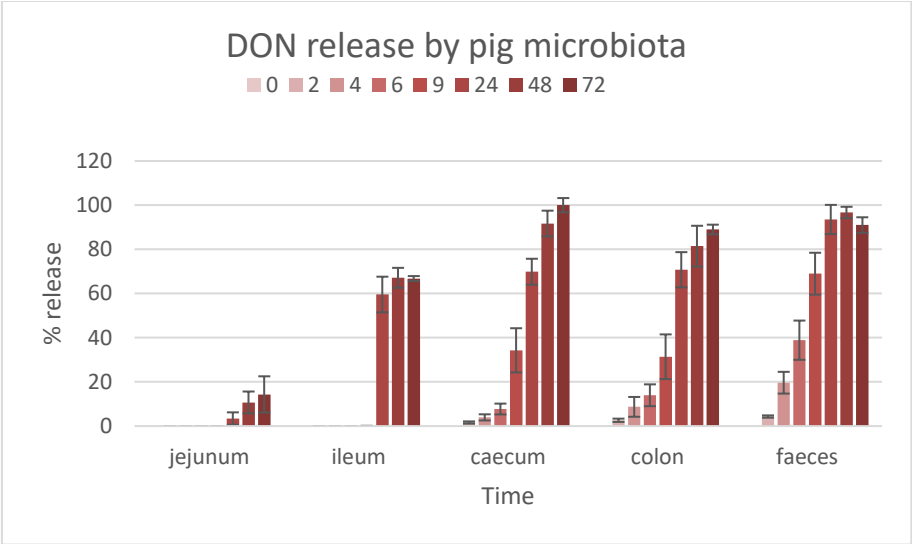


Figure 19: Percentage release of DON by pig fecal microbiota during different time points from the different gut regions was observed

In order to understand which bacterial intestinal species are responsible for mycotoxin metabolism, strain selection was carried out. The result showed major influence of *Bifidobacterium adolescentis* and *Bacteroides thetaiotamicron* [30]. Hence, the most important contributors to mycotoxin release were identified. Anaerobic growth curves of bacterial growths in the presence of DON mycotoxins were observed for the two bacterial strains. It was concluded that the masked mycotoxins have no effect on the bacterial growth [30].
To understand further about xenobiotic toxicity, Caco-2 cell monolayer models were used as gut model systems. The Caco-2 monolayer models could facilitate studies on xenobiotic transport, metabolism and toxicity. Intestinal organoids mimicking the mouse ileal model system was exposed to DON and comparative images were produced in order to observe crypt development compared to the control in vitro models (figure 20).

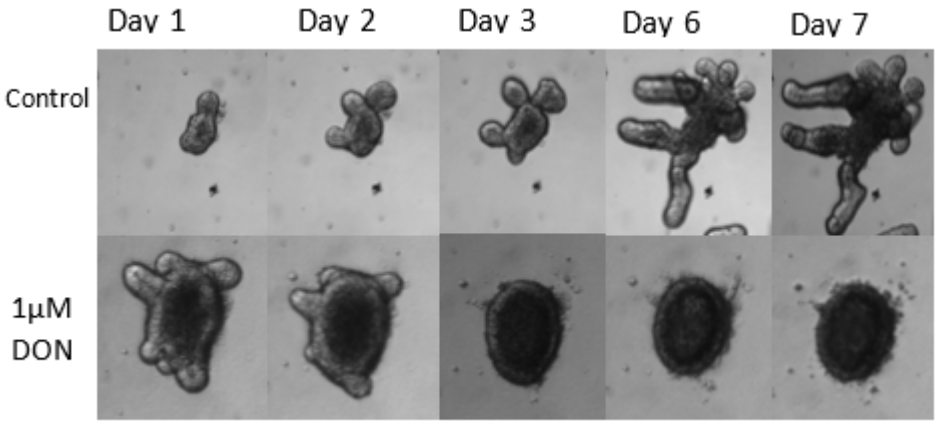


Figure 20: Mouse ileal organoids exposed to DON observed on different time points

Mycotoxins have a high potential for future research regarding their role in intestinal disorders. More studies would aid in understanding more about the potential targets of mycotoxins in the intestine.

4.6 Presentation 6: Functional characterisation and modulation of human gut microbiome by Patricia Lepage (INRA Research Center, FR)

The main areas of research include understanding the gut ecology, meta-omic technologies, data analysis and integration using biostatistical methods and anaerobic cultivation of fecal samples to learn about the bacterial consortia. Two major research work were predominantly discussed; functional and metabolic adaptation of Coriobacteriaceae bacteria in the context of lipid metabolism and understanding he role of gut microbiome in anticancer immunotherapy [31].

Coriobacteriaceae are the family of bacteria that have a role in type-2 diabetes, high triglycerides and cholesterol formation. They possess important metabolic functions including conversion of bile acids, steroids or phytoestrogens. Three Coriobacteriaceae strains namely, *Collinsella aerofaciens*, *Egerthella lenta* and *Enterorhabdus mucosicola* are known to be associated with metabolic disorders in humans *[32]*. To understand if the metabolic potential of this bacterial family influences host lipid metabolism, germ free mice were exposed to each of the 4 bacterial strains (*Atopobium parvulum*, *Collinsella aerofaciens*, *Eggerthella lenta* and *Enterorhabdus mucosicola*) with control diet, high-fat diet and bile acid (BA) diets. High fat diet was observed to trigger significant increase in the body weights of all housing groups, whereas the bile acid diet did not influence the body weights of the animals. CORIO mice (5 weeks old) fed with bile acid rich diet had a different body composition with increased fat mass [33].

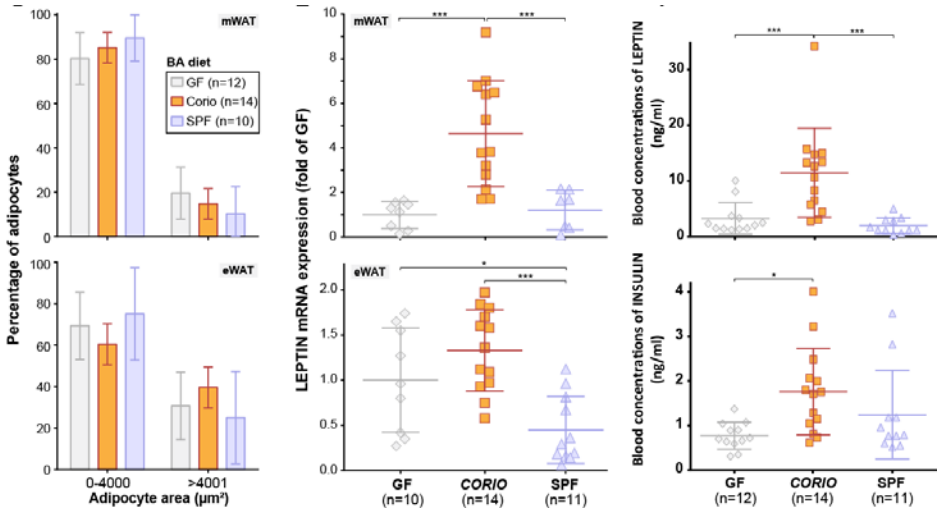
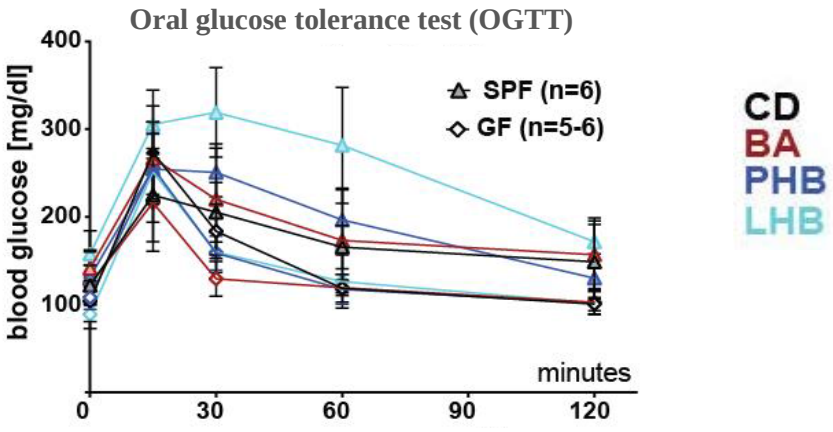


Figure 21: Bar plots showing significant increase in the adipocyte size of BA diet on germ free (GF), CORIO and complex microbiota (SPF) mice, the figures in the middle show alterations in WAT mass accompanied by high leptin gene expression and the figure on the right show increased blood insulin and leptin levels. CORIO mice fed with BA diet showed signs of metabolic disturbances

No significant increase in adipocytes size was observed (see figure 21). Increased WAT mass accompanied by higher gene expression of leptin in mesenteric (mWAT) and epididymal (eWAT) WAT were observed and increased leptin and insulin blood levels were seen in the CORIO mice fed with (BA) diet (see Figure 21). These alterations in the metabolite levels indicate the effect of the diet on the health of the mice models [33]. The Coriobacteriaceae-induced increase in WAT mass under BA fed was hypothesised to be a result of high fat cell size or hypertrophy or increased fat cell number or hyperplasia. In order to obtain detailed information about possible pathophysiological cellular mechanisms associated with increased WAT mass, non-targeted proteome analysis of (eWAT) collected from BA-fed mice was performed. The results showed 12 of the CORIO-specific proteins to be related to lipid metabolism. In conclusion, CORIO mice were characterised by systemic hypercholesterolemia and increased cholesterol levels in (iWAT) when fed BA diet and intestinal Coriobacteriaceae has the ability to modulate host metabolism, particularly in lipid homeostasis mechanism [33].

Further, to study the impact of fat source, dietary supplementation with primary bile acids in a combination with fat enrichers from different fat sources was conducted on germ-free (GF) and (SPF) rats. Higher body weights in SPF mice than GF was observed and both high fat diets (lard-based high fat diet and palm oil) increased body weights when combined with BA for 8 weeks and SPF mice were observed to have a higher increase in WAT mass compared to palm oil diet. Oral glucose tolerance test (OGTT) showed impaired glucose tolerance in lard-fed diet SPF mice when compared to palm oil fed mice. Lard diet was observed to have a detrimental impact on host metabolism when in combination with bile acids, but only in the presence of endogenous gut microbes [33].



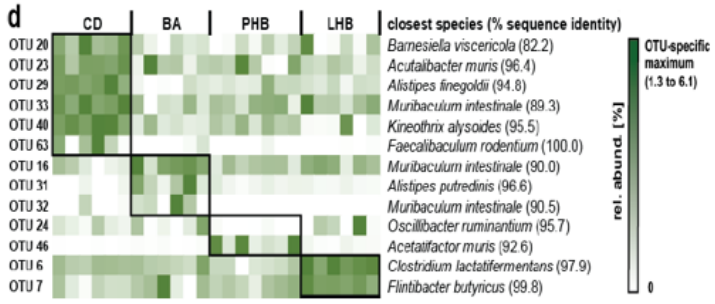


Figure 22: Results from impaired glucose tolerance in lard-fed SPF mice when compared to palm oil group (top figure) and heat map showing relative abundances of dietary group-specific OTUs (bottom figure)

Bacterial community profiling was then conducted to observe the diet induced shifts in the gut bacterial profiles using caecal samples (figure 22). Relative abundances of OTUs were observed in control diet, Bile acids, palm oil and lard-based high-fat diet (PHB and LHB respectively) fed mice. LHB feeding was seen to be associated with increased bacterial species richness. Furthermore, metatransciptomics pipeline was followed on 18-week caecal content of mice to produce a mouse metagenome catalog. The mouse metagenome catalog (iMGMC) was observed to increase the number of aligned reads, on an average, 2 million (mRNA) reads were successfully mapped onto 180,412 genes from the iMGMC [34].

Mouse Gene Catalog (MGC)

Integrated mouse gut metagenome catalog (iMGMC)

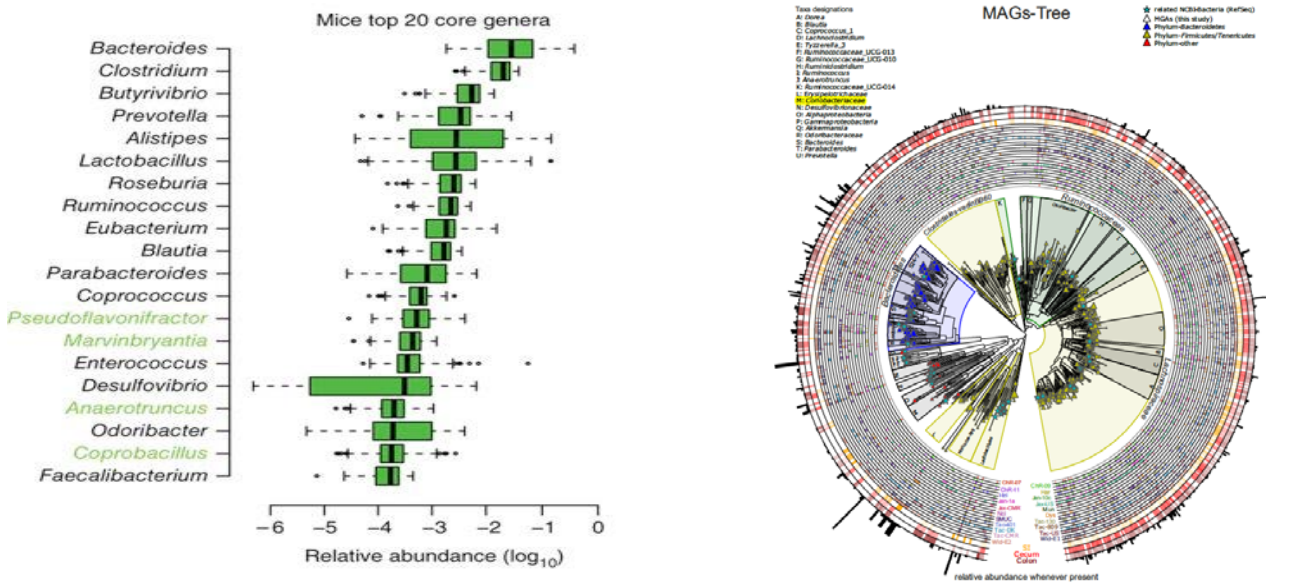


Figure 23: The top 20 most abundant core bacterial genera in mouse gut microbiota (left figure) and MAGs-tree constituting 4.6 million nonredundant genes; 660 MAGs / 1.323 16S rRNA and DNA from fecal samples of 298 mice (right figure)

Next important translational research in the lab involved studying the role of gut microbiome in anticancer immunotherapy. Antibody anti-CTLA4 (αCTLA4) (IFX) plays a role in destroying the tumour cells and its efficacy has been known to require the presence of specific gut bacteria. αCTLA4 have been observed to efficiently reduce tumour cell sizes in the presence of bacterial species, such as*, B. thetaiotaomicron*, *B. fragilis* and combination of Burkholderia sp. and *B. fragilis*. Anti-tumour response of CTLA-4 in the presence of the previously mentioned gut bacterial species have been well characterised [35]. In melanoma patients, 8-22% of treated patients receiving (IFX/Ipi) limumab showed an improvement and hence reduction in the tumour

levels. 3 bacterial enterotypes; Firmicutes, Bacteroidetes and Prevotella were observed in melanoma patients at baseline (see figure 24) [36].

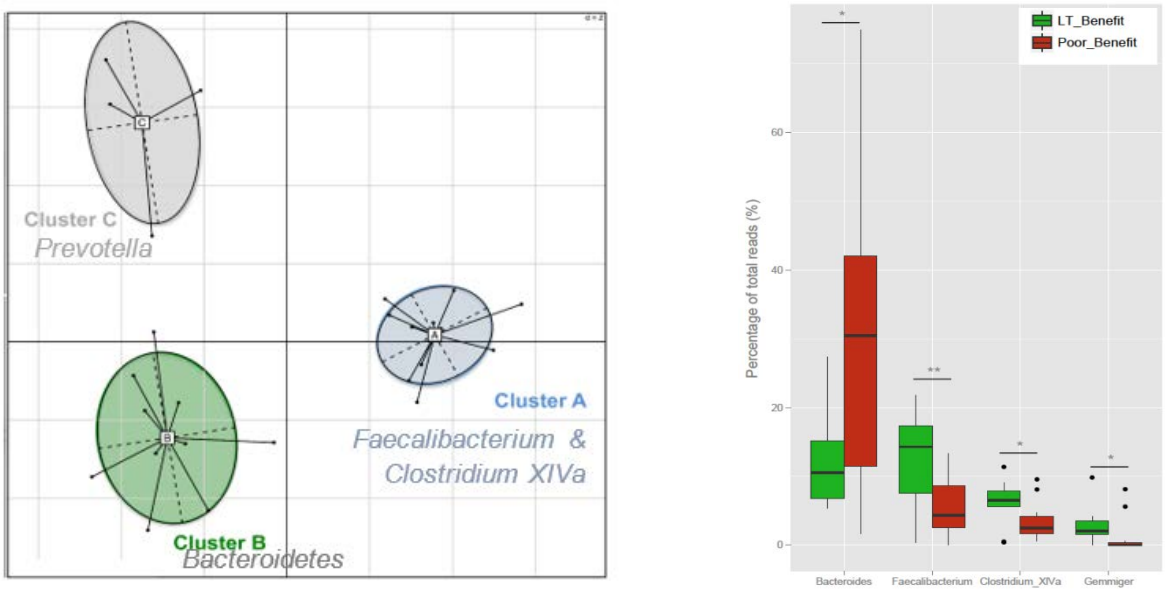


Figure 24: Inter-class principal component analysis representing the clusters of 3 dominant bacterial genera (left figure). Boxplot of the percentages of four dominant genera differentially represented between both groups, Bacteroides, Faecalibacterium, Clostridium XIVa and Gemmiger (right figure) (LT_Benefit = long term benefit)

Role of gut microbiota as an efficacy predator of ipilimumab treatment was observed and high percentage of Bacteroidetes showed poor benefit to IPI whereas, high Faecalibacterium showed long-term benefit to IPI treatment to melanoma patients. Clearly, a link was found between Ipilimumab-mediated colitis to microbial dysbiosis. High Bacteroidetes were observed to be linked to Colitis protection in some patients [36].

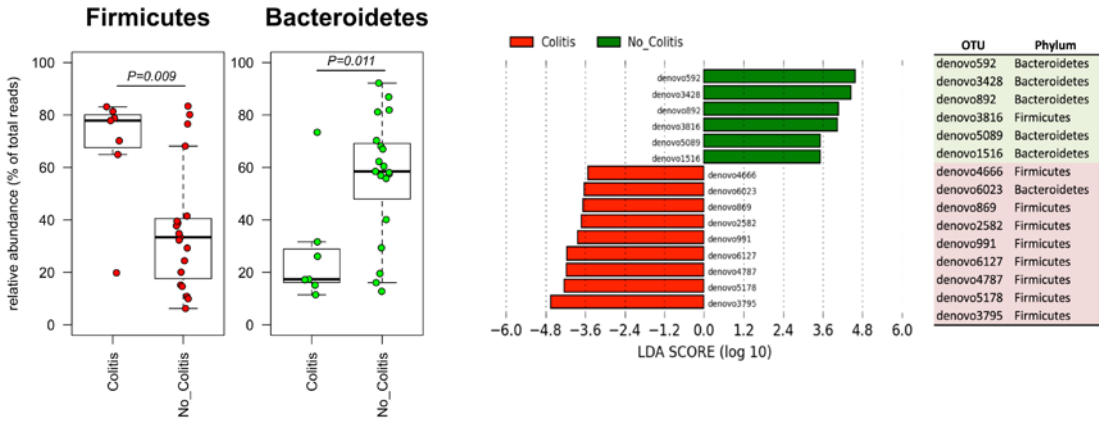


Figure 25: Boxplot of relative abundance of the two dominant phyla Firmicutes and Bacteroidetes at baseline between patients prone to or resistant to ipilimumab-induced colitis (left figure). OTUs predictive of colitis development during ipilimumab treatment (right figure)

Some specific OTUs were found to have functions in predicting immune-mediated colitis associated with Ipilimumab in melanoma patients (see figure 25). This study proved to confirm the intestinal microbiota influence on systemic immunity and anti-tumour immunity [36]. It is still unknown regarding the mechanism of gut microbiota composition that could have an impact on a distant tumour and the possible relation between the immune checkpoints and microbiota composition.

4.7 Presentation 7: The fecal metabolome as a functional readout of the gut microbiome (WebEx) by Dr. Cristine Menni (King's College London, UK)

Metabolomics is one of the new high throughput 'omic technologies that has the ability to measure endogenous metabolites in cells, tissues and other matrices such as blood, urine, saliva and feces. This omics method encourages comprehensive and simultaneous systemic determination of metabolite levels and their changes with respect to time. Blood metabolomics aid in the identification of useful biomarkers for prediction or diagnostic purposes. Fecal metabolomics on the other hand, provide further insight into the interplay between the host, intestinal microbiome and diet.

The aim of the research work was to provide a comprehensive description of the fecal metabolome including associations with age, gender and obesity, host genetic influences and dependencies with the gut microbiome. The study involved fecal metabolome profile of twin human donors followed by bacterial profiling using 16S sequencing data [37]. 786 fecal sampling from a population-based twins study (TwinsUK), was carried out and from their metabolome profiling data, 894 out of 1116 identified metabolites were known. It has been already studied that the number of metabolites in fecal samples is higher than in blood. Apparent age dependent separation of metabolites and obesity associated metabolite profiling have already been known [37].

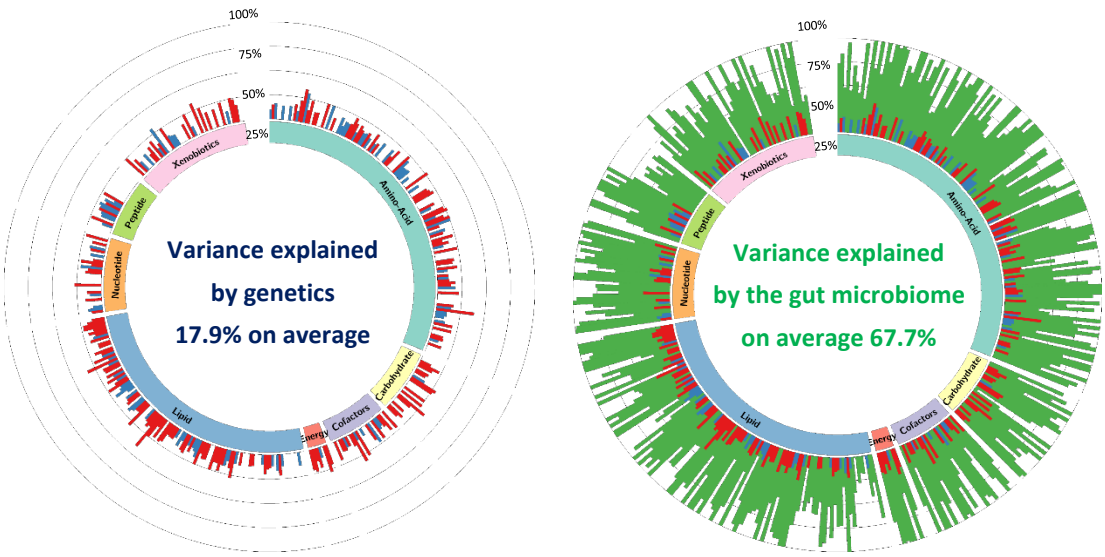


Figure 26: The fecal metabolome, on examining 1,116 metabolites from 786 individuals from a population-based twin study (TwinsUK), was found to be only modestly influenced by host genetics (17.9%) (left figure). The fecal metabolome largely reflects gut microbial composition, explaining on average 67.7% of its variance (right figure)

Variances of various classes of metabolites associated with the gut microbiome have been observed to be 67.7% on an average (see figure 26). Alpha and beta diversity of the fecal metabolome associated microbes are shown in the following figures (figure 27). In conclusion, the fecal metabolites are influenced by host genetics, they provide a functional readout regarding the gut microbiome and functional annotation for the intestinal microbes [37].

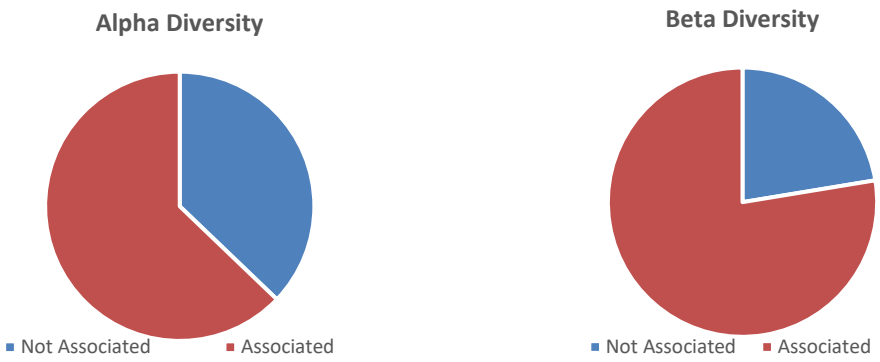


Figure 27: Pie charts showing fractions of alpha diversity and beta diversity which are explained by gut-associated and non-gut-associated microbes.

4.8 Presentation 8: Using PBK modelling to predict plasma concentrations of intestinal microbial metabolites of xenobiotics by Dr. Karsten Beekmann (Wageningen University & Research, NL)

The intestinal microbiome is considered as one metabolically active organ on its own. It is known to have metabolic capacity equivalent to liver. The research work aimed at developing in vitro methods in order to study chemical compound-microbial interactions. The study involved determination of reaction rates of chemical metabolic processes using physiologically based kinetic modeling methods (PBK) and assessment if adverse effects of the chemicals on the intestinal microbiome (using metabolomics). This was necessary to also understand interspecies and interindividual variations.

The PBK models comprised of compartmentalisation describing the kinetics of different organs. This model would then be used in reverse dosimetry (see figure 28). PBK based reverse dosimetry would translate the in vitro concentration response data to in vivo dose response curve in order to predict adverse effects. This method helps in risk assessment strategies. The methodology for the in vitro experiments involved anaerobic small batch incubation of individual and pooled fecal samples from rats, mouse, pigs and human donors, followed by enrichment and washing of samples and hence analyses by LC-MS/MS, UPLC based methods.

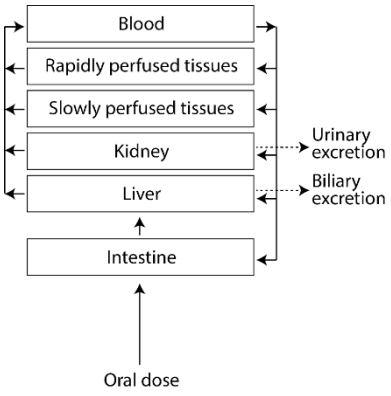


Figure 28: Schematic diagram of the PBK model used for reverse dosimetry-based prediction of in-vivo concentration-response using in vitro dose-response data

Two research topics were addressed during this presentation; the role of gut microbial and mammalian co-metabolism in the toxicity of zearalenone & the role of gut microbiota in the estrogenicity and related health effects of the isoflavone daidzein (EFSA CONTAM Panel, 2017, EFSA Journal 15(7):4851). Zearalenone is a mycotoxin produced by *Fusarium* sp. that contaminate crops like wheat, maize, barley, etc. They are estrogenic compounds that result in reprotoxic effects and infertility. Humans have been known to be the most sensitive species due to high production of a-zearalenol (a-ZEL), which has the highest estrogenic potential. Fecal slurry incubation methodology was optimised for rat, pig and human models in order to obtain a linearity in the fecal slurry concentration over time (unpublished).

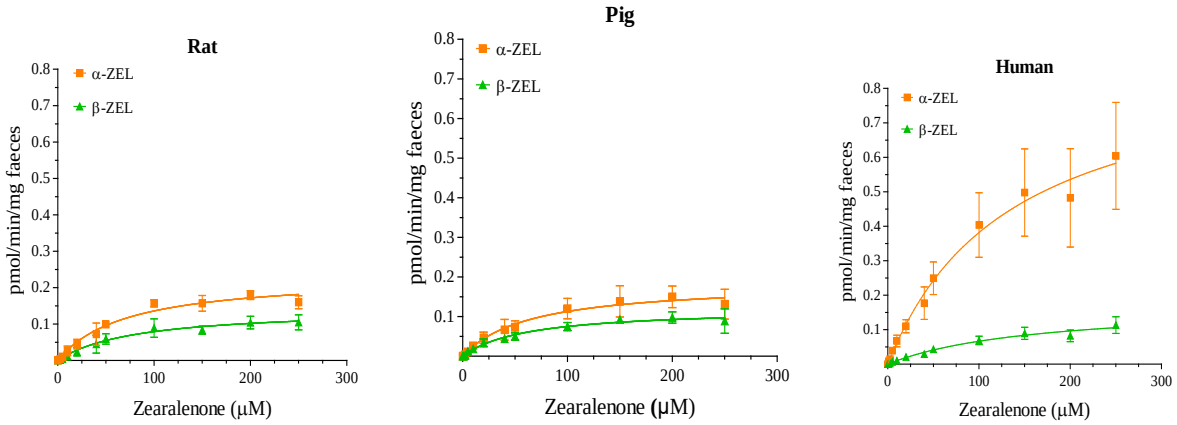


Figure 29: Concentration-dependent formation of α-ZEL and ß-ZEL in incubations with pooled fecal samples of rat, pig and human respectively (unpublished)

After in vitro anaerobic fecal cultivation from rat, pig and human slurries, formation of a-ZEL and ß-ZEL were observed (see figure 29). It was concluded that humans have the highest a-ZEL:ß-ZEL ratio compared to the other animal models as shown in the figures. Furthermore, a comparison between the de/toxification activity of ZEN mycotoxins by intestinal microbiome and liver was conducted (figure 30).

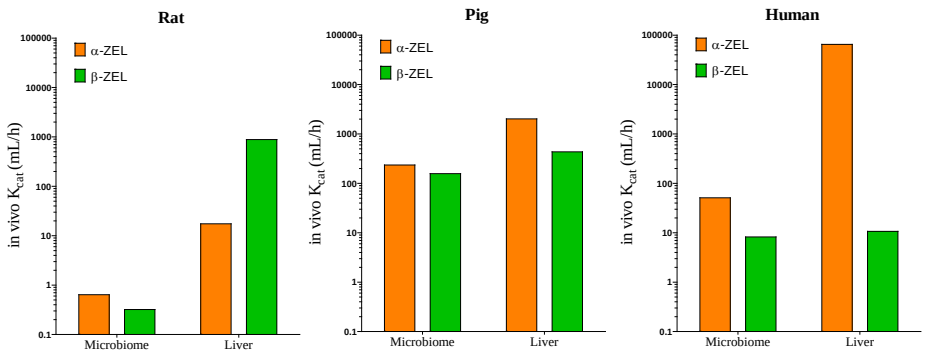


Figure 30: Comparison of the calculated in vivo kcat from microbial and hepatic formation of α-ZEL and ß-ZEL from ZEN in rats, pigs and humans respectively (unpublished).

In the next study was performed to better understand the role of gut microbiota in the estrogenicity of isoflavone daidzein, interindividual differences of intestinal microbial metabolism were analyzed. The study involved in vitro cultivation of rat fecal slurries, optimisation of linearity of slurry concentration over time and

adaptation at low concentrations. Kinetics of microbial metabolite formation (like DHD, (S)-equol and O-DMA) were determined using Michaelis-Menten kinetics (see table 1) (unpublished).

Table 1: Kinetic parameters for formation of Daidzein gut microbial metabolites

	V_{max} (µmol/h/g feces)	K_m (µM)
DHD	0.35	1.69
(S)-equol	0.28	1.08
O-DMA	0.04	2.42

PBK model was standardised in order to predict the (S)-equol concentration in the plasma after daidzein ingestion. The in-silico model system contained compartments depicting the intestinal microbiome (figure 31). The predicted concentrations of daidzein and (S)-equol in the presence and absence of microbiota (and associated metabolism) in the rat plasma after the ingestion of daidzein were found to be in-line with the concentrations reported in literatures.

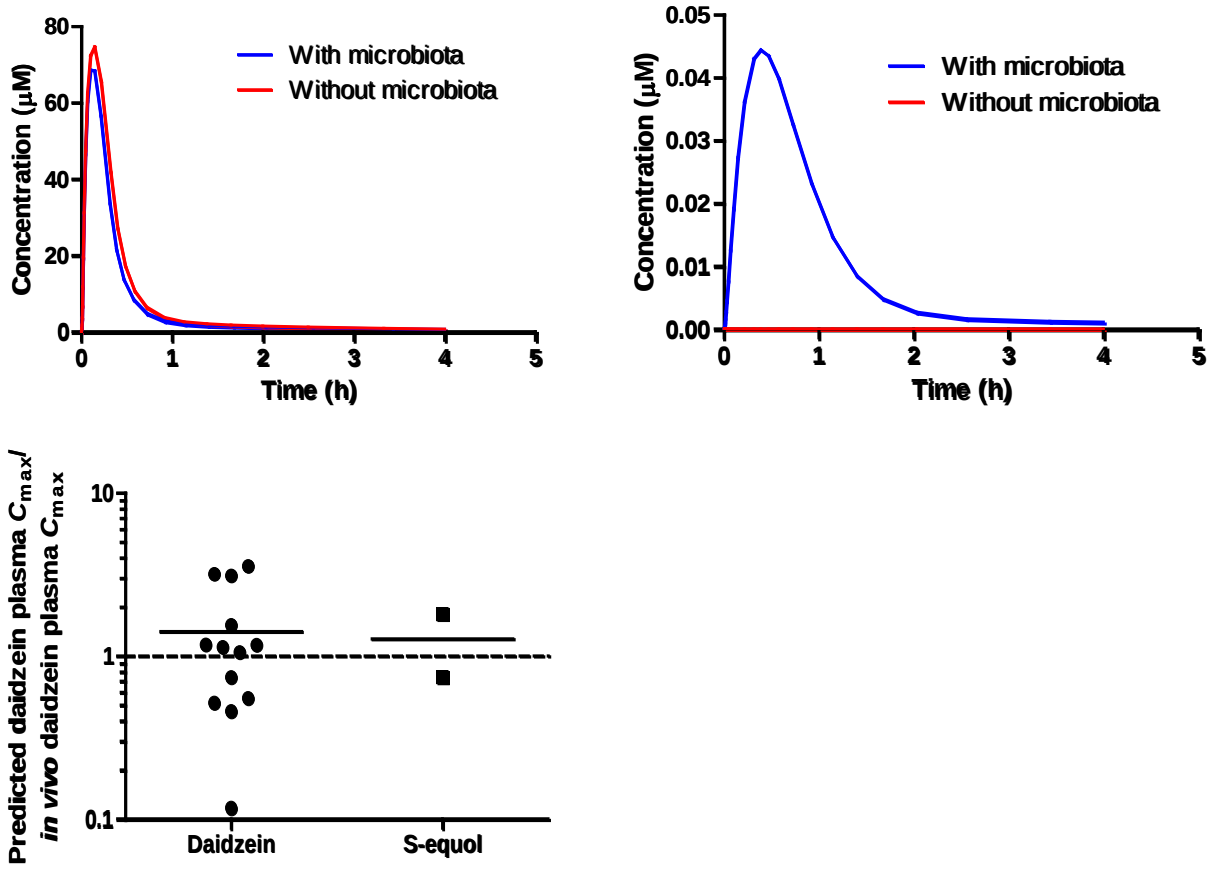


Figure 31: The curves PBK model predicted plasma concentrations of S-equol (left) and daidzein (right) upon oral dosing of 20 mg kg^{-1} (bw) daidzein (top figures). Predicted plasma Cmax compared to in vivo Cmax of daidzein and S-equol upon oral dosing of daidzein (bottom figure)

4.9 Presentation 9: Functional analysis of the gut microbiome using in vitro methodologies by Dr. Frédéric Moens (ProDigest, BE)

The motivation behind the in vitro models was to setup complete simulation of the entire gastrointestinal tract. This involved in vitro compartment models that mimic stomach, small intestine and colon. This would aid in understanding the digestion, absorption, microbial fermentation in the colon, host-microbiome interactions, survival of probiotics, and many more. Also, in order to understand complex interactions between the host and its microbes would need in vitro methods as the in vivo sampling would not be feasible.

Figure 32: The image shows (SHIME®) system (Simulator of the Human Intestinal Microbial Ecosystem)

The various difficulties in order to understand the intestine and its functions include the use of invasive methods (like endoscopy) to get direct access to the region, in most cases only the input and output data are available and not the underlying intestinal process, high difficulty to get mechanistic insight, etc. To address such difficulties, alternative methods serve the best as they do not pose ethical issues and have higher possibilities to get a true insight into the intestinal processes.
In vitro gut simulator TWINSHIME® was developed by ProDigest to simulate the human intestinal microbial ecosystem (see figure 32, 33). Understanding the microbial functionality through metabolism-associated outcomes by exposure to xenobiotics would aim to provide a link between the composition and functional output. This solved the need for in vitro models that simulate inter- and intra-individual microbiota variation to obtain a mechanistic understanding of microbial xenobiotic metabolism.

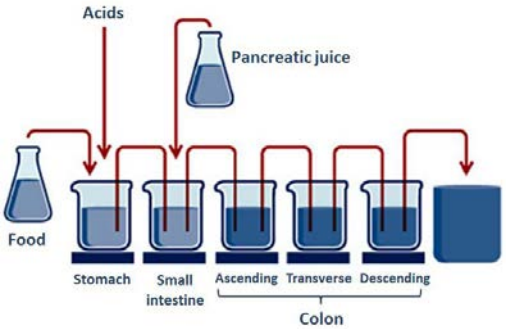
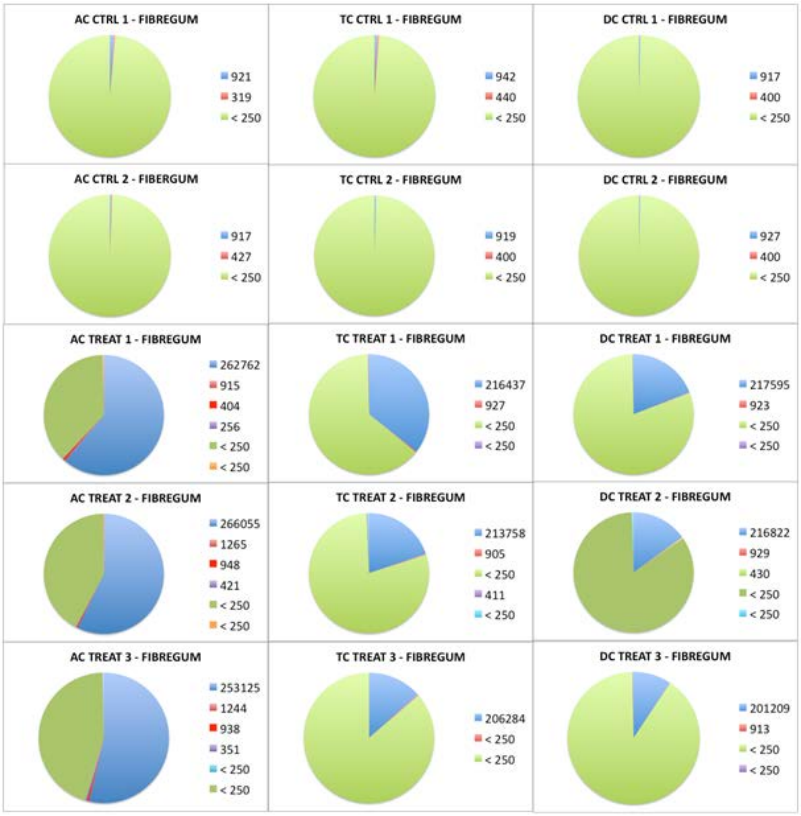


Figure 33: Schematic diagram of the working of a SHIME® in vitro cultivation method performed to mimic the in vivo gut construct

Modeling of intestine using (SHIME®) technology involved simulation of GI tract physiology, different regions of GI tract, lumen, mucus and colon microbiota derived from a healthy donor. This method encourages mode of action (MoA) studies and also deeper understanding to xenobiotic metabolism. Some complex challenges could be the different dynamics between the different intestinal regions, various gradients in the GI tract with respect to microbial colonisation, activities, shift in populations, pH, metabolic process, etc. Between the

colonic region, the proximal colon is known to be saccharolytic and the distal colon, proteolytic in function. The solution to such challenges would be to separately model the different colon regions in order to study the intestinal microbiome in detail. Phylogenetic characterisation of the SHIME® colon microbiota using the (HITChip) method revealed diverse and unique microbiota in different regions [38].
Furthermore, to understand the prebiotic fiber fermentation in the GI tract and its effects, SHIME® technology was used. Prebiotic treatment was used in order to study the cause-consequence relationships by eliciting a change using one factor (i.e. prebiotic fiber). Residual fiber Arabinogalactin was observed to be mainly fermented in traverse and descending colon. For the fiber analysis, size-exclusion chromatography method was used (see figure 34).



(Green = background, Blue = fiber)

Figure 34: The figure shows a gradual decrease in Arabinogalactan fermentation.

The relative abundances of the overall mucosal microbiota were observed where L-SHIME indicates the old model and M-SHIME is the model with lumen and mucosal microbiota included (figure 35). Mucus seemed to be predominantly colonised by Firmicutes species that were much less present in the old luminal model, L-SHIME. On conducting the phylogenetic analysis of the microbes, it was observed that the mucus is specifically colonised by Clostridium species and is devoid of Bacteroidetes and Proteobacteria [38]. The mucus layer was found to be colonised by probiotic Lactobacilli. This species-specific colonisation of the mucosal compartment could be understood using M-SHIME technology [39].

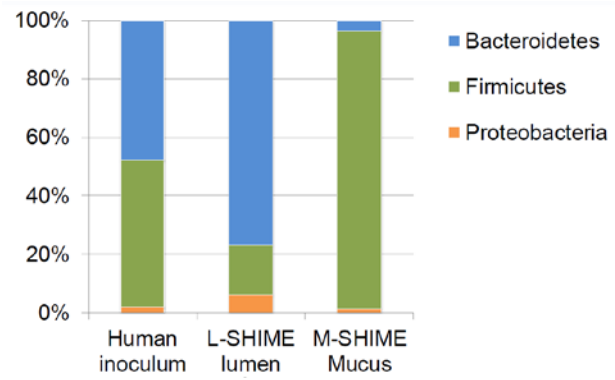


Figure 35: Relative abundances of overall mucosal microbiota showed highly specific colonisation by Firmicute bacterial phyla.

8-prenylnaringen (8-PN) is known as the strongest estrogen-like substance in nature. To compare the production of 8-PN in vitro and in vivo, SHIME® technology for fecal microbiota incubation was used. The study involved fecal samples from female donors with high, moderate and low 8-PN production (see figure 36). The 8-PN production of these in vitro samples were compared to the 8-PN quantified from urine samples (in vivo samples).

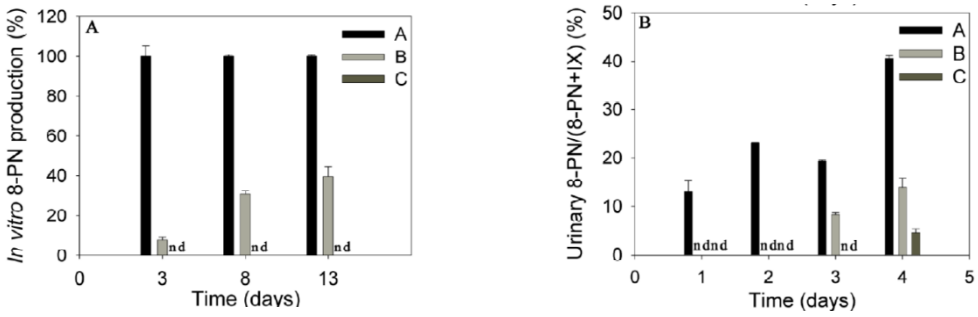


Figure 36: The box plots show 8-PN production in vitro (left figure) compared to in vivo (urinary) production (right figure)

SHIME Technology could aid in mimicking luminal and mucosal microbiota, gaining insight regarding region-specific fermentation of fibers (prebiotic), understanding mucosal colonisation by probiotics and translating the in vivo functionality in vitro. This model proves to be a big step in the application of alternative methods to gain in-depth knowledge regarding the complexity of in vivo processes.

4.10 Presentation 10: Investigation of host-microbiota co-metabolism as a new strategy for biomarker discovery – New chemical biology tools for Metabolomics analysis of the gut microbiome using in vitro methodologies by Prof. Daniel Globisch (Uppsala University, SE)

Discovery of biomarkers/metabolite patterns has a huge influence in the fields of toxicology and pharmacology. The investigation of microbiota-derived metabolites aims to pave the way to the identification of biomarkers. Gut microflora is known to have an eminent impact on the host physiology, personality and drug efficacy. Human-gut microbiota co-metabolism provides in depth understanding regarding the various interactions and their role in health and disease [40].

LC-MS based metabolome profiling methods are very well known in order to analyze metabolites in various biological matrices. Some of the challenges of this method of detection are lack of tools for selective investigation, structural identification of unknown metabolites, selective investigation of microbial metabolites & quantitative analysis of metabolites in the pmol to (fmol)HRMS range.

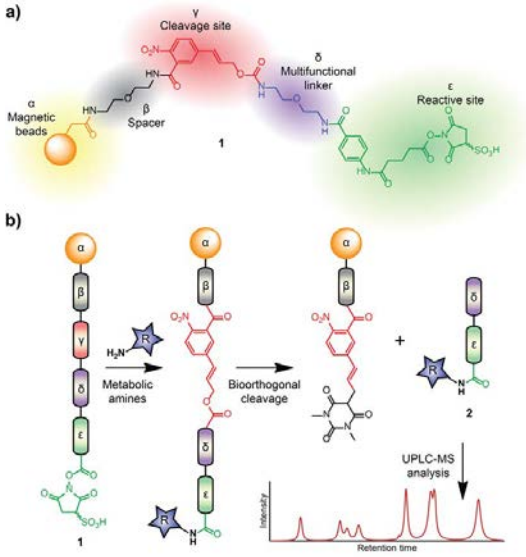
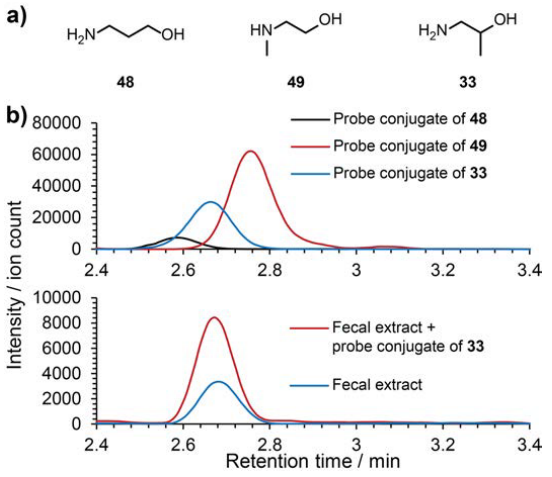


Figure 37: a) Chemical probe design. The reactive site bearing an amine-specific moiety (green) is connected by a multifunctional linker (purple) to the p-nitrocinnamyloxycarbonyl-cleavage site (red). This probe is conjugated to magnetic beads (orange) by a spacer (black). b) The general workflow for analysis of metabolic amines using the chemical probe [41]

Use of chemo selective probes helps in enrichment of the metabolites of interest, see figure 37 [42]. The procedure involves selection of metabolites by binding it to probes and removal of unreacted metabolites followed by LC-MS analysis of bound metabolites [41]. This methodology was applied on human fecal samples in order to study metabolites present, that would provide in-depth knowledge about the microbiome-associated metabolites (selective metabolites of interest) that bind to the chemoselective probes for further analyses by Mass spectrometry technique. The fecal metabolites identified included microbiome related, endogenous metabolites and many others.

A probe-based method for mass spectrometric analysis containing *p*-nitrocinnamyloxycarbonyl (Nac) as a new cleavage site was successfully designed and synthesised. Coupled to magnetic beads, the chemical probe enabled direct extraction of metabolites from human samples. The chemoselective probe was applied to the analysis of human fecal samples, resulting in the discovery of four metabolites previously unreported and confirmation of the presence of medically relevant gut microbiota-derived metabolites [41].



	Fecal metabolites[20]	Association with disease[19,21-22]
Microbial	*N*-Acetylcadaverine	Crohn's disease / IBD
	N-Acetylputrescine	Lung cancer
	5-Aminovaleric acid	
	Ammonia	Bacterial virulence
	Cadaverine	Lung cancer
	Methylamine	Rectal cancer
	Phenethylamine	Crohn's disease / IBD
	Piperidine	
	Putrescine*	Lung cancer
	Tyramine	
Endogenous	1,3-Diaminopropane*	Lung and bladder cancer
	Ethanolamine	
	Glycine	
	Histamine	
	L-(Iso)leucine	
	L-Lysine	
	L-Alanine	
	1-Aminoacetone	Diabetes
Others	**1-Aminopropan-2-ol**	
	2-Amino-3-methylbenzoic acid	

Figure 38: The figure/table on the right metabolites detected using the chemical probe and their disease association. Metabolites mentioned in bold were detected in significantly high amounts compared to the standards.

Further, in order to identify unknown bioactive molecules produced by gut microbiota in the human host, chemoselective probes were used to immobilise magnetic beads for analysis of carbonyls in human fecal samples from pancreatic cancer patients (figure 38). The carbonyl-containing compounds like aldehydes and ketones are reactive metabolites that can be produced by a wide range of gut bacteria. These aldehydes and ketones produced by microbes have been reported to have beneficial effects (by destroying potential growth of pathogenic strains) and deleterious effects. These metabolites have been known to be endogenously produces as a result of oxidative stress, are a common diet component, and have been linked to diabetes, cancer and neurodegenerative diseases. 112 carbonyl metabolites were detected in the fecal samples and as expected, all the gut microbiome-produced metabolites were quantified. The chemoselective probe-based method facilitated the detection of metabolites in the femtomole (fmol) range because of increased mass spectrometric sensitivity (see figure 39). This enabled identification of multiple metabolites derived from microbiota metabolism and linked to disease development [42].

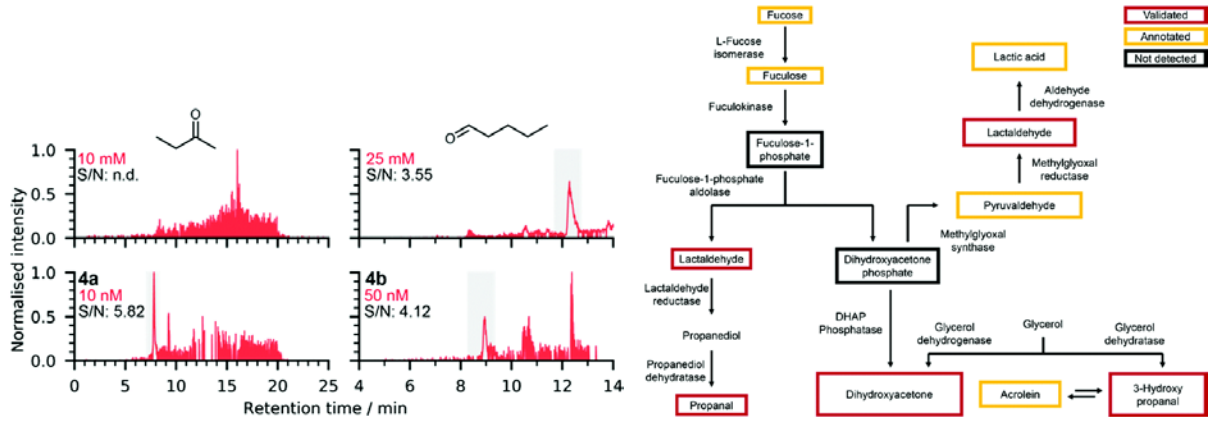
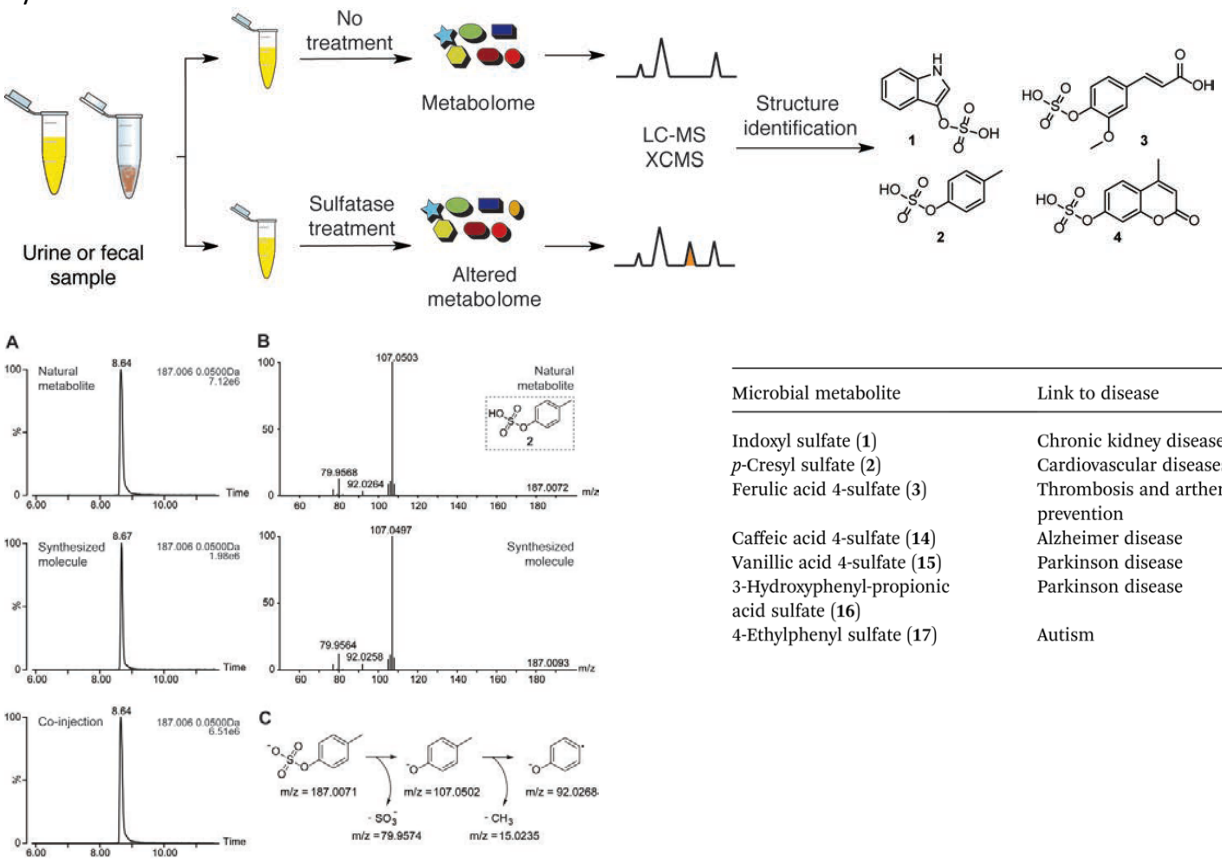


Figure 39: The figure on the left depicts limit of detection comparison of unmodified butanone and valeraldehyde with their corresponding conjugates** 4a/4b. **The figure on the right shows examples of detected metabolites in the fucose degradation, methylglyoxal, and glycerol conversion pathway. Metabolites validated with internal standards are highlighted in red, metabolites identified based on the chemical formula in yellow and carbonyl-containing metabolites not detected in black.

In a further study, selective investigation of the O-sulfated metabolite co-metabolism of bacteria and their human host was conducted. Analysis of human urine and fecal samples resulted in the detection of 206 sulfated metabolites and successful confirmations of the chemical structures of 36 sulfated metabolites including unknown and commonly reported microbiota-derived sulfated metabolites. The results enable targeted investigation of other metabolite classes as well as the discovery of biomarkers for diseases affected by microbiota.



Microbial metabolite	Link to disease
Indoxyl sulfate (**1**)	Chronic kidney disease (CKD)
p-Cresyl sulfate (**2**)	Cardiovascular diseases (CVD)
Ferulic acid 4-sulfate (**3**)	Thrombosis and artherosclerosis prevention
Caffeic acid 4-sulfate (**14**)	Alzheimer disease
Vanillic acid 4-sulfate (**15**)	Parkinson disease
3-Hydroxyphenyl-propionic acid sulfate (**16**)	Parkinson disease
4-Ethylphenyl sulfate (**17**)	Autism

Figure 40: Workflow of the targeted metabolomics-driven identification and mass spectrometric analysis of sulfated metabolites including the chemical structure of four representative gut microbiota-derived metabolites 1–4 identified in human samples (top figure). Example of sulfate ester validation in urine with p-cresyl sulfate (2). (A) UPLC-MS chromatogram comparison of urine sample vs. the synthesised standard molecule; (B) MS/MS fragmentation pattern of 2 in urine sample vs. the synthesised standard molecule (bottom left figure). Table with identified sulfated metabolites derived from microbial metabolism and link to disease development (bottom right figure) [43]

A final study regarding Glucuronidation, that plays an important role in human clearance metabolism and glucuronidated metabolites that are linked to microbiota–host co-metabolism was conducted (figure 40). A combination of an untargeted metabolomics analysis and enzymatic metabolic conversion for the selective detection of glucuronide conjugates by UPLC-MS/MS was done using human urine samples. The results enable selective identification of glucuronidated molecules and to discover unknown natural metabolites. In total, 191 metabolites in a single sample including microbiota-derived compounds as well as previously unidentified molecules, were identified [43].

Further, as hydrolyzed metabolite *p*-cresol (a gut microbiota derived metabolite) cannot be detected at physiological concentrations in either positive or negative MS mode, it was identified as the most significantly altered glucuronidated feature in a bioinformatic analysis [44]. The tentative structure of *p*-cresyl glucuronide was determined according to HRMS, fragmentation pattern and comparison with internal fragmentation spectra. Co-injection experiments with the synthesised metabolite led to an identical retention time and fragmentation pattern, thus validating the correct structure (see figure 41) [44].

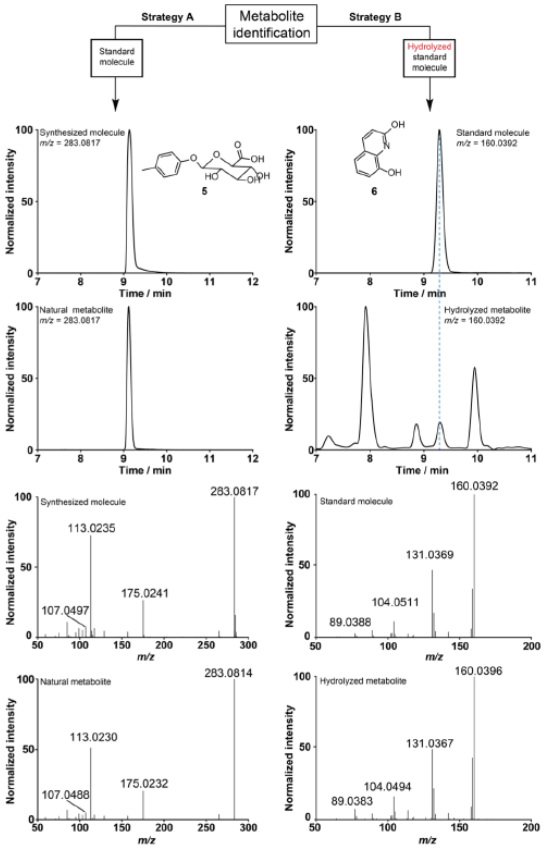


Figure 41: Strategies for identification of metabolite structures. Strategy A: co-injection of a synthetic internal standard; strategy B: structure determination through co-injection of hydrolyzed internal standard [44]

The presentation involved research outcomes including biomarker discovery for microbiome-host co-metabolism, chemical synthesis of microbiome-associates metabolites and the use of chemoselective probes to identify and chemically synthesise important metabolites.

5. BREAK OUT GROUP DISCUSSIONS

The main motivation behind the break-out group discussion was to unravel what is unknown and yet to be explored and what are the known research questions that have already been addressed regarding the gut microbiome associated metabolic capacity. The scientists of the present Workshop on the gut-microbiome mediated metabolism in the field of health and diseases got together to summarise the potential issues have remained unaddressed. Following are the report summaries of the two break-out groups.

5.1 Break-out group A

The aim of this group was to discuss the already known research outcomes related to the gut microbiome associated metabolic capacity. A publication from Gerard Clarke provides a good collection of examples of microbiome metabolism [5]. The review includes integration of knowledge regarding the contribution of gut microbiome in xenobiotic metabolism and major chemical biotransformation reactions carried out by specific bacterial enzymes. This contributed to understanding the microbial capacity and role in the host health and disease. Another important publication made by Michael Zimmermann includes results from genome wide screening of gut bacteria to reveal the drug-metabolising genes and their products. The research work aimed at providing an outline of the drug-metabolising activity of human gut bacteria and discovered that about 2/3rd of the drugs are metabolised by at least one bacterial strain. Findings like these promote better understanding of microbiome drug metabolism and eventually, metabolism-related microbiome-host interactions [45].

Some of the other well-known research findings involving the direct microbiome-based drug interactions include metabolic outcomes and products of microbial metabolism, (de)toxification reactions, increase in metabolite potency and prodrug activation mechanisms of the gut microbiome. Research questions related to the gut microbial activity on biotransformation of parent compounds, drug metabolism and metabolic reactions of Phase II metabolites entering the GI tract via the biliary secretions have been extensively addressed. Furthermore, bacterial enzymes that carry microbiome-specific metabolic reactions including hydrolytic enzymes, lyases, sulfatases, azoreductases, acetyltransferase, methyltransferases, enzymes for bile acid conversion, epoxidase, certain radical enzymes (eg. SAM and B_{12} dependent), glycosidase and nitroreductases have been well known and characterised. Other microbiome associated reactions that have been identified include tyrosine metabolism, glycerol [46], mucin production, deacetylation, hydroxylation caused by polyphenols, acetate/propionate production, glucuronidation function and short chain fatty acids (SCFA) metabolism caused by microbial dysbiosis.

Certain other findings have also addressed microbiome associated neurotransmitter production, bacterial biotransformation on fiber-rich diets, competition among bacterial strains for substrates, species-specific antibiotic resistance effecting the metabolic reactions, sharing of toxins within species, metabolic reactions involving single and multiple steps requiring several species and necessity for the presence of gene. It is known that some microbial metabolisms do not require the presence of specific species but more effectively the presence of the respective gene/protein that proves the functionality.

Contribution of some host associated factors also have impact on the microbiome-drug interactions. Such factors that have been known to play a key role in influencing the microbial metabolism include enterohepatic circulation, transit time of the drug, dietary components (such as high fiber-based diet) having the ability to alter the drug bioavailability, acute vs chronic exposure of the drug, increased permeability, host response and immune system.

The above mentioned portrays a short summary of the discussion within the first group regarding the 'majorly' already known research findings in the field of gut microbial metabolism.

5.2 Break-out group B

The main purpose of the second group was to discuss the knowledge gaps and the possible issues in the field of microbiome-associated metabolic processes.

- Few of the uncertainties are; 1) the exact definition of the metabolic capacity of the microbiome. 2) What exactly does one look for regarding the metabolic functionality of the microbiome? 3) Lack of knowledge regarding the interindividual variability. 4) Prediction of microbial properties in the individuals, the areas that the predictions should be made in and impact of the variability of these predictions on the microbiome functionality. 5) Are the biomarkers really necessary for xenobiotic metabolism identification or can the metabolites be directly measured?
- Other unanswered research questions involve; 1) The characterisation of dysbiosis considering the interindividual variability in the gut microbial composition. 2) Why certain group of patients do not respond well to a certain treatment? 3) Identification of clinical markers that integrate the microbiome with the physiological characteristics. 4) What is considered to be a healthy/normal gut microbiome composition. 5) Ability to impact microbiome alteration by immunotherapy and other than the diet. 6) What other host exposure influences changes in the microbial metabolic mechanisms? Apart from all these questions, even less is known regarding how a certain dysbiosis could be related to specific infection/diseased condition (modes of action studies) and how some dysbiosis could be beneficial while others not. To learn more about the functionality of the microbial metabolism, approaches to integrate gene frequency (list of genes) data are required. This also leads to the fact that a lot of bacteria have not been characterised and their genes are still not annotated.
- Bacterial competition for substrates is necessary to be considered to understand the microbial functionality with respect to the metabolic processes. Not only the bacterial gut colonisers, but there is a lack of knowledge regarding the functionality and composition of other organisms known to colonise the gut such as fungi, protists, archaea and viruses.

Unfortunately, it is unknown if the samples that are currently being used for microbiome research purposes in order to better understand the microbiome associated (or related) metabolism are truly representative of what really happens in vivo. There is hardly any information regarding the precise duration the microbiome takes to recover and get back to the 'normal' state following an insult such as antibiotic treatment. Another question that need to be considered is the interplay between the small intestine, large intestine and bile acids and how to derive samples to understand the different interactions.
Some of the other really important issues that need to be addressed are the efficiency of a complex microbiome to be able to degrade/metabolise a certain compound and if its biomarkers could be identified. More research is necessary in order to understand topics like characterisation of a microbial dysbiosis, how bad could adverse effects for risk assessment be and how could it be measured, differentiation between metabolic pathways in an aerobic and anaerobic environment
Unaddressed research questions like these need to be addressed in order to have a strong knowledge regarding the capacity and functionality of the gut microbiome in order to excel in the fields of toxicology and pharmaceutics.

6. CONCLUSIONS AND RECOMMENDATIONS

The Experts Meeting concluded with a list of questions that need to be addressed to guide research to gain a better knowledge in the areas of gut microbiome metabolic activity. Addressing these questions would provide the opportunity for applications in the fields of Pharmacology and Toxicology. Many unresolved questions in the field of gut microbiome research aiming at the understanding of the metabolic capacity of the intestinal microbiome were put forward during the course of the 2-days workshop. Trying to address the questions to find appropriate research solutions was the key objective of ECETOC Workshop.

The CEFIC LRI ELUMICA research project aims at developing a systematic understanding of the various metabolic reactions and the capacity of the intestinal microbiome and interindividual variability in humans and murine (rats) models. The project involves studies in order to elucidate the contribution of gut microbiome associated metabolism in rats and to provide a foundation to further investigate in vitro. The other aims of the project involve development of in vitro models to study the gut microbial metabolism of chemical probes and hence understand the interspecies and interindividual differences of toxicological relevance.

The goal of the ECETOC workshop was successfully met by inviting and gathering experts in the field of gut microbiome research and discussing about the already addressed, ongoing and future research topics. A huge amount of inputs and information from various perspectives regarding one common topic was obtained during the workshop. In addition to the great presentations by the experts who talked about their areas of expertise also an interactive session was conducted during the Workshop.

The interactive Break-out session involved active participation of everybody at this ECETOC meeting. The session involved open discussion and simultaneously, report writing where all the points that were discussed or mentioned were recorded. An integrating discussion at the end of the break-out session took place in order to combine and compare all the topics that were covered by both the groups formed during the workshop.

The session aimed at not only accumulating adequate knowledge and insights but also the complete involvement of everyone who was involved in the 2-day workshop. This not only enabled everyone to understand each other's perceptions regarding the gut microbiome research, but also the different techniques that were used by different labs to investigate the, 'metabolic capacity of gut microbiome'.
Overall, the workshop ended on a good note, enabling potential future collaborations among the experts and their respective labs and the efforts of conducting the workshop fulfilled its motive, contributing significantly to paving the way for a successful CEFIC LRI ELUMICA project.

ABBREVIATIONS

ELUMICA	Elucidating microbial metabolic capacity
HMP	Human Microbiome Project
MetaHIT	Metagenomics of Human Intestinal Tract
GI	Gastrointestinal
OECD	Organisation for Economic Co-operation and Development
PBK	Physiologically based kinetics
IVIVE	In vitro to in vivo extrapolation
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid chromatography–mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
PCA	Principle component analysis
CMC	Carboxymethyl cellulose
SCFA	Short chain fatty acids
IDO	Indoleamine-2,3-dioxygenase
UC	Ulcerative colitis
DSS	Dextran sulfate sodium
qPCR	quantitative PCR
UHPLC-HRMS	Ultra-high-performance liquid chromatography-high-resolution mass spectrometry
PCoA	Principle coordinate analysis
PBS	Phosphate-buffered saline
DON	Deoxynivalenol
DON3Glc	Deoxynivalenol-3-glucoside
ZEN	Zearalenone
Caco-2 TC7	Cellosaurus cell line
HT2	Fusarium mycotoxin
HT2-Glc	HT-2 toxin-3-O-β-glucoside
DOM-1	Deepoxy-deoxynivalenol
eWAT	Weight of epididymal white adipose tissue
mWAT	Weight of mesenteric white adipose tissue
iWAT	Weight of inguinal white adipose tissue
BA	Bile acid
GF	Germ free
SPF	Specific pathogen free
OGTT	Oral glucose tolerance test
OTU	Operational taxonomic unit
iMGMC	Integrated mouse gut metagenome catalog
MGC	Mammalian gene collection
PHB	Poly-β-hydroxybutyrate
mRNA	messenger RNA
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
IFX	Infliximab
IPI	Ipilimumab
DHD	Dihydrodaidzein
(S)-equol	An intestinally derived metabolite of isoflavone daidzein
O-DMA	O-Desmethylangolensin
bw	body weight
SHIME	The Simulator of the Human Intestinal Microbial Ecosystem

MoA	Modes of action
HITchip	Human Intestinal Tract chip
8-PN	8-Prenylnaringenin
fmol	Femtomole
HRMS	High Resolution Mass Spectrometry

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APPENDIX B: WORKSHOP PROGRAM

Monday, 8 July'2019

12:00-13:00	**All**	Registration and lunch
13:00-13:10	**Olivier de Matos, Ben van Ravenzwaay**	Welcome
13:10-13:30	**Saskia Sperber**	ELUMICA - Elucidating Microbial Metabolic Capacity
13:30-13:50	**Aishwarya Murali**	Influence of the gut microbiome on plasma metabolite patterns
13:50 - 14:10	**Jonathan Swann**	Contribution of the gut microbiota to host endogenous and xenobiotic metabolism
14:10-14:45	**Coffee Break**	
14:45-15:00	**Olaf Kelber**	Influence of the microbiota on the phytochemical constituents of natural products and vice versa – metabolic approaches
15:05-15:25	**Silvia Gratz**	Analysis of gut microbial xenobiotic metabolism in vitro
15:25-15:45	**Patricia Lepage**	Functional characterisation and modulation of human gut microbiome
15:45-16:05	**Cristina Menni**	The fecal metabolome as a functional readout of the gut microbiome (WebEx)
16:05-16:20	**Coffee Break**	
16:20-16:40	**Karsten Beekmann**	Using PBK modelling to predict plasma concentrations of intestinal microbial metabolites of xenobiotics
16:40-17:00	**Frederic Moens**	Functional analysis of the gut microbiome using in vitro methodologies
17:00-17:20	**Daniel Globisch**	Identification of gut microbiota derived metabolites
17:20-17:30	**End of day 1 (Closing remarks)**	
19:00-22:00	**Dinner**	

Tuesday, 9 July'2019

09:00-12:00	Two break out groups -	1. Major areas of known microbiome metabolic capacity (what we know for sure)
		2. Information / Knowledge gaps
09:00-10:30	Each group worked on the respective topics for 1.5 hours	
10.30-11.00	**Coffee Break** (and topic reversal)	
11.00:12:00	Completion of data set from the other group	
12:00-13:00	**Lunch Break**	
13:00-13:30	Presentations by both groups	
13:30-14:00	Consensus building and conclusion	
14:30	**End of day 2 (Closing remarks)**	

APPENDIX C: ORGANISING COMMITTEE

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