Investigating the Role of Different Dietary Patterns on Gut Metabolites

Thesis submitted by
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Abstract

This thesis investigates the interplay between dietary patterns and gut metabolites, focusing on short-chain fatty acids (SCFAs) and bile acids (BAs), key contributors to gut metabolism. It addresses the need to move beyond reductionist, nutrient-specific approaches by examining how whole dietary patterns shape metabolic outcomes. Using advanced analytical techniques, including novel Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS), this work evaluates gut-related metabolites across contrasting diets.

Two primary studies frame this research. The first examines SCFA and BA concentrations in free-living vegans and omnivores within a cross-sectional cohort. Significant metabolic differences emerged between these groups. Vegans showed elevated SCFA concentrations in biofluids, reflecting their high-fibre diets, whereas omnivores exhibited increased secondary bile acids in faecal samples, likely linked to higher fat intake. These results highlight the distinct effects of plant-based versus omnivorous dietary patterns on gut-derived metabolites and their potential implications for health.

The second study investigates the acute metabolic effects of contrasting dietary patterns in a randomised, controlled, crossover trial. By monitoring SCFA and BA concentrations following healthy and unhealthy diets, this study captures temporal fluctuations in metabolite profiles. SCFA levels varied with dietary intake and displayed notable inter- and intra-individual variability. For BAs, temporal variation appeared stronger than dietary effects. Integrating metabolomic and dietary data, this work emphasises the dynamic and personalised nature of gut metabolite responses among individuals consuming the same diet.

Overall, this thesis advances understanding of the complex interactions between diet, host metabolism, and the microbiota. Recognising the role of metabolite variability is essential for interpreting metabolic pathways and developing tailored dietary recommendations. By elucidating how dietary patterns shape gut metabolites under real-life and controlled conditions, this research contributes to future applications in precision nutrition and supports the development of metabolite-informed dietary strategies.

Declaration of Originality

I certify that this thesis, and the research to which it refers, are entirely the products of my own work. Any ideas, data, or quotations from the work of others, whether published or unpublished, are fully acknowledged and cited in accordance with the standard referencing practices of the discipline. I solemnly affirm that I have adhered to the ethical guidelines and policies of the institution, as is naturally expected in the noble pursuit of research integrity.

The following samples or datasets were provided by other people:

- Samples for Chapter 3 and Chapter 5 were provided by Professor Oluf Pedersen and his team, who conducted a cross-sectional cohort study at the University of Copenhagen, Denmark.

-Faecal bile acids results presented in Chapter 5 were analysed by Dr Adesola Bello, as part of her PhD Thesis named "Investigating gut-microbiota mediated polymerisation of deoxycholic acid in humans"(1) submitted at Imperial College London.

- Samples from Chapter 4 and Chapter 6 were obtained from a randomised clinical trial conducted and managed by the Nutrition Section at Imperial College London, led by Professor Gary Frost and his team.

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List of Abbreviations

12 DHCA	12 Dehydrocholic acid
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
A. faecalis	Alcaligenes faecalis
AA	African Americans
AB	After breakfast
ABST	Apical bile salt transporter
ACE	Acetonitrile
AD	After dinner
ADI	Average daily intake
ADME	Absorption, distribution, metabolism, and elimination
AL	After lunch
ALA	α-Linolenic acid
ALT	Alanine Aminotransferase
Apocholic acid	8(14),(5β)-Cholenic acid-3α, 12α-diol acid
ASBT	Apical sodium-dependent bile acid transporter
BAs	Bile acids
BCAAs	Branched-chain amino acids
BSCFA	Branched short-chain fatty acid
BMI	Body mass index
BP	Blood pressure
BSH	Bile salt hydrolase
C10:0	Capric acid
°C	Celcius
C12:0	Lauric acid
C14:0	Myristic acid
C14:1,n-5	Myristoleic acid
C15:0	Pentadecylic acid
C16:0	Palmitic acid
C16:1,n-7	Palmitoleic acid
C17:0	Heptadecanoic acid
C18:0	Stearic acid
C18:1,n-7	Vaccenic acid
C18:1,n-9	Oleic acid
C18:2,n-6	Linoleic acid
C18:3,n-3	α-Linolenic acid
C18:4,n-3	Stearidonic acid
C20:0	Arachidic acid
C20:1,n-11	Gadoleic acid
C20:4,n-6	Arachidonic acid
C20:5,n-3	Eicosapentaenoic acid
C22:0	Behenic acid
C22:1,n-11	Cetoleic acid
C22:1,n-9	Erucic acid
C22:5,n-3	Docosapentaenoic acid
C22:6,n-3	Docosahexanoic acid

C24:0	Lignoceric acid
C24:1,n-9	Nervonic acid
CA	Cholic acid
CCK	Cholecystokinin
CDCA	Chenodeoxycholic acid
CE	Collision energy
CHO	Carbohydrates
CKD	Chronic kidney disease
CRC	Colorectal cancer
CRF	Clinical research facility
CS	Cholesterol
CV	Cross -validation
CVD	Cardiovascular diseases
D3	Day 3
D4	Day 4
DASH	Dietary approaches to stop hypertension
DCA	Deoxycholic acid
DHCA	3,7,12 Dehydrocholic acid
DI	Dietary intervention
DP	Dietary pattern
E. coli	Escherichia coli
EI	Electron ionization
EPA	Eicosapentaenoic acid
ESI	Electrospray ionisation
ER	Endoplasmic reticulum
FA	Fasting
FAs	Fatty acids
FDR	False discovery rate
FMO3	Flavin monooxygenase 3
FXR	Farsenoid X receptor
g	Gram
GC	Gas Chromatography
GC- FID	Gas chromatograph flame ionization detector
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GCUDCA	Glycoursodeoxycholic acid
GDCA	Glycodeoxycholic acid
GHCA	Glycohyocholic acid
GLCA	Glycolithocholic acid
GLP-1	Glucagon like peptide 1
GPR	G-protein-coupled-receptors
h	Hours
H2O	Water
HbA1C	Haemoglobin Glycated
HCC	Human hepatocarcinoma
HDAC	Histone deacetylase

HDL	High density lipoprotein
HOMA2 %B	Homeostatic Model Assessment to estimate beta cell function in type 2 diabetes
HOMA2 %S	Homeostatic Model Assessment for insulin sensitivity
HOMA2 IR	Homeostatic Model Assessment for insulin resistance
IBAT	Ileal Bile Salt Transporter
IBD	Inflammatory bowel disease
ID	Identification
ILCA	Isolithocholic acid
IPA	2-propanol
IQR	Interquartile range
IR	Insulin Resistance
IS	Internal standard
IU	International unit
LA	Linoleic acid
LC	Liquid Chromatography
LCA	Lithocholic acid
LDL	Low density lipoprotein
m	Meter
m/z	Mass to charge
MCA	Murocholic acid
MeOH	Methanol
mg	Milligram
min	Minutes
mm	Millimetre
mM	Millimole
MMC	Migrating motor complex
mL	Millilitre
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MRP	Multidrug-resistance-associated protein
MRP2	Multidrug resistance-associated transporter 2
MRP4	Multidrug resistance-associated transporter 4
MS	Mass spectrometry
MUFAs	Monounsaturated fatty acids
NASH	Non-alcohol related steatohepatitis
NCDs	Non-communicable diseases
ng	Nanogram
nm	Nanometer
nM	Nanomolar
NorDCA	3α,12α, 23 Nor Deohycholic acid
OGTT	Oral glucose tolerance test
OLTT	Oral lipid test tolerance
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
OSTα	Organic solute transporter alpha
OSTβ	Organic solute transporter beta
PC	Principal component

PCA	Principal component analysis
pmol	Picomole
PUFAs	Polyunsaturated fatty acids
PYY	Peptide tyrosine tyrosine
QC	Quality control
RA	Rural Africans
RCF	Relative Centrifugal Force
RS	Resistant starch
RT	Retention time
SCFAs	Short chain fatty acids
SD	Standard deviation
SFAs	Saturated fatty acids
SIM	Selected ion monitoring
TCA	Tricarboxylic cycle
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
TFAs	Trans-fatty acids
TG	Triacylglycerol
THCA	Taurohyocholic acid
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
UCNA	Ursocholanic acid
UDCA	Ursodeoxycholic acid
µL	Microliter
µm	Micrometer
µM	Micromolar
µmol	Micromole
UPF	Ultra processed food
VIT	Vitamin
VLDL	Very low-density lipoprotein
V	Volts
WHO	World Health Organization
y	Year
βMCA	β-Murocholic acid

1.General introduction

Note: Parts of this chapter are based on the following published research that I authored:

- Brignardello J, Fountana S, Garcia-Perez I, Posma M J, et al. 2021. "Characterization of diet-dependent temporal changes in circulating short-chain fatty acid concentrations: A randomized crossover dietary trial". Am J Clin Nutr. 2022. Nov; 116(5): 1368–1378. PMID: 36137188.
- Brignardello J, Holmes E, Garcia-Perez I."Metabolic Phenotyping of Diet and Dietary Intake". Adv Food Nutr Res. 2017;81:231-270 (Book Chapter). PMID: 28317606

1.1 Transforming nutritional research: beyond macronutrients to metabolic insights

Food intake is recognised as one of the primary sources of nourishment for the body, supplying the nutrients required to meet its biological needs. Historically, the study of diet focused primarily on individual nutrients, rather than adopting a holistic approach that considered whole food consumption within the context of dietary patterns (2). Similarly, this shift in perspective is part of broader changes in the field of food and nutrition science. Traditionally, food was assessed only in terms of its macronutrient content, overlooking the metabolic effects of these "nutrients." However, advances in biological, chemical, and nutritional sciences have provided evidence to support a more integrated approach. This modern perspective considers not only food intake and dietary patterns, but also factors such as eating time, diet quality, the metabolic contributions of the gut microbiome, and the production of microbiome-derived metabolites (3).

1.1.1 Importance of investigating dietary patterns over isolated nutrients in nutrition studies

Diet is widely recognised as one of the most important factors in both the prevention and development of non-communicable diseases (NCDs), including obesity, diabetes, cardiovascular diseases (CVD), cancer, and other metabolic disorders (4). Historically, diet studies primarily focused on individual nutrients or food groups to investigate the relationship between diet and disease outcomes. However, this reductionist approach has been criticised for posing several methodological limitations (5).

Firstly, food and nutrients are rarely consumed in isolation: they are typically part of complex meals in which various nutrients and dietary compounds interact. These interactions can be inhibitory, synergistic, or competitive. For example, iron and zinc compete for the divalent metal transporter 1 (DMT1), with zinc reducing iron's bioavailability (6, 7). Moreover, dietary phytates found in plant-based diets further complicate nutrient absorption by decreasing the bioavailability of both zinc and iron (8). Secondly, the health effects of an individual nutrient may be small and challenging to detect in isolation. In contrast, the combined effects of multiple nutrients from a variety of foods can have a more significant and measurable impact. For example, while reducing saturated fatty acid intake is a standard recommendation for CVD prevention, studies have shown that adopting overall dietary patterns (DPs) is more effective in preventing CVD than focusing solely on reducing a single type of fatty acid (7, 9).

Given these complexities, investigating dietary patterns (DPs) offers a more comprehensive and realistic approach to understanding the relationship between diet and health. This approach enables the identification of food combinations that contribute to either positive or negative health outcomes, ultimately providing more effective dietary recommendations for the prevention and management of various diseases (10).

1.1.2 Comparative analysis of dietary patterns: evolution, nutritional impact, and health outcomes

Over the past century, dietary patterns have undergone substantial transformations influenced by sociocultural, economic, and technological changes (11). Traditional diets, which were primarily plant-based with minimal processed foods, have shifted towards greater consumption of refined sugars, fats, and ultra-processed foods (UPFs) (12).

Various epidemiological studies have provided evidence to elucidate the optimal diet, health maintenance, and disease prevention (13-16). These studies have led to the development of different dietetic approaches and population-based recommendations, including the Mediterranean, Nordic, Healthy, Vegetarian, Vegan, and Dietary approaches to stop hypertension (DASH) diets, among others (17). Conversely, epidemiological research on diets has also identified dietary patterns (DPs) that may be detrimental to health (10, 17).

Omnivorous DPs are characterised by the consumption of both plant and animal products, generally providing a wide range of nutrients. However, omnivorous DPs can be classified as either healthy or unhealthy (Figure 1.1). For instance, the Mediterranean dietary pattern is widely recognised for its health benefits, largely attributed to its emphasis on diverse plant-based foods rich in polyphenols, olive oil, fish, and a low intake of red meat and poultry(16). In contrast, the Western dietary pattern is associated with adverse health outcomes over the long term. This DP is characterised by a high intake of refined sugar, UPFs, and animal fat, alongside a low intake of fibre, fruits, and vegetables (18, 19).

Plant-based DPs can be subdivided into vegetarian and vegan dietary patterns. Several nutritional organisations have provided perspectives and guidelines on adopting a vegan diet (20-23). According to these sources, a well-planned vegan diet is capable of meeting the nutritional needs of healthy individuals (24). The nutritional differences between omnivores and vegans have been extensively documented. A healthy vegan diet is typically characterised by a high intake of fruits and vegetables, low sodium and saturated fat consumption, and the exclusion of animal products and their derivatives (25). Both vegan and omnivorous DPs have been the focus of extensive research over many years (26-28). While evidence from epidemiological studies is robust, most findings emphasise the health benefits of vegetarian diets—particularly for cardiovascular health—over those of omnivorous DPs (29, 30).

1.1.3 Dietary intake assessment methods and associated challenges

Dietary intake data is typically collected using various methods, such as weighed food records, food frequency questionnaires, food diaries, and 24-hour recall methods (31). Table 1.1 summarises the primary methods used to measure food and/or nutrient intake. These techniques provide information on eating habits, portion sizes, and food and nutrient consumption; however, the data can be inaccurate due to factors such as food composition tables for estimating nutrients, other food contents, and energy (32, 33). Moreover, dietary assessments often require careful validation, significant time, highly skilled personnel, cognitive ability from the respondent, and the researcher's expertise to ensure reliable results (34).

A significant challenge in nutritional sciences is the accurate assessment of dietary intake at both individual and population levels. Misreporting is estimated to range from 21.5% to 67% in free-living individuals (35), with unhealthy foods often underreported and fruits and vegetables overreported (36). Additionally, the underreporting of dietary energy intake is particularly problematic among obese and overweight individuals, likely due to the increasing prevalence of obesity and the stigma associated with it (37, 38).

Current tools for dietary intake assessment present significant challenges in determining whether dietary effects are genuinely present at both the population and individual levels. Researchers face the dilemma of discerning whether an observed lack of effects is due to inherent limitations in the physiological impact of the diet itself, poor compliance with dietary recommendations, or the high interpersonal variability in individual responses to the same diet (39).

Accurate reporting of dietary intake is essential for developing appropriate nutritional recommendations and evaluating the impact of diet on health and disease. By understanding the complex interactions between nutrients and dietary compounds, as well as the effects of different dietary patterns on metabolism, more effective strategies can be devised for the prevention and management of various diseases at both individual and population levels.

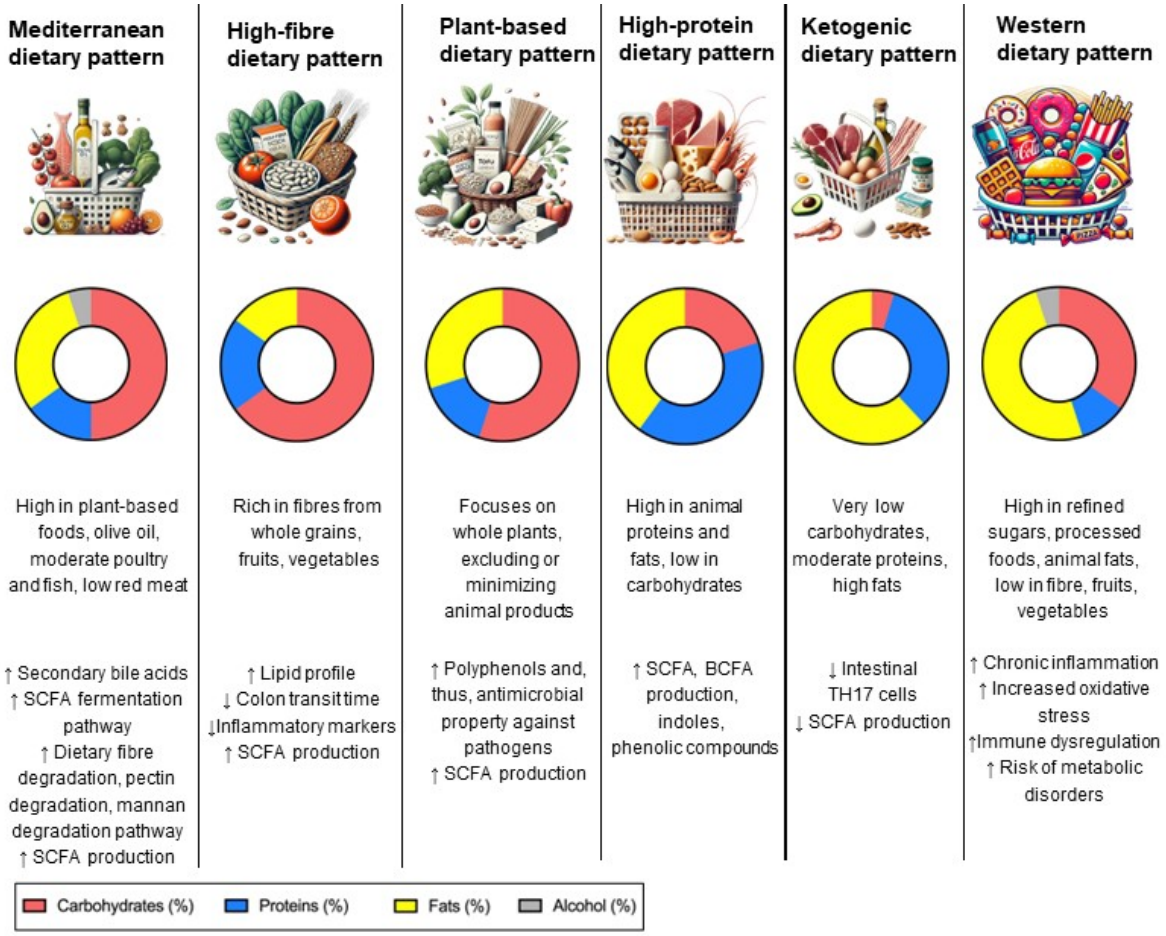

Figure 1.1 Macronutrient distribution of different dietary patterns, diet characteristics, gut metabolite production, and effects on the gut. Each column represents a specific dietary pattern: Mediterranean, high-fibre, plant-based, high-protein, ketogenic, and Western dietary patterns. The pie charts represent the distribution of the macronutrients (carbohydrates, protein, lipids) and alcohol content for each diet. The figure illustrates the characteristics of each dietary pattern and the consequent effects on metabolite production. Upward arrows indicate an increase in metabolites, whereas downward arrows denote a reduction in metabolites. This detailed overview explains how various dietary patterns can influence gut metabolites, offering insights into their possible effects on overall health and well-being. SCFA, short chain fatty acids; BSCFA, branched chain fatty acids; TH17, T helper 17 cells; TMA, trimethylamine; TMAO, trimethylamine N-oxide. Figure adapted and modified from Fig 1, reference (10).

Table 1. 1 Common dietary record methods employed for diet study. Information compiled from (31).

Method	Brief Description	Strengths	Weaknesses
Weighed Food Records	It involves weighing every food item before consumption and recording the details.	High precision in portion sizes.	High respondent burden Risk of misreporting. Expensive. Limited food composition data.
Estimated Food Records	Portion sizes are estimated using household measures, photos, or models .	Lower respondent burden than weighed food records. Widely used method.	Inaccurate estimation of portion sizes. Risk of misreporting. Expensive. Limited food composition data.
24-Hour Recall	Retrospective method that recalls the food intake in the last 24 hours Interviewer may use portion sizes using household measures, photos, or models for better estimation.	Lower respondent burden than weighed food records. Widely used method.	Inaccurate estimation of portion sizes. Risk of misreporting. Expensive. Limited food composition data.
Multiple Pass Recall	An enhanced 24-hour recall method involving multiple passes to improve recall accuracy and capture all consumed items.	Improved precision compared to standard 24-hour recall. Low respondent burden. Suitable for large-scale surveys. It can be administered by telephone.	Estimation of portion sizes. Memory-dependent. Potential bias in reporting.
Food Frequency Questionnaire (FFQ)	A questionnaire that collects data on the frequency of consumption of a list of foods over a specific period.	Low respondent burden. Suitable for large-scale surveys. It can be self-completed. It can be emailed.	Potential over-reporting of “healthy” foods. Estimation of portion sizes. Requires validation against reference methods.
Food Diaries	Participants record detailed descriptions and amounts of all foods and beverages consumed over a certain period, typically 7 days.	Provides detailed intake data. It can help identify eating patterns. Useful for detailed analysis of diet at the individual level.	High respondent burden. Time consuming entry and analysis. Potential underreporting or omissions.

1.2 Dietary Patterns, nutrients and gut metabolites

Different studies have unequivocally demonstrated the effect of dietary patterns and food interventions on health outcomes (10). These associations have been pivotal in the development of nutritional guidelines and dietary recommendations for the population. However, an increasing number of studies are adopting a more holistic approach to evaluate dietary intake and its metabolic effects on the human body (3). The impact of eating patterns should not be reductionist, focusing solely on associations with macronutrient composition, anthropometric outcomes, or the presence or absence of diseases.

In the past, nutritional deficiencies and undernutrition were major public health challenges affecting many countries worldwide. These issues, combined with the scientific knowledge and technology available at the time, shaped the prevailing approach to nutrition (11, 40). However, over the past two decades, this approach has evolved to address emerging public health challenges. The integration of new technologies and advancements in scientific knowledge has introduced new perspectives and participants to the "nutritional science table".

Over the past two decades, the understanding of food, dietary patterns, and metabolism has evolved significantly due to advances in knowledge about the gut microbiota and its influence on human physiology, revealing new insights into energy metabolism and obesity (41-43). In parallel, the study of gut metabolites has further expanded our understanding of food and dietary patterns, offering invaluable contributions to the field of human metabolism (44-47).

The influence of dietary patterns and their components, such as fibre and fats, on gut metabolites has been widely studied, particularly regarding short-chain fatty acids (SCFAs), bile acids (BAs), and their metabolic effects on human health (Figure 1.1). For instance, in a study involving American Africans and rural Africans, participants switched their traditional diets for two weeks (typically Western and predominantly plant-based, respectively). Both groups experienced diverse changes in metabolites following the dietary intervention, particularly in faecal SCFAs and bile acids (18).

1.2.1 Dietary fibre: definitions, physiological roles, and research complexities

Based on the Codex Alimentarius definition, fibre is defined as carbohydrate polymers consisting of ten or more monomeric units that cannot be digested by enzymes in the human small intestine (48). This includes naturally occurring edible carbohydrate polymers found in food, those derived from raw food materials through physical, enzymatic, or chemical processes that demonstrate a positive physiological impact on human health, and synthetic carbohydrate polymers that have been proven to confer health benefits (48). This definition includes non-starch polysaccharides, resistant starch, resistant oligosaccharides, lignin, and other compounds when associated with polysaccharides in the

plant cell wall. The investigation of the effects of fibre on health requires caution, as studies on its health benefits encompass different types of fibres. These include synthetic fibres that consist of a single molecule, extracted fibres naturally found in plant-based foods containing one or a limited number of fibres, single foods with specific types of fibres, and high-fibre diets that include a wide variety of fibres from diverse foods with complex food matrices. Therefore, the study and translation of findings reporting health benefits must be approached carefully, with consideration of the type of study (e.g., *in vitro*, *in vivo*, animal models, or human trials) and the study design.

Multiple health benefits of fibre have been reported in human health. For instance, a 2019 meta-analysis examined the impact of dietary fibre intake on disease risk, analysing 185 studies that included 135 million person-years of data from prospective studies and 58 clinical trials with 4.635 participants (49). Observational data suggested a 15–30% reduction in mortality from all-cause cardiovascular diseases when comparing populations with high fibre intake to those with low fibre intake. The greatest reduction in disease risk was observed when daily fibre intake ranged between 25 and 29 g, which aligns with population recommendations for fibre intake (48). However, most populations fail to meet these recommendations, with average fibre intake estimated at approximately 20 g in the UK, 16.2 g in the United States, and 12.5 g in Chile (50, 51).

Fibre, as a food component, has potential applications in therapeutic interventions for medical conditions that require dietary modifications, such as gastrointestinal disorders, cardiovascular diseases, and kidney diseases (50). Its consumption is associated with multiple effects, including improved digestion and nutrient absorption, modulation of lipaemic metabolic responses, and enhanced glycaemic control. Additionally, fibre plays a role in regulating plasma cholesterol levels by reducing bile acid absorption, influencing gut transit time, and altering microbiota composition and metabolism (50). These reported effects are strongly influenced by the physicochemical characteristics of fibre, which play a crucial role in promoting health benefits. Fibre is present in various types of food (Table 1.2).

Solubility in fibre refers to its ability to dissolve in water and its affinity for water, in contrast to insoluble fibres (52). Examples of soluble fibres include pectins, dextrins, alginates, and galacto-oligosaccharides (Table 1.2). The reported beneficial effects of soluble fibres include improved nutrient absorption and the regulation of gastric emptying. However, it is important to note that fibre in food is a complex mixture of both soluble and insoluble fibres, each with distinct physiological effects that cannot be predicted solely based on their solubility properties (50).

Viscosity is a property typically associated with soluble fibres, referring to their ability to thicken or form a gel-like consistency when water is absorbed, which influences the flow of digestive contents. This property alters transit time in the upper gut, slowing gastric emptying and modulating small intestinal transit (53). Other associated effects of dietary fibre consumption include delayed digestion, increased satiety in humans, and reductions in postprandial glycaemia and lipaemia (50).

Table. 1. 2 Physicochemical characteristics of common dietary fibres found in food. , modified from (50).

Fibre Type	Common Sources	Solubility	Viscosity	Fermentability
Cellulose	All green plant cell walls	Insoluble	Non-viscous	Low
Lignins	All green plant cell walls	Insoluble	Non-viscous	Low
Arabinoxylans	Wheat, psyllium	Low to Medium	Medium	High
β-Glucans	Oat, barley, fungi	Low to Medium	Medium to High	High
Galactomannans	Guar gum, fenugreek	Medium to High	Medium to High	High
Pectins	Fruits, vegetables, legumes	High	Medium to High	High
Inulin	Cereals, fruits, vegetables	Medium to High	Low to High	High
Galacto-oligosaccharides	Pulses (e.g., beans, peas, lentils)	High	Low	High
Dextrins	Cereals (e.g., wheat dextrins)	High	Non-viscous to Low	High
Alginate	Seaweed	High	High	Low
Methylcellulose	Synthesized	High	High	Non-fermentable
Resistant Starch (RS-1)	Whole grains, legumes, raw fruits, vegetables	Insoluble	Non-viscous	High
Resistant Starch (RS-2)	Cereals, raw legumes, raw fruits, vege,,s	Low	Non-viscous	High
Resistant Starch (RS-3)	Cooking and cooling of any starch source	Low	Non-viscous to Low	High
Resistant Starch (RS-4)	Synthesized (e.g., acylated starches)	Low to High	Low to Medium	High
Resistant Starch (RS-5)	Synthesized (e.g., amylose and stearic acid)	Low	Low	Low

Fibre fermentability refers to the ability of fibre to be fermented by various gut microbiota species, often referred to as keystone species (54). For instance, *Ruminococcus bromii* is capable of degrading certain types of resistant starch. The fermentability of fibre is typically assessed using *in vitro* models; however, these characteristics do not always correspond to outcomes observed in *in vivo* models. In *in vivo* conditions, the capacity to produce SCFAs in stool and to measure hydrogen through breath testing presents challenges in terms of reproducibility and interpretation. Nevertheless, the intake of foods rich in highly fermentable fibre has been associated with higher concentrations of SCFAs in faecal samples in humans (55).

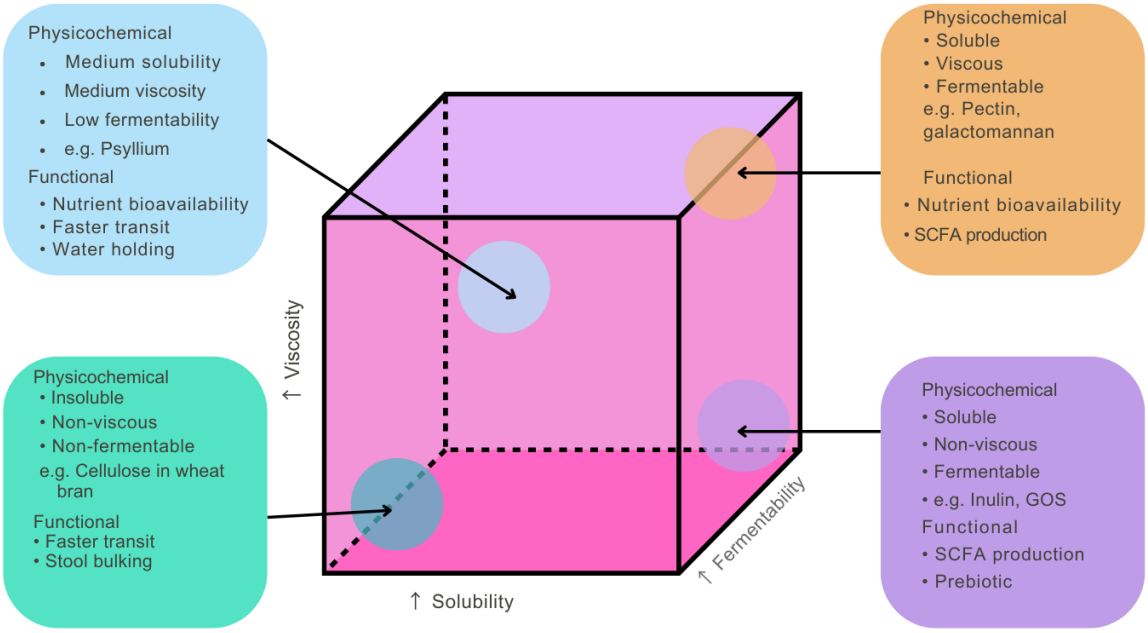

Figure 1. 1 Spectrum of physicochemical characteristics of dietary fibre. Physicochemical properties of dietary fibre (solubility, viscosity and fermentability) provides a diversity of effects on the gastrointestinal level. The combination of these properties seems to determine which effects will be exerted at the gut. For example, fibres located in the bottom left corner in light green (non-viscous, insoluble, non-fermentable) are primarily involved in regulating bowel transit time. Those located in the bottom right corner in light purple (soluble, non-viscous, fermentable) are associated with promoting gut microbiota health and fermentation processes. Meanwhile, fibres positioned in the top right corner in light orange (soluble, viscous, fermentable) contribute to gut microbiota balance, fermentation, and the absorption of nutrients. Fibres located between these points are likely to have a mix of these functions. GOS stands for galacto-oligosaccharides, and SCFA refers to short-chain fatty acids. Figure modified from Figure 3, reference (50)**.**

Recent research has highlighted that, irrespective of the characteristics and properties of fibres, individual differences in the microbiome play a key role in regulating responses to various fibres. For instance, resistant starch is well known for its fermentability; however, it affects microbiota composition differently among individuals, leading to variations in SCFA responses observed in faecal samples (56).

1.2.2 Dietary fats digestion and BAs

In a normal diet, fat intake should contribute 20 to 35 per cent of daily calories (57). Based on an approximate intake of 2,500 kcal, this corresponds to a fat intake ranging between 55 and 100 g. Dietary fat is primarily consumed as triacylglycerols (TGs), with smaller amounts derived from phospholipids (1–2 g/day), cholesterol (300–400 mg/day), and fat-soluble vitamins. Additionally, some endogenous lipids reach the intestine through biliary secretion, epithelial desquamation, and dead bacteria from the

intestinal microbiota. These endogenous lipids can account for approximately 1–2 g/day of cholesterol and 20 g/day of phospholipids, exceeding the intake from dietary sources (58).

The digestion and absorption of lipids is a highly efficient process that occurs in the small intestine, specifically in the duodenum and proximal jejunum. More than 95 per cent of dietary TGs and over 60 per cent of CS are absorbed, with only 2 to 5 g of lipids excreted in the stool daily. Due to their hydrophobic nature, lipids exhibit low solubility in the aqueous intraluminal environment (58). The primary challenge in lipid absorption lies in converting these water-insoluble nutrients, present as emulsion droplets, into a water-miscible form capable of reaching the epithelial surface for absorption. As a result, the mechanisms involved in lipid digestion and absorption differ significantly from those of other nutrients.

1.2.2.1 Gastric and intestinal lipid digestion

Lipid digestion is an intraluminal process that begins with the action of acidic lipases, specifically lingual and gastric lipases. Lingual lipase is active within a pH range of 4.5 to 5.4, allowing its activity until food reaches the stomach. The chief cells in the gastric fundus produce gastric lipase, an enzyme that operates effectively in an acidic environment with a pH range of 3 to 6. This enables the enzyme to remain active after entering the duodenum, continuing its function until the pancreas releases bicarbonate to neutralise the acidic gastric chyme or until trypsin degrades the enzyme itself. Gastric lipase hydrolyses TGs with medium-chain fatty acids (FAs) more rapidly than those with long-chain FAs. It preferentially acts on the 3rd carbon of the TG molecule, releasing diacylglycerol (DG) and free fatty acids (FFA), which play a stabilising role in the lipid emulsion within the duodenum (58).

The duodenum is where three fluid flows converge: gastric chyme, pancreatic juice, and bile secretion. The mixture of these fluids is essential for the proper digestion of lipids, including dietary and biliary CS. This combination significantly alters the interaction between the aqueous-based phase and the fatty components within the lumen. It is hypothesised that a fluid equilibrium, or "continuum," is established among the breakdown of the oil-based phase (emulsion), the incorporation of breakdown products into bile acid micelles, and their movement towards the epithelium for absorption (58, 59).

1.2.2.2 Micelles formation and BAs

Micelles are found in low concentrations in aqueous solutions, with BAs present as monomers in low concentrations. However, when their concentration exceeds a threshold known as the "critical micellar concentration" (~2.5 mM), BAs aggregate to form small micelles (3–20 nm). In bile, BAs combine with PL and CS to form mixed discoidal micelles. The enzymatic digestion of dietary lipids produces long-chain FAs, lysophospholipids, CS, vitamins, monoglycerides, and glycerol. These components are incorporated into the mixed micelles in the lumen, resulting in their enlargement and transformation

into spherical structures, referred to as expanded micelles. These fat-soluble compounds cannot independently diffuse through the stationary hydrophilic aqueous layer adjacent to the intestinal lining. Combined micelles, which are rich in BS and act as surfactants, facilitate their movement towards the surface of the duodenal-jejunal epithelium. Upon contact with the plasma membrane of the brush border, these substances are released (58, 59)

Medium-chain fatty acids (MCFAs), being more hydrophilic, do not require incorporation into micelles to cross the unstirred water layer. Conversely, when the concentration of digestion products in the aqueous phase is high while the concentration of BAs remains low, liquid crystalline vesicles (liposomes) with a diameter of 40–60 nm can form. These processes may play a crucial role in how enterocytes absorb long-chain fatty acids (LCFAs) and monoglycerides in individuals with BS deficiencies. Moreover, as micelles release digestion products at the surface of the epithelium, their structure disassembles, leaving BAs within the intestinal lumen to be reused in the formation of new micelles. This cycle continues until the BAs reach the terminal ileum, where they are reabsorbed and re-enter the enterohepatic circulation (60).

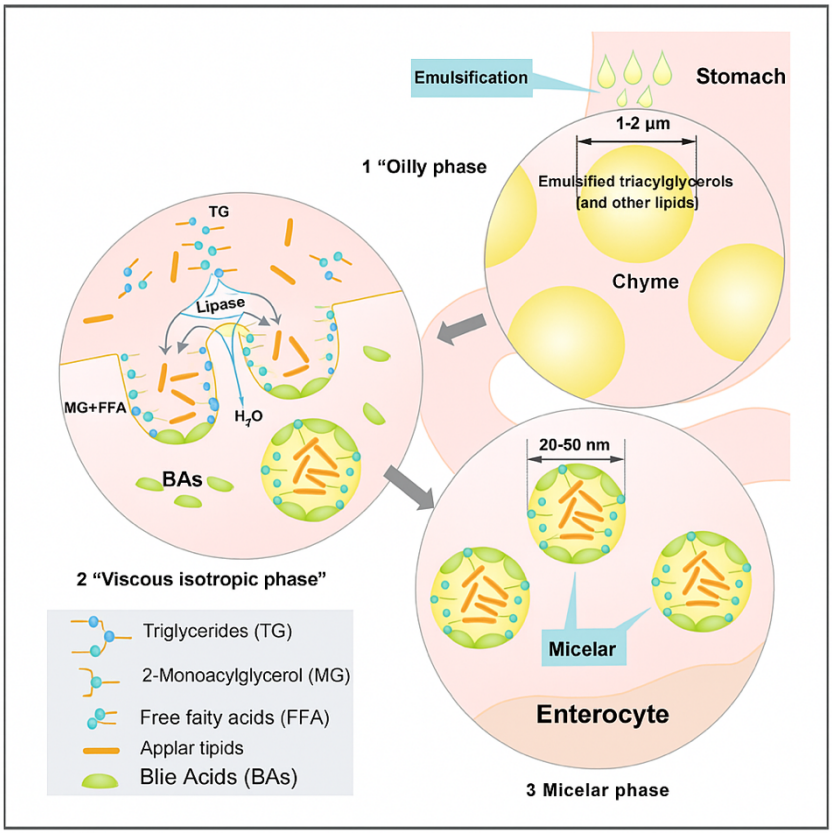

Figure 1. 2 Digestion of fat molecules in small intestine lumen and micelles formation.

1.2.2.3 BAs and fat intake

It has been described that, after a meal, nutrients reach the small intestine, where they are detected by enteroendocrine cells (58). This triggers the release of cholecystokinin (CCK), which induces

gallbladder contraction to release bile into the duodenum (58). The consumption of high-fat or mixed-fat meals elicits postprandial responses to fat that involve multiple processes, including BA secretion, digestion, and absorption. These responses exhibit distinct kinetics and mechanisms depending on the fat content and type (58).

In high-fat meals like oral lipid test tolerance (OLTT), postprandial plasma levels of BAs rise significantly in the postprandial state. An OLLT study has shown a threefold increase in primary and secondary BAs, a 4.3-fold increase in glycine-conjugated BAs and a 5-fold increase in taurine-conjugated BAs in plasma (61). The postprandial bile acid response is linked to the fat content in the meal to aid in fat absorption. The most significant increases were in conjugated BAs, which are efficiently reabsorbed and recirculated through the enterohepatic circulation. The presence of fat leads to more prolonged bile acid secretion and absorption compared to meals with little fat (61, 62).

In a mixed meal containing carbohydrates, proteins, and fats, the BA response is still primarily driven by the fat content, although it differs slightly from that of a purely high-fat meal. BAs respond to both the fat content and the mixed composition of the meal. Research indicates that a mixed meal (e.g., 28% fat, 16% protein, and 56% carbohydrates) induces a threefold increase in total BAs (63). Plasma BA levels rise gradually and remain elevated for a longer duration due to delayed fat digestion compared to an OLTT. Additionally, the consumption of mixed meals containing fats can result in higher BA secretion than an OLTT (62).

1.2.3 Short Chain Fatty Acids

SCFAs are essential metabolites produced by the fermentation of dietary fibres by the gut microbiota (64). The fermentation of complex polysaccharides generates these metabolites as end products, along with gases such as carbon dioxide, hydrogen, and methane. SCFAs, consisting of one to six carbons, are classified as saturated aliphatic organic acids. Acetate (C2), propionate (C3), and butyrate (C4) are the most abundant forms, typically present in faecal samples at a molar ratio of 3:1:1(65). According to faecal sample analyses, the concentrations of acetate, propionate, and butyrate are approximately 52 mM, 14 mM, and 9 mM, respectively (66). In contrast, significantly lower concentrations have been observed in peripheral blood, measuring 79 µM, 5 µM, and 4 µM, respectively (65). Other organic acids, including caproate (C6), valerate (C5), and formate (C1), as well as succinate and lactate, are found in considerably smaller amounts (65, 67). Nondigested proteins or peptides may also serve as substrates, contributing to the remaining <5% of microbial SCFA production of SCFA (68). Microbial protein fermentation generates a variety of metabolites, including amines, indoles, phenols, thiols, and branched-chain fatty acids, many of which can negatively impact epithelial and barrier function, as well as the gut and metabolic health of the host (69-71).

1.2.3.1 Absorption, transport and systemic distribution of SCFAs

SCFA production occurs in the caecum and proximal colon, with rapid absorption in the colon, as only 5–10 per cent are excreted in faeces. SCFAs can be absorbed through the apical membrane of colonic epithelial cells via four proposed mechanisms: bicarbonate exchange at a 1:1 ratio (72);. sodium-coupled monocarboxylate transporter 1 (SMCT1) (73); hydrogen-coupled monocarboxylate transporters (MCT1, MCT2, and MCT3); and non-ionic diffusion of protonated SCFAs (74, 75).

SCFAs absorbed in the caecum, ascending colon, or transverse colon are transported via the superior mesenteric vein, whereas those absorbed in the descending colon and sigmoid colon enter the inferior mesenteric vein. Both veins ultimately drain into the portal vein, delivering SCFAs to the liver (75, 76). Research investigating SCFA dynamics during major upper abdominal surgical procedures has revealed that acetate is the most abundantly absorbed SCFA in the liver. It also achieves the highest peripheral concentration (19–160 µmol/l) compared to propionate (1–13 µmol/l) and butyrate (1–12 µmol/l) (76, 77). SCFAs are present in the blood of the liver, portal vein, and peripheral circulation. Their levels in the systemic bloodstream fluctuate depending on the rate at which they are produced and absorbed in the gut, as well as the influence of dietary intake on their production (75, 78).

1.2.3.2 SCFA receptors

The mechanisms proposed for SCFA receptors are primarily twofold: gene expression through the inhibition of histone deacetylase (HDAC) and signalling via G-protein-coupled receptors (GPRs). Acetate, propionate, and butyrate have been shown to signal through surface free fatty acid receptors, specifically GPRs. GPR41, GPR43, and GPR109A are expressed in various tissues, including epithelial cells, adipose tissue, and immune cells such as neutrophils, dendritic cells, macrophages, and lymphocytes, including T-cells. Activation of these GPRs inhibits cAMP-dependent signalling pathways while simultaneously triggering alternative pathways, such as mTOR signalling, which occurs downstream of GPR41, GPR43, and GPR109A (79). These GPRs are expressed in diverse tissues and exhibit different affinities for specific SCFAs, contributing to the wide range of physiological roles associated with SCFAs (80). GPR41 is expressed across numerous tissues, whereas GPR43 is predominantly found in lymphatic tissues and immune cells. Both GPR41 and GPR43 interact with acetate, propionate, and butyrate, while GPR109A primarily responds to butyrate. Additionally, GPR43 activation by SCFAs induces cell-type-specific effects. Acetate and propionate have also been shown to modulate blood pressure through olfactory receptor 51E2, which is expressed in vascular smooth muscle cells and the kidney (81, 82).

Butyrate and propionate exert their effects, in part, by inhibiting histone deacetylase (HDAC) activity (83). SCFAs can enter cells either through passive diffusion or via specific transmembrane carriers,

such as sodium-coupled monocarboxylate transporter 1 (SMCT1) and monocarboxylate transporter 1 (MCT1). Once inside the cell, SCFAs can bind to and inhibit intracellular HDACs. Furthermore, at low concentrations, butyrate and propionate increase histone acetylation by being metabolised to acetyl-CoA, which serves as an acetyl donor for histone acetyltransferases (84). Butyrate can also directly donate an acetyl group for histone acetylation, influencing gene expression in intestinal epithelial cells, macrophages, dendritic cells, and lymphocytes (83, 85). Research into butyrate's effects on immune and non-immune cells has demonstrated changes in pathways related to the cell cycle, differentiation, antibacterial responses, inflammation, fatty acid metabolism, and oxidative stress (83, 85).

Different molecular targets illustrate how varying gradients of SCFAs can influence diverse tissues. Cells with both immune and non-immune functions mediate a wide range of processes at the mucosal barrier, regulating immunity both locally and in distal organs.

1.2.3.3 Formation of SCFAs

SCFAs are produced through a shared metabolic process (Figure 1.4). This process begins with non-fermentable carbohydrates undergoing hydrolysis. Depending on the number of carbon atoms in the carbohydrate, this hydrolysis occurs either via glycolysis or the pentose-phosphate pathway (86, 87). Acetate is produced through two distinct routes. The primary pathway involves the hydrolysis of acetyl-CoA, which is derived from non-fermentable carbohydrates. The secondary pathway, known as the Wood-Ljungdahl pathway, accounts for approximately one-third of acetate production. In this process, carbon dioxide is converted into carbon monoxide, which is subsequently methylated to produce acetate (88-90).

The synthesis of propionate occurs via three distinct metabolic routes, each requiring specific bacterial species (91). The succinate carboxylate pathway is the most common mechanism, in which phosphoenolpyruvate (PEP) is converted into oxaloacetate. This compound then progresses through several intermediary stages, including succinate and methylmalonate, before ultimately forming propionate. Bacteria from the *Firmicutes* and *Bacteroidetes* phyla are known to utilise this metabolic pathway. Within the *Veillonellaceae* and *Lachnospiraceae* families, the acrylate pathway facilitates the fermentation of lactate into acrylate and, subsequently, propionate. Concurrently, the propanediol pathway, found in *Proteobacteria* and the *Lachnospiraceae* family, enables the transformation of deoxy sugars into propionaldehyde, then propionyl-CoA, and ultimately propionate (91, 92). The primary pathway for butyrate production involves the conversion of butyryl-CoA to butyrate via the acetyl-CoA pathway, which also generates acetate as a by-product (87, 91, 93).

1.2.3.4 Short-Chain Fatty Acids: modulators of immune and metabolic responses

SCFAs are present in the bloodstream and can be absorbed by various organs, where they function as both signalling molecules and metabolic substrates. Among these SCFAs, butyrate is particularly significant, playing crucial roles in modulating immune responses and contributing to the prevention of various diseases (92). SCFAs play a key role in maintaining intestinal barrier integrity, reducing inflammation, and promoting regulatory T cell differentiation, thereby protecting against diseases such as inflammatory bowel disease, asthma, and allergies (92). Furthermore, their influence extends to extra-intestinal health, impacting conditions such as non-alcoholic fatty liver disease, multiple sclerosis, and type 1 diabetes by modulating immune and metabolic responses (92). SCFAs, particularly propionate, are involved in appetite regulation through their role in stimulating peptide YY and glucagon-like peptide-1 (GLP-1). They also contribute to energy homeostasis by modulating neural activity that regulates appetite, thereby helping to maintain energy balance in the body (93, 94). SCFA levels can be influenced by diet, antibiotics, and microbial diversity. They have potential therapeutic roles in managing and preventing immune-mediated diseases and infections by modulating the microbiome and regulating the immune system (92).

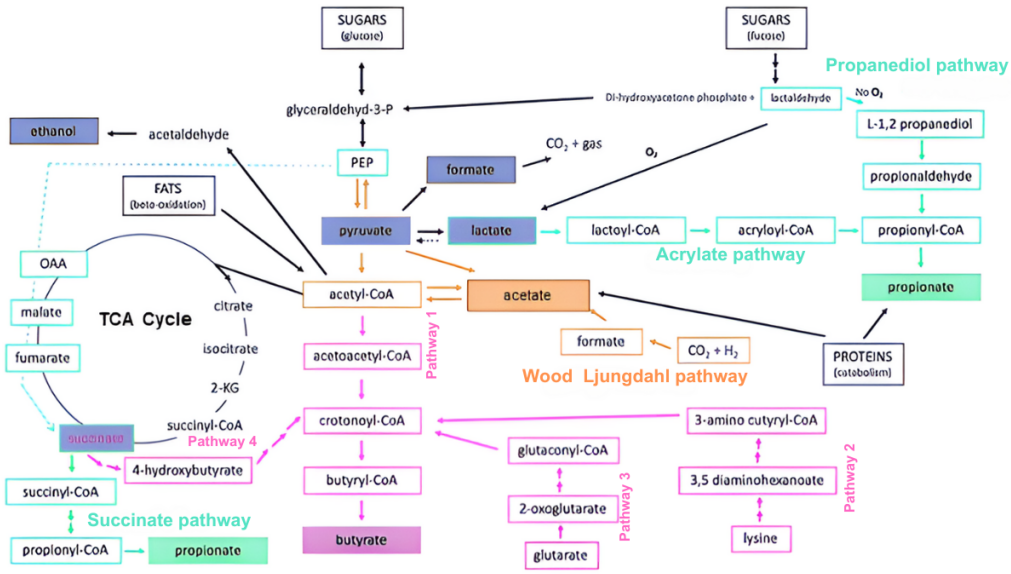

Figure 1. 3 Biochemical pathways for the synthesis for the three key SCFAs: acetate (orange), propionate (green) and butyrate (pink). Acetate (2C) is synthesized in microorganisms through the acetyl-CoA and Wood–Ljungdahl pathways. Propionate (3C) is produced via the succinate, acrylate, and propanediol pathways. Butyrate (4C) synthesis involves four distinct pathways: acetyl-CoA (pathway 1), lysine (pathway 2), glutarate (pathway 3), and succinate (pathway 4). Under aerobic conditions, the tricarboxylic acid (TCA) cycle operates in a modified branched form to generate propionate via the succinate pathway. Intermediate metabolites, such as lactate and succinate, are highlighted in blue. Figure adapted from Figure 1, reference (95).

1.2.4 Bile acids

BAs are amphipathic molecules with a steroid backbone (96). The synthesis of primary BAs occurs predominantly in hepatocytes and is initiated by the hydroxylation of cholesterol, catalysed by CYP7A1 (cholesterol 7 alpha-hydroxylase or Cytochrome P450 7A1). These primary BAs are then conjugated with one of the amino acids, glycine or taurine (97). Primary BAs are further metabolised by the gut microbiota into secondary BAs, while tertiary BAs are formed in the liver and gut through the modification of secondary BAs (98, 99).

Primary BAs primarily consist of cholic acid (CA) and chenodeoxycholic acid (CDCA), while secondary BAs include deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), among others (100). Each BA possesses distinct chemical properties and biological activities. Following meal ingestion, BAs are released from the gallbladder into the duodenum to facilitate the digestion and absorption of dietary lipids and fat-soluble vitamins A, D, E, and K (101).

Due to the inherent properties of lipids, a significant challenge in their absorption lies in the conversion of these non-polar compounds into an emulsion or a hydrophilic form, such as micelles, which can effectively reach the surface of the gut epithelium for absorption. Bile acids are critical in facilitating this process.

1.2.4.1 Bile secretion

Bile secretion is stimulated by secretin and cholecystokinin, which causes the gallbladder contraction. Approximately 750 ml of bile is secreted daily; it is an isotonic fluid with plasma containing bilirubin, lecithin (phosphatidylcholine), cholesterol, and BAs. The primary bile acids, cholic and chenodeoxycholic acids, can be transformed by intestinal bacteria into secondary bile acids, lithocholic and deoxycholic acids, the latter of which is easily reabsorbed at the ileal level and can also be found in bile (96, 102). Cholic acid and chenodeoxycholic acid each represent 40% of the bile acids, while deoxycholic acid accounts for 20% of the total bile acid pool (103). These acids are conjugated with the amino acids glycine and taurine to form conjugated bile salts, a process that enhances their hydrophilicity (Figure 1.5). The concentration of bile salts in the duodenum is elevated during the first 30 minutes following food intake due to the initial contraction of the gallbladder, which releases its contents into the lumen; subsequently, the concentration decreases and stabilises (103).

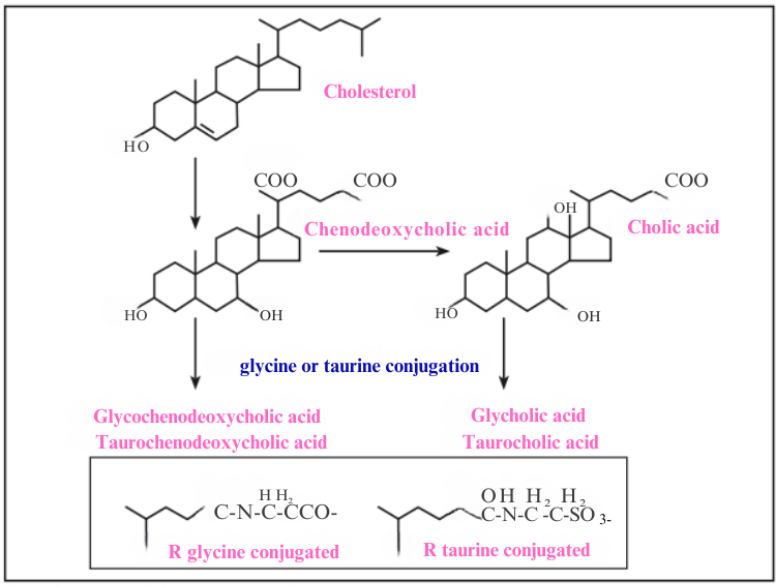

Figure 1. 4 Chemical structure of cholesterol, primary bile acids and conjugated.

BAs are reabsorbed in the terminal ileum through the action of a sodium-dependent active transporter known as the ileal bile salt transporter (IBAT), also referred to as the apical sodium-bile acid transporter (ASBT) (103). Following reabsorption, BAs enter the circulation, are taken up by the liver, and are subsequently excreted back into the bile. Consequently, a proximal-to-distal gradient of BAs is established in the intestine, with concentrations ranging from 8 to 10 mM in the jejunum to 0.9 to 2 mM in the distal ileum (103). The enterohepatic circulation of BAs (Figure 1.6) occurs two to three times during the digestion of a meal, or six to ten times per day. Despite the total bile acid pool being relatively small, comprising only 2 to 4 g, their rapid recycling accounts for the high volume of BAs secreted into the duodenum, which ranges from 12 to 40 g per day (102). This secretion is not continuous throughout the day but instead occurs in relation to meal consumption. The half-life of bile salts (BS) is approximately two to four days; any losses of BS are compensated by de novo synthesis, which produces about 0.6 g per day, representing between 15% and 30% of the total bile acid pool (103, 104).

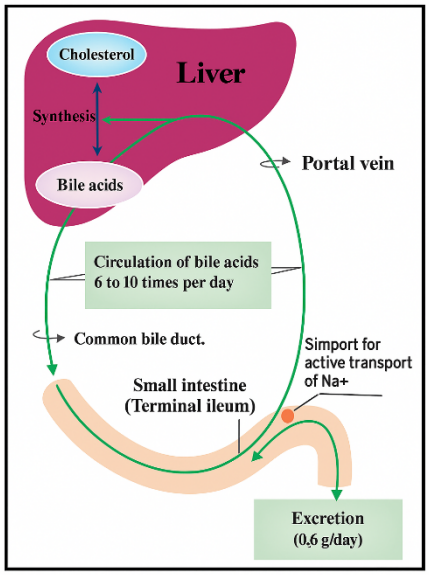

Figure 1. 5. Enterohepatic cycle of bile acids.

1.2.4.2 Synthesis and chemical properties of bile acids

In humans, primary bile acids, specifically cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesised by hepatocytes through the 7-hydroxylation of cholesterol mediated by a specific hydroxylase enzyme (58, 105, 106). The activity of this enzyme is regulated by the end products of the synthesis process, which exert feedback inhibition on further bile acid synthesis. From this initial compound, and through successive hydroxylation reactions, cholyl-CoA is produced, serving as the precursor for all BAs. Bile salts are formed when the terminal carboxyl group of the activated side chain reacts with the amino group of glycine or taurine. These bile salts are amphipathic and exhibit greater hydrophilicity compared to cholesterol, thereby enabling the formation of micelles that assist in the absorption of fats and fat-soluble vitamins within the intestine (58). Unconjugated BAs possess a pKa of approximately 5. Conjugation with taurine or glycine results in a lower pKa for bile acids, increasing their solubility in water while reducing their ability to permeate lipid membranes (103). C27 bile alcohols are conjugated with sulfate, while C27 bile acids are conjugated with taurine. C24 bile acids are predominantly conjugated with taurine or glycine, and, to a lesser extent, with glucuronate, N-acetylglucosamine, or sulfate (106). Within the physiological pH range, most bile acids exist in their deprotonated form; however, when the acid concentration is elevated, they predominantly exist in the protonated form (bile acids). Ionised bile salts exhibit greater amphipathicity, enhanced water solubility, and increased emulsifying capacity relative to protonated bile acids, facilitating the formation of mixed micelles (103). Within the pH range of 5 to 7 in the duodenal lumen, conjugation with glycine or taurine enhances the relative proportion of ionised bile salts compared to that of protonated bile acids (107).

1.2.4.3 Bile composition

Hepatic bile constitutes an isotonic fluid with an electrolyte composition resembling that of plasma. The electrolyte composition of vesicular bile differs from that of hepatic bile; this alteration occurs due to the reabsorption of a significant portion of inorganic anions, such as chloride and bicarbonate, across the gallbladder epithelium. Consequently, as a result of water reabsorption at this anatomical location, the concentration of total solutes in bile increases from 3 to 4 g/100 ml in hepatic bile to 10 to 15 g/100 ml in vesicular bile (58).

The primary solutes in bile, expressed as a molar percentage, comprise bile acids (80%), lecithin along with smaller amounts of other phospholipids (16%), and unesterified cholesterol (4%). In lithogenic bile, the cholesterol concentration can increase to 8-10%. Other constituents include conjugated bilirubin, proteins (such as immunoglobulins and serum albumin), metabolites of specific hormones and proteins metabolised in the liver, electrolytes, mucus, as well as various drugs and their metabolites. The total secretion of hepatic bile is approximately 500 to 600 mL per day. Numerous substances that are either taken up or synthesised by hepatocytes are secreted into the bile canaliculi, where the canalicular membrane forms microvilli and is associated with actin microfilaments, microtubules, and other contractile elements. Prior to their secretion into bile, many substances taken up by hepatocytes are conjugated, whereas others, including phospholipids, certain primary bile acids, and some portion of cholesterol, are synthesised de novo (58).

1.2.4.4 Enterohepatic circulation of bile acids

Following their secretion into bile, bile acids (BAs) are recycled in the small intestine. During the enterohepatic cycle, BAs are absorbed from the intestinal lumen and subsequently transported to the liver via the portal vein, where they are reabsorbed by hepatocytes and secreted once more into bile (102, 105). The principal mediator of the intestinal absorption of bile salts is the sodium-dependent bile salt cotransporter (ASBT), which is expressed in the apical region of enterocytes, predominantly in the terminal ileum and to a lesser extent in the jejunum (103). The hepatic recovery of BAs from the portal vein is incomplete; their bile acid concentration can fluctuate from 10 to 80 µM in comparison to the peripheral circulation levels (2-10 µM) (108).

Enterohepatic circulation reduces faecal loss of bile acids to a mere 3-5% per day, thereby minimising the need for de novo synthesis. Bile acids present in the systemic circulation that evade the enterohepatic cycle are filtered in the glomeruli and reabsorbed by transporters in the proximal convoluted tubule (109). The cyclical pooling of BAs between the liver and intestine can be modulated by dietary patterns, specifically the consumption of lipids and carbohydrates (101, 110).

1.2.4.5 Role of bile acids

BAs are integral to both nutrient digestion and systemic metabolic regulation, acting as emulsifiers in the digestive tract and as signalling molecules throughout the body (3). BAs are pleiotropic signalling molecules and agents that rapidly activate nuclear receptors, such as the farnesoid X receptor (FXR), Takeda G protein-coupled receptor 5 (TGR5), and cell signalling pathways that regulate lipid, glucose, energy metabolism, and gut permeability (111-114). Additionally, it has been proposed that BAs relay messages of ingestion and energy availability to the peripherical tissues (43). At the intestinal level, BAs are known to have an effect on modulating pancreatic enzymes and being released by cholecystokinin (CCK) release (115). Moreover, they have antimicrobial capacity that prevent bacterial overgrowth in the small intestine (116).

The dual roles of BAs in emulsification and signalling demonstrate their crucial function as mediators of nutrient digestion and systemic metabolic adaptation. Their detergent-like activity ensures efficient lipid absorption, while their signalling functions through receptors like TGR5 translate nutrient availability into coordinated metabolic responses across tissues (3).

1.3 Brief overview of metabolic phenotyping in nutritional research

Note: This section is based on the following published research that I authored:
- Brignardello J, Holmes E, Garcia-Perez I."Metabolic Phenotyping of Diet and Dietary Intake". Adv Food Nutr Res. 2017;81:231-270 (Book Chapter). PMID: 28317606

Nutrition studies in clinical settings and epidemiological cohorts have unequivocally demonstrated significant associations between the consumption of different dietary patterns and health outcomes. However, despite these advances, studying these associations remains a complex and challenging endeavour, particularly in real-world settings where accurate dietary assessment is often affected by factors such as misreporting and the variability of eating behaviours. Addressing these challenges requires the exploration of new methodologies and approaches to fill the existing gaps to enhance and improve the understanding of the impact of dietary patterns on health.

Metabolic phenotyping encompasses the comprehensive simultaneous profiling of multiple metabolites within a biological system, as well as the temporal changes induced by factors such as genetic, environmental, and pharmaceutical influences (117). Through the application of advanced analytical techniques, a high-density data set can be generated to characterise the biochemical composition of a diverse array of samples, including human biofluids. This methodology is also applicable in nutritional research, specifically to assess the changes in multiple metabolites that occur before, during, and after dietary interventions. Metabolic phenotyping can elucidate and enhance our understanding of the intricate interactions between food intake and metabolism (39). The primary applications of metabolic phenotyping are illustrated in Figure 1.7.

By leveraging thousands of analysed metabolites that provide information on physiological status, diet intake, metabolism, and environmental exposure, traditional standardised nutritional advice for individuals can be transformed into personalised nutritional management. This approach considers the unique responses of the individual, lifestyle, culture, environment, and phenotype, allowing a more tailored nutritional strategy.

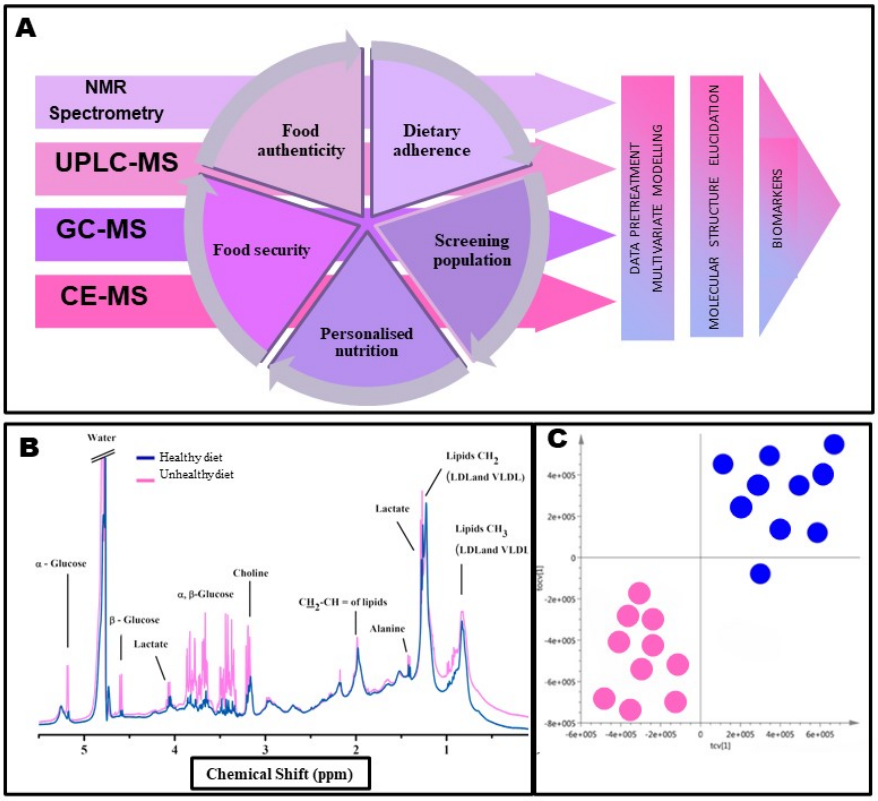

Figure 1. 6 Applications and workflow of metabolic phenotyping in selected nutritional and food areas. A) Common analytical techniques used in nutritional and food settings and summarised metabolomics workflow. B) Illustration of 1D NMR spectra with corresponding identification of metabolites. C) Illustration of multivariate analysis, principal component analysis (PCA) for subjects who consumed a healthy and unhealthy diet (blue, healthy diet and pink, unhealthy diet). NMR, Nuclear Magnetic Resonance; UPLC-MS, ultra-performance liquid chromatography mass spectrometry; GC-MS, gas chromatography mass spectrometry; CE-MS, capillary electrophoresis mass spectrometry. Figure adapted and modified from Figure 1, reference (39).

Biofluid samples, such as plasma, serum, urine, and faecal samples, are fundamental in metabolic profiling, including cell culture media analyses. These samples are typically analysed using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy or various forms of chromatography, including liquid, gas, and capillary chromatography, coupled with mass spectrometry. These analytical approaches can be categorised into two distinct types: untargeted and targeted methods. Untargeted approaches provide a comprehensive metabolic profiling of all components within a sample, whereas targeted methods focus on quantifying specific, pre-selected metabolites (39).

Analytical instruments exhibit varying degrees of sensitivity, with some requiring elaborate sample preparation protocols, while others provide quicker and more consistent results at the cost of reduced sensitivity. Consequently, it is crucial to understand the advantages and limitations of each technique to determine the most suitable method for a given sample type, experimental setup, and research objective. This understanding allows researchers to make informed decisions when selecting the optimal analytical approach for their specific needs. See Table 1.3 (39).

Table 1. 3 Summary of main employed analytical techniques considering sample preparation, sensitivity, advantages and limitations. Information extracted from (39).

Analytical Technique	Type of Compounds Analysed	Sample Preparation	Sensitivity	Advantages	Limitations
^{1}H NMR	Small molecules, metabolites, lipids.	Minimal preparation: typically involves simple dilution and pH adjustment.	Moderate Sensitivity: Requires higher concentrations for detection.	Non-destructive, high reproducibility, provides structural information, broad selectivity.	Lower sensitivity compared to MS, requires larger sample volumes, limited detection of inorganic ions.
UPLC-MS / LC-MS	Broad range of metabolites, including polar, non-volatile compounds, and lipids.	Often requires filtration, dilution, and sometimes extraction; minimal derivatisation.	High Sensitivity: Extremely sensitive, especially for low-abundance metabolites.	High sensitivity, minimal sample volume required, excellent chromatographic resolution.	Less robust than NMR, time-consuming, challenging novel compound identification.
GC-MS	Volatile organic compounds, metabolites.	Requires derivatisation to make compounds volatile; involves extraction and concentration.	High Sensitivity: Very sensitive, capable of detecting low levels of volatile compounds.	High sensitivity, good separation resolution, comprehensive databases for metabolite identification	Requires sample derivatisation, slower analysis time, non-recoverability of samples
CE-MS	Ionic and highly polar metabolites	Requires minimal preparation, usually dilution and sometimes derivatisation	Moderate to High Sensitivity: High sensitivity for ionic and polar metabolites, but variability in reproducibility.	High separation capabilities, rapid detection, low sample volume	Poor reproducibility, less suitable for complex biological samples

It is important to highlight that no single analytical technique is capable of providing an analysis of the entirety of compounds present in biological samples, food, dietary supplements, pharmaceutical derivatives, food chemicals, microbiome metabolites, nutrients, and phytochemicals. Each platform offers a different information set and partially represents a segment of the metabolome. Combining multiple analytical platforms for metabolic profiling can yield a more integrative and holistic understanding of the compounds studied within nutritional contexts. However, the selection of the analytical technique will be dictated by practical considerations, including the type of molecules to be detected, the time required for result analysis, and economic feasibility (39).

1.3.1 Chromatography and mass spectrometry: techniques for separation and analysis.

Analysing biofluid samples containing complex mixtures of compounds often requires advanced separation techniques. A variety of established methods are available, each designed to suit the specific physical and chemical properties of the sample and its components. For instance, centrifugation separates materials based on density differences, while electrophoresis utilises gel media to isolate compounds according to their charge and size. Chromatography exploits the diversity of interactions between analytes and both mobile and stationary phases, whereas distillation separates substances based

on their boiling points. The selection of the method depends on the properties of the sample and the analytes of interest, ensuring an efficient approach to sample analysis (118).

Mass spectrometry, in contrast, is a highly versatile tool for identifying and quantifying compounds by measuring their mass-to-charge (m/z) ratios. A mass spectrometer consists of three key components: an ion source, a mass analyser, and a detector, which can be tailored to specific analytical needs. The process begins with ionisation, where molecules in the sample are converted into charged gas-phase ions, either positive or negative. These ions are directed into the mass analyser, which separates them based on their m/z values. Once separated, the ions reach the detector, which measures their intensities and records the data as peaks in a mass spectrum. This spectrum provides valuable information about the identity and abundance of the compounds present in the sample (118).

Stable isotope-labelled internal standards are frequently employed in analyses to enhance accuracy. These standards address potential variability in ionisation efficiency and sample preparation, thereby enabling precise quantification. When combined, chromatography and mass spectrometry constitute a powerful analytical pair. Chromatography provides robust separation of complex mixtures, while mass spectrometry facilitates precise molecular identification and quantification. Together, these techniques serve as essential tools in chemical and biological research, allowing for the elucidation of the complexities of molecular systems and the uncovering of detailed insights into their structures and functions (118).

1.3.2 Ultra performance liquid chromatography coupled to mass spectrometry in biofluids analysis

LC component separates analytes based on their interactions with the stationary and mobile phases. Reverse-phase chromatography, the most widely used LC method, employs a hydrophobic stationary phase, typically a silica column modified with hydrocarbon chains (e.g., C18), alongside a polar mobile phase. Molecules with higher hydrophobicity bind more strongly to the stationary phase and elute more slowly. Gradually, elution is often employed to optimise separation, where the composition of the mobile phase changes gradually over time, improving the retention and resolution of analytes with varying polarities.

Following separation, analytes are introduced into the mass spectrometer, where ionisation occurs—most commonly through electrospray ionisation (ESI). This gentle ionisation technique applies a high voltage to convert liquid samples into charged droplets. As the droplets evaporate, charged molecules remain and are analysed based on their mass-to-charge ratio (m/z). The mass spectrometer is capable of detecting both negatively and positively charged ions, depending on the operational mode. Upon data analysis by a computer, a three-dimensional chromatogram is generated that illustrates the various molecules detected according to their retention time. Molecules with the longest retention times are

typically non-polar, whereas highly polar molecules exhibit shorter retention times. Each retention time corresponds to a spectrum that indicates the mass-to-charge ratio of individual ions. (119-121). See Figure 1.8 (118-120).

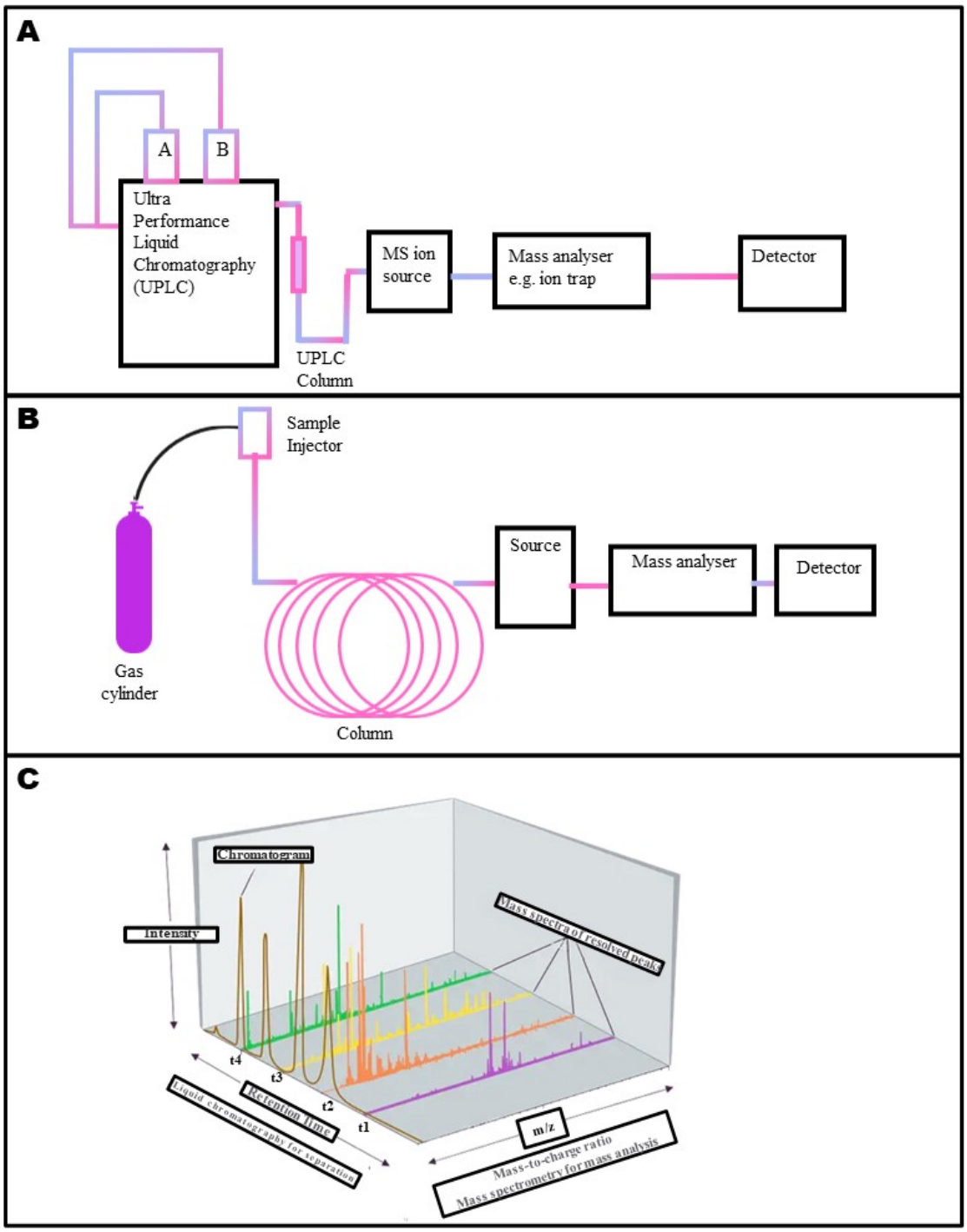

Figure 1. 7 A scheme of UPLC-MS and GC-MS. A) Diagram of UPLC- MS. B) Diagram of GC-MS. C) 3D picture of a chromatogram of elements.

1.3.3 Gas chromatography-mass spectrometry (GC-MS) in biofluids analysis

GC-MS is a powerful analytical technique widely used for identifying and quantifying volatile, low molecular weight compounds, particularly in the analysis of biofluids (118, 121). It combines two fundamental processes: gas chromatography (GC) for separating compounds and mass spectrometry (MS) for identifying and quantifying them. This combination has made GC-MS an essential tool in metabolomics and biomarker discovery (118).

In the GC stage, a sample is injected and carried through the system by an inert gas, such as nitrogen or helium, which acts as the mobile phase. The gas transports the sample through a column coated with a stationary phase, typically composed of a polymer or a liquid film on a solid support. As the sample moves through the column, its components separate based on their volatilities and interactions with the stationary phase. This separation is achieved using a temperature-programmed gradient that ranges from 120 to 400 °C, allowing the temperature to increase gradually to ensure that analytes with varying boiling points are efficiently eluted (118).

Once analytes are separated, the compounds pass into the MS system for ionisation. A commonly used method is electron impact (EI) ionisation, where high-energy electrons fragment the molecules into distinctive ions (119). These ions are sorted in the mass analyser, often a quadrupole, based on their mass-to-charge ratio (m/z). The detector then records these ions and produces a mass spectrum that serves as a unique fingerprint for each metabolite. By combining retention time data from GC with mass spectral data from MS, GC-MS achieves exceptional accuracy and sensitivity, even when analysing complex biological samples. This analytical approach is comparable in many respects to UPLC-MS in terms of its ability to produce precise and reliable data (118, 119).

1.3.4 Data processing and preparation for statistical analysis

Data processing is a critical step in metabolomics research using analytical platforms such as LC-MS and GC-MS. Whether in targeted or untargeted workflows, this process transforms complex raw instrumental outputs into structured numerical datasets suitable for statistical analysis. The quality and reproducibility of the findings depend heavily on robust and well-documented data processing procedures. The general workflow typically includes preprocessing, peak detection (feature extraction), alignment, normalisation, and finally data matrix generation (122, 123).

In untargeted metabolomics, thousands of features are simultaneously measured without prior knowledge of their identity, making data processing especially challenging. Conversely, targeted approaches, which were primarily used in this thesis, involve the quantification of a preselected set of known metabolites, simplifying downstream data handling and interpretation. Nevertheless, both approaches require careful data curation to ensure analytical and biological validity.

The first critical step is preprocessing, where raw LC-MS or GC-MS data undergo initial cleaning and transformation. This includes noise reduction, baseline correction, and smoothing of the chromatographic and spectral data. Noise may originate from various sources, including electronic interferences, chemical background, or matrix effects, all of which can obscure real signals (122). Baseline correction is particularly important for accurate peak integration, as it removes background drift and ensures that peak intensities reflect true analyte concentrations. The software used for preprocessing often includes different platforms that are instrument-specific.

Following preprocessing, the data undergo feature detection, the identification of chromatographic peaks that correspond to chemical compounds. In LC-MS, features are typically defined by their retention time (RT) and mass-to-charge ratio (m/z), while in GC-MS, fragmentation patterns from electron ionisation are used alongside retention times. In targeted workflows, this process involves extracting ion chromatograms for pre-specified transitions, whereas in untargeted workflows, software automatically scans for all potential peaks. The objective is to capture real biological signals while avoiding false positives, which can arise from noise, isotopes, adducts, or in-source fragmentation (124).

Alignment is a critical step in untargeted LC-MS and GC-MS workflows, where a large number of unknown features are detected across samples without predefined identities. Due to minor variations in chromatographic conditions between runs, the same metabolite may elute at slightly different times, leading to misalignment of peaks. Without alignment, these features may be incorrectly assigned as distinct compounds across samples, compromising the consistency and comparability of the dataset. To address this, alignment algorithms are applied to correct retention time shifts and ensure that the same features are matched accurately across the entire dataset (125). In contrast, targeted analyses focus on a predefined set of compounds with known RT and m/z. As such, alignment is generally less critical in these workflows because the integration windows are already set based on expected values (124).

Following alignment (when necessary), normalisation is applied to correct for technical variability and to make samples more directly comparable. In targeted LC-MS and GC-MS, normalisation typically involves the use of internal standards, stable compounds spiked into all samples at known concentrations, which allow correction for variation in sample preparation, injection volume, and instrument sensitivity. The peak area of each target analyte is divided by the area of its corresponding internal standard to produce a normalised value (124).

In untargeted workflows, where internal standards may not be available for every feature, alternative normalisation strategies are employed. These include total ion current (TIC) normalisation, probabilistic

quotient normalisation (PQN), and quality control (QC)-based normalisation methods. These approaches aim to reduce non-biological variability and ensure that observed differences in feature intensities reflect underlying biological variation. Finally, after these steps, a data matrix is obtained that can be statistically analysed. In both targeted and untargeted workflows, rows represent individual samples, while columns correspond to known concentration compounds (targeted) analysis or detected features (untargeted) that can be statistically analysed using different approaches (123).

More details about the analytical strategies employed in this thesis can be found in Chapter 2, Section 2.3. Additionally, the appendix sections provide information about data processing and preparation for data analysis for the targeted methods used.

1.3.5 Statistical analysis

These analytical methods involve analysing multiple samples that can assess different variables simultaneously for the same sample. In targeted analyses, multiple metabolites are evaluated from one sample. Occasionally, decreasing the dimensionality and converting the data into more easily interpretable information becomes necessary. For that, multivariate analysis can extract information from complex datasets, understanding the relationship of multiple variables. Simultaneously, univariate statistics focus on the evaluation of one characteristic. Both types of approaches are relevant for metabolic phenotyping and nutritional sciences.

1.3.5.1 Univariate analysis

This analysis can provide a general perspective on potentially significant compounds or metabolites. Additionally, it can offer a certain level of confidence derived from the results obtained through multivariate models. The Student's t-test, a parametric approach, can be employed to evaluate a null hypothesis regarding the difference between the means of two groups. In contrast, analysis of variance (ANOVA) allows for the comparison of means across more than two groups. Both statistical techniques require the assumption of a normal distribution.

In the case of non-parametric testing, the Wilcoxon Rank-Sum Test (Mann-Whitney U Test) is utilised to compare the medians of two independent groups. The Wilcoxon Signed-Rank Test is applied when comparing two related samples, matched samples, or repeated measurements on a single sample, in order to assess whether their population mean ranks differ. The Kruskal-Wallis test compares the medians of three or more independent groups, whereas the Friedman test compares the medians of three or more paired or matched groups.

In the results analysis, missing values can often be a challenge for data analysis. In that case, the Skillings-Mack test can be used to balance the block design of missing data (126). The p-value is

calculated to determine whether to reject or accept the initial hypothesis based on the predefined significance level. Considering the large number of variables in metabolomics datasets, repeated testing may occur, potentially leading to erroneous positive or negative outcomes. A multiple testing correction was implemented to address this concern by determining the false discovery rate (FDR) using the Benjamini-Hochberg method. This approach helps to minimise the likelihood of obtaining misleading results due to the extensive testing involved in metabolomics analyses (127).

1.3.5.2 Multivariate analysis

Metabolic phenotyping analysis generates highly complex datasets, characterised by their multi- or even mega-variate dimensions, which can make interpretation particularly challenging. Consequently, multivariate statistical methods are fundamental for identifying patterns within the data and linking these to specific variables of interest, such as dietary patterns or health status.

1.3.5.3 Unsupervised methods- principal component analysis (PCA)

Principal Component Analysis (PCA) is a commonly used unsupervised projection method that offers a comprehensive perspective on extensive datasets. This unsupervised projection method identifies the biggest orthogonal directions of variation in the multivariate space of the data, and can reveal latent structure in the data without previous knowledge of data labels. It illustrates the similarities and disparities amongst observations whilst also uncovering information about the metabolite characteristics responsible for the observed patterns. This mathematical procedure reduces dimensionality by extracting orthogonal principal components (PCs) that capture the majority of the variance within the original data. Each PC, constructed as a linear combination of the initial variables, accounts for the maximum variance in descending order, with subsequent PCs explaining variance not addressed by preceding ones. Nevertheless, as PCA differentiates solely on the basis of variance, confounding factors may overshadow the results, particularly when the variance within groups surpasses the variance between the groups of interest.

When data is transformed into PCs, it generates two matrices: scores and loadings. The scores matrix represents the samples' coordinates within the PCA model, while the loadings matrix reflects the variances of the original variables that contribute to these scores. The directions in both matrices are aligned, enabling interpretation by examining corresponding directions in the loadings space. In a scores plot, each data point corresponds to an individual sample, allowing for the detection of metabolic separation if differences exist between metabolic profiles.

Additionally, outliers can be identified as points lying beyond the 95% confidence interval of the modelled variation in Hotelling's T2 region. However, it is important to exercise caution before excluding any outliers from the analysis.(122, 123).

1.3.5.4 Supervised methods- Orthogonal partial least squares- discriminant analysis (O-PLS-DA)

O-PLS-DA is a supervised method for pattern recognition that uses projection to model the relationships between dependent and independent variables. It effectively separates predictive variation from non-predictive variation orthogonal to the response. This technique divides the matrix into two components: a predictive component, which models the variable variation associated with the response (class information), and a non-predictive component, which is orthogonal and uncorrelated to this information. By enhancing relevant variation and reducing unrelated variation, O-PLS-DA improves the robustness of the analysis. When the class information is qualitative (such as diet) rather than quantitative (such as age), discriminant analysis is applied.(122, 128). Similar to PCA, O-PLS-DA produces a score plot for subsequent analysis. The quality of the models is typically explained using goodness-of-fit parameters, such as the proportion of explained (R^2) and predicted (Q^2) variance. Both statistics are calculated using the difference-squared (residual) totals between observed and predicted values, evaluated in X (predictors, commonly spectral intensities) or Y (the response, e.g., class labels). R^2 ranges from 0 to 1; higher values indicate a better fit. By contrast, Q^2 evaluates how well the model fits held-out samples (via cross-validation or an external test set) and thus quantifies goodness of prediction. By definition, R^2 is always higher than or equal to Q^2, and when these parameters are closer to 1, it indicates a better model. In unsupervised analyses, R^2X and Q^2X are calculated on X. In supervised analysis, the goodness of predictions is calculated with respect to the outcome, R^2Y and Q^2Y (123)

The primary tuning parameter in PCA and OPLS-DA is the number of components. The decision usually considers the use of cross-validation to maximise the goodness of fit to the left-out data, particularly Q^2. As an acceptance criterion, it is recommended to increase the explanation of the variance by at least 5 % in Q^2 before adding a new component in both unsupervised and supervised analyses. This recommendation is arbitrary and can be adjusted; however, when Q^2 starts to decrease, it indicates that noise is being modelled, and the model is overfitting. The number of components must always be lower than the number of samples or variables in the dataset. Overfitting is particularly problematic in supervised models. It can be prevented by limiting the number of components (the increase in the number of components increases the risk of overfitting) or by applying additional resampling techniques, such as cross-validation.

1.4 Scope of this thesis: hypothesis and aims

Diet plays a key role in gut metabolite production, as nutrients act as substrates for metabolism by the gut microbiota (e.g., in the case of SCFAs) or contribute to the release and metabolism of bile acids (BAs). Some studies have reported differences in gut metabolites, such as SCFAs and BAs, between free-living vegans and omnivores using serum and faecal samples. However, research simultaneously exploring serum, urine, and faecal samples in these populations under free-living conditions is currently unexplored. Furthermore, as mentioned earlier, dietary records pose significant challenges in free-living populations, with rates of misreporting ranging from 21.5% to 67% (35) . The effect of different dietary patterns on gut metabolites under controlled inpatient dietary conditions also remains unexplored, and the influence of these dietary patterns on gut metabolism is not yet well-understood.

The hypothesis of this work is that specific dietary patterns will be reflected in blood, urine and faecal gut metabolites. Metabolic differences related to these dietary intakes will be assessed by understanding the differences in chemical and nutritional compositions.

The overall aim of this project is to understand how the consumption of specific dietary patterns, based on their nutritional composition, influences metabolic changes in the biofluids of healthy volunteers. This project will generate valuable data related to SCFA production, BAs metabolism, their interactions with host-microbial metabolomes and global profiles in urine, blood and faeces according to the dietary pattern consumed by healthy individuals exposed to vegan, omnivores, healthy and unhealthy dietary patterns.

The specific aims of this project included

1. To compare the concentrations of SCFAs of vegans versus omnivores, considering the nutritional composition of each dietary pattern (Chapter 3);

2. To compare the temporal changes in SCFA concentrations in response to two omnivorous dietary interventions that differed in nutritional composition under inpatient controlled conditions. (Chapter 4);

3. To compare the concentrations of BAs of vegans versus omnivores, considering the nutritional composition of each dietary pattern (Chapter 5);

4. To compare the temporal changes in BA concentrations in response to two omnivorous dietary interventions that differed in nutritional composition under inpatient controlled conditions (Chapter 6);

An overview of the structure of this thesis is provided in Figure 1.9:

Chapter 1

Introduction
Dietary patterns and health and diseases.
The role of fibre, fats and gut metabolites
Analytical techniques employed in Metabolic phenotyping and statistical approaches.

Chapter 2

Material and methods employed in this thesis

Chapter 3

Short-chain fatty acids concentrations in free-living vegans and omnivores.
This chapter evaluates and analysed the differences between SCFAs in omnivores and vegan, studying three different biofluids (serum, urine and faeces) using a novel GC-MS method

Chapter 4

Circulating short-chain fatty acids concentrations after the intake of two contrasting dietary patterns under controlled conditions.
This chapter evaluates the levels of SCFA after the consumption of two extreme dietary patterns that are 25% and 100% aligned with the healthy eating recommendations of the WHO/SCFA were evaluated in serum samples after each meal and 24 hours urine samples using a novel GC-MS method.

Chapter 5

Bile acids concentrations in free-living vegans and omnivores.
This chapter evaluates and analysed the differences between BAs in omnivores and vegan, studying three different biofluids (serum, urine and faeces) using a novel LC-MS method.

Chapter 6

Circulating bile acids concentrations after the intake of two contrasting dietary patterns under controlled conditions
This chapter evaluates the levels of BAs after the consumption of two extreme dietary patterns that are 25% and 100% aligned with the healthy eating recommendations of the WHO. BAs were evaluated in serum samples after each meal and time spots urine samples using a novel LC-MS method.

Chapter 7

General discussion, suggestions and new challenges to explore

Appendixes

Published articles, congress presentations, awards and recognitions, list of supplementary material for different chapters, figures, and tables

Figure 1. 8**.** Overview of the structure of this thesis work**.**

2. Materials and Methods

Parts of this chapter are based on the following published articles that I authored:

- Brignardello J, Fountana S, Garcia-Perez I, Posma M J, et al. 2021. "Characterization of diet-dependent temporal changes in circulating short-chain fatty acid concentrations: A randomized crossover dietary trial". Am J Clin Nutr. 2022. Nov; 116(5): 1368–1378. PMID: 36137188.
- Calder N, Walsh K, Olupot-Olupot P, Ssenyondo T, Muhindo R, Mpoya R, Brignardello J, et al."Modifying gut integrity and microbiome in children with severe acute malnutrition using legume-based feeds (MIMBLE): A pilot trial". Cell Rep Med . 2021 May 18;2(5):100280. PMID: 3409588

For this thesis, two different studies were investigated. The first study involved a cohort of free-living vegans and omnivores, while the second study was a randomised crossover trial that compared two contrasting dietary patterns under controlled conditions.

For each study, short-chain fatty acids (SCFAs) and bile acids (BAs) were analysed from biofluids using targeted methods, employing gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), respectively.

2.1 Study 1: "Gut-related metabolites concentrations in free-living vegans and omnivores

2.1.1 Study design and subjects

Biofluid samples were obtained from a cross-sectional study that recruited volunteers between December 2013 and November 2014. This study was conducted in collaboration with the Pedersen Group from Copenhagen University, Denmark, which had previously published work related to this cohort (129-131). For this particular cohort, 78 omnivores and 82 vegans were recruited through local Danish newspapers and online advertisements.

The inclusion criteria considered vegans and omnivores eligible if they were in good health, aged between 18 and 65 years, and had maintained a stable weight (±1 kg, as determined through an interview) for at least two months prior to the study. Vegan volunteers were eligible to participate if they had adhered to a vegan diet for at least one year. Pregnant and lactating women were excluded from the study. Volunteers were also excluded if they had undergone antibiotic treatment within three months prior to the study, had known gastrointestinal disorders, exhibited symptoms at the time of participation, or were following a medically prescribed diet.

The study adhered to the principles of the Declaration of Helsinki and received approval from the Regional Committee on Health Research Ethics for the Capital Region of Denmark (J.no. H-3-2012-145) (129). Informed consent was obtained from all participants.

2.1.2 Sample collection

Blood and urine samples were taken after 10 hours of overnight fasting. Blood samples were collected via venepuncture of the antecubital vein, separated into serum, and stored at -80°C until analysis. Urine samples were taken as first-morning voids and similarly stored at -80°C until analysis. Participants collected faecal samples at home; subsequently, the research team collected them, preprocessed them, and stored them at -80 °C.

2.1.3 Anthropometry

Height and weight were measured. Height was measured using a wall-mounted stadiometer (ADE MZ10023, ADE, Germany), recording the value to the nearest 0.5 cm, with participants not wearing shoes. Weight was measured using an electronic scale (TANITA WB-110MA, Tanita Corporation of America, USA), with participants dressed in light clothing or underwear after voiding their bladders. Body mass index (BMI) was calculated by dividing the weight (in kg) by the square of the height (in metres) (129).

2.1.4 Dietary records

Dietary intake was assessed using a self-reported 4-day weighed food diary, which included two weekdays and two weekend days within the same week (1). All foods were weighed to the nearest 0.1 g using a precision scale (ProScale XC-2000, HBI Europe, Erkelenz, Germany). Dietary data were accurately collected and validated for 150 participants. Nutrient intake was analysed using the Dankost Pro software (version 1.5.49.21), which utilised the Danish Food Composition Databank, comprising 1,049 food items (www.frida.fooddata.dk). For vegan recipes unavailable in the software, qualified nutrition experts developed them based on foods with complete data in the database. The average daily intake (ADI) of macronutrients and micronutrients was calculated as follows (130):

ADI = ((average on working days × 4) + (average on weekend days × 3))/7

2.2 Study 2: "Circulating gut-related metabolite concentrations after the intake of two contrasting dietary patterns under controlled conditions"

2.2.1 Study design and participants

This was a sub-study from a previous study that was a randomised, controlled, crossover trial to evaluate the impact on metabolism of different diets classified according to their healthiness levels (132). Nineteen healthy individuals (aged 21 to 65 years; nine women and ten men) were selected from the healthy volunteer database of the UK National Institute for Health Research (NIHR)/Wellcome Trust Imperial Clinical Research Facility (CRF). The number of participants included in the study was determined based on literature reports of observed variations in circulating SCFA concentrations between two or more contrasting diets, where participant numbers typically ranged from 10 to 18 (133-135). The body mass index (BMI) of participants was distributed between 20.4 and 33.3 kg/m² (132). (see Table 4.2, Chapter 4). Participants were deemed ineligible for the study if they had any clinically significant medical conditions, experienced a weight change of 3 kg or more in the previous two months, were on prescription medications, smoked, used illicit substances, or showed abnormalities in medical assessments, electrocardiograms, or screening blood tests. Pregnant or breastfeeding women were also excluded. Full details of the inclusion and exclusion criteria are provided in the supplementary materials (Appendices 1 and 2). This study received approval from the London–Brent Research Ethics Committee and was conducted in accordance with the Declaration of Helsinki (13/LO/0078).

2.2.2 Randomisation and masking

Each participant received one dietary intervention during each inpatient stay, with the sequence of interventions assigned randomly throughout the study period. A team member not directly involved in the study performed the randomisation for each participant's diet. Sequentially numbered envelopes containing one of the four dietary interventions in random order were sealed and assigned to participants. For each new individual enrolment, a researcher opened the corresponding envelope. Dietary interventions could not be masked from researchers and patients, but data researchers were masked from the randomisation order.

2.2.3. Dietary interventions

Patients attended the CRF for an inpatient period of 72 hours on four different occasions. Diets were provided over three consecutive days, and samples were collected on day three (Figure 2.1), considering the changes produced in the microbiome following dietary pattern consumption and its impact on metabolite production (136). The processes of absorption, distribution, metabolism, and elimination (ADME) were completed by the majority of the dietary metabolites. Therefore, the kinetics of food metabolites could vary, contributing differently to blood and urine metabolome composition (137). A minimum wash-out period of five days was required for each participant to avoid any potential

carryover effect from another diet delivered previously. For instance, in a kinetic study, tartaric acid, a grape biomarker, had been shown to have urinary excretion a few hours after grape consumption (138). Acetic acid had been observed to reach its peak in blood six hours after consuming pectin with a low-fibre breakfast, with its concentration reduced two hours after reaching its peak (64, 139). Similarly, it has been observed that BAs changed their plasma concentrations after the intake of different types of meals over a period of 90 minutes post-meal (140).

A qualified dietitian developed the dietary interventions based on the WHO's healthy eating guidelines (141). Diet 1 was the most aligned with these recommendations, whereas diet 4 showed the least adherence (Table 2.1). Various non-communicable diseases (NCDs) were associated with dietary patterns that are energetically dense and low in fibre (142, 143). Diet 1 was high in vegetables, fruits, and fibre, while being low in saturated fats, sugars, and salt. In contrast, diet 4 had a different energy density, and diet 1 provided three times the amount of fibre compared to diet 4 (Table 2.1).

Participants were instructed to consume all the food provided for each meal or snack and were allowed to drink water as required; however, this was not quantified. In each visit, the relevance of consuming all the food provided and not leaving the CRF was explained to keep each participant closely monitored. All patients provided informed consent to participate in the study. Food adherence was assessed using a food scale, where food was weighed before and after the food was provided. Participants were permitted to engage in minimal physical activity, such as walking slowly from the hospital bed to the toilet.

Table 2. 1. Dietary content for the healthy diet provided to the 19 volunteers.

Meal type (time)	Diet 1	Amount g	Nutrient intake
Breakfast (09:00)	Whole wheat cereal Semi-skimmed milk Wholemeal bread, toasted Margarine Egg, hard-boiled	60 150 60 10 60	Kcal 664, Protein (g) 26.5, Fat (g) 20.8, Carbohydrate (g) 99.1, Starch (g) 70.0, Total sugars (g) 29.2, Non-starch polysaccharides (g) 11.9, Total dietary fibre (g) 14.9, Saturated fats (g) 5.8, Monounsaturated fats (g) 6.7, Poly-unsaturated fats (g) 6.2, Trans fat (g) 0.81, Cholesterol (mg) 240, Sodium (mg) 724, Potassium (mg) 892, Calcium (mg) 316, Iron (mg) 10.18, Vitamin D (ug) 1.87, Vitamin B12 (ug) 1.7, Vitamin C (mg) 8
Morning Snack (11:00)	Apple, Granny Smith	150	
Lunch (13:00)	Salmon, steamed Jacket potato Garden peas, boiled Carrots, boiled Broccoli, bolied	150 200 60 60 100	Kcal 783, Protein (g) 49.8, Fat (g) 20, Carbohydrate (g) 109.8, Starch (g) 2.8, Total sugars (g) 28.4, Non-starch polysaccharides (g) 13.6, Total dietary fibre (g) 18.1, Saturated fats (g) 3.4, Monounsaturated fats (g) 7.2, Poly-unsaturated fats (g) 5.8, Trans fat (g) -, Cholesterol (mg) 81.0, Sodium (mg) 355, Potassium (mg) 1232, Calcium (mg) 129, Iron (mg) 3.25, Vitamin D (ug) 13.05, Vitamin B12 (ug) 6, Vitamin C (mg) 57
Afternoon Snack (15:00)	Grapes	150	
Dinner (18:00)	Chicken breast, grilled Whole wheat pasta Peppers Onions Tomato pasta sauce	125 150 80 40 150	Kcal 814 , Protein (g) 63.2, Fat (g) 18.2, Carbohydrate (g) 106, Starch (g) 84.7, Total sugars (g) 19.6, Non-starch polysaccharides (g) 9.5, Total dietary fibre (g) 12.9, Saturated fats (g) 3.7, Monounsaturated fats (g) 5.4, Poly-unsaturated fats (g) 6.8, Trans fat (g) 0.75, Cholesterol (mg) 117.7, Sodium (mg) 1289, Potassium (mg) 1829, Calcium (mg) 214, Iron (mg) 5.36, Vitamin D (ug) 1.17, Vitamin B12 (ug) 0.6, Vitamin C (mg) 67
Evening Snack (21:00)	Whole bread, toasted Margarine, polyunsaturated	80 10	

Table 2. 2 (Continuation): Dietary content for the unhealthy diet provided to the 19 volunteers.

Meal type (time)	Diet 4	Amount g	Nutrient intake
Breakfast (09:00)	Sugar-coated cereal Whole milk White bread, toasted Butter	60 150 60 10	Kcal 728, Protein (g) 21.6, Fat (g) 28.6, Carbohydrate (g) 102.2, Starch (g) 56.4, Total sugars (g) 45.8, Non-starch polysaccharides (g) 3.3, Total dietary fibre (g) 3.6, Saturated fats (g) 17.7, Monounsaturated fats (g) 7.1, Poly-unsaturated fats (g) 1.5, Trans fat (g) 0.77, Cholesterol (mg) 63.2, Sodium (mg) 550, Potassium (mg) 661, Calcium (mg) 474, Iron (mg) 6.15, Vitamin D (ug) 0.21, Vitamin B12 (ug) 1.6, Vitamin C (mg) 3.
Morning Snack (11:00)	Greek yoghurt	125	
Lunch (13:00)	Pork sausages, fried Potato waffles, grilled Cola	125 120 330	Kcal 891, Protein (g) 21.6, Fat (g) 45.9, Carbohydrate (g) 103, Starch (g) 12.1, Total sugars (g) 65.3, Non-starch polysaccharides (g) 3.3, Total dietary fibre (g) 4.4, Saturated fats (g) 14.9, Monounsaturated fats (g) 11.2, Poly-unsaturated fats (g) 2.5, Trans fat (g) 0.3, Cholesterol (mg) 69.1, Sodium (mg) 1105, Potassium (mg) 354, Calcium (mg) 244, Iron (mg) 1.75, Vitamin D (ug) -, Vitamin B12 (ug) 1.7, Vitamin C (mg) -
Afternoon Snack (15:00)	Milk Chocolate	45	
Dinner (18:00)	Beef burgers, fried Potato waffles, grilled Processed cheese Tomatoes Cola	100 120 30 100 330	Kcal 1070, Protein (g) 45.1, Fat (g) 51.5, Carbohydrate (g) 112.4, Starch (g) 41.7, Total sugars (g) 45.1, Non-starch polysaccharides (g) 4.3, Total dietary fibre (g) 5.6, Saturated fats (g) 21.6, Monounsaturated fats (g) 15.2, Poly-unsaturated fats (g) 2, Trans fat (g) 1.41, Cholesterol (mg) 142.8, Sodium (mg) 1411, Potassium (mg) 863, Calcium (mg) 396, Iron (mg) 5.01, Vitamin D (ug) 2.05, Vitamin B12 (ug) 3.4, Vitamin C (mg) 17.
Evening Snack (21:00)	White bread, toasted Butter	80 10	

2.2.4 Sample collection

Serum and urine samples were obtained from a vegan and omnivore cohort study at Copenhagen University, Denmark (129, 130). Serum and urine samples from the controlled diet study were obtained from a previously published study (132).Timed blood, spot urine, and 24-hour urine samples were collected on the third day of each diet to allow for diet adaptation before measuring the dietary response in biofluids (136). Blood samples were collected 2 hours after breakfast (AB), after lunch (AL) and after dinner (AD) on day 3 (D3). Fasting (FA) samples were collected on day 4 (D4). Serum samples were obtained within 15 min from whole blood samples and stored at -80 °C (Figure 2.1). On day 3, urine was collected throughout the day in three distinct periods: morning (0900–1300 h; cumulative sample 1), afternoon (1300–1800 h; cumulative sample 2), and evening through to the following morning (1800–0900 h; cumulative sample 3). A 24-hour urine sample was created by combining these individual samples (Figure 2.1). Urine samples were stored at -80 °C and centrifuged before analysis. Samples were not exposed to freeze-thaw cycles prior to SCFA analysis (78).

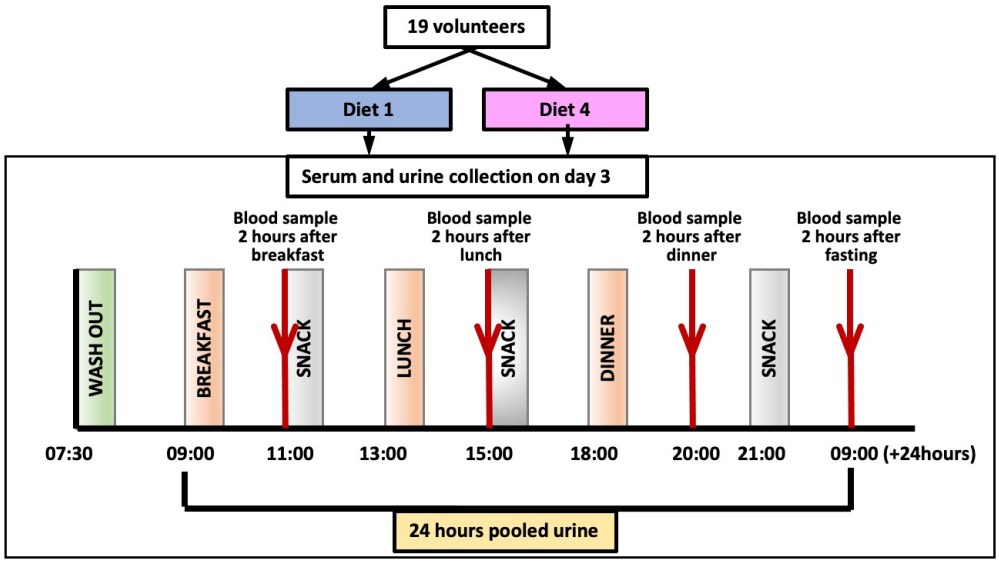

Figure 2. 1 Flowchart of sample collection for serum and urine. Samples were obtained after consuming a healthy (Diet 1) and an unhealthy diet (Diet 4). SCFAs from both biofluids were analysed with GC-MS and LC-MS. Modified from (78).

Additionally, for LC-MS analysis, urine samples were collected at different time points as follows:

Table 2. 3. Urine collection times.

Urine sample time group	Mealtime	Time	Total hours of collections
1	Breakfast to Lunch	9:00 to 13:00	4 hours
2	Lunch to Dinner	13:00 to 18:00	5 hours
3	Dinner to Fasting	18:00 to 9:00 (+24 hours)	15 hours

2.3 Analytical strategies

2.3.1 SCFA targeted method for GC-MS analysis in serum and urine samples

A total of five short SCFAs, three methyl-branched SCFAs (branch chain fatty acids) and two hydroxyl carboxylic acids were analysed by GC-MS using an in-house method developed by Dr Sofia Fountana (144), adapted from Moreau et al. (145) and recently published by Brignardello et al.(78).

2.3.1.1 Materials

All analytical standards were prepared using standards and solvents purchased from Sigma–Aldrich. Acetic acid, propionic acid, isobutyric acid, butyric acid, 2-methylbutyric acid, isovaleric acid, valeric acid, caproic acid, L-lactic acid, (S)-2-Hydroxybutyric acid, and methyl stearate were used as internal standards (IS) (CAS number 85769). The analytes of interest were dissolved in water before preparing the stock solution. Hydrochloric acid (HCl) was used for acidification. Extraction of acids was done with Tert-Butyl Methyl Ether (MTBE). The derivatisation step was carried out with N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide with 1%-tert-Butyldimethylchlorosilane (MTBSTFA + 1%TBDMCS) (78).

GC-MS consumables were purchased from Agilent Technologies: liner (5062- 3587), blue screw caps with red silicone, regular amber vials, silanised amber vials, and deactivated glass inserts (see Appendix 3).

GC-MS analyses were performed using an HP-5MS capillary column (30m, 0.25mm, 0.25 µm) on a 7890B GC, equipped with a 7693A automatic liquid sampler and coupled to a 7010 MS/MS, all from Agilent Technologies. Data preprocessing and compound identification were performed using Agilent MassHunter Workstation Software for Quantitative Analysis (QQQ) (Refer to Appendix 3).

2.3.1.2 Sample preparation

A volume of 100 µL of sample was suspended in 500 µL methyl tert-butyl ether (MTBE) with 100 ppm of methyl stearate as an internal standard (IS) to correct injection variability across

the various sample batches and to compensate for analytical drift or minor instability (78). After, each sample was acidified with 0.01% hydrochloric acid to precipitate sample proteins. A short homogenisation step was included by vortexing each tube for 3 seconds individually, followed by a further 20 minutes using a plate vortex, after which samples were centrifuged for 5 minutes at 4 °C at 10000 rcf (78). Immediately, 90 µL of supernatant was transferred to a silanised vial, and 60 µL of derivatiser *N*-tert- Butyldimethylsilyl- *N*-methyltrifluoroacetamide with 1 % tert-Butyldimethylchloro-silane were added. The samples were then incubated for 45 min at 60 °C. Before GC-MS analysis, 70 µL of the sample was taken into a regular vial with a deactivated insert (78) (see Appendix 5 for laboratory protocol).

For analysis, quality control (QC) samples were prepared by pooling individual samples. These QC samples were run in the instrument between each of the 10 study samples. Analytical calibration curves were prepared from a stock solution and subjected to the same steps as sample preparation. All analytes and IS were included. The ranges of the calibration curves for each compound were shown (Table 2.4 and Appendix 6 for preparation).

Table 2. 4 Compounds and calibrations curve ranges for GC-MS.

Compound	Concentration Range (µM)	Compound	Concentration Range (µM)
Acetic acid	0.8-400	Isovaleric acid	0.08-20
Propionic acid	0.04-20	Valeric acid	0.04-4
Isobutyric acid	0.04-20	Caproic acid	0.02-10
Butyric acid	0.04-20	Lactic acid	80-4000
2-methylbutyric acid	0.04-20	2-Hydroxybutyric acid	4-2000

2.3.1.3 GC-MS analysis in serum and urine

The analysis used for serum and urine for SCFAs was published by Brignardello et al. (14). The GC system used for these analyses was an Agilent 7890B instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a 7693 automatic liquid sampler (Agilent Technologies, Palo Alto, CA, USA). The GC was fitted with an HP-5 MS UI inert capillary column (30 m × 0.25 mm × 0.25 µm film thickness; Agilent J&W Scientific Inc., CA. USA) and helium was used as the carrier gas at 1.5 mL/min. Each injection was made in split mode with an injection of 1 µL and an injector temperature of 250 °C (78). A glass liner ultra-inert, splitless, single taper and glass wool (5190-2293, Agilent Technologies, Palo Alto, CA, USA) was used to avoid any possible column contamination with non-volatile elements (78). The oven temperature program was optimised with an initial temperature of 40 °C and a final

temperature of 300 °C. The total run time of the method was 9 minutes. The GC system was connected to an Agilent 7010 MS/MS triple quadrupole mass spectrometric detector with an electron ionisation (EI) source, operating at 70 eV. The source temperature was maintained at 230 °C whereas the temperature of both quadrupoles was set at 150 °C. The helium quench gas flow was set at 2.25 mL/min and the nitrogen collision gas flow at 1.5 mL/min. A solvent delay of 2 minutes was used. Analyses were performed in positive MRM mode, optimising collision energy for each selected transition during method development. Data acquisition was performed using Mass Hunter workstation software for QQQ (Agilent Technologies, Palo Alto, CA) (14). SCFA concentrations obtained from 24-hour urine samples were normalised to the total volume collected to account for differences in urinary osmolality partially (78). The protocol for sample analysis using the GC-MS instrument was presented step by step in Appendix 7.

2.3.1.4 Targeted data processing

All the studied metabolites were analysed with Agilent Mass Hunter Workstation Software for Quantitative Analysis. Peaks were inspected individually and corrected manually according to their retention times as quality checks, including for IS. The obtained area under the curve was normalised with the respective intensity of the IS (78). Therefore, using calibration curves, resultant values were expressed in concentration levels using µmol/L as a unit. The steps to use the setup software using the information from SCFAs used in the method were in Appendix 8, and data obtention from samples analysis were in Appendix 9.

2.3.2 SCFA targeted method for GC-MS analysis in faecal samples

Faecal samples obtained from the vegans and omnivores study were analysed by GC-MS using a published method for detecting SCFA in faecal samples (146) transferred into a single quad instrument as part of the thesis of Dr Sofia Fountana (144). This method identified and quantified acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid in adults and children. This method was adapted from Garcia- Villalba (146) and later used for analysis in human samples (147).

2.3.2.1 Materials

Water (CHROMASOLV™ LC-MS) was sourced from Fisher Scientific. Phosphoric acid, ethyl acetate, 4-methylvaleric acid as the IS, and all SCFA chemical standards were supplied by Sigma Aldrich. Consumables for the GC-MS/MS analysis, such as liner, blue screw caps with red silicone, regular amber vials, silanised amber vials, and deactivated glass inserts (5183-

2072), were acquired from Agilent Technologies. See section 2.3.1.1 for SCFA standards. (see Appendix 4 for consumable details and CAS numbers).

GC-MS analysis was performed using a DB-WAXetr capillary column (30m, 0.25mm, 0.25 µm) on a 7890A GC equipped with a 7693A automatic liquid sampler, coupled to a 5977A single quadrupole mass selective detector instrument. The instruments and column were from Agilent Technologies. Data preprocessing and compound identification were done using the Agilent MassHunter Workstation Software for Quantitative Analysis (QQQ).

2.3.2.2 Sample preparation

100 mg of faeces was weighed and mixed with 1 mL of water containing 0.5% phosphoric acid. After acidification, the samples were vortexed for 2 minutes and centrifuged for 10 minutes at 18,000 × rcf at 4°C. The organic extract from the faecal water was obtained by adding 1 mL of ethyl acetate, followed by centrifugation for 10 minutes at 18,000 × rcf at 4°C. Subsequently, 600 µL of the organic phase was transferred to a glass vial, and 4-methylvaleric acid was added as an internal standard (IS) to achieve a final concentration of 500 µM.

For analysis, quality control (QC) samples were prepared by pooling organic phases obtained from 15 faecal samples in the same manner as the protocol suggested. These QC samples were run in the instrument between each 10 study samples. A stock solution was prepared in ethyl acetate as a serial dilution ranging from 2µM to 10 mM for all the interest analytes and IS for calibration curves.

2.3.2.3 GC- MS analysis in faecal samples

The method analysis employed for faecal SCFA analysis was the same as that of Calder et al.(147). Metabolite analysis was conducted using an Agilent 7890A GC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a high-polarity DB-WAXetr fused silica capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies 122-7332LTM), which was coated with polyethylene glycol (147). Helium was used as a carrier for the column with a flow rate of 1 mL/min. A volume of 1 µL was injected in splitless mode with a temperature of 250 °C. A glass liner with a glass wool plug was used to prevent contamination with non-volatile material from faecal samples. The column temperature was initially 90 °C, then it was increased to 150 °C at a rate of 15°C /min, then to 170°C at 5°C/min, and finally to 250 °C at 20 °C/min and this temperature was maintained for 2 min. The total run time of the method for faeces was 14 min. The GC system was connected to an Agilent 5977A mass spectrometer, equipped with a single quadrupole detector and an EI source operating at 70 eV.

The system was set to scan the m/z range from 30 to 250. The ion source temperature was set at 230°C, the quadrupole temperature at 150°C, and the transfer line temperature at 280°C. A solvent delay of 3.5 minutes was applied. The target ions for SIM in this method were acetic acid (m/z 60), propionic acid (m/z 74), isobutyric acid (m/z 88), butyric acid (m/z 73), isovaleric acid (m/z 87), and valeric acid (m/z 73)(147). SCFAs were identified based on the retention times of standard compounds.

2.3.2.4 Targeted data processing

As mentioned in section 2.3.1.4, faecal SCFAs were analysed with Agilent Mass Hunter Workstation Software for Quantitative Analysis. Peaks were inspected individually and corrected manually according to their retention times as quality checks, including for IS. The obtained area under the curve was normalised with the respective intensity of the IS. Therefore, using calibration curves, resultant values were expressed in concentration levels using µM as a unit.

2.3.3 BAs targeted method for LC-MS analysis in serum and urine samples

Using the method published by Sarafian et al., 59 BAs and 16 deuterated internal standards (DIS) were considered for these analyses in serum, urine and faeces (98).

2.3.3.1 Materials

UHPLC-MS-grade Chromasolv™ solvents, including acetonitrile (ACN) and 2-propanol (IPA), were obtained from Honeywell International Inc. (USA). UHPLC-UV-grade water (H2O) was sourced from Fisher Scientific (Loughborough, UK). These solvents were used to prepare mobile phases and sample extraction solutions. Additionally, ammonium acetate and acetic acid were used as additives to adjust the pH to 4.15 of mobile phase A. These consumables were purchased from Sigma-Aldrich (Dorset/Gillingham, UK) (see Appendix 3 for consumable details and CAS numbers).

BAs standards such as allolithocholic acid, 3,7-diketocholanic acid, 3,7,12-dehydrocholic acid, 3,7,12-glycodehydrocholic acid, 3,7,12-taurodehydrocholic acid, 3α-hydroxy-7,12-diketocholanic acid, 3α-hydroxy-7-ketolithocholic acid, 3α-hydroxy-12-ketolithocholic acid, 3-ketocholanic acid, 5α-cholanic acid-3,6-dione, 5α-cholanic acid-3α-ol-6-one, 5-cholenic acid-3β-ol, 8(14),(5β)-cholenic acid-3α,12α-diol, 9(11),(5β)-cholenic acid-3α-ol-12-one, glycocholic acid, glycohyodeoxycholic acid, glycoursodeoxycholic acid, glycoursocholanic acid, isolithocholic acid, lithocholenic acid, taurolithocholic acid, taurohyodeoxycholic acid,

tauroursocholanic acid, and tauroursodeoxycholic acid were obtained from Makaira Ltd. (London, England).

Additional BAs standards including α-muricholic acid, β-muricholic acid, ω-muricholic acid, 3,6-diketocholanic acid, 3,12-diketocholanic acid, 3α-hydroxy-6,7-diketocholanic acid, 3α,12α,23-nordeoxycholic acid, 3-dehydrocholic acid, 5β-cholanic acid-3β,12α-diol, 5β-cholanic acid-3α,6α-diol-7-one, 5β-cholenic acid-7α-ol-3-one, cholic acid, deoxycholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid, glycolithocholic acid, glycohyocholic acid, hyocholic acid, hyodeoxycholic acid, isodeoxycholic acid, lithocholic acid, murocholic acid, taurocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid, taurohyocholic acid, tauro-α-muricholic acid, tauro-β-muricholic acid, tauro-ω-muricholic acid, ursodeoxycholic acid, and ursocholanic acid were acquired from Steraloids Inc. (Newport, Rhode Island, USA).

Deuterated bile acid standards were used as IS. Ursodeoxycholic acid- d4, β-hyodeoxycholic acid-d4, chenodeoxycholic acid-d5, glycolithocholic acid-d4, glycodeoxycholic acid-d4, glycoursodeoxycholic acid-d4, taurolithocholic acid-d4, taurochenodeoxycholic acid-d4, tauroursodeoxycholic acid-d5, taurodeoxycholic acid-d4, and taurocholic acid-d5 were purchased from Medical Isotopes Inc., Pelham (New Hampshire, USA) while lithocholic acid-d4, cholic acid-d4, glycocholic acid-d4, deoxycholic acid-d4 and glycochenodeoxycholic acid -d4 were purchased from Steraloids Inc., Newport (Rhode Island, USA).

2.3.3.2 Sample preparation

For serum sample preparation, 100 µL of the sample was taken, and 280 µL of methanol (MeOH) was added, followed by thorough vortexing. The mixture was then centrifuged at 14,000 rcf for 15 minutes at 4ºC. After centrifugation, the mixture was incubated at -20ºC for 20 minutes. Following incubation, 190 µL of the supernatant was carefully taken, 10 µL of the DIS was added, followed by another round of vortexing. The required volume for LC-MS analysis was then taken (100 uL), and the remaining supernatant was saved as a backup.

For urine sample preparation, 100 µL of the sample was combined with 100 µL of methanol (MeOH) and vortexed thoroughly. The mixture was then centrifuged at 14,000 g for 15 minutes at 4ºC. After centrifugation, 10 µL of the DIS was added to the supernatant and vortexed again. The required volume for LC-MS analysis was taken, and the remaining supernatant was saved as a backup.

QC samples were prepared by pooling individual samples. The instrument was used to analyse these QC samples after every 10 study samples to ensure consistency. Calibration curves were generated from a stock solution in a 2:1:1 H2O:ACE:IPA mixture for both standards and DIS. The calibration curves included all analytes and DIS, with concentrations ranging from 1 μM to 0.5 nM.

Table 2. 5 Complete experimental details for selected ion/reaction monitoring (SIM/SRM) experiments conducted on bile acid standards.

MRM #	BA ID	IS for normalisation	NAME	Formula	M (g/mol)	*m/z* (M-H)	transitions	CE (eV)
1	**BA1**	IS60	Ursocholanic Acid	$C_{24}H_{40}O_2$	360.3	359.28	359.29>359.28	10
2	**BA3**	IS60	5-Cholenic acid-3β-ol	$C_{24}H_{38}O_3$	374.28	373.274	373.27 > 373.26	10
	BA2	IS60	Lithocholenic acid		374.28			10
	BA4	IS60	3-Ketocholanic acid		374.28			10
3	**BA7**	IS60	Allolithocholic acid	$C_{24}H_{40}O_3$	376.3	375.29	375.29 > 375.28	10
	BA6	IS60	Isolithocholic acid		376.3			10
	BA5	IS60	Lithocholic acid		376.29			10
4	**BA9**	IS64	3α,12α, 23-Nordeoxycholic acid	$C_{23}H_{38}O_4$	378.28	377.27	377.27 > 377.26	10
6	**BA10**	IS64	9(11), (5β)-Cholenic acid-3α-ol-12-one	$C_{24}H_{36}O_4$	388.26	387.25	387.25 > 387.24	10
	BA11	IS63	5α-Cholanic acid-3, 6-dione		388.26			10
	BA12	IS65	3,7-Diketocholanic acid		388.26			10
	BA14	IS64	3,12-Diketocholanic acid		388.26			10
	BA13	IS63	3,6-Diketocholanic acid		388.26			10
7	**BA15**	IS64	8(14),(5β)-Cholenic acid-3α, 12α-diol	$C_{24}H_{38}O_4$	390.28	389.26	389.26 > 389.25	10
	BA16	IS65	5β-Cholenic acid-7α-ol-3-one		390.28			10
8	**BA17**	IS63	5α-Cholanic acid-3α-ol-6-one		390.28	389.27	389.27 > 389.26	10
	BA18	IS65	3α-Hydroxy-7 Ketolithocholic acid		390.28			10
	BA19	IS64	3α-Hydroxy-12 Ketolithocholic acid		390.28			10
9	**BA20**	IS63	Murocholic acid	$C_{24}H_{40}O_4$	392.29	391.28	391.28 > 391.27	10
10	**BA32**	IS62	Ursodeoxycholic acid		392.28	391.28	391.28 > 391.27	10
	BA25	IS63	Hyodeoxycholic acid		392.29			10
	BA22	IS64	5β-Cholanic acid-3β, 12α-diol		392.29			10
11	**BA24**	IS65	Chenodeoxycholic acid		392.29	391.28	391.28 > 391.27	10
	BA23	IS64	Deoxycholic acid		392.29			10
	BA21	IS64	Isodeoxycholic acid		392.29			10
15	**BA26**	IS66	3,7,12 Dehydrocholic acid	$C_{24}H_{34}O_5$	402.24	401.26	401.26 > 401.25	10
16	**BA27**	IS66	3α-Hydroxy-7,12-DiketoCholanic acid	$C_{24}H_{36}O_5$	404.26	403.25	403.25 > 403.24	10
17	**BA28**	IS66	3α-Hydroxy-6,7-DiketoCholanic acid		404.26	403.25	403.25 > 403.24	10
18	**BA31**	IS66	12 Dehydrocholic acid	$C_{24}H_{38}O_5$	406.27	405.26	405.26 > 40.5.25	10
	BA29	IS66	5β-Cholanic acid-3α, 6α-diol-7-one		406.27			10
	BA30	IS66	3 Dehydrocholic acid		406.27			10
19	**BA33**	IS66	ω Muricholic acid	$C_{26}H_{40}O_5$	408.29	407.28	407.28 > 407.27	10
	BA34	IS66	β Muricholic acid		408.29			10
	BA37	IS66	α Muricholic acid		408.29			10

MRM #	BA ID	DIS for normalisation	NAME	Formula	M (g/mol)	*m/z* (M-H)	transitions	CE (eV)
20	**BA36**	IS66	Hyocholic acid		408.29	407.28	407.28 > 407.27	10
	BA35	IS66	Cholic acid		408.29			10
22	**BA38**	IS67	Glycoursocholanic acid	$C_{26}H_{43}NO_3$	417.32	416.32	416.32 > 74	40
23	**BA39**	IS67	Glycolithocholic acid	$C_{26}H_{43}NO_4$	433.32	432.31	432.31 > 74	40
25	**BA41**	IS68	Glycochenodeoxycholic acid	$C_{26}H_{43}NO_5$	449.31	448.31	448.31 > 74	40
	BA42	IS69	Glycodeoxycholic acid		449.31			40
26	**BA43**	IS70	Glycoursodeoxycholic acid		449.31	448.31	448.31 > 74	40
	BA40	IS69	Glycohyodeoxycholic acid		449.31			40
29	**BA44**	IS71	3,7,12-Glycodehydrocholic acid	$C_{26}H_{37}NO_6$	459.26	458.25	458.25 > 74	40
30	**BA49**	IS71	Glycohyocholic acid	$C_{26}H_{43}NO_6$	465.31	464.28	464.28 > 74	40
31	**BA46**	IS71	Glycocholic acid		465.3	464.3	464.3 > 74	40
32	**BA47**	IS72	Tauroursocholanic acid	$C_{26}H_{45}NO_4S$	467.31	466.3	466.3 > 80	60
34	**BA48**	IS72	Taurolithocholic acid	$C_{26}H_{45}NO_5S$	483.3	482.29	482.29 > 80	60
36	**BA50**	IS74	Tauroursodeoxycholic acid	$C_{26}H_{45}NO_6S$	499.3	498.29	498.29 > 80	60
	BA51	IS75	Taurohyodeoxycholic acid		499.3			60
	BA53	IS75	Taurodeoxycholic acid		499.3			60
37	**BA52**	IS73	Taurochenodeoxycholic acid		499.3	498.29	498.29 > 80	60
40	**BA54**	IS77	3,7,12 Taurodehydrocholic acid	$C_{26}H_{39}NO_7S$	509.25	508.24	508.24 > 80	60
41	**BA58**	IS77	Tauro-ω-Muricholic acid	$C_{26}H_{45}NO_7S$	515.29	514.28	514.28 > 80	60
	BA56	IS77	Tauro-β-Muricholic acid		515.29			60
	BA57	IS77	Tauro-α-Muricholic acid		515.29			60
42	**BA55**	IS77	Taurohyocholic acid		515.29	514.28	514.28 > 80	60
43	**BA59**	IS77	Taurocholic acid		515.29	514.28	514.28 > 80	60

Identification (ID) short codes, corresponding deuterated internal standards for normalisation, full names, molecular weights, collision energies (CE), and SIM/SRM transitions for all 57 bile acid standards. Bile acids with similar collision energies, m/z values, and SRM/SIM transitions were grouped into the same SRM window. For DIS short code IDs, please refer to Table 4.4. Modified and adapted from (1, 98, 148).

Table 2. 6 Comprehensive experimental details for selected ion/reaction monitoring experiments for internal standards used in bile acid analysis.

MRM #	IS ID	NAME	Formula	M (g/mol)	M/Z (M-H)	transitions	CE (eV)
5	**IS60**	Lithocholic acid-d4	$C_{24}H_{36}O_3d_4$	380.32	379.09	379.09 > 378.08	10
12	**IS63**	β Hyodeoxycholic acid-d4	$C_{24}H_{40}O_4d4$	396.6	395.06	395.06 > 395.05	10
	IS62	Ursodeoxycholic acid-d4		396.6			10
13	**IS64**	Deoxycholic Acid-d4	$C_{24}H_{36}O_4d_4$	396.6	395.06	395.06 > 395.05	10
14	**IS65**	Chenodeoxycholic Acid-d5	$C_{24}H_{35}O_4d_5$	397.6	396.07	396.07 > 396.06	10
21	**IS66**	Cholic Acid-d4	$C_{24}H_{36}O_5d_4$	412.6	411.03	411.03 > 411.02	10
24	**IS67**	Glycolithocholic acid-d4	$C_{26}H_{39}NO_4d_4$	437.65	436.08	436.08 > 74	40
27	**IS70**	Glycoursodeoxycholic acid-d4	$C_{26}H_{39}NO_5d_4$	453.65	452.05	452.05 > 74	40
28	**IS68**	Glycochenodeoxycholic acid -d4	$C_{26}H_{29}NO_5d_4$	453.65	452.2	452.2 > 74	40
	IS69	Glycodeoxycholic acid-d4	$C_{26}H_{39}NO_5d_4$	453.65			40
33	**IS71**	Glycocholic Acid-d4	$C_{26}H_{39}NO_6d_4$	469.65	468.06	468.06 > 74	40
35	**IS72**	Taurolithocholic Acid-d4	$C_{26}H_{41}NO_5Sd_4$	487.75	486.02	486.02 > 80	60
38	**IS73**	Taurochenodeoxycholic Acid-d4	$C_{26}H_{41}NO_6Sd4$	503.73	502.03	502.03 > 80	60
	IS75	Taurodeoxycholic Acid-d4	$C_{26}H_{40}NO_6Sd_4$	503.73			60
39	**IS74**	Tauroursodeoxycholic acid-d5	$C_{26}H_{40}NO_6Sd5$	504.73	503	503 > 80	60
45	**IS77**	Taurocholic Acid-d5	$C_{26}H_{39}NO_7Sd_5$	520.3	519.01	519.01 > 80	60

The identification (ID) short codes, full names, molecular weights, collision energies (CE), and SIM/SRM transitions for all 16 deuterated internal standards. Internal standards with similar collision energies, m/z values, and SRM/SIM transitions were grouped into the same SRM window. Modified and adapted from (1, 98, 148).

2.3.3.3 LC-MS analysis in serum and urine

The method used for these BAs analyses was developed by Sarafian et al. (98). BAs analysis was conducted using an ACQUITY ultra-performance liquid chromatography (UPLC) system coupled with a Xevo triple quadrupole (TQ-S) mass spectrometer. Liquid chromatography was carried out on an ACQUITY BEH C8 column (1.7 µm, 100 mm × 2.1 mm), maintained at 60°C. The mobile phase solvent A consisted of a mixture of 1:10 ACN and H2O with 1 mM ammonium acetate, and its pH was adjusted to 4.15 using acetic acid. Mobile phase solvent B was a 1:1 mixture of ACN and IPA. The chromatographic gradient followed previously published protocols. Mass spectrometry was conducted in negative ionisation mode (ESI-), with the following settings: capillary voltage at 1.5 kV, cone voltage at 60 V, source temperature at 150°C, desolvation temperature at 600°C, desolvation gas flow rate at 1000 L/h, and cone gas flow rate at 150 L/h. A total of 57 bile acid species were quantified using MRM, including 36 non-conjugated, 12 taurine-conjugated, and 9 glycine-conjugated bile acids. The transitions for each bile acid and the deuterated internal standards were described by Sarafian et al. (98), but this data were confirmed again when the method was transferred and implemented in a new UPLC instrument.

2.3.3.4 Targeted data preprocessing

MS data from the targeted LC-MS analysis was acquired using Waters MassLynx software version 4.1 and pre-processed with Waters TargetLynx software version 4.2. For the optimisation of data retrieved from MassLynx, a method was created for each DIS, including their corresponding BAs, as detailed in Table 2.5 and Table 2.6. This approach allowed for more efficient data acquisition and calculation, reducing the computational resources required. It also enabled faster processing when manually selecting chromatogram areas for BAs and DIS in TargetLynx. Due to the high number of samples analysed for BAs, a quick reference chromatogram guidance by MRM was designed to identify each chromatogram peak correctly and reduce manual errors (Appendix 10).

2.3.4 BAs targeted method for LC-MS analysis in faecal samples

2.3.4.1 Sample preparation

BAs from faecal samples were analysed by Dr. Adesola Bello as part of method development for targeted BA analysis in faeces during her PhD thesis (1). Accordingly, the method described here reflects the work she conducted for the analysis of faecal samples. For these analyses, the samples were prepared using an improved protocol developed by Mullish et al. (149) for monomeric BA profiling. Although, originally designed for an untargeted method, the protocol was adapted for this targeted analysis. Approximately 1 gram of stool was placed into a 2 ml Eppendorf tube, which was sealed with parafilm wax. The parafilm was then slightly pierced, and the tubes were frozen overnight at -80°C.

Following this, the samples were freeze-dried using a VirTis Benchtop Pro (SP Scientific) until they were completely dry. Next, 50 mg of the freeze-dried stool sample was transferred into 2 ml tubes containing around 0.05 g of 1.0 mm zirconia beads (Biospec Products, USA) and 1 ml of a 2:1:1 solution. The sample was homogenised using a bead beater (Precellys 24, Bertin Instruments, France) for three 30-second intervals. The homogenised sample was centrifuged at 4°C for 20 minutes at 16,000 x rcf (Eppendorf Centrifuge 5430, Hamburg, Germany). Subsequently, 500 µl of the supernatant was filtered using 0.45 µm pore size nylon Corning® Costar® Spin-X® filters (Massachusetts, USA). The spin filters were centrifuged for 15 minutes under similar conditions, and the resulting filtrate was collected and transferred into vials (Waters, UK) for subsequent LC-MS analysis (1).

2.3.4.2 Sample analysis and targeted data preprocessing

The same analytical conditions used for urine and serum were used for sample analysis, while data preprocessing was conducted similarly as urine and serum.

2.4. Statistical analysis

2.4.1 Univariate analysis

Initially, data were explored descriptively for each group and biofluid. The normality of the data was then assessed between groups using the Shapiro-Wilk test (150). None of the SCFA or BA concentrations in serum, urine, or faeces were normally distributed. Results were expressed as the median with interquartile range (IQR). Data for groups within each biofluid were compared using a non-parametric approach, specifically the Wilcoxon Rank- Sum test, with adjustments made using the False Discovery Rate (FDR)(127).

Heatmaps were studied for data sets in vegans and omnivores, including dietary data and metabolites. Spearman correlation was performed and adjusted using the FDR (127). Significance was defined as $p < 0.05$ for all tests. Statistical analyses were carried out in the R software environment (151). Heatmap images and related analyses were generated using Python programming language (152). Other graphs were produced for SCFAs and BAs in GraphPad Prism 8 version 8.4.2 (GraphPad Software, USA).

2.4.2 Multivariate analysis

Data for vegans and omnivores was analysed using Simca 17.1 (Umetrics, Umea, Sweden). Principal component analysis (PCA) was conducted to visualise sample clustering and evaluate the validity of the included run quality control (QC). Models were considered valid with positive R^2X and Q^2Y values and a difference of a maximum of 0.3 units between the two variables. Additionally, supervised analysis with OPLS-DA (Orthogonal projections to latent structures discriminant analysis) was performed to

identify the features that differentiate between the groups. The OPLS-DA models were validated using CV-ANOVA, which evaluates the null hypothesis that there is no significant difference between the residuals of the validated model and a randomly fitted model based on the same dataset (153).

2.5 Figures

Figures from Chapter 1, as well as schematic summary figures for Chapters 3, 4, 5, and 6 were designed using Canva (updated October 2024)

3. Short-chain fatty acid concentrations in free-living vegans and omnivores.

Note: For this chapter, SCFAs will be referred to by their systematic names in their protonated forms based on their chemical structures.

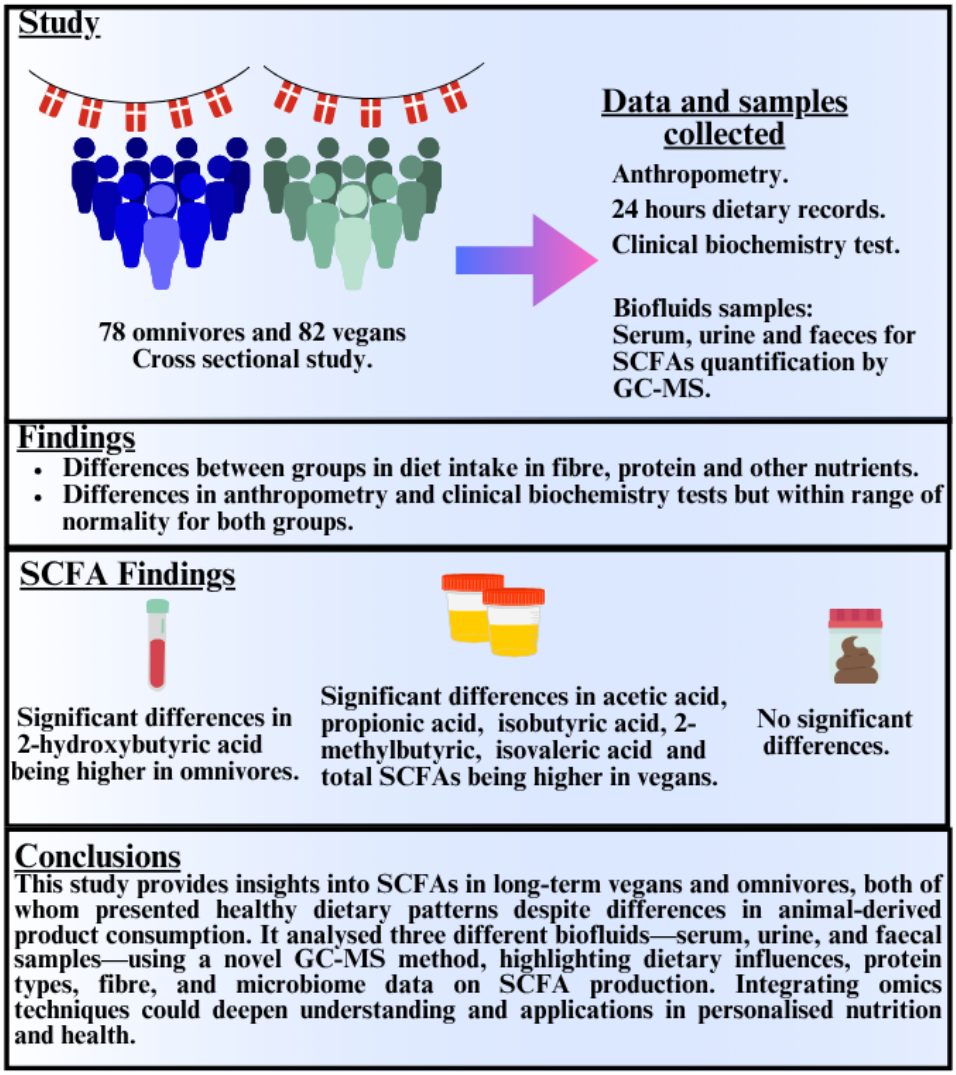

Figure 3. 1 Schematic Summary of Chapter 3.

3.1 Introduction

During the last ten years, veganism as a dietary preference has increased globally due to the different health benefits associated, such as a reduction in the risk of developing some cancers, type 2 diabetes and cardiovascular diseases (13, 27, 143, 154-158). Moreover, environmental issues, sustainability and animal welfare have also influenced the rise of veganism within the population (159, 160).

Several nutrition bodies have published their opinions and recommendations regarding the consumption of a vegan diet (20-23). It has been reported that a well-planned vegan diet can meet all the nutritional requirements of healthy individuals (24). Nutritional differences between omnivores and vegans have been well-documented (25). A healthy vegan dietary pattern is characterised by a high intake of fruits and vegetables, low sodium intake, low intake of saturated fat, alongside a non-consumption of animal products and their derivatives (25). A high intake of fibre has been associated with increased production of SCFAs, which is one of the characteristics of a vegan dietary pattern (161).

Diet plays a key role in the production of SCFAs in the gut. Their production results from non-digestible carbohydrates, undigested proteins, and amino acids (69, 162). The consumption of different dietary patterns (Mediterranean, vegan, omnivorous, caloric restrictive, etc.) and their subsequent production of SCFAs have been studied in various biofluids (163-167). However, these studies have often limited their metabolite analysis to acetate, butyrate, and propionate, thereby neglecting the potential role of other SCFAs and related metabolites that may vary between dietary patterns and contribute to metabolism.

Nonetheless, there remain gaps in our understanding of how a vegan dietary pattern, compared to an omnivorous dietary pattern, can impact metabolism and SCFA production in different serum, urine, and faecal samples. To explore the production of SCFAs, biofluid samples from a cohort of free-living vegans and omnivores were studied (see Chapter 2, section 2.1 for details of this study). A novel GC-MS targeted technique was employed to quantify SCFAs and related metabolites in serum and urine (78). A previously described GC-MS method for six SCFAs was used for faecal samples (146).

3.2 Hypothesis

The regular consumption of a vegan dietary pattern, compared to an omnivorous dietary pattern, will result in differences in the concentrations of SCFAs in serum, urine, and faeces.

3.3 Aims

The main aim of this study was to compare the concentrations of SCFAs of vegans versus omnivores, considering the nutritional composition of each dietary pattern.

3.4 Objectives

The specific objectives of this study were as follows:

1. To provide baseline sociodemographic and clinical characteristics of participants adhering to both dietary patterns.
2. To conduct multivariate analyses of SCFAs from different biofluids in relation to dietary patterns.
3. To compare the concentrations of SCFAs between vegan and omnivorous participants in each biofluid.
4. To assess the dietary intake of participants and investigate its relationship with SCFA concentrations in different biofluids.
5. To correlate relevant dietary information with SCFA production and concentration in each biofluid.

3.5 Materials and methods.

As previously discussed in Chapter 2, the samples for this study were obtained from a cross-sectional study that recruited 78 omnivores and 82 vegans (129-131). Serum and urine samples were collected after 10 hours of overnight fasting, while faecal samples were collected at home. Anthropometric information was obtained by measuring weight and height (129). Dietary records were self-reported, and data from 4 days of food weight were collected and processed (130).

SCFAs in serum and urine were quantified using a novel technique that included 10 metabolites, a total of five short SCFAs, three methyl-branched SCFAs (branch chain fatty acids) and two hydroxyl carboxylic acids. This method was previously validated and published by Brignardello et al (78). For faecal SCFAs, a previously validated method was employed that identified and quantified acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid in adults and children. This method was adapted from Garcia- Villalba (146) and later used for analysis in human samples (147).

3.6 Results

3.6.1 Population and diet characteristics

Anthropometric results were compared between the groups (Table 3.1 and Table 3.2). BMI, height, weight, body fat percentage, systolic blood pressure, and diastolic blood pressure were not significantly different between the groups. Differences in fasting clinical biomarkers of glucose metabolism, such as HOMA2%S, HOMA2-IR, HOMA-IR, and insulin, were not significantly different between groups. By contrast, plasma glucose and HbA1c levels were significantly higher in the omnivorous group (P_{adj} = 0.005 and $P_{adj} = 4 \times 10^{-5}$, respectively). The lipid profile showed significant differences in total

cholesterol and LDL cholesterol ($P_{adj} = 3.8 \times 10^{-5}$ and $P_{adj} = 7.21 \times 10^{-6}$, respectively), while VLDL cholesterol, HDL cholesterol, and triglycerides were not different between groups. Haemogram parameters, such as leucocytes and lymphocytes, were significantly lower in vegans ($P_{adj} = 0.017$ and $P_{adj} = 0.005$, respectively). Ferritin concentrations were significantly higher in the omnivorous group ($P_{adj} = 8.01 \times 10^{-4}$). Concentrations of cobalamin ($P_{adj} = 3.5 \times 10^{-9}$), TMAO ($P_{adj} = 1.74 \times 10^{-6}$), choline ($P_{adj} = 0.04$), and carnitine ($P_{adj} = 2.78 \times 10^{-3}$) were significantly higher in omnivores, while betaine concentration was higher in vegans ($P_{adj} = 2.78 \times 10^{-3}$).

The nutrient characteristics of the two dietary groups were compared (Table 3.3). Macronutrients such as fat intake in grams ($P_{adj} = 2.4 \times 10^{-3}$), energy from fats ($P_{adj} = 2 \times 10^{-3}$), protein ($P_{adj}= 1.27 \times 10^{-17}$ and $P_{adj} = 3.96 \times 10^{-9}$), and sodium ($P_{adj} = 5.77 \times 10^{-6}$) were significantly higher in omnivores. In contrast, carbohydrates ($P_{adj} = 2.64 \times 10^{-12}$ and $P_{adj} = 4.11 \times 10^{-5}$), fibre ($P_{adj}= 5.85 \times 10^{-10}$), starch ($P_{adj} = 0.0154$), biotin ($P_{adj} = 1.56 \times 10^{-4}$), and the DASH score ($P_{adj} = 1.58 \times 10^{-10}$) were significantly higher in the vegan group.

The amino acid content of dietary intake was compared between groups, focusing on those that had been reported to contribute to SCFA and BSCFA production through fermentation in in vitro models (168-170). The following amino acids were compared between groups being all of them significantly lower in vegans (see Table 3.3): alanine ($P_{adj}=2.71x10^{-9}$), glutamic acid ($P_{adj}= 9.93x10^{-7}$), glycine ($P_{adj}=9.93x10^{-7}$), histidine ($P_{adj}= 1.58x10^{-10}$), isoleucine ($P_{adj}= 2.93x10\text{-}^{13}$), leucine ($P_{adj}=6.19x10^{-12}$), lysine ($P_{adj}=1.29x10^{-17}$), methionine ($P_{adj}= 1.02x10^{-17}$), phenylalanine ($P_{adj}= 4.73x10^{-8}$), proline ($P_{adj}= 6.12x10^{-12}$), serine ($P_{adj}= 1.71x10^{-9}$), threonine ($P_{adj}= 3.6797x10^{-11}$) and valine ($P_{adj}= 1.646x10^{-11}$).

Table 3. 1 Anthropometric and biochemical characteristics of omnivores and vegans[1].

	Omnivores	Vegans	P value[2]	$P_{adj.}$ value
n	78	82		
Gender				
Female	39	43		
Male	39	35		
Vegan duration (y)		5.5(2.7-8)		
Age (y)	30.0(25.4-36.7)	28.3(24.9-35.6)	0.54(‡)	0.68
BMI (kg/m2)	22.1(20.3-23)	20.93(19.7-22.2)	0.03(‡)	0.07
Height (cm)	174(170-184)	174(168-180.3)	0.43(‡)	0.57
Weight (kg)	66.6(60.55-75.43)	62.75(57.8-71.93)	0.04(‡)	0.08
Fat Percent	19.46(5.5)	19.74(6.01)	0.76(§)	0.87
Systolic blood pressure (mm/Hg)	118(113-125)	119(112-127)	0.81(‡)	0.89
Diastolic blood pressure (mm/Hg)	74(67-80)	73(67.-80)	0.94(‡)	0.68
Glucose Metabolism				
Plasma Glucose (mmol/L)	5.2(4.8-5.3)	4.9(4.7-5.3)	0.005(‡)	0.01
HOMA2 %B	78(22.1)	82.5(19.3)	0.202 (§)	0.34
HOMA2 %S	131(95-162)	127(107-160)	0.62(‡)	0.74
HOMA2 IR	0.77(0.62-1.06)	0.79(0.63-0.94)	0.62(‡)	0.42
Insulin (pmol/L)	41(31-55)	41.(33.75-50)	0.93(‡)	0.94
HbA1c (%)	5.2(5-5.3)	4.9(4.7-5.2)	$4x10^{-5}$(‡)	$2.2x10^{-4}$
Liver and Lipid Metabolism				
ALT (IU/L)	26(21-35)	25(20-34)	0.85	0.908
Cholesterol (mmol/L)	4.4(3.8-5)	3.9(3.4-4.3)	$3.8x10^{-5}$(‡)	$2x10^{-4}$
VLDL (mmol/L)	0.4(0.3-0.5)	0.4(0.3-0.5)	0.21(‡)	0.34
LDL (mmol/L)	2.6(0.79)	2.05(0.53)	$7.21x10^{-6}$ (§)	$5.95x10^{-5}$
HDL (mmol/L)	1.4(1.2-1.6)	1.4(1.1-1.52)	0.11(‡)	0.21
Triglycerides (mmol/L)	0.8(0.7-1.1)	0.9(0.81.1)	0.18(‡)	0.33
CRP-HS (mg/L)	0.4(0.12-0.8)	0.2(0.7-0.6)	0.006(‡)	0.01

[1]Data is expressed in mean (standard deviation) or median (25th percentile, 75th percentile), according to their normality distribution. [2]P-values were calculated using Student's t-test (§) or Wilcoxon rank-sum test (‡) and adjusted using False Discovery Rate (FDR). Significance was accepted at P adj <0.05.

Table 3. 2 Anthropometric and biochemical characteristics of omnivores and vegans[1] (Continuation).

	Omnivores (n=78)	Vegans (n= 82)	P value[2]	P_{adj-} value
Haemogram profile				
Haemoglobin (mmol/L)	8.9(0.74)	8.8(0.8)	0.32(§)	0.49
Leukocytes (10^3/µL)	2.6(2.1-3.2)	2.1(1.7-2.5)	0.01(‡)	0.04
Lymphocytes(10^3/µL)	1.9(1.6-2.3)	1.7(1.45-2.1)	0.005(‡)	0.01
Monocytes (10^3/µL)	0.4(0.3-0.4)	0.3(0.3-0.4)	0.21(‡)	0.34
Neutrophils (10^3/µL)	2.9(2.4-3.8)	2.8(2.3-3.3)	0.34(‡)	0.49
Nutrition biomarkers, TMAO& TMAO precursors				
Vitamin D (ng/mL)	65(53.5-78)	57(38.7-71)	0.002(‡)	0.01
Ferritin (ng/mL)	51.5(36.75-135.8)	40.5(23-70.3)	0.0008(‡)	0.003
Albumin (g/L)	51.6(3.2)	51.1(3.7)	0.37(§)	0.52
Cobalamin (ng/L)	324(250.8-415.5)	190(157.8-292.5)	$2.32x10^{-10}$ (‡)	$3.83x10^{-9}$
TMAO (µmol/L)	4.02(2.6-5.8)	2.6(1.9-3.5)	$1.74x10^{-7}$ (‡)	$1.91x10^{-6}$
Choline (µmol/L)	9.6(8.1-10.6)	8.4(7.6-10.1)	$1.5x10^{-2}$(‡)	0.04
Betaine (µmol/L)	28.9(20.2-36.8)	50.7(39.2-63.1)	$1.68x^{-12}$ (‡)	$5.54x10^{-11}$
Carnitine (µmol/L)	29.8(24.7-35.1)	25.6(2.9-30.8)	$6.5x10^{-4}$ (‡)	$3.06x10^{-3}$

[1]Data is expressed in mean (standard deviation) or median (25th percentile, 75th percentile), according to their normality distribution. [2]P-values were calculated using Student's t-test (§) or Wilcoxon rank-sum test (‡) and adjusted using False Discovery Rate (FDR) . Significance was accepted at P adj <0.05.

Table 3. 3 Omnivores and Vegans Diets Characteristics [1].

	Omnivores (n=78)	Vegans (n= 82)	P value[2]	P_{adj} value
Energy (kcal)	2335(1916-2769)	2391(1927-2960.5)	0.523 (‡)	0.54
Proportion of fat	32.3(5.6)	27.0(7.7)	$4.02x10^{-6}$ (§)	$5.7x10^{-6}$
Energy from fat (kcal)	757(596-904)	586(453-791)	$1.77x10^{-3}$(‡)	0.0021
Fats (g)	85(68-104)	68(52-95)	$2.11x10^{-3}$	0.0024
Proportion of protein	15.7(15.-17.8)	12.1(10.5-13.1)	$7.72x10^{19}$ (‡)	$1.27x10^{-17}$
Energy from proteins (kcal)	359(315-478)	267(213-349.)	$1.92x10^{-9}$(‡)	$3.96x10^{-9}$
Proteins (g)	86(79-113)	65(53-86)	$7.67x10^{-9}$(‡)	$1.5x10^{-8}$
Proportion of carbohydrate	50(6.1)	59.2(7.6)	$4x10^{-13}$ (‡)	$2.64x10^{-12}$
Energy from carbohydrates (kcal)	1098 (925-1331)	1393(1073-1742)	$3.24x10^{-5}$(‡)	$4.11x10^{-5}$
Carbohydrates (g)	275(143-333)	348(268-436)	$3.24x10^{-5}$(‡)	$4.11x10^{-5}$
Saccharose (g)	27(19-39)	28(18-43)	0.6999 (‡)	0.6999
Starch (g)	89(67-113)	111(79-143.)	0.014 (‡)	0.0154
Fibre (g)	29(24-36)	43(34-57)	$2.13x10^{-10}$ (‡)	$5.85x10^{-10}$
Biotin (µg)	28(17-40)	37(28-50)	$1.2x\ 10^{-4}$ (‡)	$1.56x10^{-4}$
Sodium (mg)	2497(1827-3220)	1751(1158-2384)	$3.95x10^{-6}$(‡)	$5.77x10^{-6}$
DASH score	30(27-32)	33(31-34)	$5.27x10^{-11}$(‡)	$1.58x10^{-10}$
Fruits (g/day)	171(88-249)	273(157-464)	$2.1x10^{-5}$(‡)	$2.89x10^{-5}$
Vegetables (g/day)	217(153.-298)	326(227-501.95)	$1.1x10^{-6}$(‡)	$1.73x10^{-6}$
Nuts and legumes (g/day)	15(7-47)	74(32-124)	$7.27x10^{-10}$(‡)	$1.71x10^{-9}$
Whole grain (g/day)	135(93-220)	154(88-224)	0.49(‡)	0.5216
Alanine (mg)	2859(2295-3701)	1872(1344-2501)	$1.23x10^{-9}$ (‡)	$2.71x10^{-9}$
Glutamic acid (mg)	11354(9263-13693)	8567(5746-11013)	$5.97x10^{-7}$ (‡)	$9.93x10^{-7}$
Glycine (mg)	2462(1944-3324)	1721.(1301-2391)	$6.02x10^{-7}$ (‡)	$9.93x10^{-7}$
Histidine (mg)	1555(1332-2040)	1039(735-1352)	$5.23x10^{-11}$ (‡)	$1.58x10^{-10}$
Isoleucine (mg)	2841(2427-3514)	1728(1230-2078)	$3.56x10^{-14}$ (‡)	2.93x10-^{13}
Leucine (mg)	4687(3965-5700)	2928(2101-3545)	$1.23x10^{-12}$ (‡)	$6.19x10^{-12}$
Lysine (mg)	3913(3145-5044)	1922(1363-2294)	$1.17x10^{-18}$ (‡)	$1.29x10^{-17}$
Methionine (mg)	1370(1102-1672)	613(439-856)	$3.08x10^{-19}$ (‡)	$1.02x10^{-17}$
Phenylalanine (mg)	2795(2379-3422)	1998(1428-2457)	$2.58x10^{-8}$ (‡)	$4.73x10^{-8}$
Proline (mg)	4288(3458-5235)	2470(1826-3504)	$1.30x10^{-12}$ (‡)	$6.12x10^{-12}$
Serine (mg)	2961(2523-3653)	2013.71(1484-2584)	$7.10x10^{-10}$ (‡)	$1.71x10^{-9}$
Threonine (mg)	2240(1844-2813)	1435(1039-1858)	$9.81x10^{-12}$ (‡)	$3.67x10^{-11}$
Valine (mg)	3524(2969-4273)	2170(1549-2846)	$3.97x10^{-12}$ (‡)	$1.64x10^{-11}$

[1]Data is expressed in mean (standard deviation) or median (25th percentile, 75th percentile), according to their normality distribution. [2]P-values were calculated using Student's t-test (§) or Wilcoxon rank-sum test (‡) and adjusted using False Discovery Rate (FDR). Significance was accepted at P adj <0.05.

3.6.2 Multivariate analysis

3.6.2.1 PCA models comparing SCFAs concentrations of vegans and omnivores

Multivariate analysis was performed using the vegan and omnivorous serum SCFAs datasets. PCA was conducted, and a close clustering of the QCs of SCFAs quantified in serum was observed(Figure 3.2A). This finding confirmed the stability of the GC-MS instrument throughout the run and reproducibility of the assay. The model explained 74.5% of the variance with a low ability to predict 4%. The plot of SCFA in serum did not show a clear separation between vegans and omnivores due to the high variability between the groups that were independent of the diet.

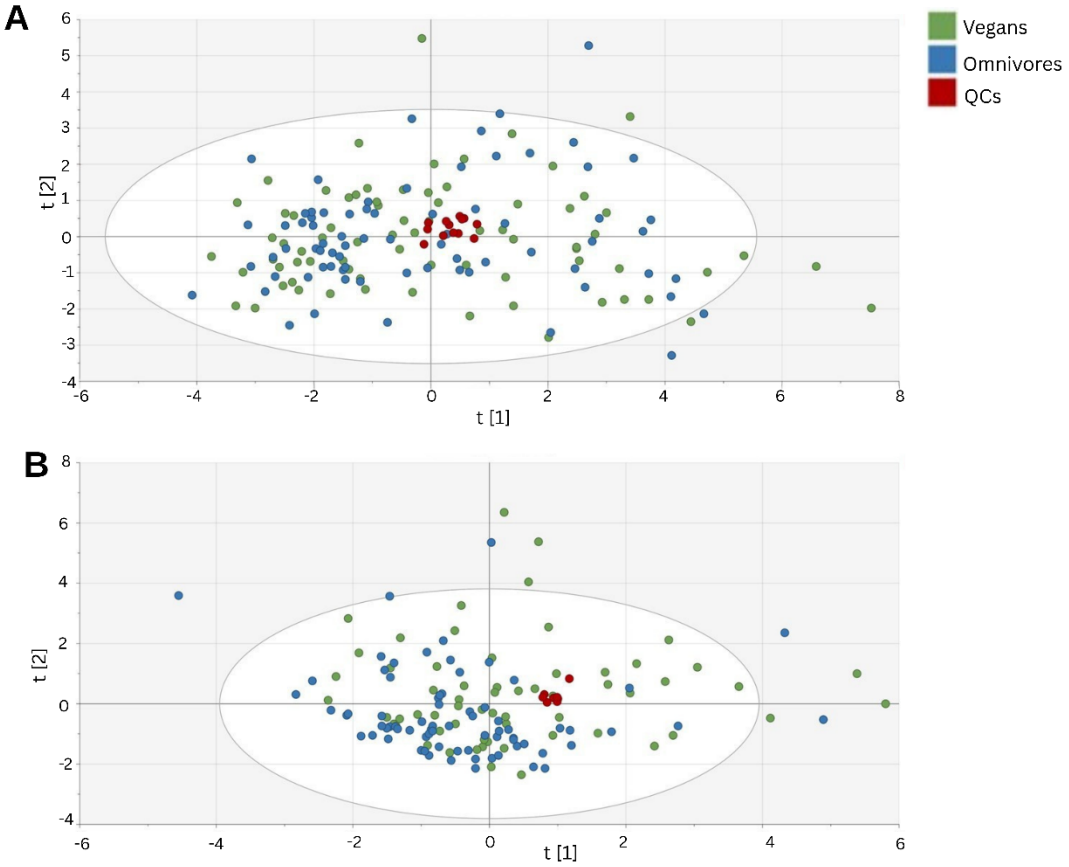

Figure 3.2 PCA score plot of serum and urine SCFA profiles for vegans and omnivores. Vegans (green), omnivores (blue) and quality control (red) in serum and urine. Models are comprised of three principal components. Ellipse Hotellin's T2 (95%). (A) SCFAs in serum R^2X= 0.745, Q^2X= 0.0409. (B) SCFAs in urine R^2X= 0.617, Q^2X=0.032.

PCA was performed in the vegan and omnivorous urine SCFAs datasets (Figure 3.2B), but no clear separation between groups was found. The QCs samples were clustered to ensure the stability and performance of the instrument. The model explained 61.7% of the variance with a low ability to predict 3.2%. The high variability in SCFA results could explain this. Therefore, diet may not explain the high variability in both the biofluids.

3.6.2.2 OPLS-DA models comparing SCFAs concentrations of vegans and omnivores

A supervised analysis was performed with OPLS-DA to analyse the features responsible for the discrimination between vegans and omnivores in serum and urine (Figure 3.3). In the serum, no separation was found between the omnivores and vegans (Figure 3.3A). The model explained 55.6% of the variance in the X-matrix (SCFA serum concentrations). This suggested the model captured a moderate amount of the underlying data structure. However, the model's ability to discriminate between the groups is weak. The R^2Y value was only 0.209, meaning just 20.9% of the variance in the Y-matrix (the group classification) was explained by the model. This finding suggested a lack of a strong relationship between the measured SCFAs and the dietary group (vegan or omnivore) distinction. Furthermore, the predictive ability of the model, as indicated by the Q^2 value of 0.177, was very low. The low Q^2 value indicated that the model lacked reliable predictive power and was not capable of accurately classifying new samples into the correct dietary group.

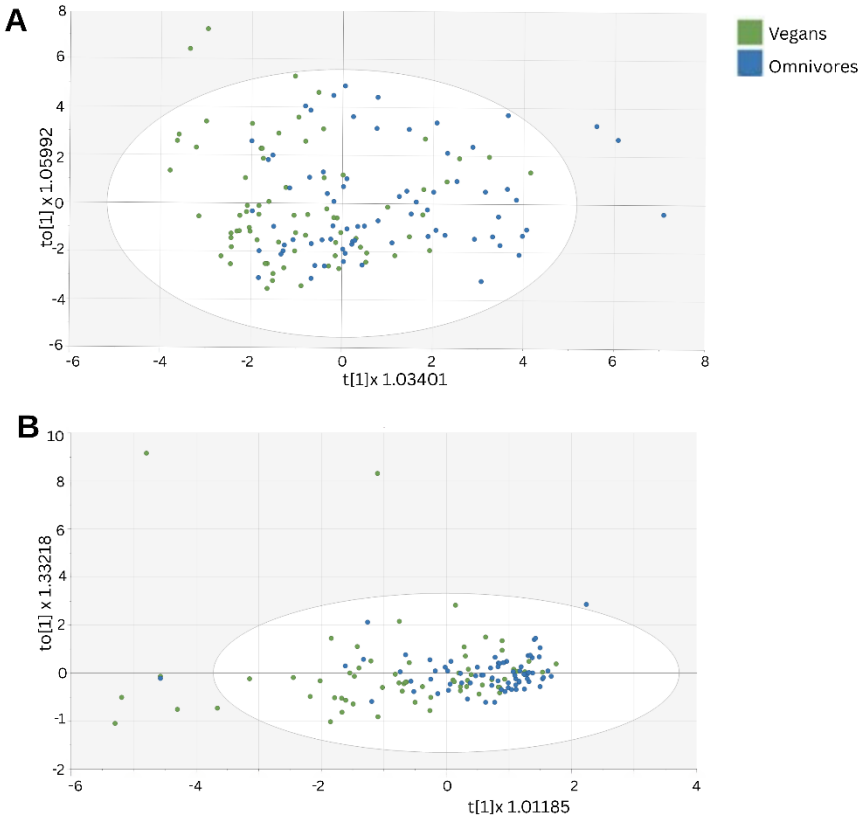

Figure 3. 3 OPLS-DA score plot of serum and urine SCFA profiles for vegans and omnivores. Cross-validated OPLS-DA score plots of SCFAs concentrations comparing vegans (green) and omnivores (blue). The models are comprised of one predictive (t[1]) and one orthogonal (to[1]) principal component. Ellipse; Hotellin's T2 (95%).(A) serum SCFA concentrations $R^2X = 0.556$, $R^2Y = 0.209$, $Q^2 = 0.177$.(B) Urine SCFA concentrations $R^2X = 0.366$, $R^2Y = 0.193$, $Q^2 = 0.114$.

The urine model (Figure 3.3B) showed a similar outcome. It explained 36.6% of the variance in the X-matrix ($R^2X = 0.366$), but only 19.3% of the variance in group classification ($R^2Y = 0.193$). The predictive capability was also very poor ($Q^2 = 0.114$), confirming that urinary SCFA concentrations did not provide a reliable basis for discriminating between vegans and omnivores.

Omnivores tended to cluster together on the left ellipse, whereas vegans were distributed throughout the entire ellipse. This suggested that omnivores had homogenous SCFA concentrations, whereas vegans exhibited greater variability, spreading across the score plot and overlapping with omnivores. A few strong outliers from vegan and omnivore samples were found in the serum and urine in this dataset, reflecting a strong biological variation for SCFAs quantification.

3.6.2.3 Multivariate analysis in faecal SCFAs

As was shown in section 3.6.5, no significant differences were found between faecal SCFAs in vegans and omnivores. Therefore, a multivariate analysis was not performed for this dataset.

3.6.3 SCFA concentrations in serum

The concentrations of SCFAs and related metabolites were compared between the groups. 2-hydroxybutyric concentration was the only significant metabolite found to be higher in omnivores than in vegans ($P_{adj} = 7x10^{-5}$). Other SCFAs did not show any significant differences, including total SCFA concentrations (see Table 3.4).

Table 3. 4. Comparison of serum SCFA between omnivores and vegans [1].

Serum SCFA	Omnivores (n=74)	Vegans (n=75)	P-value	P_{adj}-value
Acetic acid (µmol/L)	42.09(22.6-94.64)	52.04(23.2-136.87)	0.12	0.57
Propionic acid (µmol/L)	2.08(1.08-4.04)	2.4(1.28-4.19)	0.54	0.66
Isobutyric acid (µmol/L)	0.33(0.19-0.55)	0.31(0.13-0.46)	0.26	0.57
Butyric acid (µmol/L)	1.21(0.49-2.38)	1.51(0.51-3.42)	0.47	0.66
2-methylbutyric acid (µmol/L)	0.28(0.23-0.32)	0.27(0.24-0.33)	0.51	0.66
Isovaleric acid (µmol/L)	0.14(0.11-0.18)	0.16(0.11-0.21)	0.28	0.57
Valeric acid (µmol/L)	0.14(0.08-0.19)	0.13(0.07-0.21)	0.88	0.97
Caproic acid (µmol/L)	0.32(0.23-0.54)	0.33(0.23-0.49)	0.98	0.98
Lactic acid (µmol/L)	1598(1301-1819)	1680(1338-2012)	0.31	0.57
2-hydroxybutyric acid (µmol/L)	33.44(26.74-45.9)	25.81(19.76-32.54)	$7x10^{-6}$	$7x10^{-5}$
Total serum SCFAs (µmol/L)	1711.01(731.02-1932.27)	1834.61(1462.23-2169,78)	0.26	0.57

[1] Values expressed in median and interquartile range. Comparisons of groups were analysed by Mann- Whitney test and False Discovery Rate (FDR). Significance was accepted at (P_{adj} <0.05).
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3.6.4 SCFA concentrations in urine

SCFA concentrations were compared between omnivorous and vegan groups. Acetic (Padj= $1.03x10^{-3}$), propionic (P_{adj}= 0.04), isobutyric (P_{adj}= $1.03x10^{-4}$), 2-methylbutyric (P_{adj} = $1.03x10^{-4}$), isovaleric (P_{adj}= $1.07x10^{-4}$) and total SCFAs in urine (P_{adj} =.0.003) were higher in vegans compared to omnivores (see Table 3.5).

Table 3. 5 Comparison of urine SCFA between omnivores and vegans [1].

Urine SCFA	Omnivores (n=74)	Vegans (n=71)	P- value*	P_{adj}-value
Acetic acid (µmol/L)	28.97(34.11- 86.82)	52.31 (34.11- 86.82)	$2.8x\ 10^{-5}$	$1.03x10^{-3}$
Propionic acid (µmol/L)	0.8(0.49-1.31)	1.07(0.62-1.65)	0.02	0.04
Isobutyric acid (µmol/L)	0(0-0.05)	0.05(0-0.16)	$1.3x10^{-5}$	$1.03x10^{-4}$
Butyric acid (µmol/L)	0.06(0-0.5)	0.1(0-0.41)	0.5423	0.7457
2-methylbutyric acid (µmol/L)	0.19(0.15-0.27)	0.28(0.21-0.58)	1.9x10-5	$1.03x10^{-4}$
Isovaleric acid (µmol/L)	0.04(0.04-0.06)	0.07(0.04-0.11)	$3.9x10^{-5}$	$1.07x10^{-4}$
Valeric acid (µmol/L)	0.15(0.12-0.19)	0.15(0.1-0.2)	0.7	0.85
Caproic acid (µmol/L)	0.33(0.27-0.41)	0.33(0.26-0.4)	0.88	0.88
Lactic acid (µmol/L)	31.06(0-115.5)	58.58(10.35-164.8)	0.06	0.09
2-hydroxybutyric acid (µmol/L)	4.05(3.29-5.44)	4.1(3.39-5.4)	0.88	0.88
Total urine SCFAs (µmol/L)	70.39(48.06-171.70)	134.04(68.31-313.67)	0.001	0.003

[1] Values expressed in median and interquartile range. Comparisons of groups were analysed by Mann-Whitney test and False Discovery Rate (FDR). Significance was accepted at (P_{adj}<0.05)

3.6.5 SCFA concentrations in faeces

The differences between the concentrations of the studied SCFAs in faeces were not significant between the two groups. (Table 3.6). Similar findings have been reported previously in vegan subjects (167, 171).

Table 3. 6 Comparison of faeces SCFA between omnivores and vegans[1]

Faecal SCFA	Omnivores (n= 75)	Vegans (n=74)	P- value
Acetic acid (µmol/L)	1283.14(838.37-1793.76)	1429.27(1042.72-1862.24)	0.4
Propionic acid (µmol/L)	532.17(336.36-829.1)	559.54(373.09-898.06)	0.8
Isobutyric acid (µmol/L)	47.96(25.4-73.9)	35.1(17.3-66.57)	0.08
Butyric acid (µmol/L)	722.12(371.9-937.15)	715.67(421.42-1075.68)	0.6
Isovaleric acid (µmol/L)	66.21(24.5-115.53)	43.96(11.95-92.76)	0.08
Valeric (µmol/L)	84.61(51.87-133.81)	80.87(30.87-132.89)	0.2
Total faecal SCFAs (µmol/L)	3885.6(2377.12-11536.55)	3979.05(2619.26-5637.83)	0.64

[1] Values expressed in median and interquartile range. Comparisons of groups were analysed by Mann- Whitney test.

3.6.6 Correlations between SCFAs concentrations in biofluids and nutrients

Correlations between dietary information of both groups, omnivores, and vegans, were correlated separately with SCFAs from serum, urine, and faeces (Figure 3.4). Fibre, proteins, and specific amino acids were studied due to their capacity to produce SCFAs in "in vitro" models (162, 168-170). Biotin was considered due to its relation described with glucose metabolism and 2 hydroxybutyric acid, since high circulating levels of 2 hydroxybutyric acid and low levels of biotin had been associated with insulin resistant states (172). The DASH score (14) was included due to its role in the classification of dietary patterns (that were characterised by low in salt, fat, and processed food intake and high consumption of fruit and vegetables) and its effect on blood pressure (BP).

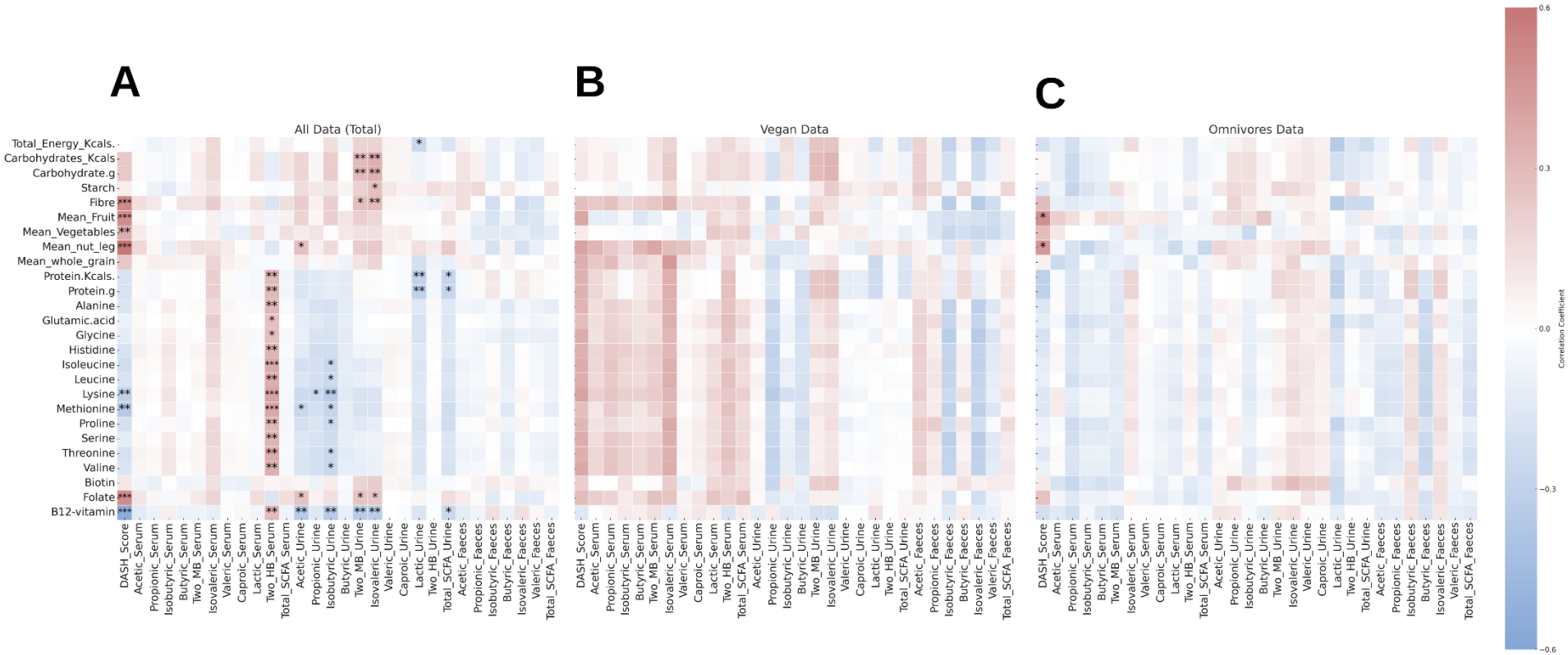

Figure 3. 4 Correlations between serum, urine and faecal SCFAs in omnivores and vegans, and nutrients with SCFA production capacity in in vivo and in vitro models
. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively. Key: (A) vegan and omnivore data, (B) vegan data and (C) omnivore data.

As was presented in Figure 3.4A, a few significant correlations were found between the data of both groups and serum SCFAs. All significant correlations were classified as weak (173). Notably, despite adjustments, lysine intake exhibited a highly significant correlation with 2-methylbutyric acid ($R= 0.4062$, $P_{adj} = 7.3x10^{-4}$). A similar case was observed for methionine, where its intake was significant for 2-methylbutyric ($R= 0.39$, $P_{adj} = 9.2x\ 10^{-4}$). No significant correlations were found between SCFAs and dietary information from vegans (Figure 3.4B) or omnivores (Figure 3.4C).

3.6.7 Correlations between SCFA concentrations in serum, urine, and faeces.

Exploratory correlation analyses of SCFAs within the same biofluid and between different biofluids were performed. These analyses were performed in the whole group and independently for each diet group.

3.6.7.1 Correlations between serum SCFAs and SCFAs from all biofluids

Correlations between serum SCFAs in both groups (Figure 3.5A) showed a clear correlation and significant pattern between acetic, propionic, isobutyric, butyric, 2- methyl butyric, 2- hydroxybutyric, isovaleric, valeric, and caproic acids. Lactic acid and total SCFAs levels were positively and significantly correlated, respectively. Few negative and significant correlations were found between biofluids. A weak correlation was found between acetic acid in urine and acetic acid in serum ($R= 0.24$, $P_{adj} = 0.016$). In the case of 2-hydroxybutyric acid, a weak correlation was found between serum and urine ($R= 0.33$, $P_{adj} = 2.28x10^{-4}$). No correlations were found between serum and faeces.

In vegans (Figure 3.5B), similar correlation patterns were found in both groups. The strongest correlations were found between acetic, propionic, isobutyric, butyric, 2- methylbutyric, 2-hydroxybutyric, isovaleric, valeric, and caproic acids. The concentration of 2-hydroxybutyric acid was weakly correlated with its concentration in serum and urine ($R= 0.372$, $P_{adj} = 0.007$). No correlations between serum and faeces were observed.

Correlations among omnivorous SCFAs have revealed some positive and significant results (Figure 3.5C). Within serum, strong positive correlations were found between acetic, propionic, isobutyric, butyric, 2- methyl butyric, 2- hydroxybutyric, isovaleric, valeric and caproic acid. A weak correlation was found between 2-hydroxybutyric acid in serum and urine ($R= 0.34$, $P_{adj} = 2.27x10^{-4}$).

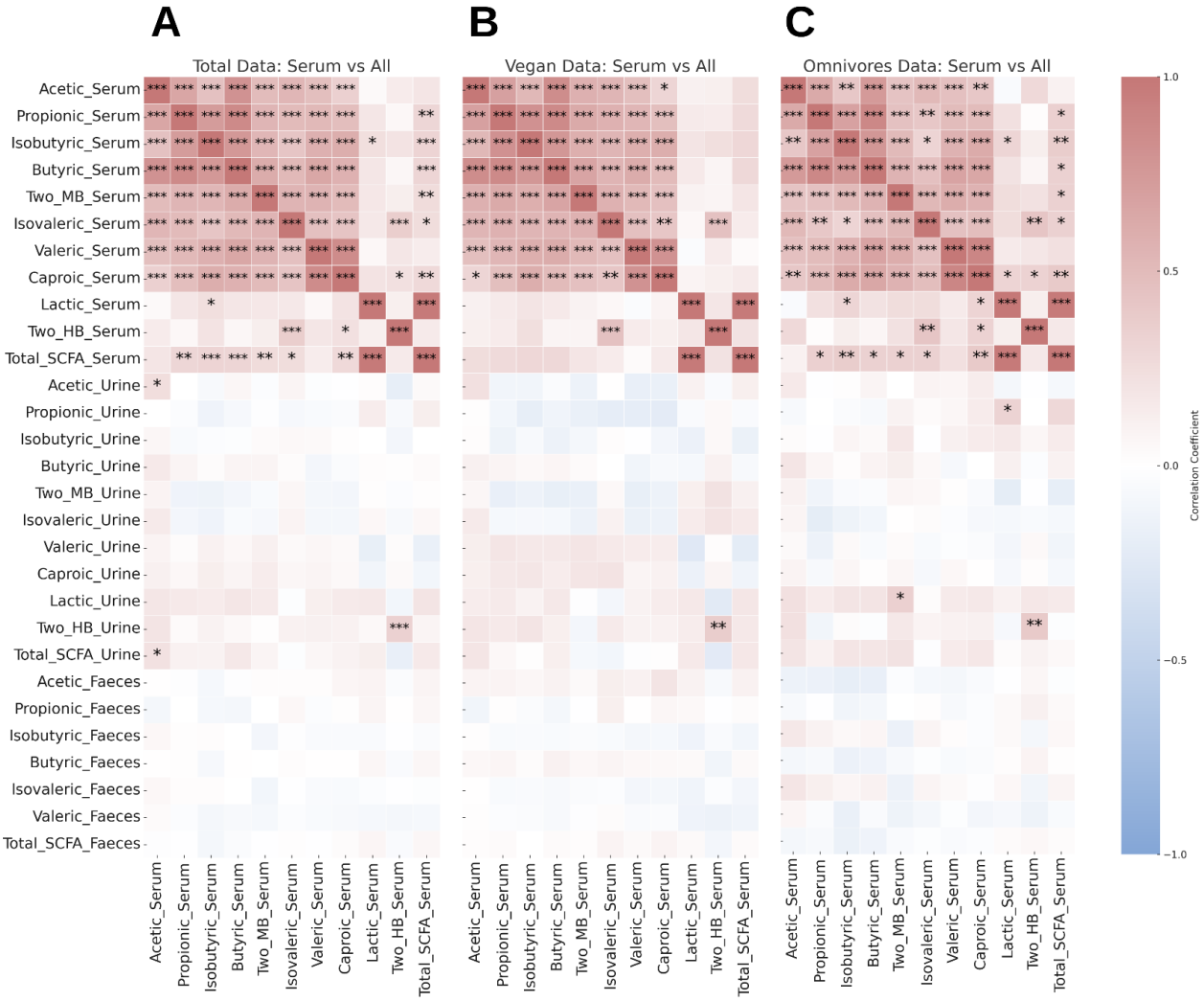

Figure 3.5 Correlations between serum SCFAs and serum, urine, and faecal SCFAs of vegans and omnivores. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively. Key: (A) vegan and omnivore data, (B) vegan data and (C) omnivore data.

3.6.7.2 Correlations between urine SCFAs and SCFAs from all biofluids

Correlations among all groups in serum and urine had been previously described in Section 3.6.7.1. Within urine in both groups, a clear pattern among the metabolites was not identified; however, several significant correlations were observed, including butyric acid and isobutyric acid (R= 0.46, P_{adj} =1.10x10 ^{7}), isovaleric acid and 2- methyl butyric (R= 0.86, P_{adj} = 8.72x10^{-41}), caproic acid and valeric acid (R= 0.91, P_{adj} = 1.84x10^{-53}), total SCFAs and lactic acid (R= 0.87, P_{adj} =2.68x10^{-42}) (Figure 3.6A). No correlations were found within the faeces.

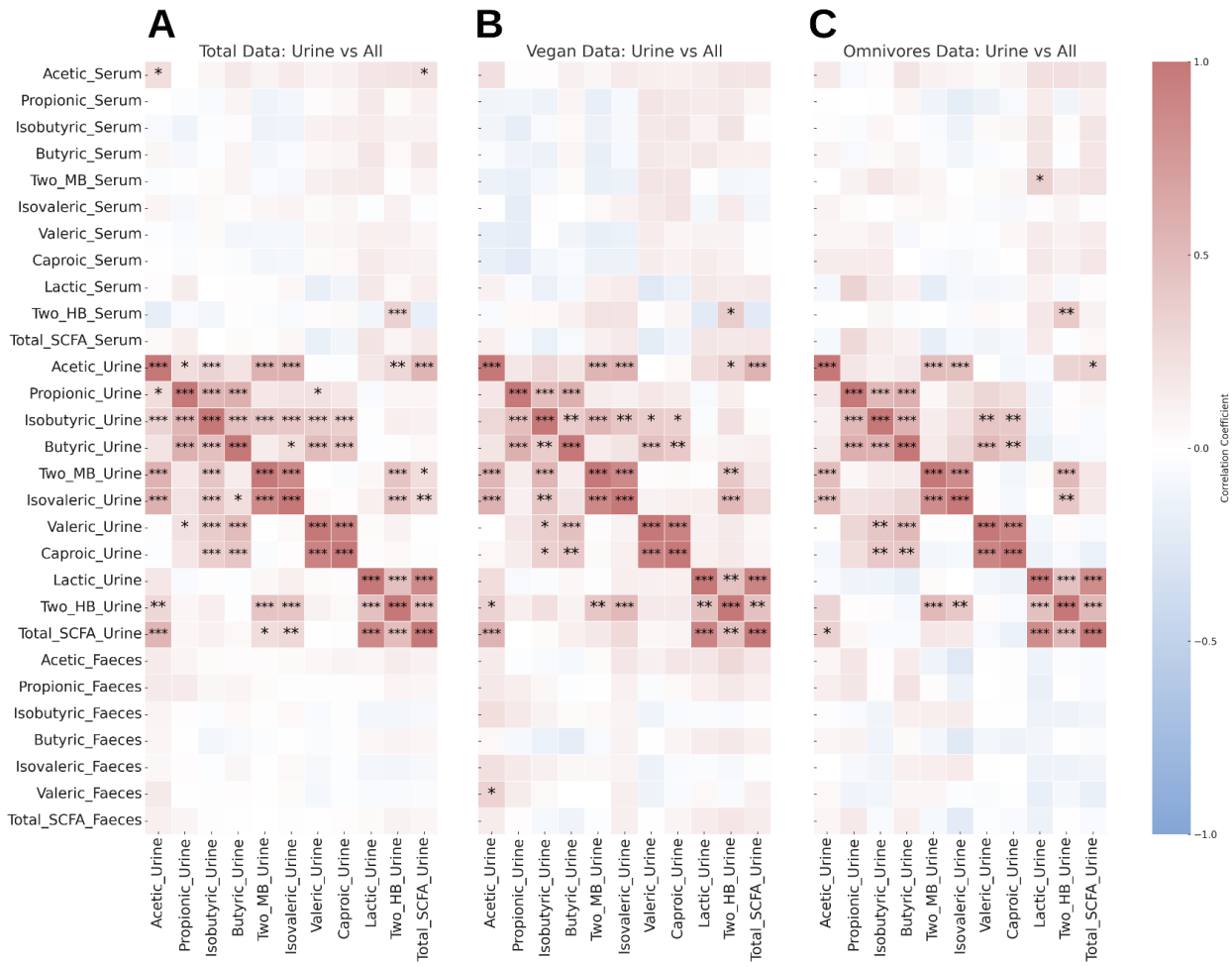

Figure 3.6 Correlations between urine SCFAs and serum, urine, and faecal SCFAs of vegans and omnivores. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively. Key: (A) vegan and omnivore data, (B) vegan data, and (C) omnivore data.

Vegans had high correlations in urine between isovaleric acid and 2-methylbutyric (R= 0.86, P_{adj} =8.55x10^{-21}), caproic acid and valeric acid (R= 0.91, P_{adj} = 2.98x10^{-27}), total SCFAs and lactic acid (R= 0.91, P_{adj} = 2.82x10^{-26}). A weak correlation in faeces was found between valeric acid and acetic acid in urine (R= 0.33, P_{adj} = 0.03) (Figure 3.6B).

In omnivores high correlations were found in urine between isovaleric acid and 2- methyl butyric (R= 0.83, P_{adj} = 2.23x10^{-17}), caproic acid and valeric acid (R= 0.901, P_{adj} = 6.41x10^{-24}), total SCFAs and lactic acid (R= 0.84, P_{adj} = 5.84x10^{-18}). Notably, no metabolite correlations were observed between faeces and urine. (Figure 3.6C).

Although most correlations within urine had the same pattern across the groups, there were two sets of correlations that seemed to be influenced by the dietary pattern. Correlations between 2-methylbutyric acid, isovaleric acid, and isobutyric acid were observed in both the groups and vegans. However, this correlation was not observed in omnivores.

3.6.7.3 Correlations between faecal SCFAs and SCFAs from all biofluids

Correlations between serum and faeces, urine, and faeces were previously described in sections 3.6.7.1, and 3.6.7.2, respectively, for all the studied groups.

Most of the faecal metabolites (Figure 3.7A) presented a positive correlation within them for both groups (vegans and omnivores), being the highest between acetic acid and propionic acid (R=0.77, P_{adj} =5.0x10^{-27}), butyric and acetic acid (R= 0.86, P_{adj} = 1.70x10^{-41}), acetic acid and total SCFAs (R= 0.93, P_{adj} = 5.45x10^{-59}), propionic and total SCFAs (R=0.88, P_{adj} = 1.50x10^{-44}), butyric and total SCFAs (R= 0.95, P_{adj} =1.51x10^{-69}).

Vegans results presented a similar correlation pattern compared to both groups (Figure 3.7B). In the same fashion, the highest correlations were found between acetic acid and propionic acid (R= 0.70, P_{adj} = 7.91x10^{-11}), butyric and acetic acid (R= 0.83, P_{adj} = 2.59x10^{-18}), acetic acid and total SCFAs (R= 0.90, P_{adj} = 8.39x10^{-26}), propionic and total SCFAs (R= 0.86, P_{adj} = 2.99x10^{-20}), butyric and total SCFAs (R= 0.94, P_{adj} = 3.62x10^{-32}) (Figure 3.7B)

Omnivores correlations were similar to previous described results for the same metabolites (Figure 3.7C), being higher between acetic acid and propionic acid (R= 0.70, P_{adj} = 7.91x10^{-11}), butyric and acetic acid (R= 0.83, P_{adj} = 2.59x10^{-18}), acetic acid and total SCFAs (R= 0.90, P_{adj} = 8.39x10^{-26}), propionic and total SCFAs (R= 0.86, P_{adj} = 2.99x10^{-20}), butyric and total SCFAs (R= 0.94, P_{adj} = 3.62x10^{-32}).

Correlations did not show any significant association when SCFA data were explored between serum and faecal SCFA for both groups and independently. In the same manner, associations between faecal and urine SCFA did not show any significant correlation in both groups and omnivores. Vegans presented only one weak correlation (R=0.33, $P_{adj=}$ 0.025) between faecal valeric and serum acetic SCFA. No other significant correlations were found.

Most of the correlations presented similar patterns across the three studied groups, but some results suggested an influence of dietary patterns. This was the case for isobutyric and propionic, isovaleric, propionic, isobutyric, total SCFAs, isovaleric acid, and total SCFAs. All of these correlations were higher when data were analysed, including both groups, compared to vegans and omnivores, due to an increase in sample size (174). The same correlations were slightly lower in vegans than in both data correlations, and most were not found when data were analysed exclusively for omnivores.

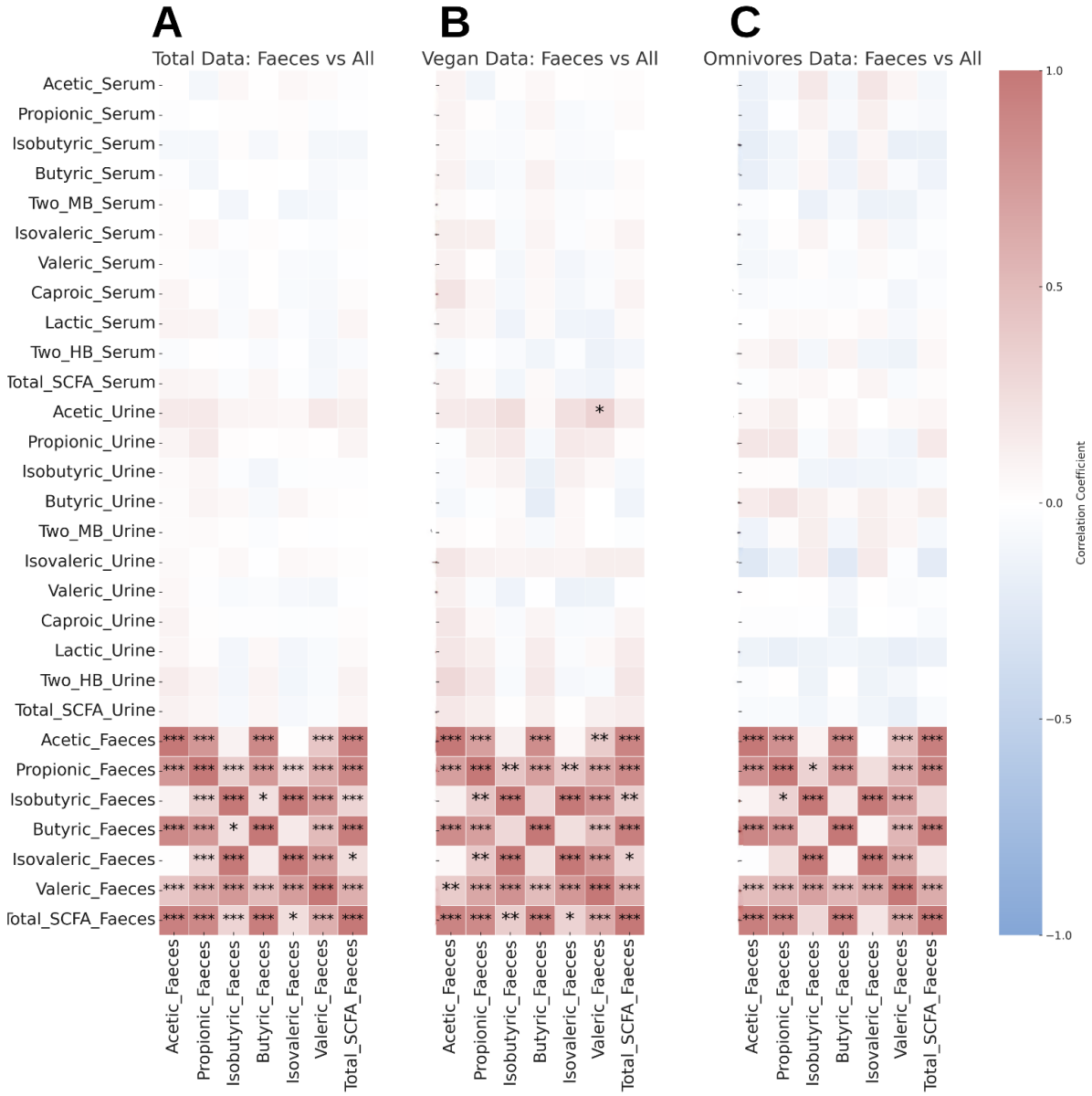

Figure 3. 7 Correlations between faecal SCFAs and serum, urine, and faecal SCFAs of vegans and omnivores. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively. Key: (A) Vegan and omnivore data, (B) vegan data and (C) omnivore data.

3.7 Discussion

To the best of my knowledge, no previously published research has studied SCFA metabolism in vegans and omnivores by analysing three different biofluids, considering the set of chosen SCFAs employing a novel GC-MS technique (78). In this observational clinical trial, although differences in anthropometric and biochemical parameters were found, the results remained within the normal range. Nutrients impacting SCFA metabolism were significantly different between the groups; however, the DASH score was comparable in both groups. Notably, 2-hydroxybutyric acid levels in serum were significantly higher in omnivores. Conversely, in urine acetic acid, propionic acid, isobutyric acid, 2-methylbutyric acid, isovaleric acid, and total urinary SCFAs were significantly higher in vegans. No significant differences were observed in faecal SCFAs.

The consumption of a vegan dietary pattern has increased in recent years (158-160). Beneficial effects on health have been associated with its consumption (27, 143, 154-157). For example, a recent randomised clinical trial involving identical twins reported cardiometabolic effects after the consumption of a vegan dietary pattern for 8 weeks (175). The study of SCFAs in different dietary patterns has been limited to a few metabolites, such as acetate, butyrate, and propionic acid, thus limiting our understanding of other related SCFA metabolites and the potential impact of diet on their metabolism. It is not well understood how SCFAs and related metabolites could differ in the serum, urine, and faeces of vegans and omnivores. A group of vegans and omnivores were studied (see Chapter 2 for details) to understand how SCFAs vary in biofluids and the potential role dietary patterns play in this process. Serum and urine samples were analysed using a novel GC-MS technique (78, 144), whereas faeces were analysed using a previously published GC-MS technique (146).

3.7.1 Differences between vegan and omnivorous populations.

Differences between anthropometry, biochemical analysis, dietary characteristics, and SCFAs in serum, urine, and faeces were explored to understand the effect of diet on SCFA metabolism.

In this study, anthropometric data were collected and analysed. BMI and weight were not significantly different between vegans and omnivores. Although vegans exhibited a BMI that was 2 points lower, these values are considered within the normal range (176). Previous epidemiological studies, such as the Adventist Health Study-2 and Epic – Oxford study, have reported a lower BMI in vegans than in omnivores (28, 177, 178). These studies have found that BMI was lower in vegans and incrementally higher in vegetarian and non-vegetarian populations; however, it is important to acknowledge that these studies were epidemiological and included larger sample sizes, with omnivorous dietary patterns being less healthy in comparison to vegans.

Systolic and diastolic blood pressure were not significantly different between the groups in our study, and the median values were in the range of normality for blood pressure in adults, according to the recommendation for blood pressure (179).

3.7.2 Diets and biochemical biomarkers in vegans and omnivores

Increasing evidence suggests that diets low in animal protein and high in vegetable fibre are associated with protection against obesity, diabetes, and cardiovascular diseases (13, 154, 155, 177). Regarding biomarkers linked to glucose metabolism, fasting glucose and HbA1c levels were significantly lower in vegans, with both groups falling within the normal ranges as defined by the NHS criteria for these biomarkers (180, 181). Previous evidence has shown that the type of carbohydrate consumed is related to insulin sensitivity (182-184). In the case of sucrose consumption, it seems to be more detrimental compared to regular starch intake (182, 184-186). In vegan diets, carbohydrate consumption is characterised by a low glycaemic index and high fibre content (25, 26). These carbohydrate types are associated with beneficial effects on insulin sensitivity (133, 184, 187). However, in both groups, HOMA results associated with beta-cell function, insulin sensitivity, and insulin sensitivity were within the recommended normal ranges (188) without significant differences between vegans and omnivores, despite their differences in starch and fibre consumption. This suggests that neither group presented evidence of glucose metabolism disturbance.

It has been established that a vegan dietary pattern provides a cardioprotective lipid profile, characterised by lower total cholesterol and lower low-density lipoprotein (LDL) levels (26, 189). In the study cohort, participants had followed a vegan diet for at least two years, with a median duration of five years. This extended period may contribute, alongside a higher intake of fibre, to the lower LDL and cholesterol levels observed in the vegan cohort, which were within the normal range as defined by NICE criteria (190). A significant reduction in leukocyte counts has been previously reported in studies comparing the immunological parameters of vegans versus non-vegans (191). In addition, after four weeks of a vegan intervention diet in patients with rheumatoid arthritis, a reduction in leukocyte counts was observed (192). Similarly, lymphocyte counts have been reported to be significantly lower in vegans compared to non-vegans (191). Other blood parameters were not significantly different, except for ferritin levels, which were lower in vegans, but within normal levels. This finding has been previously described in the literature (193, 194). Cobalamin intake of this vegan cohort has been previously described, being lower in vegans (129). Moreover, cobalamin levels in serum for both groups were found to be within the clinical range of normality (130).

Trimethylamine-N-oxide (TMAO) in serum was found to be higher in omnivores within this cohort, a result that aligns with previous findings associated with non-vegetarian dietary patterns (195). The

literature indicates that regular intake of animal protein is correlated with several microbial genera that elevate TMAO levels, a proatherogenic compound that increases the risk of cardiovascular disease (163). However, diets characterised by high fish consumption and rich in polyunsaturated fats may mitigate the adverse effects of TMAO (196). Additionally, a diet high in fibre and low in glycaemic index may reduce TMAO metabolism through alterations in the intestinal microbiome (197, 198).

Carnitine levels were observed to be higher in omnivores in this study; this finding is anticipated, as it is a common characteristic reported for meat eaters, and it correlates with TMAO concentrations in both blood and urine (199). Furthermore, the regular consumption of meat may facilitate gut microbial production that utilises carnitine as a precursor for TMAO (199, 200). In the case of choline, consumption was significantly higher in the omnivore group, with a P adjusted value close to 0.04, which remains statistically significant. An inverse relationship has been established between a plant and nut-rich diet and TMAO production (199). Paradoxically, nuts and legumes serve as important protein sources in vegan diets and contain substantial amounts of choline (195). These phenomena may be elucidated by a proposed mechanism wherein gut microbiota convert choline into trimethylamine (TMA, a precursor of TMAO) at differing conversion rates in individuals adhering to vegan diets (201). In this study, the vegan cohort had an intake of legumes and nuts that was five times higher than that of the omnivores, and TMAO concentrations were correspondingly lower in the vegans. Previous studies have reported inconsistent results between vegans and omnivores (200, 202). However, these discrepancies in TMAO levels may be attributed to variations in the characteristics of the studied participants, inclusion criteria, and duration of exposure to a vegan dietary pattern.

Betaine is regarded as a precursor to TMAO (195) and is widely found in a diverse array of foods, including shellfish, flour, grains, and certain vegetables (203-205). In this cohort, serum levels of betaine were higher in vegans, aligning with previous research that has reported similar findings for this population (206).

These findings suggest that both groups were relatively healthy in terms of their BMI classification, with no evident alterations in their glucose and lipid metabolism. Blood biomarkers for both groups were within normal ranges, despite the significant differences reported in dietary intake for certain nutrients (Table 3.3).

3.7.3 Dietary intake differences between groups

According to the dietary records completed by the study participants on four different occasions, differences were found in the distribution of macronutrients, starch, fibre, biotin, sodium, and the DASH score. Total energy and sucrose intake were not significantly different between groups. Fibre intake

was lower in omnivores compared to vegans, with average intakes of 28.92 g and 43.32 g, respectively. Interestingly, the fibre intake of omnivores fell within the range of dietary fibre recommendations for the population, which is between 25 g and 35 g daily (50). Vegan and omnivore diets can vary in their macro- and micronutrient composition, particularly regarding protein and fibre intake (191, 207-209). The DASH score was calculated based on food and nutrient consumption, which classified vegans and omnivores from the Danish cohort within the higher score classification; the median score for both groups exceeded the quintile cut-off point (a score of 29 points). This indicates that the type of food consumption, irrespective of dietary pattern, complied with the dietary DASH guidelines (210). The studied group of vegans exhibited a higher DASH score than omnivores (33 points versus 30 points, expressed in the median).

It has been reported that vegans consume more vegetables, fruits, nuts, and legumes than omnivores (16). However, it is important to highlight that following a vegan diet does not necessarily imply that it is a healthy diet due to the diversity of ultra-processed vegan products available or due to the higher consumption of refined carbohydrates (211). Emerging vegan dietary patterns that conflict with traditional vegan dietary guidelines may reflect a lack of knowledge regarding how to follow a healthy vegan diet (211).

It is important to note that participants were trained to complete their self-administered 4-day weighted records to reduce inaccuracies and misreporting that may be associated with dietary questionnaires (212). Subsequently, dietary information was calculated using the Danish Food Composition Databank. These dietary records were collected at four different time points and provided information about the accepted daily intake (ADI) of macronutrients and micronutrients. Moreover, this data represents the average consumption over the 4 days. As a limitation, this dietary data does not precisely represent food consumption from the day prior to the collection of biofluid samples. Therefore, the results should be interpreted with caution.

Until now, the effect of non-digestible carbohydrates (NDCs) on the production of SCFAs has been extensively studied (75, 93, 213, 214). However, the role of specific amino acids in modulating the production of SCFAs and branched short-chain fatty acids (BSCFAs) in humans remains inadequately understood. This is primarily due to the fact that various dietary compounds can interfere with the metabolic processes involved in the production of these metabolites derived from amino acids (68, 215). Although, the mechanism for the production of SCFAs using undigested proteins and amino acids as a substrate has been widely described in vitro models through the fermentation capacity of specific bacteria genera recovered from the gut (162, 168-170, 216). The amino acid content of dietary intake was compared between groups, especially those amino acids that have been reported to contribute

through their fermentation in SCFA and BSCFAs production (168-170). It has been described that the vegan diet contains less proteins and, subsequently, fewer amino acids compared to non-vegan and omnivore diets (191, 207-209).

SCFAs production in fasting serum can be influenced by the consumption of fibre, protein, and amino acids from the previous day (217). Previous studies have shown the effect of dietary compound consumption on SCFAs concentrations after a period of time or with specific diets. For example, the consumption of 20 g of pectin fibre can increase the concentration of acetic acid during fasting in healthy subjects (64). In addition, the consumption of barley kernels rich in amylose and β-glucans can increase butyric acid levels in the morning (218). A study examined the effect of three types of fermentable fibres on the production of short-chain fatty acids (SCFAs) in subjects. One study investigated the effects of three types of fermentable fibres on SCFA production in subjects, revealing that not all fermentable fibres are equally effective in stimulating SCFA production. This finding underscores the significance of an individual's microbiota composition in determining their response to specific dietary supplements (219).

3.7.4 Concentrations of 2-hydroxybutyric in serum

It is important to emphasise that the studied group exhibited significant differences in biochemical parameters for fasting plasma glucose and HbA1c. However, from a clinical perspective, these results remained within the normal range. Additionally, a limitation of this study is the absence of microbiome data, which precludes an exploration of whether certain SCFA differences could potentially be attributed to variations in the abundance of SCFA-producing microorganisms (94).

The role of 2-hydroxybutyric acid in metabolism remains ambiguous. This metabolite is synthesised in the liver from 2-ketobutyrate through threonine catabolism or via methionine and serine metabolism (220). Elevated levels of 2-hydroxybutyric acid during fasting are associated with impaired glucose metabolism. Positive correlations between 2-hydroxybutyric acid in serum, dietary proteins, and dietary amino acids (including their precursors) were significant when data from both groups were aggregated. These findings were not reported when correlations were examined within the groups independently; however, this discrepancy may be related to the increased sample size rather than a phenomenon linked to specific dietary patterns (174).

Levels of 2-hydroxybutyric acid, protein, and amino acid intake, including precursors, were higher in omnivores than in vegans. Previous studies have reported that vegan diets contain a lower protein content than omnivorous diets, with self-reported questionnaires used as quantification methods, despite their inherent inaccuracies (207-209, 212). Furthermore, high concentrations of 2-hydroxybutyric acid

in serum have been associated with low plasma biotin concentrations in insulin-resistant and severely obese patients (221). In this study, biotin intake was significantly higher in vegans than in omnivores (27.61 µg versus 37.21 µg, mean, P_{adj} value = $1.5x10^{-4}$), presumably due to the rich biotin food consumed in a vegan diet alongside some dietary supplements (222). The average levels of biotin intake were below the adequate intake for omnivores (30 µg/d) (223); however, this does not necessarily indicate low plasma levels or a potentially impaired glucose metabolism, nor does it imply an inflammatory phenotype in the omnivore group (221). Glucose metabolism parameters were evaluated in both groups and were classified as normal.

3.7.5 SCFA concentrations in urine

The excretion of SCFAs in the first void urine showed some differences between the two groups, with higher levels observed in vegans compared to omnivores. Specifically, acetic acid concentrations in the urine were elevated in vegans, who reported a median fibre intake of 43.32 g per day. Previous studies have indicated that acetic acid levels in 24-hour urine samples were higher in subjects consuming a diet containing 20 g of pectin daily, relative to a control group (64).

Propionic acid was detected at significantly higher concentrations in vegans than in omnivores. Elevated propionic acid levels in urine have been reported following the consumption of diets rich in vegetables and fruits by healthy individuals (224). Isovaleric, isobutyric, and propionic acids have been described as potential biomarkers in urine for diabetes, whereas 2-methylbutyric acid has been quantified in urine (224, 225). The presence of these metabolites at low concentrations in the urine appears to be normal among healthy individuals, regardless of dietary habits; however, their specific functions and implications remain to be elucidated.

A notable aspect of SCFA production in both groups was the alteration in the production pattern of these metabolites across different biofluids. Higher concentrations of SCFAs were detected in serum, while urine exhibited lower concentrations. Emerging research has shed light on the role of SCFAs in the kidneys and their modulation of inflammation and oxidative stress, both of which are implicated in the pathogenesis and progression of chronic kidney disease (CKD) (81, 226). Following the identification of G-protein coupled receptors (GPCRs) for SCFAs in the kidneys, further investigations are being conducted to enhance understanding of their metabolic processes and roles in health (227). Recent studies have suggested a function for the Olfr78 receptor for acetate and propionate in mice (designated as OR51E2 in humans), which is expressed in various tissues, including the renal artery and juxtaglomerular complex (82). Nonetheless, the impact of these receptors on urinary SCFA concentrations and their physiological roles remains unclear. The contrasting concentrations of these metabolites in serum and urine may imply that, following hepatic metabolism and entry into the

bloodstream, alterations occur at the renal level, resulting in an inverse pattern compared to serum. This shift in biofluid concentration may correlate with normal physiological responses potentially influenced by the microbiome and diet. Consequently, the production of metabolites that could be pro-inflammatory and pro-oxidant at the renal level may contribute to the development of CKD (228).
The urine samples analysed were from the first void, and it was noted that less than 0.05% of SCFAs are typically excreted in urine in healthy individuals (139). The excretion rate of these metabolites in urine may reflect the cumulative effects of fasting, overnight fermentation, and various physiological processes regulated by circadian rhythms and the microbiome (229, 230).

3.7.6 SCFA in faecal samples

Faecal SCFAs did not present significant differences between the vegan and omnivorous groups. These findings are aligned with results reported previously by Wu et al. for vegans and omnivores using proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) as an analytical technique (167). Similarly, no differences in faecal SCFAs have been reported between vegans and omnivores using a gas chromatograph flame ionisation detector (GC-FID) (231). The mechanism underlying the absence of differences in faecal SCFAs concentrations between groups is not clear, but it has been suggested that the microbiota of western vegan populations is restrictive and that SCFA production does not increase linearly with fibre consumption (167). In animal models, it has been observed that feeding mice a low-fibre diet over generations resulted in the loss of microbiota and taxa that could not be restored after a subsequent switch to a high-fibre diet in later generations (232). Conversely, it has been proposed that bacterial activity and functionality may adapt to the dietary habits of the host, and consequently, microbial fermentation did not differ between the two groups (231). SCFAs are predominantly absorbed by colonocytes; thus, SCFA concentrations in faeces do not accurately reflect the synthesis of SCFAs (233).

Multivariate analysis for SCFAs in the serum and urine did not show a clear difference between the groups. An overlap of metabolites between the vegan and omnivorous participants was observed in the loading plots for both biofluids. This finding may be attributed to the overall healthiness of the diets and the health status of participants in both dietary groups, despite their differing dietary patterns.

As mentioned previously, this study has some limitations. Participants were instructed on how to complete their dietary records, and this information did not provide an accurate representation of the immediate response of food intake and the circulating metabolite concentrations found in the analysed biofluids. Furthermore, as these participants were free-living individuals, their food intake may have been underestimated in prior reports (212). Additionally, the cross-sectional design of the study, which

included only one time for biofluid samples, did not allow for an understanding of potential variations related to the time of day or changes over time.

3.8 Conclusions

This study is one of the very few to provide dietary information on healthy individuals who have been vegans for an average period of five years, analysing serum, urine, and faecal samples for metabolite analyses, particularly SCFAs. The comprehensive data presented in this study regarding SCFAs in various biofluids from the same individuals is crucial for understanding SCFA metabolism and the dietary influences on this process.

In this investigation, ten SCFAs and related metabolites were quantified using a novel GC-MS method that provided a broader coverage of metabolites. Previous studies that have explored the differences in SCFAs in these dietary patterns have limited their study to only one or two biofluids. The biofluids that exhibited variations between groups were serum and urine; however, the significance of sampling time for SCFA analysis in serum, urine, and faecal samples warrants further consideration. In this context, SCFA metabolism could be explored more thoroughly following meal exposure, with a clear identification of the types of fibre and proteins derived from both animal and plant sources.

More research is needed to explore the role of SCFAs and related metabolites in vegan and omnivorous dietary patterns, considering the type of protein consumed, fibre, level of diet healthiness, and microbiome data to quantify the abundance and functionality of microorganisms capable of producing SCFAs. Furthermore, the integration of other omics techniques is recommended for a more comprehensive study. A better understanding of SCFA responses in different dietary contexts is essential for further exploring their potential applications in health and disease within the framework of personalised nutrition.

4. Circulating short-chain fatty acid concentrations after the intake of two contrasting dietary patterns under controlled conditions.

This chapter was based on this published article: Brignardello J, Fountana S, Garcia-Perez I, Posma M J, et al. 2021. "Characterization of diet-dependent temporal changes in circulating short-chain fatty acid concentrations: A randomized crossover dietary trial". Am J Clin Nutr. 2022. Nov; 116(5): 1368–1378. PMID: 36137188.
Note: In this chapter, SCFAs will be referred to by their systematic names in their protonated forms based on their chemical structures.

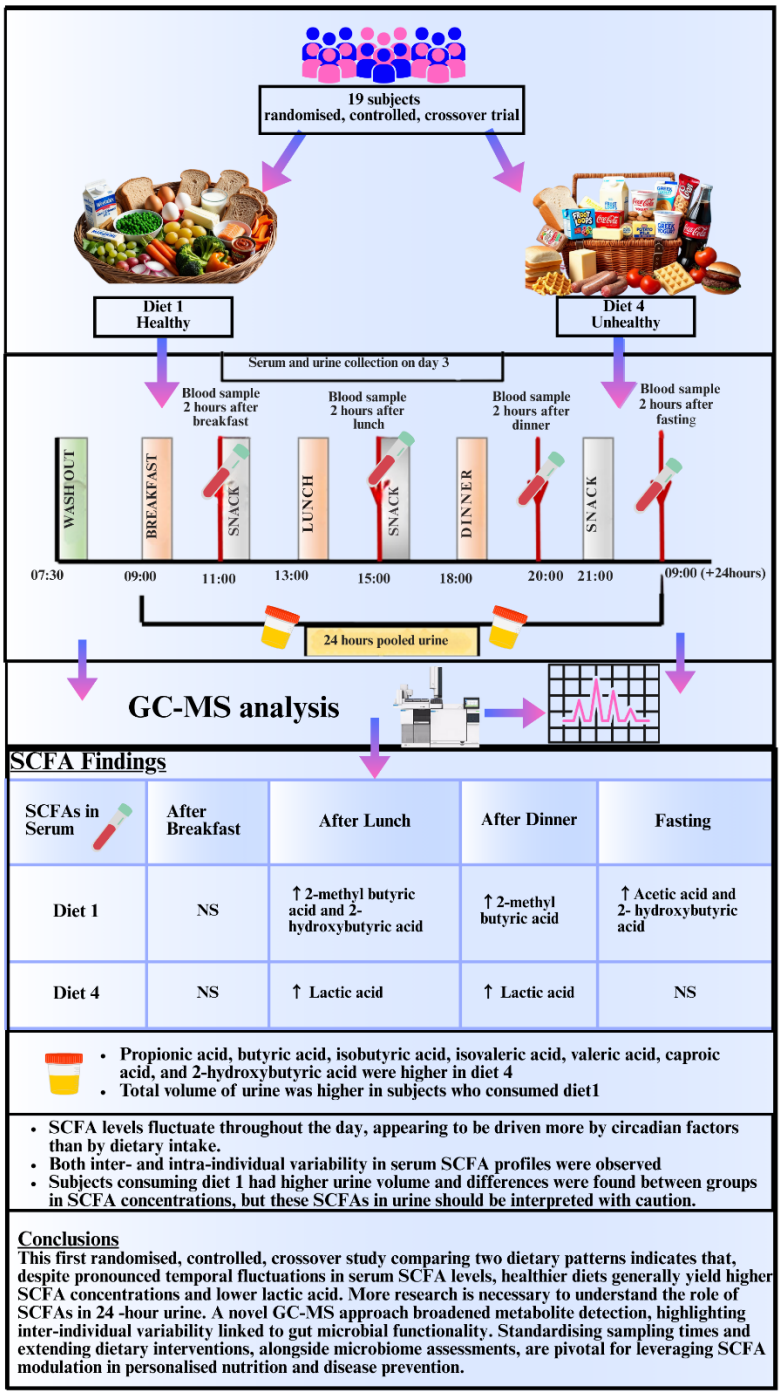

Figure 4. 1. Schematic Summary of Chapter 4

4.1 Introduction

The effect of diet on saccharolytic and proteolytic fermentation is important for understanding the relevance of short-chain fatty acids (SCFAs) in health and disease (215). Previous research has explored the differences in SCFA levels between free-living vegans and omnivores, demonstrating that dietary patterns can influence SCFA concentrations in serum and urine. These variations in SCFA levels may be attributed to factors such as carbohydrate intake and particularly the type of fibre intake. Notably, one of the most significant distinctions between omnivores and vegans is the exclusive consumption of plant proteins by vegans.

This chapter investigated SCFA production following the consumption of two contrasting omnivorous dietary patterns under controlled conditions, employing a novel (GC-MS) technique that quantifies ten metabolites, including SCFAs and related compounds (78). As mentioned in Chapter 2, this is a sub-study of previously published research that examined the impact of diets with varying healthiness on metabolism in a controlled setting. A major limitation in nutritional studies concerning dietary intake is the lack of accuracy in dietary data reporting (212, 234, 235). For instance, misreporting of energy intake assessed through 24-hour recall has been shown to range from 21.5% to 67% (212). Given this reported variability, the extent to which SCFA production may change under controlled food intake, considering different dietary patterns, remains poorly understood.

In the previously published study, four dietary interventions were designed in accordance with the WHO guidelines for healthy eating (132, 141). The nutritional interventions examined in this chapter included two extreme dietary patterns that varied in their content of fibre and protein, both of which are linked to the production of SCFAs (18, 69, 162, 236). Diet 1 (healthy) was fully aligned with the WHO healthy eating recommendations, being rich in fruits, vegetables, and fibre, while being low in saturated fats, sugars, and salt. In contrast, diet 4 (unhealthy) was low in fruits and vegetables, contained only one-third of the fibre, and was aligned with the WHO healthy eating recommendations by only 25%. Please refer to Table 4.1 for differences in dietary composition.

The two diets were provided in random order; volunteers attended the clinical research facilities four times, each for a period of 72 hours. The same food was given to the participants each day during the three-day dietary intervention period. The interventions were meticulously implemented under controlled conditions in an inpatient setting, ensuring that the same schedule was followed for each meal.

In this sub-study described in this chapter, temporal changes of SCFA-related metabolites were monitored throughout the day under controlled conditions in response to these contrasting dietary patterns and to understand the systemic exposure to SCFAs modulated by diet. This chapter has been

published in the American Journal of Clinical Nutrition under the name "Characterization of diet-dependent temporal changes in circulating short-chain fatty acid concentrations: A randomized crossover dietary trial" (78).

4.2 Hypothesis

The consumption of two omnivorous dietary patterns that differ in nutritional composition will be reflected in the concentrations of SCFAs in serum and urine.

4.3 Aims

The central aim of this study was to compare the temporal changes in SCFA concentrations in response to two omnivorous dietary interventions that differed in nutritional composition.

4.4 Objectives

The specific objectives were as follows.

1. To provide baseline sociodemographic and clinical characteristics of participants who consumed both dietary interventions.
2. To compare the SCFA serum concentrations between the two contrasting diets at four different time points over a 24-hour period.
3. To describe the variability in the response between participants in serum SCFA concentrations.
4. To evaluate 24-hour urinary SCFA concentrations and assess the potential role of diet in their excretion

4.5 Material and methods

The study design, participant recruitment, sample collection, GC-MS sample preparation, and GC-MS data analyses were described in Chapter 2 and were published by Brignardello et al. (78).

Table 4. 1 Comparison of the nutritional details of components of the two diets.

Diet 1							Diet 4						
Food	**Quantity**	**Protein g**	**Fat g**	**CHOg**	**Energy kcal**	**Fibre g**	**Food**	**Quantity**	**Protein g**	**Fat g**	**CHOg**	**Energy kcal**	**Fibre g**
Breakfast							**Breakfast**						
Weetabix	60	6	1	44	199	6	White Bread Toast	60	5	1	29	140	2
Wholemeal Bread Toast	60	7	2	30	153	5	Sweetened cereal	60	3	1	52	210	2
Semi skimmed millk	150	5	3	7	69	0	Full fat milk	150	5	5	7	95	0
Boiled Egg	60	8	6	0	86	0	Butter	10	0	8	0	74	0
Polyunsaturated margarine	10	0	2	0	80	0							
Sub total	*340*	*27*	*13*	*80*	*587*	*11*	*Subtotal*	*280*	*13*	*16*	*88*	*519*	*4*
Mid morning							**Mid morning**						
Apples	150	1	1	17	77	2	Greek Yogurt	125	7	13	6	164	0
Lunch							**Lunch**						
Peas boiled	60	4	1	6	47	3	Cola	330	0	0	36	135	0
Potatoes baked	200	5	0	45	194	5	Fried Pork Sausages	125	17	30	12	385	3
Carrots boiled	60	0	0	4	17	2	Potato Waffles	120	3	7	37	180	5
Broccoli boiled	100	3	1	3	28	3							
Salmon steamed	150	35	28	0	392	0							
Sub total	*570*	*48*	*30*	*58*	*678*	*13*	*Subtotal*	*575*	*21*	*36*	*85*	*700*	*8*
Mid afternoon							**Mid afternoon**						
Grapes, green	150	1	0	23	93	2	Chocolate, milk	45	3	14	25	210	1
Evening meal							**Evening meal**						
Wholemeal Paste	150	8	2	41	201	6	Processed Cheese	30	5	7	2	89	0
Peppers	80	1	0	2	12	2	Potato Waffles	120	3	7	37	180	5
Onions	40	0	0	3	14	1	Tomato raw	100	1	0	3	14	1
Tomato pasta sauce	150	2	6	10	98	3	Cola	330	0	0	36	135	0
Grilled Chicken	125	40	3	0	185	0	Beef Burger	100	24	11	0	193	0
Sub total	*545*	*51*	*10*	*56*	*510*	*12*	*Subtotal*	*680*	*33*	*24*	*78*	*611*	*6*
Supper							**Supper**						
Wholemeal Toast	80	9	2	39	204	7	White Bread	80	8	2	45	210	2
Polyunsaturated margarine	10	0	2	0	80	0	Butter	10	0	8	0	74	0
Sub total	*90*	*9*	*4*	*39*	*284*	*7*	*Subtotal*	*90*	*8*	*10*	*45*	*284*	*2*
Total	**1845**	**137**	**59**	**274**	**2228**	**46**	**Total**	**1795**	**85**	**113**	**327**	**2490**	**21**

4.6 Results

4.6.1 Baseline sociodemographic and clinical characteristics of participants

Samples from 19 participants were considered for this sub-study. The participants were assessed considering anthropometry and biochemical parameters (Table 4.2). The characteristics of the study group were distributed as follows: ten participants were male and nine were female, aged 29 to 65 years. The BMI of the participants ranged from normal to obese (21.1 to 33.3 kg/m^2). Glucose metabolism parameters, such as glucose and HbA_{1c}, were within the normal ranges (180, 237). Lipid metabolism parameters, including triglycerides, total cholesterol, LDL cholesterol, and HDL cholesterol levels, were screened according to the inclusion and exclusion criteria of the study (132, 238). Liver function test results, such as alanine transaminase and aspartate transaminase levels, were within the normal range (239).

Table 4.2 Baseline sociodemographic and clinical characteristics of participants.

Sex	
Male	10 (53%)
Female	9 (47%)
Age (years)	55.8 (12.6; 29–65)
Ethnic origin	
White	18 (95%)
Asian	1 (5%)
Anthropometric and biochemical parameters	
Weight (kg)	74.5 (12.5; 52.8–107.9)
BMI (kg/m^2)	25.6 (3.2; 21.1–33.3)
Glucose (mmol/L) [1]	4.8 (0.4; 4.1–5.4)
HbA_{1c} (%) [1]	5.5% (0.1, 5.1–5·8)
HbA_{1c} (mmol/mol) [1]	36.4 (0.9; 32–40)
Triglycerides (mmol/L) [2]	0.9 (0.3; 0.5–1.4)
Cholesterol (mmol/L) [2]	
Total	5.1 (0.7; 3.9–6.1)
LDL	3.1 (0.7; 1.7–4.2)
HDL	1.6 (0.4; 0.9–2.6)
Liver function tests (IU/L) [2]	
Alanine transaminase	21.2 (7.4; 12.3–40.0)
Aspartate transaminase	19.5 (3.2; 15.0–24.3)

[1] Data are in n (%) or mean (SD; range). IU, international units.[2] From plasma samples. [3] From serum samples.

4.6.2 Comparison of serum concentrations of SCFAs between the two different diets at different time points within a 24-hour period

Serum SCFA concentrations were compared over a 24-hour period, with samples collected at four time points: after breakfast (AB), after lunch (AL), and after dinner (AD) on day 3, and a fasting (FA) sample on day four following the commencement of the dietary trial (see Figure 2.1, Chapter 2).

At the AB time point, SCFA concentrations did not differ significantly between the two diets (Figure 4.2). However, AL samples were significantly higher in diet 1 for 2-methylbutyric acid (P_{adj} =5x10^{-3}) and 2-hydroxybutyric acid (P_{adj} = 1.87x10^{-2}) (Figure 4.2E and Figure 4.2J), whereas lactic acid (P_{adj} = 1.87x10^{-2}) was higher for diet 4 (Figure 4.2I).

In samples taken AD, 2- methylbutyric acid (P_{adj} = 3.05x10^{-3}) remained higher in diet 1, while lactic acid was higher in diet 4 (P_{adj}3.05x10^{-3}) (Figure 4.2E and Figure 4.2I). In the fasting samples collected on day 4, after the consumption of diet 1, concentrations of acetic acid (P_{adj} =4.19x10^{-3}) and 2-hydroxybutyric acid (P_{adj} =7.6x10^{-4}) were higher (Figure 4.2A and Figure 4.2J, respectively).

Serum concentrations of measured SCFAs and related compounds changed over time, and each SCFA showed a different trajectory pattern in response to dietary intake. For example, acetic acid levels increased AB, peaked AL, and then continued decreasing until the fasting sample on the following day. Likewise, propionic acid followed a similar pattern to acetic acid, but the concentration was ten times lower. Interestingly, butyric acid increased from almost zero from AB to AL, reached a plateau, and then rose further from AD to fasting on day 4, reflecting overnight postprandial release. Most SCFAs exhibited a similar geometric pattern, regardless of the diet type, even though their absolute concentrations differed between the two diets.

In the case of lactic and 2- hydroxybutyric acid, both metabolites showed a slight diet-dependent variation between the time trajectory patterns. These findings suggested that their trajectories were related to metabolic regulation, which was associated with the time of the day and the type of diet consumed in relation to the time of the last meal (Figure 4.2).

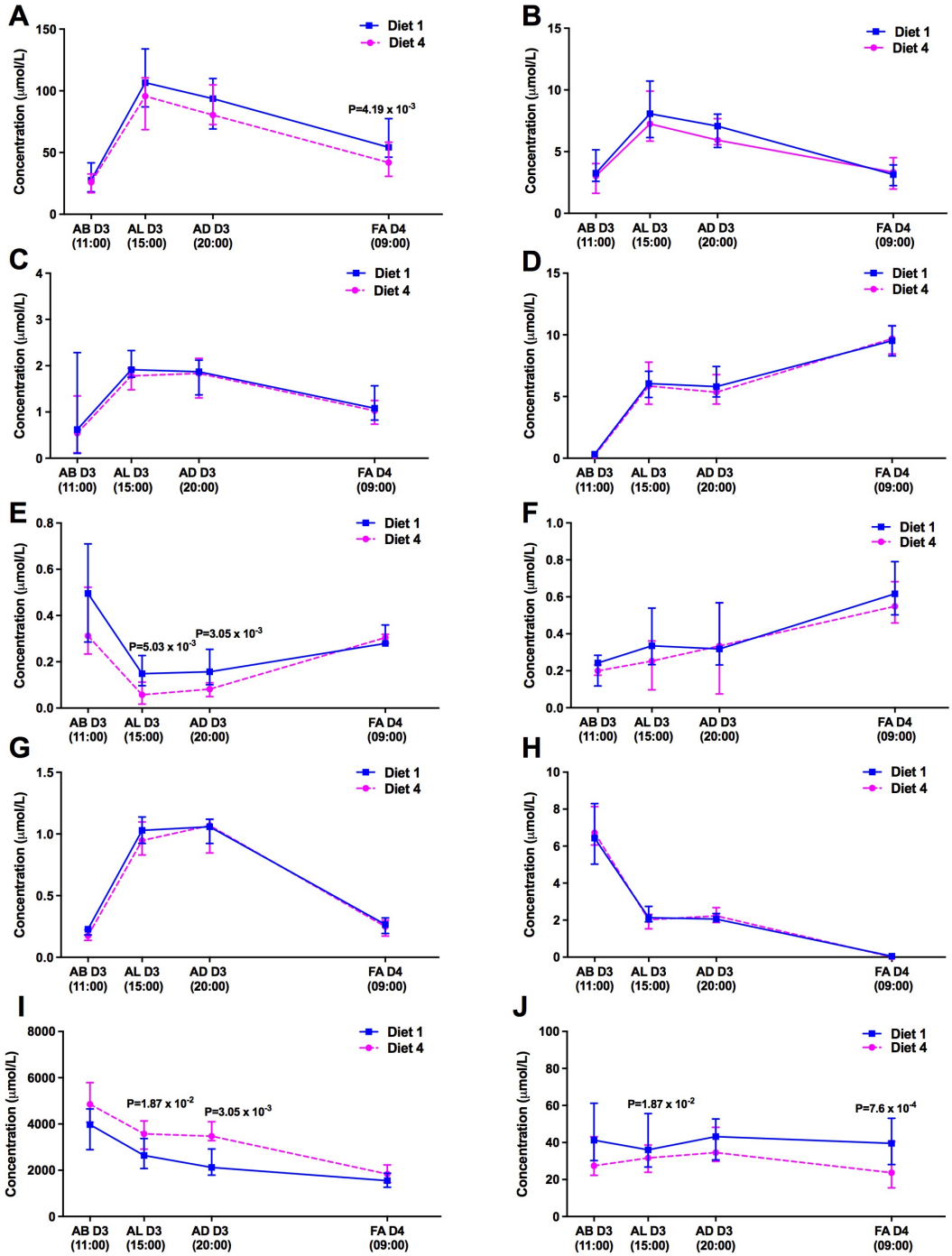

Figure 4. 2 Comparison of serum SCFA concentrations throughout the day for the same time points for diet 1 and diet 4. (A) acetic acid, (B) propionic acid, (C) isobutyric acid, (D) butyric acid, (E) 2-methylbutyric acid, (F) isovaleric acid, (G) valeric acid, (H) caproic acid, (I) lactic acid and (J) 2- hydroxybutyric. Values are shown in median and interquartile range; n = 19. Differences between the same time points for different diets were determined by Wilcoxon signed-rank test and False Discovery Rate (FDR). Significance was accepted at $P_{adj} < 0.05$.

4.6.3 Variations of serum SCFA throughout the day within and across diets

Differences between the diets were observed when SCFA concentrations were analysed at specific time points (Table 4.3). Most of the SCFAs were significantly different between the AB and AL samples and between the AB and AD ($P_{adj} < 0.05$), except for isovaleric acid, 2-hydroxybutyric acid, and isobutyric acid for diet 1 (healthy diet) and isovaleric acid, 2- hydroxybutyric acid for diet 4 (unhealthy diet). Significant differences were found between AL/AD for acetic acid ($P_{adj} = 3.05x10^{-4}$) and caproic acid ($P_{adj} = 3.53x10^{-2}$) in diet 1 and 2- hydroxybutyric ($P_{adj} = 2.1x10-3$) in diet 4. SCFA levels between AD and FA showed significant differences ($P_{adj} < 0.05$) in most SCFAs, except for isobutyric, isovaleric and 2–hydroxybutyric in diet 1 and isobutyric and isovaleric in diet 4.

In the case of AL and FA, 2- hydroxybutyric acid was the only SCFA that did not have significant differences for both diets. In the comparison of samples collected on AB versus FA were significantly higher for acetic acid ($P_{adj}= 4.7x10^{-3}$), butyric acid ($P_{adj}= 4.5x10^{-5}$), isovaleric acid ($P_{adj} = 9.65x10^{-3}$) in diet 1. In contrast, caproic acid ($P_{adj}= 4.58x10^{-5}$), lactic acid ($P_{adj}= 1.9x10^{-4}$) from diet 1 and caproic acid ($P_{adj}= 2.44x10^{-4}$), lactic acid ($P_{adj}=3.05x10^{-4}$) from diet 4 were higher in samples collected AB for both diets. 2-methylbutyric acid was significantly higher in diet 1 ($P_{adj} = 2.08x10^{-2}$) but not for diet 4, while valeric acid was significantly lower in diet 4 ($P_{adj} = 4.3x10^{-2}$), but not diet 1. See Figure 4.3 and Figure 4.4.

Table 4. 3 Summary of differences throughout the day and comparison between different time points for each diet.

SCFA	Type of diet	Differences throughout the day [1]	After breakfast versus After lunch [2,3]	After breakfast versus After dinner [2,3]	After lunch versus After dinner [2,3]	After dinner versus Fasting [2, 3]	After lunch versus Fasting [2,3]	After breakfast versus Fasting [2,3]
Acetic acid	Diet 1	$4.3x10^{-6}$	↑$2.4x10^{-4}$	↑$1.07x10^{-2}$	↓$3.05x10^{-4}$	↓$2.1x10^{-2}$	↓$3.05x10^{-4}$	↑$4.7x10^{-3}$
	Diet 4	$2.29x10^{-8}$	↑$9.1x10^{-5}$	↑$4.8x10^{-4}$	NS	↓$5.03x10^{-3}$	↓$1.54x10^{-3}$	↑$6.71x10^{-3}$
Propionic acid	Diet 1	$7.94x10^{-6}$	↑$1.07x10^{-3}$	↑$2.4x10^{-3}$	NS	↓$2.4x10^{-3}$	↓$9.18x10^{-5}$	NS
	Diet 4	$6.3x10^{-6}$	↑$3.05x10^{4}$	↑$4.8x10^{-4}$	NS	↓$5.1x10^{-3}$	↓$3.04x10^{-4}$	NS
Isobutyric Acid	Diet 1	$3.11x10^{-3}$	NS	NS	NS	NS	↓$9.18x10^{-5}$	NS
	Diet 4	$1.1x10^{-3}$	↑$1.58x10^{-2}$	↑$3.41x10^{-2}$	NS	NS	↓$3.48x10^{-3}$	NS
Butyric acid	Diet 1	$1.78x10^{-9}$	↑$1.2x10^{-4}$	↑$2.4x10^{-4}$	NS	↑$1.8x10^{-8}$	↑$1.2x10^{-4}$	↑$4.5x10^{-5}$
	Diet 4	7.25x10-9	↑$9.15x10^{-5}$	↑$3.6x10^{-4}$	NS	↑$9.7x10^{-4}$	↑$1.5x10^{-4}$	↑$2.4x10^{-4}$
2-Methylbutyric acid	Diet 1	$2.74x10^{-4}$	↓$3.93x10^{-3}$	↓$3.63x10^{-3}$	NS	↑$4.57x10^{-3}$	↑$3.05x10^{-3}$	↓$2.08x10^{-2}$
	Diet 4	$6.47x10^{-8}$	↓$1.52x10^{-4}$	↓$4.8x10^{-4}$	NS	↑$7.3x10^{-4}$	↑$1.5x10^{-4}$	NS
Isovaleric acid	Diet 1	$1.05x10^{-4}$	NS	NS	NS	NS	↑$5.79x10^{-3}$	↑$9.65x10^{-3}$
	Diet 4	$6.34x10^{-4}$	NS	NS	NS	NS	↑$7.62x10^{-4}$	↑$3.6x10^{-4}$
Valeric acid	Diet 1	$1.9x10^{-8}$	↑$1.53x10^{-4}$	↑$3.66x10^{-4}$	NS	↓$2.44x10^{-4}$	↓$9.15x10^{-5}$	NS
	Diet 4	$1.41x10^{-8}$	↑$9.15x10^{-5}$	↑$4.88x10^{-4}$	NS	↓$7.32x10^{-4}$	↓$1.53x10^{-4}$	↑$4.3x10^{-2}$
Caproic acid	Diet 1	$2.83x10^{-9}$	↓$2.75x10^{-4}$	↓$4.88x10^{-4}$	↓$3.53x10^{-2}$	↓$3.66x10^{-4}$	↓$7.65x10^{-5}$	↓$4.58x10^{-5}$
	Diet 4	$2.51x10^{-9}$	↓$3.66x10^{-4}$	↓$9.15x10^{-5}$	NS	↓$4.88x10^{-4}$	↓$1.52x10^{-4}$	↓$2.44x10^{-4}$
Lactic acid	Diet 1	$1.25x10^{-6}$	↓$1.8x10^{-2}$	↓$6.9x10^{-3}$	NS	↓$6.04x10^{-4}$	↓$9.1x10^{-5}$	↓$1.9x10^{-4}$
	Diet 4	$1.04x10^{-7}$	↓$1.1x10^{-3}$	↓$6.1x10^{3}$	NS	↓$7.6x10^{-4}$	↓$1.8x10^{-4}$	↓$3.05x10^{-4}$
2-hydroxybutyric acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	$1.34x10^{-3}$	NS	NS	↑$2.1x10^{-3}$	↓$1.8x10^{-3}$	NS	NS

[1] Differences throughout the day were analysed with the Skillings-Mack test. [2] Arrows indicate direction of SCFA concentrations accordingly to chronological order of time points.[3] Differences between different time points for the same diets were determined by the Wilcoxon signed-rank test and Hommel post hoc correction. $P_{adj} < 0.05$ = significant. NS: Not significant.

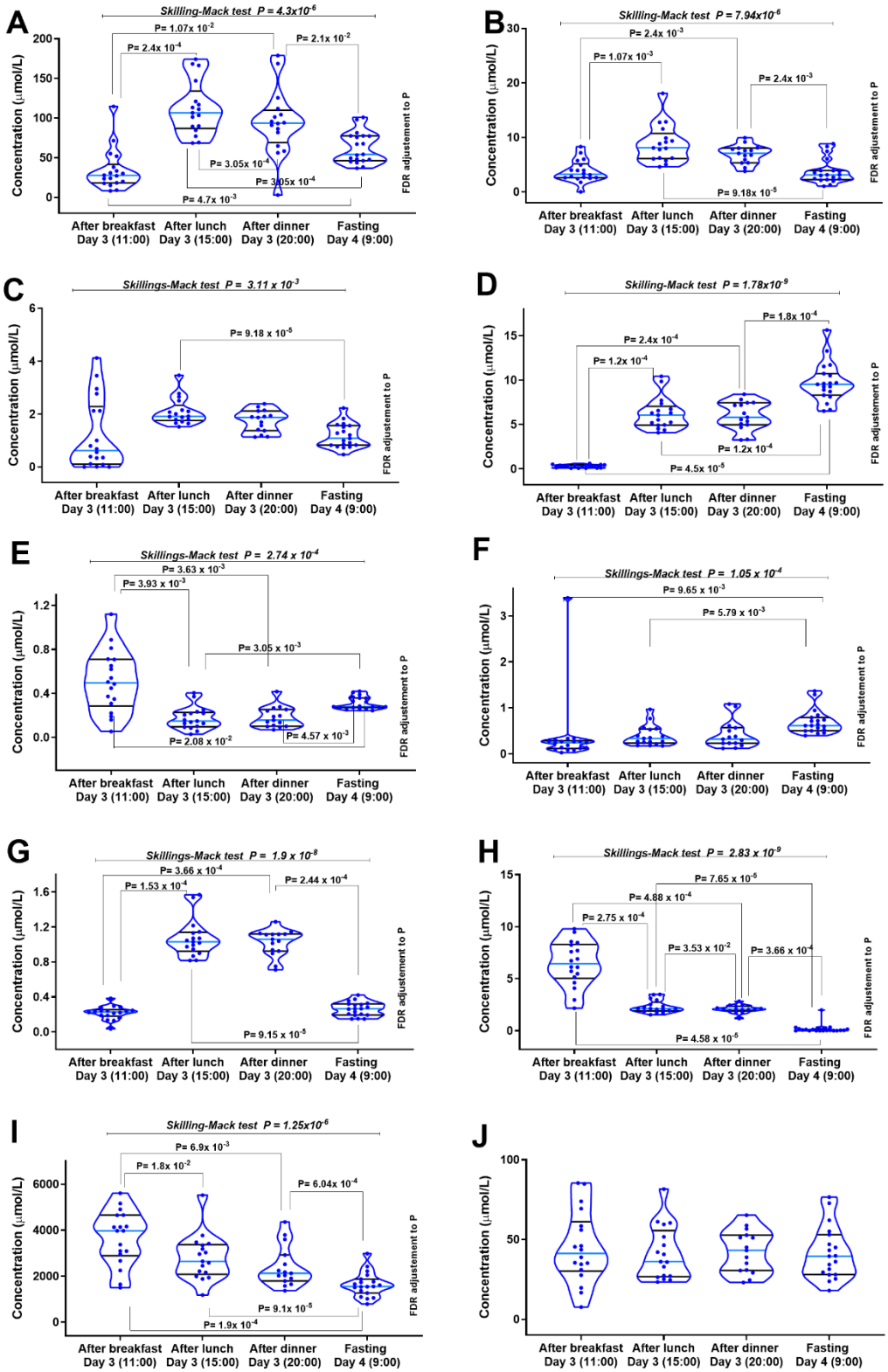

Figure 4. 3 Variations of SCFAs throughout the day within diet 1. Differences between the same time points for different diets were determined by the Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method. Significance was accepted at Padj <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) acetic acid, (B) propionic acid, (C) isobutyric acid, (D) butyric acid, (E) 2-methylbutyric acid, (F) isovaleric acid, (G) valeric acid, (H) caproic acid, (I) lactic acid and (J) 2-hydroxybutyric acid.

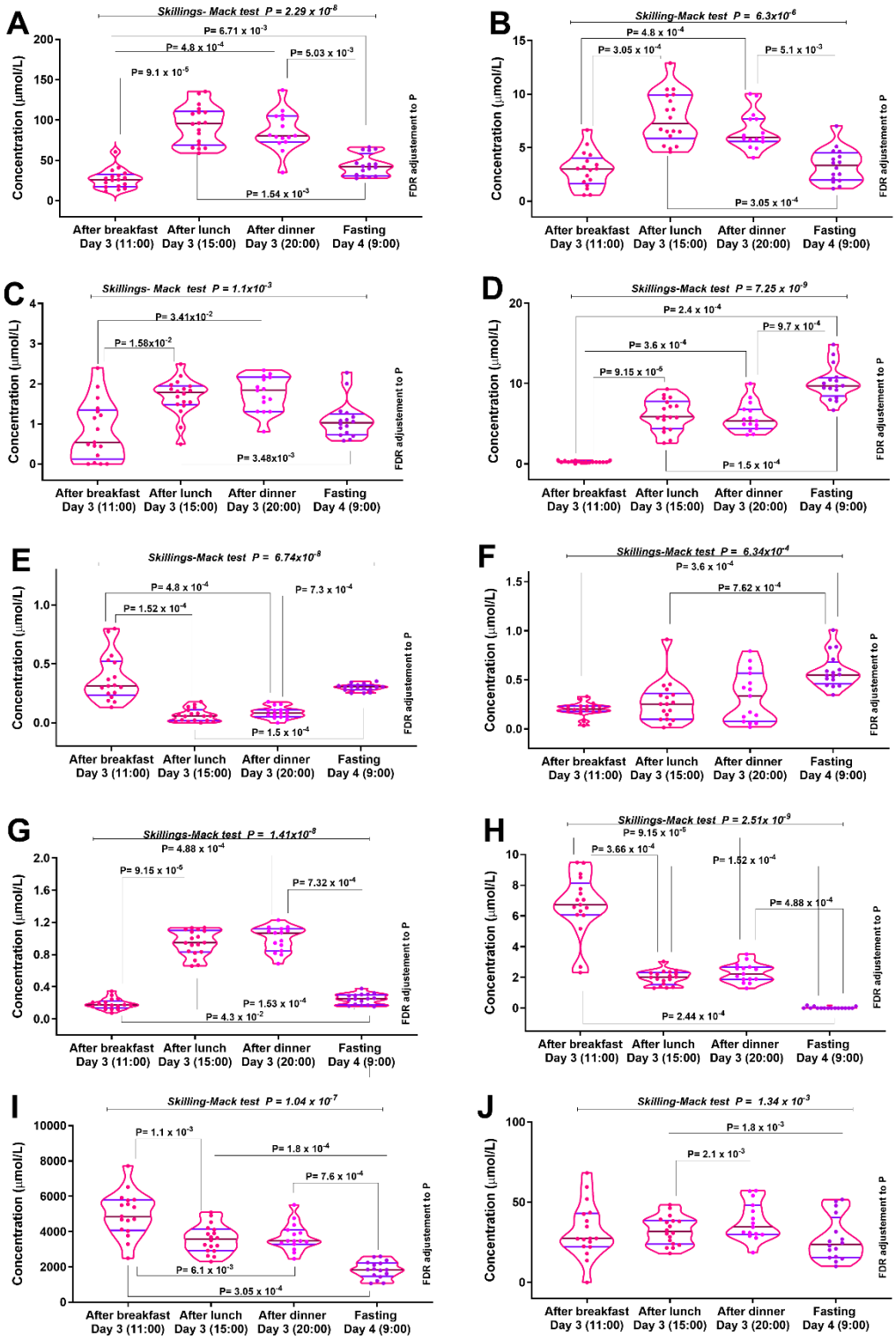

Figure 4. 4 Variations of SCFAs throughout the day within diet 4. Differences between the same time points for different diets were determined by the Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method. Significance was accepted at Padj <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) acetic acid, (B) propionic acid, (C) isobutyric acid, (D) butyric acid, (E) 2-methylbutyric acid, (F) isovaleric acid, (G) valeric acid, (H) caproic acid, (I) lactic acid and (J) 2-hydroxybutyric acid.

4.6.4 Inter-individual variability in serum SCFA profile of selected participants

SCFA serum concentrations showed different transient variations throughout the day for SCFA and time of the day (Figure 4.5). However, some participants showed a different pattern compared to the mean response. For example, acetic acid serum levels tended to be higher in diet 1 than in diet 4 for all time points, but some participants presented an opposite trend (Figure 4.5A, B, C and D). It was observed that the responses of the participant (ID 3) were different from those of the other participants (Figure 4.5E).

Acetic acid concentrations for participant ID 3 reached a maximum peak of 168.29 µmol/L AL, but dropped at the AD time point following the consumption of diet 1, decreasing to 86.48 µmol/L, below that of the other participants (Figure 4.5E). The other selected subjects showed an increase in acetic acid during the transition from the AL sample to the AD sample. At the same time, for participant ID3, the concentrations that were measured following the consumption of diet 1 were lower at the AD time-point. In addition, the same individual showed a different serum profile for propionic acid and 2-methylbutyric acid in response to dietary intake in relation to the selected responses of participants (Figure 4.5J and 4.5O), suggesting that this individual might have had a different response to diet and capacity for SCFA generation compared to the other participants. For example, the propionic acid concentrations for participant ID 3 during the transition from the AB to AD period mirrored the response pattern observed for acetic acid in diet 1 (Figure 4.5 E and J). Propionic acid in diet 1 was elevated in participant ID 3 compared to other participants (Figure 4.5F, 4.5G, 4.5H and 4.5I), peaking at 12.87 µmol/L AL and then decreasing to 9.94 µmol/L (Figure 4.5J) AD.

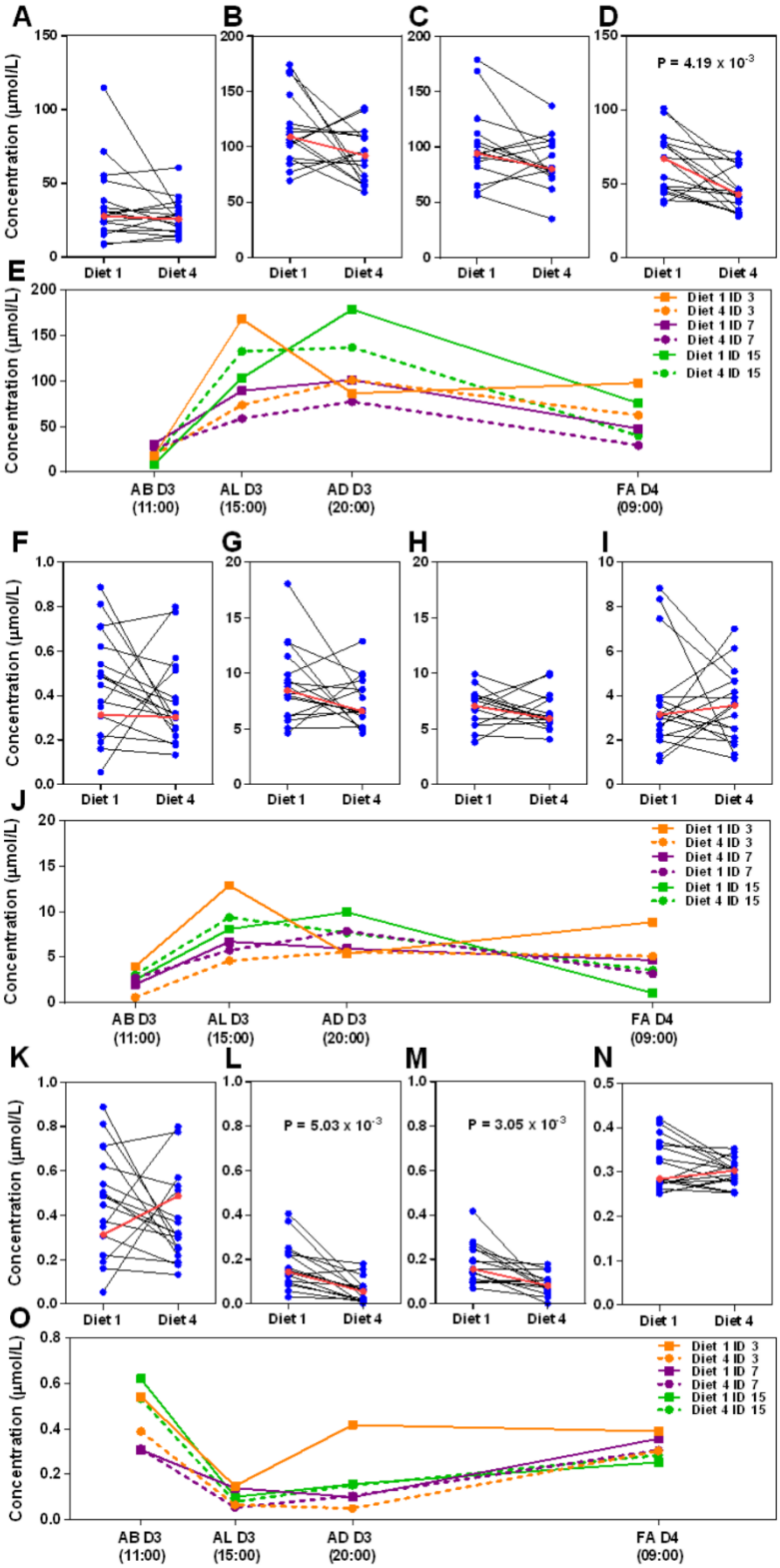

Figure 4.5 Serum variation response in selected SCFA between participants.
A) Acetic acid inter-individual variation after breakfast on day 3.
B) Acetic acid inter-individual variation after lunch on day 3.
C) Acetic acid inter-individual variation after dinner on day 3.
D) Acetic acid inter-individual variation fasting on day 4.
E) Acetic acid variation in selected participants.
F) Propionic acid inter-individual variation after breakfast on day 3.
G) Propionic acid inter-individual variation after lunch on day 3.
H) Propionic acid inter-individual variation after dinner on day 3.
I) Propionic acid inter-individual variation after dinner on day 3.
J) Propionic acid variation in selected participants.
K) 2–methylbutyric acid inter-individual variation after breakfast on day 3.
L) 2–methylbutyric acid inter-individual variation after lunch on day 3.
M) 2–methylbutyric acid inter-individual variation after lunch on day 3.
N) 2 –methylbutyric acid inter-individual variation after dinner on day 3.
O) 2-methylbutyric acid variation in selected participants.

After breakfast on day 3 (AF D3), after lunch on day 3 (AL D3) and after dinner on day 3(AD D3) and fasting on day 4 (FA D4). In inter-individual variation graphics, blue dots represent results of 19 participants and red dots and lines indicate median. Differences between the same time points for different diets were determined by Wilcoxon signed rank test and False Discovery Ratio (FDR) post-hoc correction. Significant differences are indicated by P adj <0.05

Selected propionic acid responses from individuals did not show a uniform pattern when diet 1 and diet 4 concentrations were compared (Figure 4.5J). Concentrations of 2-methylbutyric acid were significantly higher in AL (P_{adj}= 5.03 x 10^{-3}) and AD (P_{adj}= 3.05 x 10^{-3}) for diet 1 compared to diet 4 (Figure 4.5L, 4.5M). However, AB showed substantial variation between participants when the results were compared between diet 1 and diet 4. (Figure 4.5K). Once again, participant ID 3 showed a different time-related response pattern, particularly diet 1, in the levels of 2-methylbutyric acid compared to the other participants (as shown for participants ID 7 and ID 15; Figure 4.4O).

4.6.5 Intra-individual variability in serum SCFA profile of selected participants

SCFA concentrations were dynamic during the day, suggested that changes after food meal intake influenced the fluctuating changes throughout the day after hours of food intake. Acetic acid concentration steadily increased in samples taken at AL compared to AB for all participants. This suggested that sampling time affected acetic acid concentration after food intake. In case of AB, the effects of food intake were not fully reflected after two hours in serum, while the acetic acid levels after six hours of food intake were reflected in the blood independently of the diet consumed (Figure 4.5E). Then, in the interval between AL and AD, participants exhibithed a variety of responses without a clear pattern. For example, for ID 3 consuming diet 1, the concentration of acetic acid dropped from 168.3 µmol/L to 86.5 µmol/L, but for the diet in ID 7 and ID15, the concentrations of SCFA increased. In the case of diet 4, all the participants presented an increase from AL to AD. From AD to FA, ID 7 and ID 15 presented a decrease of acetic acid, while participant ID3 exhibited an opposite response, with levels rising from 86.5 µmol/L to 98.1 µmol/L.

Intra-individual variations in propionic acid between selected participants showed a similar fluctuation pattern to acetic acid, but approximately ten times lower in concentrations for each individual (Figure 4.5J). Concentrations of 2-methylbutyric followed a similar pattern in the interval of time from AB to AL for both diets. However, in the period between AL to AD, ID3, after the intake of diet 1, presented a sharp increase of concentration from 0.14 µmol/L to 0.42 µmol/L that almost reached concentrations observed at AB. The 2-methylbutyric concentrations after the intake of diet 1 in ID 7 and ID 15 were not similar during the period from AL to AD. ID 7 presented a slight reduction from 0.14 to 0.9, while ID 15 concentrations increased from 0.1 µmol/L to 0.16 µmol/L.

For the time interval from AD to AF for diet 1, ID3 presented a minimal decrease from 0.41 µmol/L to 0.39 µmol/L in 2-methylbutyric concentrations. Conversely, ID 7 and ID 15 showed an increase in their concentrations. Concentrations of 2-methylbutyric associated with diet 4, followed the same pattern of increase for all the selected subjects (Figure 4.5).

4.6.6 SCFA concentrations in urine

Results of SCFA concentrations in 24-hour urine were shown in Table 4.4. Urine SCFA concentrations were from 100 to 10000 times lower in urine than in serum, and there was an inverse relationship between serum and urine concentrations for most SCFAs. In most cases, except for lactic acid, which was directly associated with diet 4 in both urine and serum, the general trend showed that SCFA concentrations were higher in serum for diet 1, while in urine, higher concentrations of most SCFAs were linked to diet 4. These trends were statistically significant for propionic acid ($P_{adj} < 1.5x10^{-4}$), isobutyric acid ($P_{adj} < 0.044$), butyric acid ($P_{adj} < 2.2x10^{-3}$), isovaleric acid ($P_{adj} < 2x10^{-5}$), valeric acid ($P_{adj} < 2x10^{-5}$), caproic acid ($P_{adj} < 3.8x10^{-5}$), 2-hydroxybutyric acid ($P_{adj} < 2.7x10^{-5}$) (Figure 4.6B, 4.6C, 4.6D 4.6F, 4.6G, 4.6H, 4.6J). SCFA concentrations obtained from 24-hour urine samples were normalised to the total volume collected to account for differences in concentrations.

Table 4.4 Median and inter-quartile range values of each metabolite in serum and urine samples for each diet.

SCFA	Type of diet	After breakfast Day 3 (11:00)	After lunch Day 3 (15:00)	After dinner Day 3 (20:00)	Fasting Day 4 (9:00)	24 hour urine Day 3[1]
Acetic acid (µmol/L)	Diet 1	27.64(18.26-41.64)	106.7(86.99-134)	93.71(69.11-110)	54.28(46.25-77.56)	186.42(135.71-369.41)
	Diet 4	25.93(17.44-32.59)	95.7(68.57-110.6)	80.36(72.75-104.9)	42.8(31.19-62.96)	164.36(93.72-328.39)
Propionic acid (µmol/L)	Diet 1	3.25(2.6-5.16)	8.08(6.14-10.7)	7.08(5.34-8.04)	3.14(2.25-3.92)	10.92(6.81-14.75)
	Diet 4	3.03(1.63-4.03)	7.25(5.85-9.91)	5.93(5.56-7.67)	3.33(1.97-4.52)	6.57(3.80-18.74)
Isobutyric acid (µmol/L)	Diet 1	0.62(0.11-2.29)	1.92(1.75-2.33)	1.87(1.37-2.12)	1.08(0.83-1.57)	0.79(0.38-1.35)
	Diet 4	0.54(0.12-1.35)	1.78(1.48-1.95)	1.83(1.31-2.16)	1.03(0.74-1.25)	0.88(0.57-1.80)
Butyric acid (µmol/L)	Diet 1	0.33(0.12-0.44)	6.06(4.93-7.05)	5.81(4.98-7.44)	9.52(8.3-10.74)	0.75 (0.36-1.46)
	Diet 4	0.22(0.2-0.33)	5.86(4.38-7.79)	5.35(4.39-6.79)	9.7(8.46-10.72)	0.42 (0.15-1.03)
2- Methylbutyric acid (µmol/L)	Diet 1	0.5(0.29-2.29)	0.15(0.1-2.33)	0.16(0.1-2.12)	0.28(0.27-1.57)	0.10(0.03-0.34)
	Diet 4	0.31(0.23-0.52)	0.057(0.02-0.11)	0.08(0.05-0.11)	0.3(0.28-0.32)	0.24 (0.08-0.42)
Isovaleric acid (µmol/L)	Diet 1	0.24(0.12-0.28)	0.34(0.23-0.54)	0.32(0.23-0.57)	0.62(0.5-0.79)	0.28(0.20-0.46)
	Diet 4	0.2(0.18-0.23)	0.25(0.1-0.36)	0.33(0.08-0.57)	0.55(0.46-0.68)	0.33(0.21-0.46)
Valeric acid (µmol/L)	Diet 1	0.23(0.18-0.25)	1(0.92-1.1)	1.1(0.92-1.1)	0.27(0.19-0.32)	0.08(0.06-0.15)
	Diet 4	0.18(0.14-0.22)	0.95(0.83-1.1)	1.1(0.85-1.1)	0.25(0.17-0.3)	0.10 (0.05-0.19)
Caproic acid (µmol/L)	Diet 1	6.43(5.03-8.3)	2.13(1.91-2.74)	2.05(1.9-2.35)	0.05(0-0.15)	1.59 (1.20-2.17)
	Diet 4	6.72(6.06-8.14)	2.02(1.53-2.31)	2.23(1.87-2.67)	0(0-0)	1.03 (0.77-1.97)
Lactic acid (µmol/L)	Diet 1	3973(2888-4650)	2641(2077-3375)	2121(1789-2920)	1553(1263-1869)	35.06(11.46-50.69)
	Diet 4	4852(4075-5788)	3576(2916-4134)	3472(3282-4099)	1839(1465-2231)	73.86 (67.88-93.75)
2- Hydroxybutyric acid (µmol/L)	Diet 1	41.26(30.23-61.16)	36.1(26.72-55.62)	43.19(30.59-52.75)	39.52(28.05-53.1)	71.93(60.47-87.33)
	Diet 4	27.42(22.31-43.12)	31.7(23.89-38.64)	34.54(29.78-48.16)	23.72(15.54-40.56)	105.75(70.54-139.69)

[1] Urine values are expressed in µM/24 hours

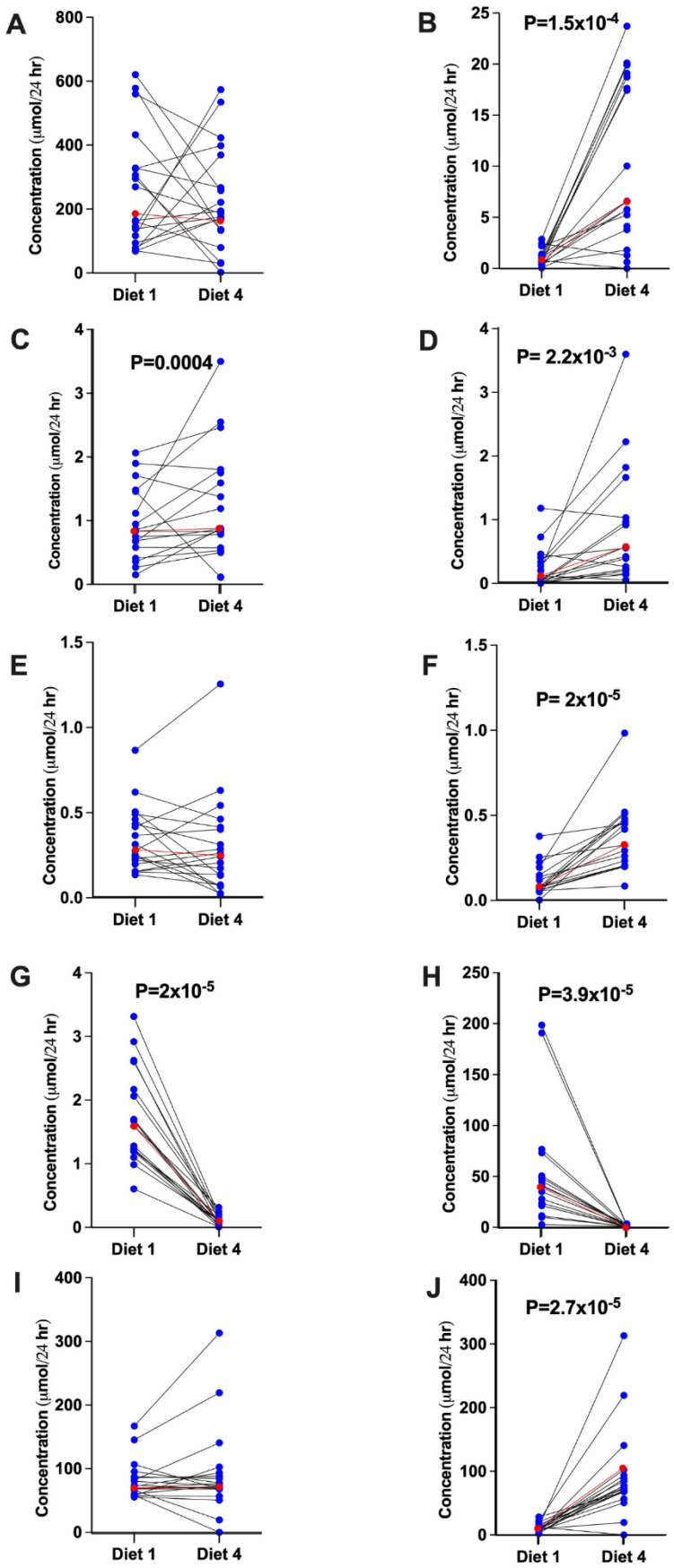

Figure 4.6 Urine samples from a 24 hour pooled collection. (A) acetic acid, (B) propionic acid , (C) isobutyric acid , (D) butyric acid ,
(E) 2- methylbutyric acid, (F) isovaleric acid , (G) valeric acid, (H) caproic acid, (I) lactic acid and (J) 2- hydroxybutyric acid.
Blue dots with lines represent results of 19 participants. Red dots and lines represent median.

Differences between 24-hour urine for different diets were determined by Wilcoxon signed rank test and False Discovery Ratio (FDR) post-hoc correction. Significance was accepted at Padj <0.05.

In addition, the urine volume significantly differed between the groups, being higher in subjects who consumed diet 1.

Table 4. 5 24-hour urine volume by diet on day 3.

	Diet 1	Diet 4	P value[2]
24 h urine volume (ml)	2640(1760-3310)	2610(1360-3100)	0.03

[1]Data is expressed in median (25th percentile, 75th percentile), according to their normality distribution. [2]P-values were calculated using Wilcoxon rank-sum test.

4.7 Discussion

This is the first study to explore SCFA production after the intake of two extreme dietary patterns under randomised, controlled, and crossover conditions. As presented in Table 4.1, fibre and protein content varied for diet and meals. At AL, 2-methylbutyric acid and 2-hydroxybutyric acid were higher in diet 1, 2- methyl butyric was higher in AD for diet 1, while lactic acid was higher in diet 4 at both AL and AD. Acetic acid and 2-hydroxybutyric acid were higher at FA when diet 1 was consumed. SCFAs were evaluated in 24- hour urine, where some metabolites were higher in diet 4; however, their metabolic relevance was unclear in this context.

However, serum SCFAs fluctuated during the day, and these changes seemed to be influenced by time rather than the specific diet intake. Faecal samples were not collected, and microbiome studies were not possible. Future studies are needed to understand the importance of sample collection for SCFA studies alongside the administration of nutritional interventions that help to modulate SCFAs and to understand how this manipulation may exert effects on personalised nutritional interventions and the management of chronic diseases.

4.7.1 Diet and time-related differences in serum SCFA concentrations

The results obtained for SCFA serum levels at different points within the day showed that their fluctuations strongly depended on the time of the day rather than the influence of meal intake during the day. Findings from in vivo models have suggested the importance of evaluating SCFAs at different times during the day at the lumen level. This approach has allowed to observe the effects on the mean crypt production rate in the distal colon of rats after the administration of different doses of SCFAs (240). Substantial differences in the circulating profiles of SCFAs were observed throughout the day, except for those samples obtained AB.

There is a gap in nutritional studies on SCFAs. Most of the time, these studies evaluate postprandial responses immediately after a nutritional intervention. This can be for a short period of time (hours) or after a long period of intervention that collects samples at specific time points (weeks) (64, 241). Studies

that evaluate postprandial responses should consider that acute periods of time may be insufficient to identify underlying changes in SCFA production, metabolism, or absorption according to the studied nutritional intervention (45, 136). On the other hand, several long-term nutritional interventions analysing SCFAs have tended to neglect detailed recording of dietary intake (241). Another challenge in SCFA studies is trying to evaluate the effect of one or a combination of dietary compounds on metabolism, which typically discards the fact that food in daily life is consumed in different food matrices. Unsurprisingly, systemic concentrations that derive from a "typical" diet appear to be different compared to SCFAs that were measured after the intake of a single dietary compound or SCFAs supplementation (64, 77, 218, 242).

In this study, differences in the serum profiles of multiple SCFAs were evident in response to two contrasting dietary patterns that varied in their nutritional composition, with differences in fibre and protein contents. Fibre contents in breakfast were notoriously different in diet 1 and diet 4, 14.9 g and 3.6 g, respectively. However, no significant differences were found between the healthy and westernised diets in SCFA from samples taken after breakfast, indicating the relevance of the time of the day for sample collection. Previous studies have shown the effects of different dietary supplements on SCFA levels in the blood of healthy and unhealthy individuals (242, 243). It has been demonstrated that fermentative processes in the gut are initiated in the colon 6 to 8 hours after food intake (244-246) and its colonic transit affects the production of several metabolites, including SCFAs (247, 248). Moreover, dietary fibres have different fermentation degrees in the human gut, for example, cellulose and lignins are less fermentable compared to pectin, oligosaccharides, resistant starch, inulin and β-glucans (48, 249). This is consistent with previous research that evaluated SCFA concentrations after dietary fibre supplementation or intake of SCFA as part of the first meal of the day (139, 218, 242). It is not surprising that changes were not observed in SCFA concentrations 2 hours after the first meal of the day, since serum concentrations are likely to have equilibrated after overnight fasting.

4.7.2 Concentrations of 2-methylbutyric acid after lunch and after dinner

Concentrations of 2- methylbutyric acid concentrations were significantly elevated in diet 1 compared to diet 4 in AL and AD. This metabolite belongs to the family of branched short-chain fatty acids (BSCFAs), with isovaleric and isobutyric acids. Their production indicates protein breakdown in the gut lumen by proteolytic fermentation by gut microbiota (162). BSCFAs are derived from branched-chain amino acids; their serum concentrations have been associated with metabolic syndrome (250-252) and leucine is a precursor of 2-methylbutyric acid (162). There is little knowledge about the metabolic role of BSCFAs in human health; however, the consumption of diets with high protein content along with a low-fibre diet has been associated with 2-methylbutyric acid production (253).

4.7.3 Lactic acid concentrations after lunch and after dinner

Lactic acid concentrations were high in postprandial samples taken from AL and AD, after the consumption of diet 4, which was characterised by a low content of fibre (13 g per day), high in sugar (25 g per day), and energetically dense (1,9 kcal/g). The study of lactic acid as a signalling molecule has become increasingly popular, specifically in its capacity to drive brown fat activity and adipose tissue development (254). These findings are similar to those described for diets with typical Westernised patterns that have been associated with increased levels of lactic acid in the blood (255, 256). Fasting serum lactic acid concentrations have been proposed as a predictive biomarker of type II diabetes (256, 257). Unhealthy diets, such as the Westernised diet that was consumed in the study, are characterised by a high content of sugar and subsequently increasing glycaemia levels (19). Glucose can be oxidised to carbon dioxide aerobically via the tricarboxylic cycle (TCA). Alternatively, glucose can be converted to lactic acid via glycolysis under anaerobic conditions (258).

4.7.4 Concentrations of 2- hydroxybutyric acid after lunch and fasting

2-hydroxybutyric acid concentrations were higher in the healthier diet (diet 1) in those samples taken AL and fasting, although these results have a high degree of inter-individual variation. However, the biological relevance of these findings remains unclear. 2- hydroxybutyric acid is produced in the liver by 2-ketobutyric acid, which is derived from threonine catabolism or methionine metabolism via cystathionine, and is metabolised to propionyl-CoA and carbon dioxide (220). A high concentration of this metabolite in children's urine is indicative of an inborn metabolic error or impaired intestinal absorption of methionine (259, 260). It seems unlikely that 2- hydroxybutyric acid or its precursors originate exclusively from dietary sources, as it is predominantly an endogenous metabolite (261, 262). Furthermore, this metabolite is associated with elevated oxidative levels, which has been shown to contribute to insulin resistance, lupus, and other comorbidities (262, 263). Previous research has linked fasting levels of 2-hydroxybutyric with the prediction of increased postprandial glycaemia levels and early β-cell pancreatic dysfunction in oral glucose tests in patients with an increased risk of diabetes (264).

4.7.5 Fasting and acetic acid concentrations

Fasting serum acetic acid levels were significantly higher in diet 1 (median of 54.3 µmol/L) compared to diet 4 (median of 42.8 µmol/L). In a fed state, acetic acid is likely to be primarily derived from the fermentation of colonic bacteria. Conversely, during periods of starvation, acetic acid production is regulated by the liver, together with ketone bodies, to ensure the delivery of suitable oxidisable substrates to tissues such as the myocardium and brain (217). The beneficial or detrimental role of acetic acid in health remains a topic of controversy, necessitating a review of discrepant findings in studies involving humans (265). Previous studies have reported that the consumption of fibre such as

pectin in a portion of 20 g/day resulted in an increased concentration of fasting acetic acid in serum (64), whereas the consumption of barley kernels with a high amylose content or high β-glucans resulted in an increase in butyric acid in fasting serum the following morning. This evidence, together with other research studies, suggests that the type of fibre plays a role in the type of SCFA production and metabolism and influences the production of specific or total SCFA produced by the gut microbiota (93, 139). In one study, non-obese diabetic mice were fed a special diet based on high-amylose maize-resistant starch modified with acetate and butyrate to release high levels of acetic acid, which improved gut integrity and decreased the levels of diabetogenic cytokines (266). Afterwards, these findings were explored in subjects with type 1 diabetes in a feasibility study that administered the same supplement. Subjects presented changes in gut microbiome composition and an increase of faecal and serum SCFAs, suggesting that dietary SCFAs may be a mechanism to improve immune profiles and tolerance alongside glycaemic control (267).

Similarly, reduced levels of acetic acid have been associated with preeclampsia, and maternal feeding of germ-free mice with acetic acid have been shown to rescue compromised foetal thymic $CD4^+$ T cells and regulatory T cell development, highlighting its potential role in obesity (268). The fermentation of non-digestible carbohydrates (NDCs) in the colon takes several hours and is dependent on the physicochemical properties of the NDCs and the host microbiota (48, 269). The impact of diet on SCFA production can be prolonged, and regular consumption of a high-fibre diet provides continuous stimulation of the colonic microbiota to produce and absorb SCFAs (65). For example, diets high in fibre have been shown to result in high levels of acetic acid compared to a diet low in fibre (64, 269).

4.7.6 SCFA concentrations patterns after diets intake

In this study, the dietary patterns consumed were aligned in different proportions with the WHO guidelines for healthy eating (141), where a diversity of NDCs such as pectins, cellulose, resistant starch, and others were consumed in diet 1 (48), while diet 4 represented a typical Westernised diet high in fat and sugar and low in fibre, fruits, and vegetables. SCFAs were monitored, and each SCFA pattern change in serum was observed to understand their variation after the intake of different meals throughout the day. Four contrasting patterns were found where the peak of specific SCFA occurred: i) after overnight fasting (butyric acid and isovaleric acid); ii) after breakfast (lactic acid, caproic acid, and 2-methylbutyric acid); iii) after lunch (valeric acid, acetic acid, propionic acid and isobutyric acid); iv) no significant differences in levels over 24-hour period (2-hydroxybutyric acid).

None of the SCFAs measured peaked their concentrations at the AB point, which supports the fact that meal intake takes approximately 6 hours or more to have a systemic effect. These responses were similar to those reported in previous studies where SCFA concentrations were quantified after food intake (64,

133). Most of the SCFA concentrations remained stable in the period of AL versus AD, suggesting that postprandial concentrations of SCFAs derived from those meals would not yet be evident in venous blood after 2 hours due to food transit time from the mouth until the colon could take between 6 to 8 hours or more (64, 270). The difference in the systemic profiles of butyric acid, which manifested peak concentrations in the overnight fasting sample, compared to the other main SCFAs, acetic acid and propionic acid, which peaked in the AL sample, was striking. This difference in the patterns of the three main SCFAs is expected, as they behave somewhat independently, as reflected by the differences in the metabolic origin of acetic acid, propionic acid, and butyric acid, and in line with the fact that these SCFAs have different metabolic functions. The anti-inflammatory effects of SCFAs are mediated by the free fatty acid receptors (FFAR), FFAR2 and FFAR3. FFAR2 (also referred to as GPR43) present a higher affinity for acetic acid and propionic acid than butyric acid, whereas FFAR3 has a higher affinity for butyric acid and propionic acid (271, 272). Both FFARs are expressed in the intestinal endocrine L cells and stimulate the release of GLP-1 and PYY. FFAR2 is also expressed in adipose tissue, and its activation by SCFAs has been shown to promote GLP-1 secretion in the gut, resulting in increased insulin sensitivity via suppression of fat accumulation in white adipose tissue (273). In addition to its expression in intestinal endocrine cells, FFAR3 (or GPR41) is also expressed in the sympathetic ganglia, and its activation leads to the release of noradrenaline (273).

One important point to highlight is that the two contrasting diets resulted in differences in the serum concentrations of some SCFAs, but these diets did not influence the overall diurnal profile of the SCFAs. For example, the highest level of SCFA was observed in the AB sample for diet 1, and peak concentrations were also observed in the AB sample for diet 4.

4.7.7 Inter-individual and intra- individual variability in serum SCFA profiles

Inter-individual differences in the temporal variation of SCFA concentrations in serum were evident, which has been previously described in other studies (77, 139, 218). This observation suggests that food mastication, digestion processes, and colonic transit times may account for some of these differences. (244, 248, 274-276). Additionally, the gut microbiome seems to be one of the most important contributors, influencing the digestion absorption processes of dietary compounds and regulating diverse physiological responses at the systemic level (277-279). The human gut microbiome is highly individualised, and this inter-individual variability has been shown to contribute to differences in glycaemic control (278). Although clear diet-dependent differences in SCFA levels in serum and urine were observed, the primary source of variation in the serum SCFAs profile was inter-individual differences in SCFAs production despite the rigorous environment and diet control.

Additionally, variations in serum SCFA concentrations throughout the day suggest that some SCFA could be influenced by the type of dietary substrate and, subsequently, by endogenous production. Under normal conditions, the major source of acetic acid in mammals is intestinal fermentation of unabsorbed carbohydrates (217, 280). Propionic acid has been proposed to be produced endogenously as a result of amino acid catabolism of threonine and methionine (281). Although propionic acid production derives from colonic fermentation, the liver extracts most of the propionic acid from portal blood (282). These metabolic processes have been confirmed by rectal infusion of acetic acid and propionic acid in equimolar amounts (283). This suggests that there is a higher production of acetic acid than propionic acid at the colonic level after the intake of both diets, which may explain the individual responses of the selected participants.

These intra-individual variations can be potentially intrinsically associated with physiological responses that can be synergistically enhanced and shaped by the intake of meals as a substrate (133). Thus, it can be speculated that variations in circulating SCFAs can be mainly attributed to the unique microbiome composition of the participants, but also to fluctuations that may be related because SCFAs are derived from colonic bacteria, and differences in microbial environments can directly influence fermentation rates of dietary substrates for SCFAs and their metabolic fate.

Some limitations of this study are related to these diets, which were designed considering the content of fibre and protein as a whole. The specific types of NDCs or amino acids contained in each meal, as well as some SCFA responses, may be attributed solely to the consumption of a high-fibre diet along with the presence of protein, rather than to specific dietary compound. Therefore, it is unknown how proteolytic and saccharolytic fermentation could influence each other processes after the simultaneous intake of specific food or dietary compounds. Nonetheless, a recent study reported that proteolytic and saccharolytic fermentation may influence non-response to lifestyle interventions, such as using exercise for diabetes prevention (284).

Moreover, faecal samples were not collected in this study, thus preventing direct associations from being made between SCFA concentrations and microbial taxonomy or metagenomic data.

4.7.8 SCFA in urine and potential diet impact

The 24-hour urine excretion of SCFAs showed a high degree of inter-individual variability even within the same diet, even though all the volunteers followed the same diet under highly controlled conditions. Despite the inter-individual differences, diets influenced SCFA excretion. Interestingly, 24- hour urine volumes from diet 1 compared to diet 4 were significantly higher, but water intake was not controlled

in this study. However, this result aligns with a previously published study in a group of individuals with a similar nutrition intervention high in fibre (64).

Higher concentrations of propionic acid, butyric acid, isobutyric acid, isovaleric acid, valeric acid, caproic acid, and 2-hydroxybutyric acid were found in 24- hour of urine from participants who consumed diet 4, which corresponds to a typical Western dietary pattern. The presence of 2-hydroxybutyric acid and lactic acid in the urine have been associated with lactic acidosis (261); however, in this study, other biomarkers were not measured to confirm this possible clinical condition associated with the consumption of diet 4. For all SCFAs, the variability in response to diet 4 was greater than that observed for diet 1.

A previous study that analysed the urinary excretion of these metabolites over a 24-hour period in healthy individuals using labelled SCFAs reported that less than 0.05 % of them are excreted in urine (139). Since the urinary SCFAs were calculated for the 24-hour sample, unlike the full serum profiles, a direct comparison between serum and urinary SCFA concentrations was difficult. Moreover, the results of SCFAs obtained from 24-hour urine should be interpreted carefully, considering that multiple urine spots were pooled together to create a 24-hour sample. Although SCFA results in urine represent the consumption of an entire diet over a day, these measurements do not provide enough information to elucidate specific food responses and modulation of SCFAs as targets for nutritional interventions.

4.8 Conclusions

This study is the first to compare the effects of two different dietary patterns under randomised, controlled, and crossover conditions that reflect SCFA changes in serum and urine. Moreover, this study used a novel GC-MS method that quantified metabolites related to SCFAs, including acetic acid, propionic acid, butyric acid, methyl-branched SCFAs, and hydroxylated carboxylic acids, providing a broader coverage of metabolites than previous nutritional studies that have evaluated and quantified SCFAs. Temporal variation in serum SCFA profiles was stronger than the dietary influence over the controlled three-day diet; nevertheless, a healthier diet was generally associated with higher levels of SCFAs and lower levels of circulating lactic acid. Despite the rigorously controlled diet and environment, inter-individual variations in both the absolute levels and serum profiles of SCFAs were detected, indicating differences in the presence or functionality of the gut microbiota. In order to fully understand the relationship between the microbiome and SCFA profiles, characterisation of the faecal microbiome and the functional capacity of the microbial community would be required.

The significant temporal differences in SCFA profiles emphasise the need for standardisation of study protocols regarding the timing of sample collection and highlight that SCFAs may exhibit their own

patterns of circulation that are chronologically regulated. Further studies are necessary to explore how the consumption of diverse dietary patterns over an extended period can modulate SCFAs, providing a better understanding of how diets impact SCFA profiles and how their manipulation may be utilised to deliver personalised nutrition to aid in the prevention and management of chronic diseases.

5. Bile acid concentrations in free-living vegans and omnivores.

Note: For this chapter, bile acid abbreviations have been used, so please find them in the Abbreviation Section (Page 17).

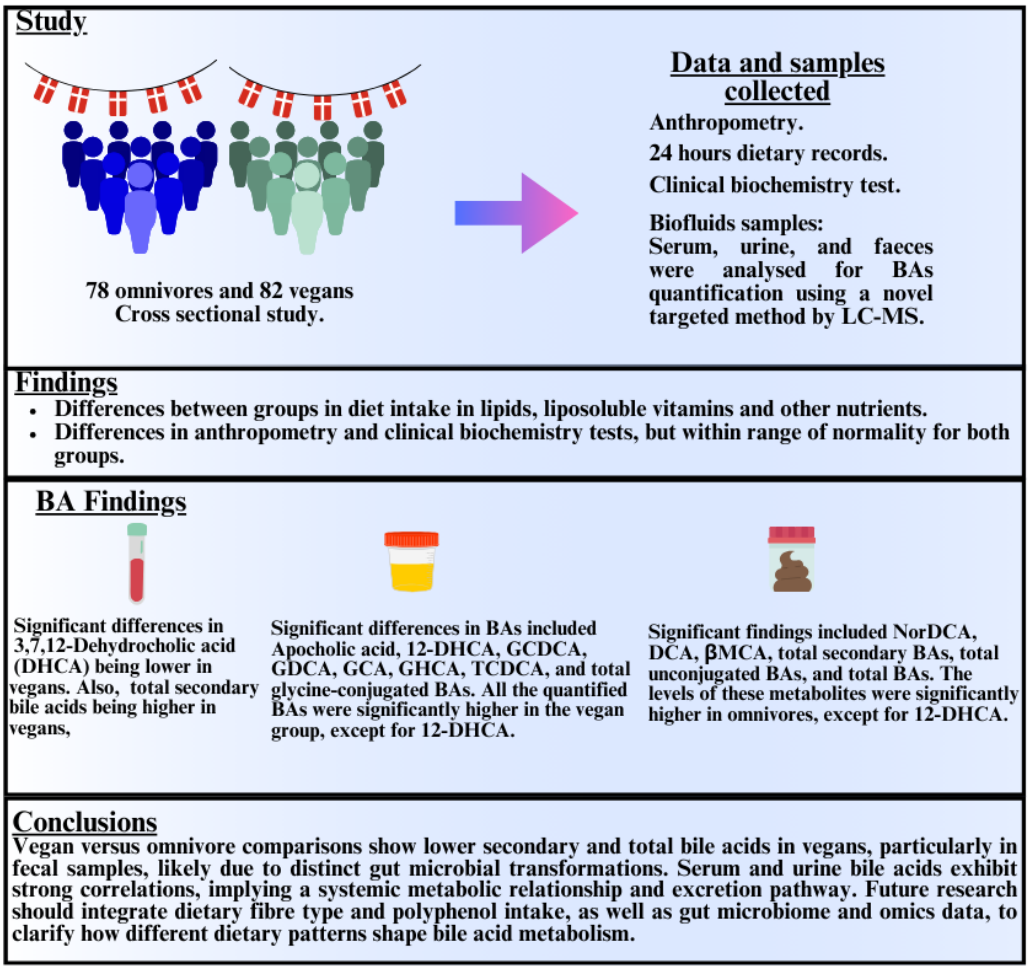

Figure 5. 1 Schematic Summary of Chapter 5.

5.1 Introduction

Veganism, as a dietary pattern, has been extensively studied for its potential health benefits, including disease prevention and improved health outcomes (13, 25, 27, 143, 154-158). Over the past decade, this dietary pattern has gained global popularity not only for its health benefits but also for broader considerations, including sustainability, environmental impact, animal welfare, and the pursuit of a healthier lifestyle (159, 160).

As previously described in Chapter 3, a well-planned vegan diet can meet all nutritional needs of healthy individuals, provided that it is properly planned (24). Diverse nutrition bodies have established their positions regarding the consumption of vegan dietary patterns (20-23). The vegan dietary pattern is well characterised by its differences from the omnivorous dietary pattern (25). Key characteristics of a vegan dietary pattern include a high intake of fruits and vegetables, low sodium intake, and low saturated fat intake, alongside the exclusion of animal products and their derivatives (25).

BAs are metabolites derived from cholesterol, which is synthesised by hepatocytes. They facilitate the formation of micelles, thereby aiding in the intestinal absorption, emulsification, and transport of nutrients, lipids, and lipophilic vitamins (96, 101). Some evidence suggests that dietary patterns can influence BA production (18, 285). Diets high in saturated fats and low in fibre, similar to Western-style diets, have been shown to trigger an increase in both primary and secondary bile acids at the colonic level (18, 285).

Increased serum BAs have been correlated with elevated glucose levels in subjects following a 75 g glucose tolerance test, demonstrating a proportionally greater increase in BAs than in glucose levels (286). An increase in the total circulating BAs has also been observed in patients with insulin resistance and diabetes (287).

It has been described that liver glucose homeostasis is affected by the activation of the farnesoid X receptor (FXR), which plays a role in glucose metabolism, energy homeostasis, postprandial glucose level modulation, and regulation of genes involved in glycogen synthesis, glycogenolysis, and gluconeogenesis (287, 288).

A study evaluated the responses to an Oral Lipid Tolerance Test (OLTT), which consisted of 150 ml of a water/fat solution (75 g vegetable fat, 69 g water, 675 kcal), 5 ml sunflower oil (4.6 g, 41.5 kcal), and 5 ml olive oil (4.6 g, 41.6 kcal). After 2 hours, total bile acids, as well as taurine- and glycine-conjugated bile acids, exhibited an early rise at 2 hours post-OLTT and remained elevated for up to 6 hours. Simultaneously, the primary bile acid CDCA significantly decreased at 4 hours after the intake of the test drink, while CA remained unchanged during the OLTT (61).

Likewise, mixed meals containing both fats and carbohydrates have been reported to induce a higher secretion of glycine-conjugated BAs and total BAs than foods composed exclusively of fats (140). Most existing studies exploring the effects of vegan dietary patterns on BAs have typically limited to analysing one or two types of biofluids (289, 290). Moreover, the ways in which a vegan dietary pattern may influence circulating BAs across different biofluids compared to an omnivorous dietary pattern, and subsequently, its impact on overall BA metabolism, is poorly understood. To investigate BAs metabolism, we studied three types of biofluid samples from a cohort of free-living vegans and omnivores (see Chapter 2, Section 2.1 for details of this research).

A novel liquid chromatography-mass spectrometry (LC-MS)-targeted technique developed by Sarafian et al. (98) was used to quantify BAs and related metabolites in serum and urine. For faecal samples, this method was adapted by Dr. Adesola Bello as part of her PhD thesis using the analytical method published by Sarafian et al. as a reference. (1, 98). This targeted method detected and quantified 145 bile acids using LC-MS. The targeted analysis included primary, secondary, and tertiary bile acids, providing a comprehensive coverage of the bile acid pool in human biofluid samples, such as serum and urine. However, 57 BAs were used for this thesis since the subjects were healthy and some BAs were species-specific (see Chapter 2, Section 2.3.3 and Section 2.3.4).

5.2 Hypothesis

The regular consumption of a vegan dietary pattern, compared to an omnivorous dietary pattern, will result in differences in the concentrations of BAs in serum, urine, and faeces.

5.3 Aims

The main aim of this study was to compare the concentrations of BAs of vegans versus omnivores, considering the nutritional composition of each dietary pattern.

5.4 Objectives

The specific objectives of this study were as follows:

1. To provide baseline sociodemographic and clinical characteristics of participants relevant adhering to both dietary patterns that are relevant in the context of BAs.
2. To conduct multivariate analyses of BAs from different biofluids in relation to dietary patterns.
3. To compare the concentrations of BAs between vegan and omnivorous participants in each biofluid.

4. To assess the dietary intake of participants and investigate its relationship with BAs concentrations in different biofluids.
5. To correlate relevant dietary information with BA production and concentration in each biofluid.

5.5 Material and Methods.

Chapter 2 describes the study design, participant recruitment, sample collection, LC-MS sample preparation, and LC-MS data analysis.

5.6 Results

Earlier, in Chapter 3, the population and dietary characteristics of vegans and omnivores were explored due to their potential impact on the metabolism of SCFAs in three different biofluids. This chapter focused primarily on BA metabolism and its quantification, considering the same dietary patterns previously described in Chapter 3. However, while some findings from Chapter 3 (Chapter 3, Table 3.1) were be referenced here, they were considered in the context of their influence on BA metabolism (62).

5.6.1 Population and diet characteristics

The anthropometric and biochemical characteristics of both omnivores and vegans, as detailed in Table 3.1 and Table 3.2 from Chapter 3, were referenced in this chapter. However, it had been described that some specific nutrients or dietary patterns can influence the stimulation of BA metabolism and,consequently, alter the BA profile in biofluids (62, 165, 289). Therefore, nutrients that may potentially have affected BA production and metabolism were considered in the dietary analysis conducted in this chapter.

As described in Chapter 3, Table 3.1 and Table 3.2 and Section "3.6.1 Population and Diet Characteristics," some differences were found in anthropometric measurements and clinical biomarkers between omnivores and vegans. However, these values were within the normal clinical ranges. Some of the biomarkers discussed previously, such as ALT, glucose, cholesterol, LDL, and vitamin D, had been linked to the production and metabolism of BAs (291-293).

Table 5.1 and Table 5.2 compared the consumption of nutrients from the diets of the two groups. These tables included nutrients that were not considered in Table 3.3 of Chapter 3. A detailed profile of fatty acids and liposoluble vitamins wereincluded in Table 5.2 for this comparison due to of their potential influence on BAs metabolism (18, 61, 62). All the nutrients considered in Table 5.1 and Table 5.2

showed significant differences ($P_{adj} < 0.05$) between groups, with the exception of total energy consumption, vaccenic acid (C18:1, n-7), α-linolenic acid (C18:3, n-3), behenic acid (C22:0), lignoceric acid (C24:0), and vitamin D (considering the reported supplementation contribution).

Table 5. 1 Omnivores and vegans diets characteristics with a potential role in BAs metabolism.

	Omnivores (n=78)	Vegans (n= 82)	P value[2]	P_{adj-} value
Energy (kcal)	2335.08(1915.86-2769.11)	2391.19(1926.7-2960.5)	0.523	0.546772727
Carbohydrate proportion	49.98(6.11)	59.2(7.56)	$4x10^{-13}$	$1.2x10^{-12}$
Carbohydrate energy (kcal)	1097.86(924.70-1330.72)	1392.96(1072.73-1742.46)	$3.24x10^{-5}$	$4.8x10^{-05}$
Carbohydrates (g)	274.46(142.88-332.68)	348.24(268.18-435.61)	$3.24x10^{-5}$	$4.84x10^{-5}$
Protein proportion	15.71(14.97-17.72)	12.07(10.45-13.09)	$7.72x10^{19}$	$3.2x10^{-18}$
Proteins energy (kcal)	358.78(315.3-478.08)	267.27(212.77-348.49)	$1.92x10^{-9}$	$3.7x10^{-09}$
Proteins (g)	86.01(75.81-113.27)	65.38(52.7-85.87)	$7.67x10^{-9}$	$1.4x10^{-08}$
Proportion of fat	32.31(5.56)	27.03(7.74)	$4.02x10^{-6}$	$6.8x10^{-6}$
Energy from fat (kcal)	756.87(596.36-903.83)	585.54(452.83-791.4)	$1.77x10^{-3}$	0.00239
Fats (g)	85.36(67.64-103.59)	67.76(51.98-94.61)	$2.11x10^{-3}$	0.0028
Saturated fatty acids (g)	29.54(22.17-36.34)	13.31(9.95-19.91)	$1.3x10^{-15}$	$4.8x10^{-15}$
Monounsaturated fatty acids (g)	27.77(23.09-36.22)	23.75(17.11-32.18)	0.00679	$8.2x10^{-3}$
Polyunsaturated fatty acids (g)	12.43(9.66-15.31)	21.53(15.91-29.19)	$4.8x10^{-11}$	$1.09x10^{-10}$
Total omega- 3 fatty acids (g)	2.16(1.64-3.13)	1.72(1-2.74)	0.02272	0.0268
Total omega - 6 fatty acids (g)	9.54(7.4-12.06)	12.98(10.45-20.29)	$6.9x10^{-6}$	$1.1x10^{-5}$
Total Trans fatty acids (g)	1.2(0.79-1.7)	0(0-0)	$1.6x10^{-25}$	$2.2x10^{-24}$
Selected dietary fatty acids (g)				
Capric acid (C10:0)	0.61(0.44-0.92)	0(0-0.08)	$9.2x10^{-20}$	$4.7x10^{-19}$
Lauric acid (C12:0)	0.91(0.64-1.26)	0.19(0.02-0.69)	$3.6x10^{-8}$	$6.4x10^{-8}$
Myristic acid (C14:0)	2.57(1.93-3.64)	0.18(0.09-0.52)	$1.6x10^{-21}$	$1.1x10^{-20}$
Myristoleic acid (C14:1,n-5)	0.32(0.23-0.43)	0(0-0)	$6.9x10^{-29}$	$3.07x10^{-27}$
Palmitic acid (C16:0)	15.61(11.94-18.61)	6.61(4.43-9.02)	$5.6x10^{-19}$	$2.6x10^{-18}$
Palmitoleic acid (C16:1,n-7,c)	1.23(0.9-1.7)	0.29(0.16-0.43)	$2.6x10^{-23}$	$2.5x10^{-22}$

[1]Data is expressed in mean ± SD or median (25th percentile, 75th percentile), according to their normality distribution. [2]P-values were calculated using the Wilcoxon rank-sum test.

Table 5. 2 Omnivores and vegans diets characteristics with a potential role in BAs metabolism (Continuation).

	Omnivores (n=75)	Vegans (n= 75)	P value[2]	P_{adj-} value
Stearic acid (C18:0)	6.29(4.83-7.95)	2.69(1.61-3.55)	$1.2x10^{-18}$	$4.7x10^{-18}$
Vaccenic acid (C18:1,n-7)	0.06(0.02-0.14)	0.06(0.02-0.09)	0.12640	0.14
Oleic acid (C18:1,n-9)	25.69(20.35-33.15)	20.87(15-28.41)	$3.5x10^{-3}$	$4.5x10^{-3}$
Linoleic acid (C18:2,n-6)	9.46(7.32-12)	12.98(10.45-20.29)	$4.6x10^{-6}$	$7.5x\ 10^{-6}$
α-Linolenic (C18:3,n-3)	1.6(1.23-2.21)	1.62(0.92-2.72)	0.86881	0.86
Arachidic acid (C20:0)	0.14(0.09-0.24)	0.11(0.04-0.21)	0.03244	0.03
Gadoleic acid (C20:1,n-11)	0.6(0.41-0.79)	0.14(0.07-0.21)	$1.9x10^{-20}$	$1.8x10^{-19}$
Arachidonic acid (C20:4,n-6)	0.04(0.02-0.08)	0(0-0)	$3.5x10^{-20}$	$1.9x10^{-19}$
Eicosapentaenoic acid (C20:5,n-3)	0.09(0-0.21)	0(0-0)	$1.5x10^{-15}$	$4.8x10^{-15}$
Behenic acid (C22:0)	0.07(0.04-0.12)	0.05(0-0.22)	0.26672	0.29
Cetoleic acid (C22:1,n-1)	0(0-0.12)	0(0-0)	$1.6x10^{-9}$	$3.2x10^{-09}$
Erucic acid (C22:1,n-9)	0.02(0-0.09)	0(0-0)	$4.8x10^{-10}$	$9.9x10^{-10}$
Docosapentanoic acid (C22:5,n-3)	0.02(0-0.06)	0(0-0)	$3.2x10^{-12}$	$8.9x10^{-12}$
Docosahexanoic acid (C22:6,n-3)	0.23(0.05-0.55)	0(0-0)	$2.3x10^{-23}$	$2.4x10^{-22}$
Lignoceric acid (C24:0)	0.04(0-0.08)	0.03(0-0.09)	0.58621	0.59
Nervonic acid (C24:1,n-9)	0.02(0-0.06)	0(0-0)	$1.9x10^{-12}$	$5.6x10^{-12}$
Liposoluble vitamins				
Vitamin A (µg)	802.86(569.5-2564.5)	534.71(333.34- 876.68)	$2.2x10^{-4}$	$3.1x10^{-4}$
Alpha-tocopherol (mg)	8.03(5.93-10.38)	13.6(10.2-18.1)	$3.2x10^{-12}$	$8.3x10^{-12}$
D3 (µg)	0.47(0.28-1.5)	0(0-0)	$3.4x10^{-28}$	$7.9x10^{-27}$
Vitamin D supplemented (ug)	4.93(2.29-10.22)	5.36(0-20.83)	0.31002	0.33
Vitamin K (mcg)	71.36(29.5-137.73)	216.83(117.39-390.18)	$5.8x10^{-11}$	$1.3x10^{-10}$

[1]Data is expressed in mean ± SD or median (25th percentile, 75th percentile), according to their normality distribution. [2]P-values were calculated using the Wilcoxon rank-sum test.

5.6.2 Multivariate analysis and BAs

5.6.2.1 PCA models comparing BAs concentrations of vegans and omnivores

Multivariate analysis was performed on BAs datasets from omnivores and vegans. PCA showed that the quality control (QC) samples were tightly clustered (Figure 5.2), confirming the reproducibility of the BA assay and the stability of the LC-MS method for BA analysis in serum, urine, and faeces. No separation was observed between the groups. A few strong outliers from both the vegans and omnivores were distributed in the first and fourth Cartesian quadrants. Low values of R^2X and Q^2X in the BAs datasets suggested a high degree of metabolite variance between participant biofluid samples that were not fully captured in the first three components.

PCA of serum BAs (Figure 5.2A) confirmed the stability of the instrument, as indicated by the close clustering of the QC samples. This model explained 23.5% of the variance, exhibited some overlap between omnivores and vegans. Additionally, the predictive ability of the model was low, with a Q2X value of 14.5%. The plot did not show a clear separation between the two dietary groups.

PCA results for urine BAs (Figure 5.2B) showed that the R^2X value explained 37% of the variance, with some overlap between omnivores and vegans. The prediction capacity of the model ($Q^2X = 15.6\%$) was low, indicating limited predictive power. Although the model identified a few strong outliers, it did not reveal a clear separation between the two dietary patterns. The QCs were clustered, indicating the stability of the instrument throughout the analysis.

PCA of faecal bile acids (Figure 5.2C) explained 35.7% of the variance ($R^2X = 0.357$), with limited predictive ability ($Q^2X = 0.204$). The loading plot showed a significant overlap between vegans and omnivores, indicating that no clear differences in bile acid profiles were detected between the groups.

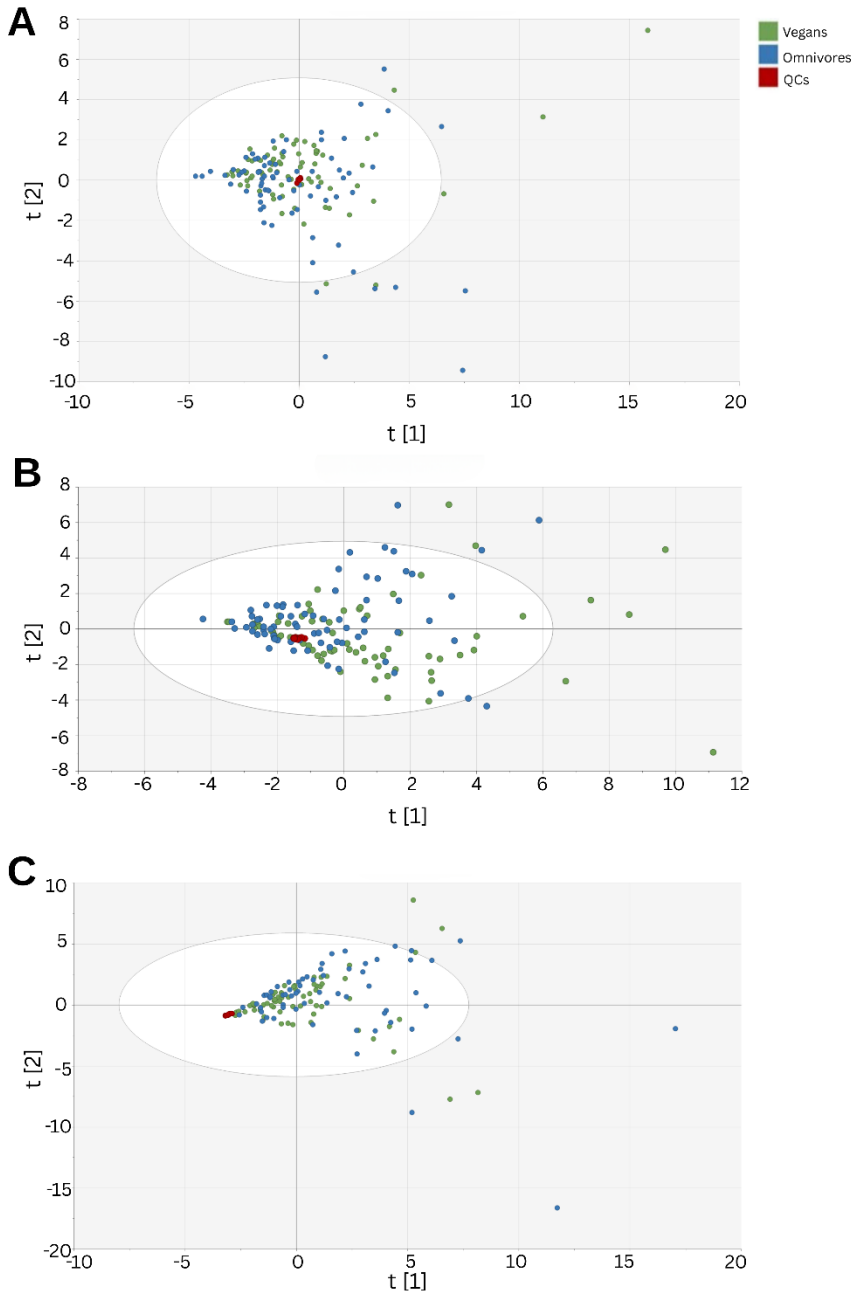

Figure 5. 2. PCA score plots of serum, urine, and faecal BAs profiles for vegans and omnivores. BAs of vegans (green), omnivores (blue), and quality controls (red) in biofluids. Models are comprised of three principal components. Ellipse; Hotellin's T2(95%). (A) Serum BAs, $R^2X = 0.235$ and $Q^2X = 0.145$. (B) BAs in urine, $R^2X = 0.379$ and $Q^2X = 0.156$. (C) BAs faeces $R^2X = 0.357$ and Q2X = 0.204.

5.6.2.2 OPLS-DA models comparing BAs concentrations of vegans and omnivores

Similarly, no further differentiation was found in the cross-validated OPLS-DA models between the vegans (green) and omnivores (blue). Metabolites from both groups were distributed in different quadrants, illustrating the diversity of results within the groups. Outliers were identified in vegans and omnivores.

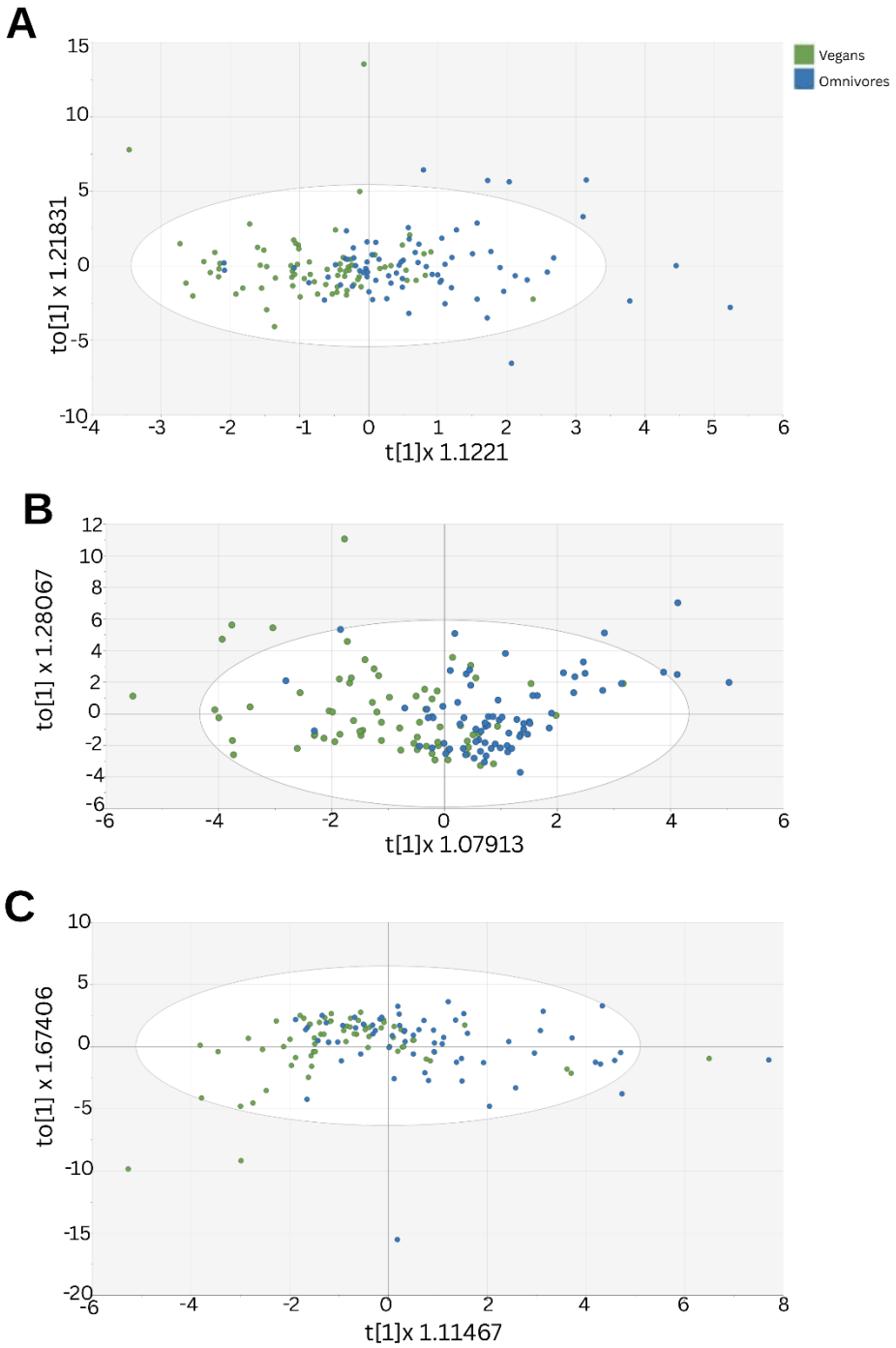

Figure 5. 3 OPLS-DA score plot of serum, urine, and faecal BAs profiles for vegans and omnivores. Cross-validated OPLS-DA score plots of BAs concentrations comparing vegans (green) and omnivores (blue). Models are comprised of 1 predictive (t[1]) and 1 orthogonal (to[1]) principal component. Ellipse; Hotellin's T2(95%).(A) BAs serum concentrations $R^2X = 0.232$, $R^2Y = 0.308$, $Q^2 = 0.193$..(B) Urine BAs concentrations: $R^2X = 0.324$, $R^2Y = 0.327$, $Q^2 = 0.207$. (C) BAs faecal concentrations $R^2X = 0.377$, $R^2Y = 0.198$, $Q^2 = -0.071$.

The OPLS-DA model for serum BA concentrations (Figure 5.3A) explained only 23.2% of the variance in the bile acid data ($R^2X = 0.232$), indicating a poor fit to the dataset. Its ability to discriminate between dietary groups was also limited, as it explained just 30.8% of the variance in group classification ($R^2Y = 0.308$). Moreover, the model showed no reliable predictive power, with a Q^2 value of 0.193, which was well below the generally accepted threshold for a robust model. Consistent with these metrics, the

score plot revealed marked overlap between omnivores and vegans, with no significant separation, suggesting that serum BA profiles were not strongly influenced by dietary pattern and may be driven by other physiological or environmental factors.

For urine BA concentrations (Figure 5.3B), the model explained 32.4% of the variance in the dataset ($R^2X = 0.324$) and 32.7% of the variance in group classification ($R^2Y = 0.327$), both of which were low and indicated a weak association between urinary BA profiles and dietary groups. The predictive ability of the model was modest ($Q^2 = 0.207$), but still below a robust threshold. The score plot again showed substantial overlap between omnivores and vegans, with no clear separation, reinforcing the limited discriminatory capacity of the model.

In faecal BA concentrations (Figure 5.3C), the model explained 37.7% of the variance in the dataset ($R^2X = 0.377$) and just 19.8% of the variance in group classification ($R^2Y = 0.198$). Importantly, the predictive ability was absent, as indicated by a negative Q^2 value ($Q^2 = –0.07$). The score plot confirmed this poor performance, showing extensive overlap between dietary groups. Together, these findings suggested that faecal BA profiles, did not clearly distinguish between vegans and omnivores, which showed considerable overlap between the groups, considering the different biofluids.

5.6.3 Comparison of BAs from omnivores and vegans in different biofluids

5.6.3.1 BAs in serum

Serum BA concentrations were compared between vegans and omnivores (Table 5.3). Two BAs were significant in the serum. 3,7,12-Dehydrocholic acid (DHCA), showed a significant difference between the two groups ($P_{adj} = 0.01$). The levels of this metabolite were significantly lower in vegans. In addition, in vegans, total secondary bile acids were significantly higher ($P_{adj} = 0.01$) than in omnivores.

5.6.3.2 BAs in urine

Urine samples were analysed and their BAs were compared between the groups (Table 5.4). The significant BAs were 8(14),(5β)-Cholenic acid-3α, 12α-diol (Apocholic acid, $P_{adj}= 0.01$), 12 Dehydrocholic acid (12- DHCA, $P_{adj} = 0.04$), Glycochenodeoxycholic acid (GCDCA, $P_{adj} = 5.98 \times 10^{-7}$), Glycodeoxycholic acid (GDCA, $P_{adj}= 0.0006$), Glycocholic acid (GCA, $P_{adj}=1.02 \times 10^{-5}$), Glycohyocholic acid (GHCA, $P_{adj} = 9.35 \times 10^{-5}$), Taurochenodeoxycholic acid (TCDCA, $P_{adj} = 0.03$) and total glycine conjugated BAs ($P_{adj} =5.71 \times 10^{-6}$). All the quantified BAs were significantly higher in the vegan group, with the exception of 12- DHCA.

5.6.3.3 BAs in faeces

The concentrations of BAs were quantified in the faecal samples. Significant findings included 3α,12α, 23-Nordeoxycholic acid (NorDCA, $P_{adj} =0.01$), Deoxycholic acid (DCA, $P_{adj} = 0.009$), β Muricholic acid (βMCA $P_{adj} = 0.01$), total secondary BAs($P_{adj} = 0.02$), total unconjugated BAs ($P_{adj} = 0.0091$), and total BAs ($P_{adj} = 0.009$). The levels of these metabolites were significantly higher in omnivores, with the exception of 12- DHCA (Table 5.5).

Table 5. 3 Comparison of serum BAs between omnivores and vegans [1].

Bile Acids ID	Serum Bile Acids	Omnivores (n=75)	Vegans (n=75)	P-value[1]	P-adjusted
BA1	Ursocholanic Acid (nM)	8.66(3.18-19.57)	9.4(4.78-16.44)	0.51	0.74
BA3	5-Cholenic acid-3β-ol (nM)	12.16(8.93-15.49)	12.6(8.8-16.64)	0.54	0.74
BA5	Lithocholic acid (nM)	19.72(15.22-28.13)	22.92(14.92-31.84)	0.19	0.49
BA6	Isolithocholic acid (nM)	9.22(4.71-18.23)	9.64(4.7-23.18)	0.29	0.57
BA7	Allolithocholic acid (nM)	2.48(1.32-5.24)	2.48(1.09-4.91)	0.81	0.84
BA18	3α-Hydroxy-7 Ketolithocholic acid (nM)	8.42(5.52-16.4)	6.32(5.24-11.2)	0.10	0.33
BA19	3α-Hydroxy-12 Ketolithocholic acid (nM)	8.82(4.87-16.07)	7.72(4.68-15.45)	0.52	0.74
BA20	Murocholic acid (nM)	63.18(28.42-139.78)	51.72(28.55-101.94)	0.44	0.74
BA22	5β-Cholanic acid-3β, 12α-diol (nM)	25.8(6.88-43.28)	23.56(9.38-39.73)	0.72	0.81
BA23	Deoxycholic acid (nM)	253.18(74.1-520.65)	177.6(62.23-446.82)	0.27	0.57
BA24	Chenodeoxycholic acid	75.02(24.78-198.7)	54.28(21.88-215.16)	0.54	0.74
BA26	3,7,12 Dehydrocholic acid (nM)	45.2(5.72-93.78)	8.36(1.8-53.7)	0.0004	0.01
BA32	Ursodeoxycholic acid (nM)	61.96(29.42-107.84)	42.52(21.72-83.68)	0.04	0.27
BA35	Cholic acid (nM)	53.68(27.52-257.52)	46.16(23.4-123.32)	0.207	0.49
BA36	Hyocholic acid	12.6(5.49-25.65)	6.72(3.95-18.52)	0.06	0.31
BA39	Glycolithocholic acid (nM)	6.48(3.83-9.28)	7.88(4.2-12.4)	0.16	0.46
BA41	Glycochenodeoxycholic acid (nM)	252.04(132.28-532.48)	441.64(174.4-984.76)	0.01	0.16
BA42	Glycodeoxycholic acid (nM)	303.28(138.49-772.03)	442.42(198.51-1401.8)	0.03	0.27
BA43	Glycoursodeoxycholic acid	32.8(15.19-68.13)	27.84(12.4-76.4)	0.68	0.79
BA46	Glycocholic acid (nM)	7904.34(1912.6-27046.64)	6621.52(1612.1-28129.34)	0.57	0.74
BA48	Taurolithocholic acid (nM)	1.28(0.93-1.8)	1.4(0.92-1.8)	0.60	0.75
BA49	Glycohyocholic acid (nM)	2561.66(858.95-6072.99)	4223.8(1482.64-11288.72)	0.04	0.27
BA50	Tauroursodeoxycholic acid (nM)	1.4(0.52-2.42)	0.92(0.34-1.69)	0.08	0.33
BA52	Taurochenodeoxycholic acid (nM)	2051.02(589.96-4821.25)	1928.68(564.88-4831.84)	0.82	0.84
BA53	Taurodeoxycholic acid (nM)	9.68(4.42-28.56)	15.36(5.28-35.76)	0.27	0.57
BA55	Taurohyocholic acid (nM)	64.1(34.55-182.75)	65.92(34.68-163.28)	0.85	0.84
BA58	Tauro-ω-Muricholic acid (nM)	3.42(1.2-9.65)	4(1.6-9.44)	0.83	0.84
BA59	Taurocholic acid (nM)	5.36(2.32-14.8)	6.92(2.36-17.04)	0.37	0.71
Total Primary BAs		20188.66(9323.37-43009.14)	28404.64(13433.58-51993.21)	0.11	0.33
Total Secondary BAs		4532.64(2671.76-9209.23)	8525.68(3961.63-14475.74)	0.0008	0.01
Total Unconjugated		754.46(342.15-1554.98)	716.36(334.89-1294.99)	0.67	0.79
Total Glycine conjugated BAs (nM)		25436(12226-49145)	33449(17533-54640)	0.08	0.33
Total Taurine conjugated BAs (nM)		2708(1516-5391)	3388(1556-6443)	0.57	0.74
Total BAs (nM)		16189.22(3509.12-39252.64)	19212(3979.16-47711.36)	0.49	0.74

[1] Values expressed in median and interquartile range. Comparisons of groups were analysed by Mann-Whitney test and False Discovery Rate (FDR). Significance was accepted at (Padj <0.05).

Table 5. 4 Comparison of urine BAs between vegans and omnivores[1].

Bile acids ID	Urine Bile Acids	Omnivores (n=75)	Vegans (n=75)	P-value [1]	P-adjusted
BA 03	5-Cholenic acid-3β-ol (nM)	0.89(0.55-1.25)	1.06(0.69-1.71)	0.14	0.19
BA 05	Lithocholic acid (nM)	1.41(0.88-2.24)	1.72(1.16-3.07)	0.02	0.06
BA 09	3α,12α, 23-Nordeoxycholic acid (nM)	3(2.84-3.55)	3.13(2.79-3.6)	0.87	0.78
BA 15	8(14),(5β)-Cholenic acid-3α, 12α-diol (nM)	1.96(1.91-2.12)	2.09(1.97-2.44)	0.004	0.01
BA 18	3α-Hydroxy-7 Ketolithocholic acid (nM)	2.85(1.7-5.62)	3.11(2.05-8.47)	0.51	0.48
BA 19	3α-Hydroxy-12 Ketolithocholic acid (nM)	2.98(2.1-6.36)	4.32(2.41-7.69)	0.14	0.19
BA 20	Murocholic acid (nM)	3.09(2.01-4.28)	2.81(2.06-4.38)	0.89	0.78
BA 22	5β-Cholanic acid-3β, 12α-diol (nM)	6.91(2.94-14.49)	5.43(2.99-15.74)	0.97	0.802
BA 23	Deoxycholic acid (nM)	18.61(8.02-37.03)	23.37(11.26-51.6)	0.07	0.12
BA 24	Chenodeoxycholic acid (nM)	2.75(1.64-4.88)	2.63(1.62-7.41)	0.34	0.35
BA 30	3 Dehydrocholic acid (nM)	4.62(2.09-11.41)	4.4(2.03-10.85)	0.94	0.802
BA 31	12 Dehydrocholic acid (nM)	1160(184.4-4241)	395.5(92.53-1869)	0.01	0.04
BA 32	Ursodeoxycholic acid (nM)	2.35(1.06-3.6)	1.32(0.55-2.58)	0.04	0.08
BA 35	Cholic acid (nM)	118.3(27.35-234.3)	52.58(20.54-211.6)	0.23	0.26
BA 36	Hyocholic acid (nM)	1.75(1-2.94)	1.9(1.01-3.67)	0.45	0.44
BA 39	Glycolithocholic acid (nM)	0.06(0.04-0.12)	0.08(0.04-0.21)	0.23	0.26
BA 41	Glycochenodeoxycholic acid (nM)	8.44(5.81-15.52)	21.15(12.43-40.97)	$2.28x10^{-8}$	$5.98x10^{-7}$
BA 42	Glycodeoxycholic acid (nM)	7.58(3.47-14.78)	14.89(6.42-32.18)	0.0001	0.0006
BA 43	Glycoursodeoxycholic acid (nM)	22.6(11.11-32.51)	27.22(12.9-48.23)	0.1005	0.14
BA 46	Glycocholic acid (nM)	59.81(36.63-103.6)	137.7(74.13-207.7)	$1.56x10^{-6}$	$1.02x10^{-5}$
BA 48	Taurolithocholic acid (nM)	0.13(0.11-0.15)	0.13(0.13-0.15)	0.02	0.06
BA 49	Glycohyocholic acid (nM)	11.28(7.42-19.89)	26.73(14.51-57.52)	0.000001	$9.35x10^{-6}$
BA 52	Taurochenodeoxycholic acid (nM)	92.82(58.42-168.1)	152.4(76.04-310.6)	0.008	0.03
BA 53	Taurodeoxycholic acid (nM)	2.28(1.94-2.99)	2.76(2.03-3.54)	0.08	0.12
BA 55	Taurohyocholic acid (nM)	62.71(37.26-151.6)	109.9(48.71-210.3)	0.04	0.08
BA 59	Taurocholic acid (nM)	3.02(1.76-5.99)	3.63(1.58-7.85)	0.32	0.34
Total Primary BAs (nM)		290.36(154.7-659.05)	426.38(216.32-822.14)	0.02	0.06
Total Secondary BAs (nM)		313.89(190.97-665.47)	474.37(191.37-1782.02)	0.04	0.32
Total Unconjugated (nM)		800.67(160.97-4052.96)	333.21(111.91-1756.45)	0.08	0.12
Total Glycine conjugated BAs (nM)		110(80.9-212)	246(142-366)	4.35x10-7	$5.71x10^{-6}$
Total Taurine conjugated BAs (nM)		170(116-391)	307(129-593)	0.01	0.056
Total Bile acids (nM)		1885(676.4-5411)	1529(700.5-2816)	0.204	0.31

[1] Values expressed in median and interquartile range. Comparisons of groups were analysed by Mann-Whitney test and False Discovery Rate (FDR). Statistical significance was accepted at (Padj <0.05).

Table 5. 5 Comparison of faecal BAs between vegans and omnivores[1].

Bile Acids ID	Faecal Bile Acids	Omnivores (n=63)	Vegans (n=68)	P-value	P-adjusted
BA04	3-Ketocholanic acid (nM/mg)	817.7(374.5-1461)	613.3(295-920.7)	0.1002	0.18
BA 05	Lithocholic acid (nM/mg)	12721(7476-17988)	8148(4992-15048)	0.03	0.07
BA 06	Isolithocholic acid (nM/mg)	1855(1260-3040)	1553(886-2985)	0.12	0.22
BA 07	Allolithocholic acid (nM/mg)	167.6(78.04-340.8)	130.4(41.35-224.8)	0.06	0.13
BA 09	3α,12α, 23-Nordeoxycholic acid (nM/mg)	3.57(2.16-7.47)	2.36(1.79-3.38)	0.003	0.01
BA 10	9(11), (5β)-Cholenic acid-3α-ol-12-one (nM/mg)	3.64(1.78-5.92)	2.88(2.22-5.05)	0.48	0.51
BA 13	3,6-Diketocholanic acid (nM/mg)	92.28(23.93-184.1)	49.65(19.57-95.72)	0.02	0.07
BA 17	5α-Cholanic acid-3α-ol-6-one (nM/mg)	4.12(1.69-7.4)	2.37(1.67-4.17)	0.07	0.14
BA 18	3α-Hydroxy-7 Ketolithocholic acid (nM/mg)	9.9(4.28-68.57)	15.43(6.53-40.51)	0.45	0.51
BA 19	3α-Hydroxy-12 Ketolithocholic acid (nM/mg)	1634(610.4-3767)	842.4(365.6-1755)	0.02	0.07
BA 20	Murocholic acid (nM/mg)	6.93(2.84-20.2)	3.56(2.17-13.29)	0.15	0.23
BA 22	5β-Cholanic acid-3β, 12α-diol (nM/mg)	722.1(184.7-15x`5)	454.4(176-817.9)	0.04	0.09
BA 23	Deoxycholic acid (nM/mg)	17143(9993-34005)	10226(5398-17922)	0.0007	0.009
BA 24	Chenodeoxycholic acid (nM/mg)	249.5(84.46-1243)	271.5(93.51-755.3)	0.84	0.72
BA 25	Hyodeoxycholic acid (nM/mg)	13.39(6.27-36.29)	12.91(7.17-20.95)	0.46	0.51
BA 29	5β-Cholanic acid-3α, 6α-diol-7-one (nM/mg)	9.83(8.02-13.48)	7.9(6.03-12.05)	0.01	0.051
BA 32	Ursodeoxycholic acid (nM/mg)	240.5(111.9-657.3	185.4(73.76-274.7)	0.01	0.051
BA 34	β Muricholic acid (nM/mg)	3.66(2.03-5.75)	2.25(1.65-3.39)	0.001	0.01
BA 35	Cholic acid (nM/mg)	98.74(31.34-493.2)	77.19(34.23-343.3)	0.65	0.59
BA 41	Glycochenodeoxycholic acid (nM/mg)	57.47(23.63-123.7)	61.6(27.7-193.9)	0.59	0.57
BA 42	Glycodeoxycholic acid (nM/mg)	28.66(10.93-103)	38.59(20.93-77.16)	0.25	0.32
BA 43	Glycoursodeoxycholic acid (nM/mg)	3.86(1.83-9.8)	3.39(1.78-9.96)	0.67	0.59
BA 46	Glycocholic acid (nM/mg)	50.98(23.13-124.5)	66.45(31.1-190.4)	0.19	0.26
BA 52	Taurochenodeoxycholic acid (nM/mg)	814(416-2301)	737(290.8-1753)	0.14	0.22
BA 53	Taurodeoxycholic acid (nM/mg)	9.04(2.38-37.63)	7.37(2.93-17.16)	0.65	0.59
BA 59	Taurocholic acid (nM/mg)	7.54(4.37-20.59)	8.42(3.45-17.6)	0.58	0.57
Total Primary BAs (nM/mg)		1323.62(635.25-6248.51)	1240.95(589.67-3850.60)	0.41	0.5
Total Secondary BAs (nM/mg)		70112.69(37324.5-134917.83)	47328.03(25000.91-81191.58)	0.005	0.02
Total Unconjugated (nM/mg)		30087.55(17529.90-66258.81)	19370.05(7254.32-38197.51)	0.0004	0.009
Total Glycine conjugated BAs (nM/mg)		145(70.5-384)	180(83.2-459)	0.42	0.5
Total Taurine conjugated BAs (nM/mg)		835(424-2258)	758(304-1904)	0.17	0.24
Total BAs (nM/mg)		46634(24248-78354)	29894(15233-50461)	0.001	0.009

[1] Values expressed in median and interquartile range. Comparisons of groups were analysed by Mann- Whitney test and False Discovery Rate (FDR). Statistical significance was accepted at (Padj <0.05).

5.6.4 Correlations between BAs concentrations in biofluids and nutrients

Dietary information and BAs concentrations from both dietary groups, vegans, and omnivores were independently correlated with the BAs concentrations in each biofluid. Nutrients such as carbohydrates, proteins, fatty acids, various food groups, and liposoluble vitamins were considered in these correlations due to their potential influence on the production of BAs in different biofluids (62, 294).

5.6.4.1 Correlations between serum BAs concentrations and dietary nutrients

Correlations between nutrients and BAs in the serum were examined. Despite analysing the data separately for each group and collectively, no significant correlations were identified for any of the variables under this study (Appendix 11, Figure 11.1).

5.6.4.2 Correlations between urine BAs concentrations and dietary nutrients

Correlations between dietary nutrients and BAs in urine were explored by analysing the data collectively for both groups, as well as separately. Upon collectively reviewing the data for both groups, some significant, yet weak correlations were detected between certain urine BAs and dietary nutrients.

Some correlations were significant when the data were explored by considering vegans and omnivores together (Figure 5.4A). Some weak correlations were found between glycolithocholic acid (GLCA) and alpha tocopherol (R= 0.35, Padj = 0.01). In addition, weak associations were found between Glycohyocholic acid (GCYCA) and capric acid (R= -0.31, P_{adj} = 0.03),GCYCA and palmitoleic acid (R= -0.35, P_{adj} = 0.01), GCYCA acid and cholesterol (R= -0.34, P_{adj} = 0.02), and GCYCA acid vitamin D3 (R= -0.39, P_{adj} = 0.003). Taurochenodeoxycholic acid (TCDCA) and palmitoleic acid (R= -0.31, P_{adj} = 0.04), total glycine conjugated BAs and retinol (R= -0.29, P_{adj} = 0.03) presented negative weak correlations. However, when correlations were examined separately for each group (vegans and omnivores), no significant associations were found (Appendix 11, 11.2).

5.6.4.3 Correlations between faecal BAs concentrations and dietary nutrients

We examined the associations between dietary information and BAs in the faecal samples. However, no significant correlations were identified in the analysed datasets (Appendix 11, Figure 11.3).

5.6.5 Correlations between significant BAs for each biofluid and dietary nutrients

We investigated potential associations between dietary nutrients and the BAs identified as significant in the comparison between groups for each biofluid (Section 5.6.3).

5.6.5.1 Correlations between significant BAs in serum and nutrients intake

After comparing the BA results from vegans and omnivores in Section 5.6.3.1, the two BAs were significant (P_{adj}< 0.05). Subsequently, these metabolites were significantly correlated with the dietary nutrients (Figure 5.4B). Weak but significant associations were found between 3,7,12 Dehydrocholic acid (DHCA) and mean intake of fruits (R= -0.33, P_{adj} = 0.005); DHCA acid and lauric acid (R= 0.25, P_{adj} = 0.03); DHCA and myristic acid (R= 0.24, P_{adj} = 0.004); DHCA and palmitoleic acid (R= 0.25, P_{adj} = 0.04); DHCA and stearic acid (R= 0.28, P_{adj} = 0.04); DHCA and trans fatty acids (R= 0.28, P_{adj} = 0.01); DHCA and cholesterol (R= 0.31, P_{adj} = 0.01); DHCA and retinol (R= 0.24, P_{adj} = 0.04); and DHCA and vitamin D3 acid (R= 0.26, P_{adj} = 0.03).

However, no significant correlations were found between these variables when the data were analysed per group, such as vegans and omnivores (Appendix 11, Figure 11.4).

5.6.5.2 Correlations between significant BAs in urine and nutrients intake

The results of the six significant BAs (P_{adj}< 0.05) from the comparison between vegans and omnivores (Section 5.6.3.2) were correlated with dietary nutrients. Weak correlations were found between the variables when the data from both groups were analysed together (Figure 5.4C).

Significant negative weak correlations were found between docosahexaenoic acid and glycine conjugated BAs (R = -0.31, P_{adj} = 0.01), arachidonic acid and glycine conjugated BAs (R = -0.31, P_{adj} = 0.009), arachidonic acid and GCDCA (R = -0.32, P_{adj} = 0.009), retinol and glycine conjugated BAs (R = -0.32, P_{adj} = 0.009), capric acid and GHCA (R = -0.32, P_{adj} = 0.009), palmitoleic acid and TCDCA (R = -0.32, P_{adj} = 0.009), cholesterol and GHCA (R = -0.33, P_{adj} = 0.009), erucic acid and GHCA (R = -0.33, P_{adj} = 0.009), palmitoleic acid and GHCA (R = -0.35, P_{adj} = 0.006), and vitamin D3 and GHCA (R = -0.39, P_{adj} = 0.001).

Significant positive correlations were weak, with the two highest correlations observed between polyunsaturated fatty acids and GHCA (R = 0.29, P_{adj} = 0.016) and polyunsaturated fatty acids and GHCA (R = 0.26, P_{adj} = 0.02).

When data from each group (vegans and omnivores) were analysed individually, no significant correlations were found (Appendix 11, Figure 11.5).

5.6.5.3 Correlations between significant BAs in faeces and nutrients intake

In the comparison of faecal metabolites between vegans and omnivores, only three metabolites and the sum of total BAs were found to be significant ($P_{adj} < 0.05$) (Section 5.6.3.3). Significant correlations were not found in these data sets (Appendix 11, Figure 11.6).

In Section 5.6.4.3, where correlations between all faecal BAs and dietary nutrients were explored, no significant associations were reported.

5.6.6 Correlations within BAs concentrations in serum, urine and faeces

Exploratory analyses were conducted to study the correlations among BAs results from different biofluids. These analyses were performed using data from the vegans and omnivores combined, as well as separately for each group.

5.6.6.1 Correlations between serum BAs and urine BAs

Strong and significant correlations were observed when data from vegans and omnivores were analysed together and independently for each group (Figure 5.5). Similar patterns of correlations were found across the data, when they were analysed for both groups (Figure 5.5A) and individually as vegans (Figure 5.5B) and omnivores (Figure 5.5C).

Strong positive and significant correlations were found between BAs from urine and serum, particularly for hyocholic acid from serum and 12 DHCA (R value= 0.63 , P_{adj}=9.15 x 10^{-17}), CA (R value= 0.65, P_{adj}= 1.14 x 10^{-17}),and 3α-Hydroxy-7 Ketolithocholic acid (R value= 0.63 , P_{adj}: 9.15 x 10^{-17}) from urine. Additionally, CDCA from serum was associated with 3α-Hydroxy-7 Ketolithocholic acid from urine (R value= 0.62 P_{adj}=3.72x10^{-16}), 3α-Hydroxy-7 Ketolithocholic acid from both biofluids correlated (R value= 0.61 , P_{adj}=2.96 x 10^{-15}).While CA from serum and CA from urine (R value= 0.65, P_{adj}=8.5 x 10^{-18}), CDCA from biofluids was positively correlated (R value= 0.61, P_{adj} = 9.5 × 10^{16}). Similar correlation patterns were found in omnivores and vegans when they were analysed independently, presented strong and moderate correlations for the same metabolites.

5.6.6.2 Correlations between serum BAs and faecal BAs

Associations between serum and faecal BAs were examined, but no significant correlations were identified in any of the studied groups (see Appendix 11, Figure 11.7).

5.6.6.3 Correlations between urine BAs and faecal BAs

The concentrations of BAs in the urine and faecal samples were analysed for correlations. However, no significant differences were identified in any of the studied groups (Figure 5.6A). Some strong correlations were found in all the data (Figure 5.6A) between hyodeoxycholic acid from faecal samples and all the urine BAs, with the exception of primary BAs, secondary BAs, unconjugated BAs, glycine conjugated BAs, and taurine conjugated Bas. The R value of these strong positive correlations ranged between 0.79 to 0.72 and P_{adj} value ranges between $5.11x10^{-22}$and $7.65x10^{-30}$. Some few strong positive correlations were found between vegans (Figure 5.6B). These correlations were between 3α-hydroxy-7 ketolithocholic acid from faeces and different BAs from urine: 3α,12α, 23-Nordeoxycholic acid, 8(14),(5β)-Cholenic acid-3α, 12α-diol, 3α-Hydroxy-7 Ketolithocholic acid, 3α-hydroxy-12 ketolithocholic acid, hyocholic acid, and GLCA. The R values ranged between 0.76 to 0.91 and P_{adj} value ranged between 4.73 $x10^{-12}$ and $2.15x10^{-24}$. No significant correlations were found between omnivores (Figure 5.6C).

5.6.7 Correlations between BAs and SCFAs

Exploratory analyses were conducted to examine correlations among BAs and SCFAs results from different biofluids, and these correlations were performed with the data from vegans and omnivores combined and separately for each group. The only significant association was found between serum BAs and SCFA levels. No other significant associations were found between BAs and SCFA from different biofluids (see Appendix 11, Figures 11.8, 11.9, and 11.10).

5.6.7.1 Correlations between serum BAs and serum SCFAs

A weak correlation was found between ursocholanic acid and 2-hydroxy butyric (R value = 0.31 and P_{adj}= 0.042) (Figure 5.7A) when the data was studied for both groups. In vegans, tauro-ω-muricholic acid and total SCFAs presented a weak correlation (R value= 0.42 and P_{adj}=0.04) (Figure 5.7B); while in omnivores, only one correlation was found between taurolithocholic acid and caproic acid (R value= 0.47 and P_{adj}= 0.005), UDCA and caproic acid (Figure 5.7C).

In summary, no correlations were found between dietary intake and BAs in serum and faeces, regardless of whether the data was analysed together or according to dietary patterns. However, in urine, weak yet significant correlations were identified between certain BAs and dietary nutrients when data from both dietary groups were analysed together. Moreover, significant correlations were found when significant BAs were considered for correlation with dietary intake. In this context, DHCA in serum BAs showed a weak correlation with some few nutrients, but secondary BAs did not present correlations. These results were obtained when the data was analysed for both groups. In urine, some few weak correlations

were found between BAs and a few nutrients when the data was analysed for both groups. In the case of faecal samples, no significant correlations were observed.

Notably, a strong correlation was observed between serum and urine BAs. However, it was important to emphasise that few strong significant correlations were found between faecal and urine BAs. Hyodeoxycholic acid from faecal samples correlated positively with most of the urine BAs when data was analysed for both dietary patterns. When correlations were explored for vegans, few strong positive correlations were found for 3α-hydroxy-7 ketolithocholic acid from faeces and few urine BAs. No correlations were found between faecal and serum BAs levels. While correlations between BAs and SCFAs were explored, only a few weak correlations were identified between serum BAs levels and serum SCFA levels. The other biofluids did not exhibit any relationship between the two metabolites.

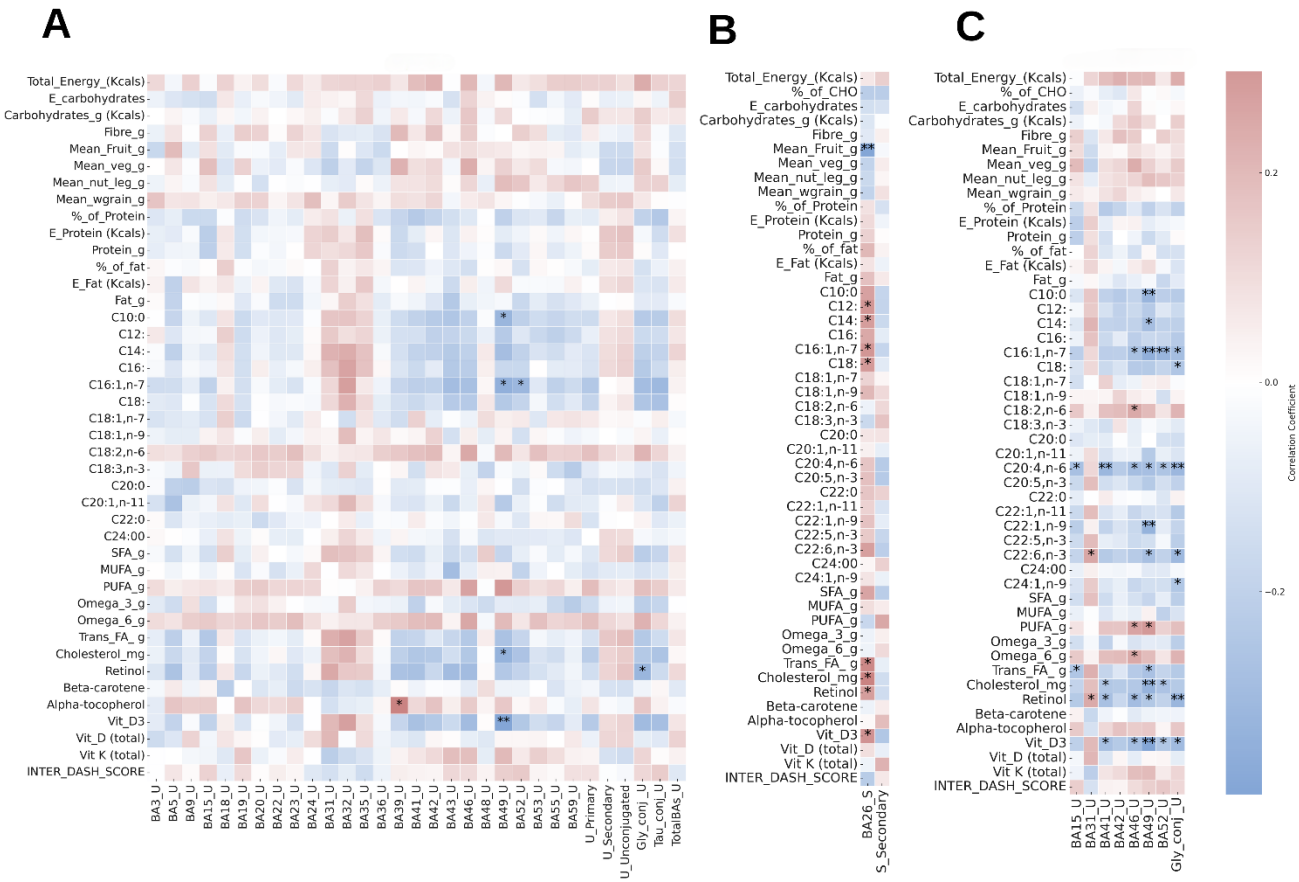

Figure 5. 4 Different correlations between variables. A) Correlations between dietary nutrients and urine BAs of omnivores and vegans. B) Correlations between dietary nutrients and significant serum BAs from omnivores and vegans. C) Correlations between dietary nutrients and significant urine BAs from omnivores and vegans. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, and $P_{adj}< 0.05$. Significance is labelled as ***, ** and * respectively.

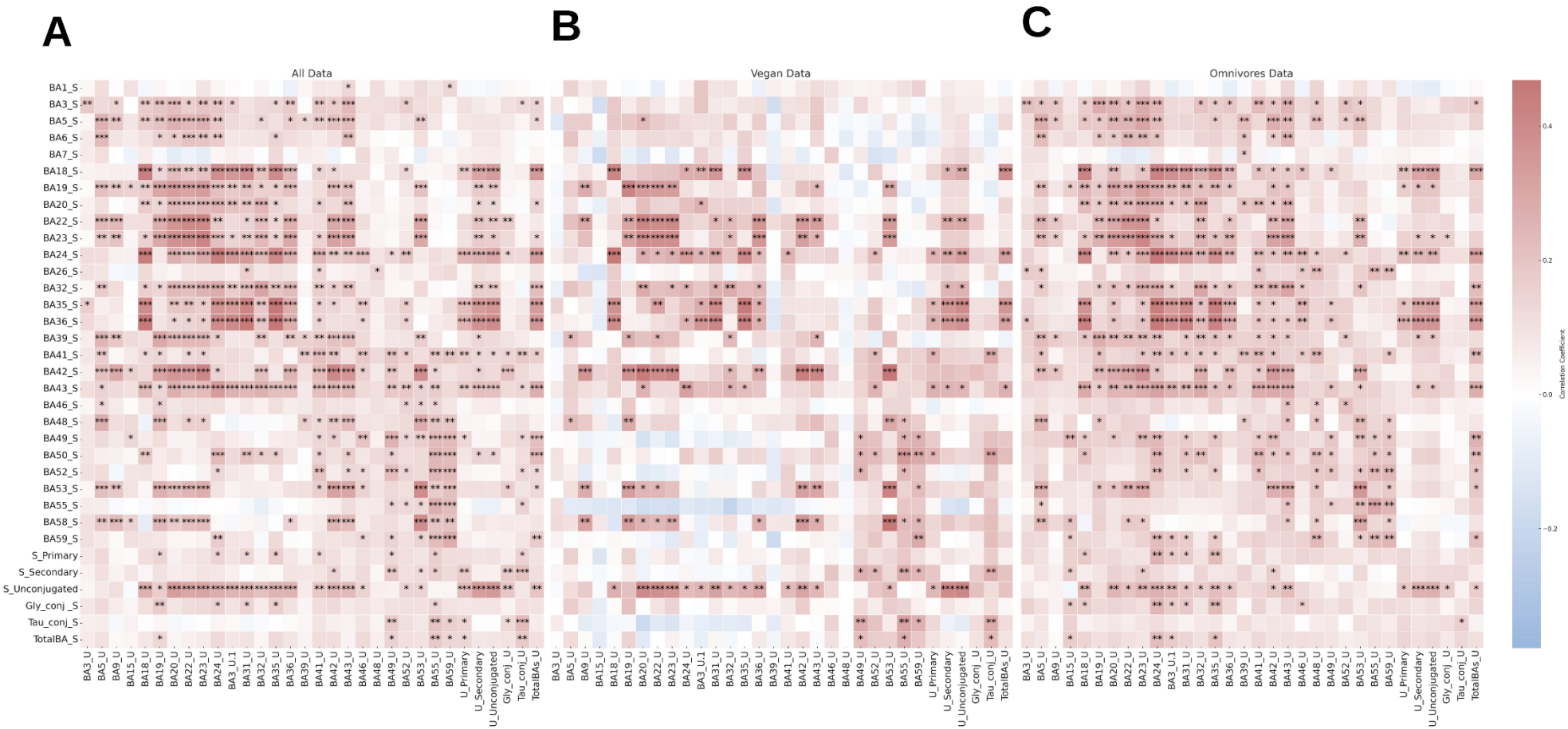

Figure 5. 5 Correlations between serum BAs and urine BAs from omnivores and vegans. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at Padj<0.001, Padj<0.01, and Padj< 0.05. Significance is labelled as ***, ** and *, respectively. (A) All data, (B) vegans and (C) omnivores.

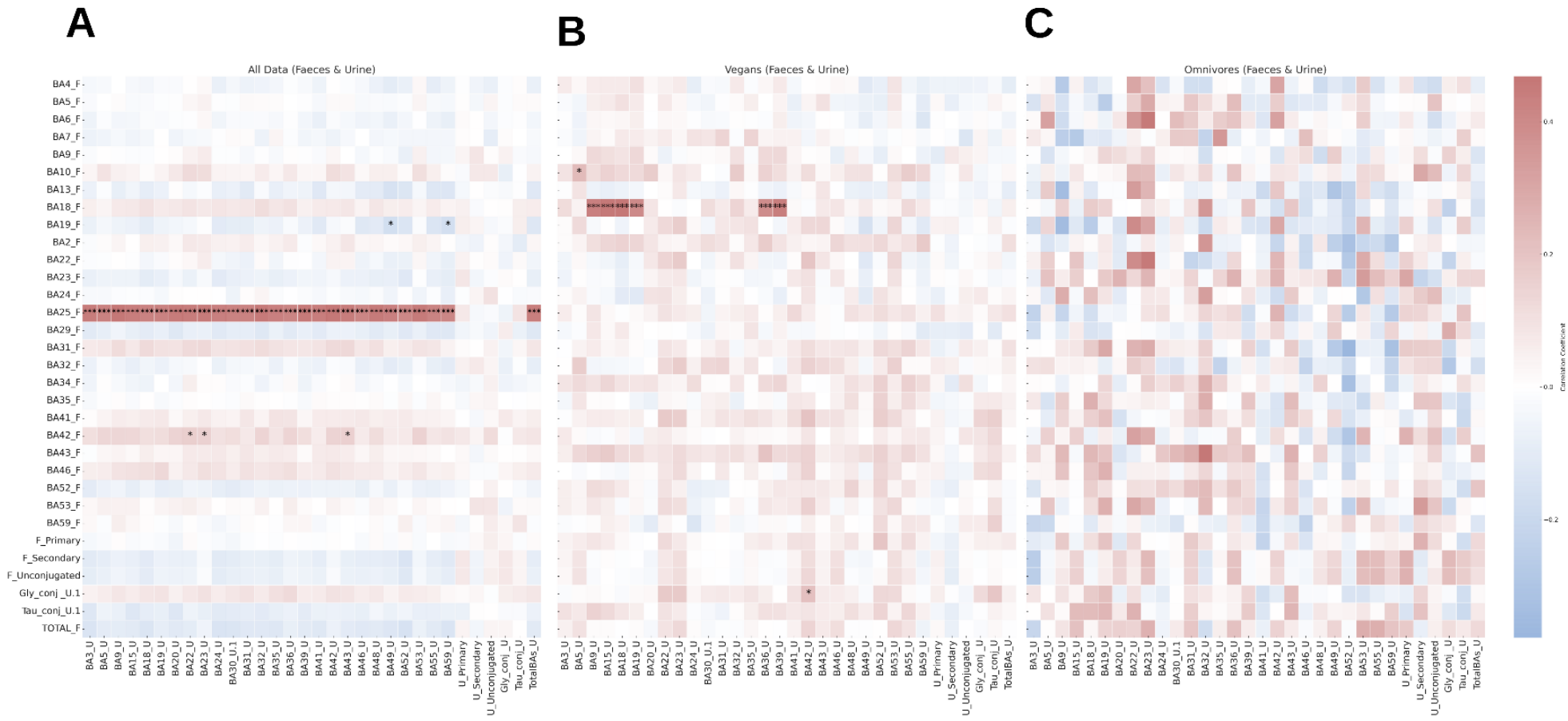

Figure 5. 6 Correlations between faecal and urine BAs from omnivores and vegans. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, and $P_{adj}< 0.05$. Significance is labelled as ***, ** and * respectively. (A) All data, (B) vegan data, (C) omnivore data.

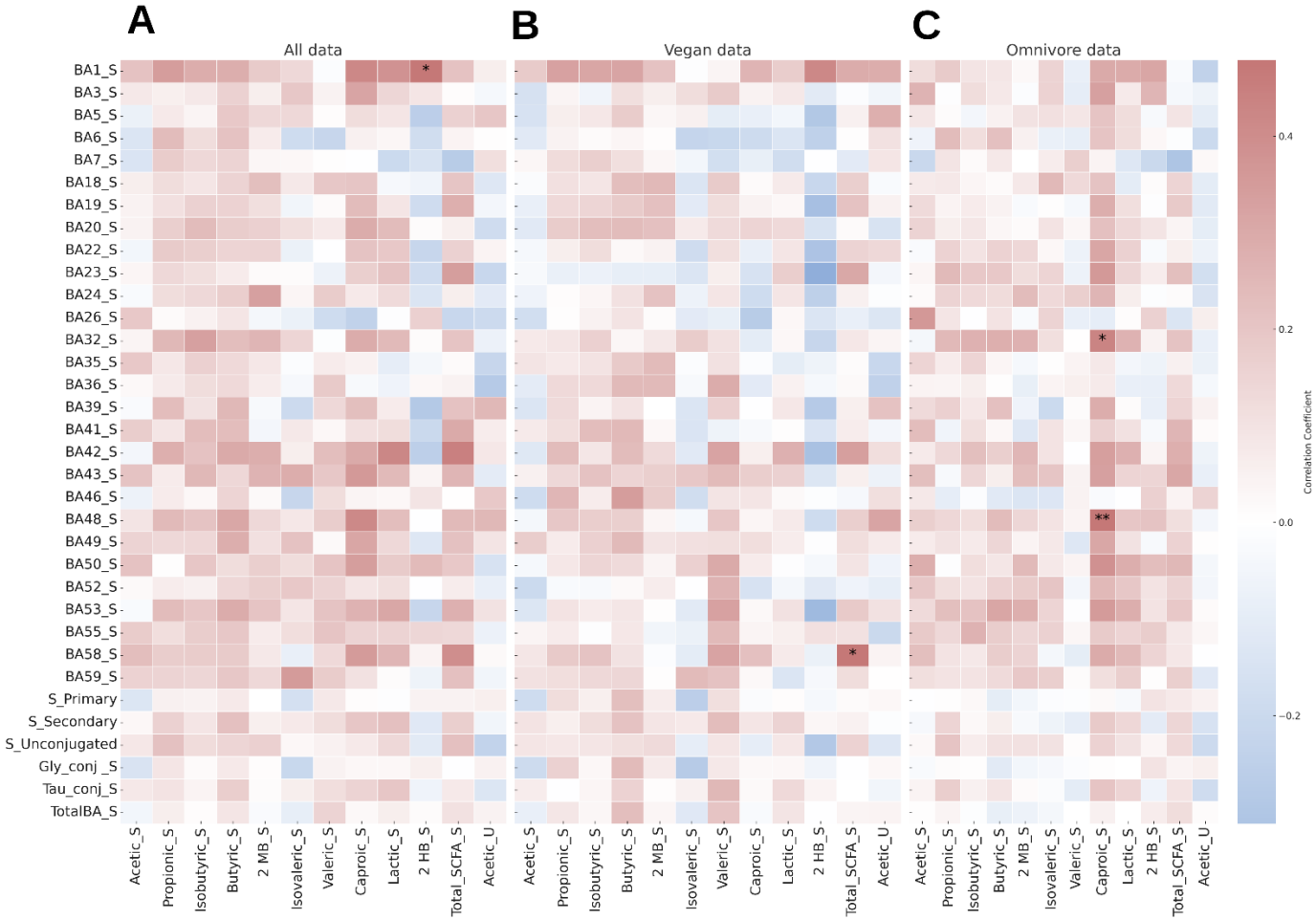

Figure 5. 7 Correlations between serum BAs and serum SCFAs of vegans and omnivores. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, and $P_{adj}< 0.05$. Significance is labelled as ***, ** and *, respectively. (A) All data, (B) vegan data, (C) omnivore data.

5.7 Discussion

Differences in the nutritional composition between vegan and omnivorous dietary patterns have been described in numerous studies (13, 25, 27, 143, 154-158). It has been previously reported that the intake of specific nutrients or dietary patterns can modulate the BAs response and concentrations (18, 61, 62). The influence of the vegan dietary pattern on BAs metabolism is poorly understood, considering the study of serum, urine, and faeces. Traditionally, vegan and omnivorous diets have been explored using one or two biofluids (289). Studies considering BA dynamics in the three biofluids in vegans and omnivores are scarce. In this sense, there is a gap that should be investigated, considering the recent and continuous increase in the consumption of vegan diets in the population (158-160). To gain a more comprehensive understanding, this study compared the impact of a vegan dietary pattern on an omnivorous dietary pattern across three different biofluids.

This study examined the biochemical parameters and dietary patterns of both groups. Notably, while there were significant differences in some biochemical parameters between vegans and omnivores, these differences were not clinically significant, as the values fell within the normal range for our groups. Conversely, there were significant differences in the dietary composition, particularly in the consumption of carbohydrates, proteins, lipids, and liposoluble vitamins; however, these met the recommended guidelines, suggesting a well-balanced diet among members of both groups (Table 5.2).

This study examined BAs in serum, urine, and faeces. Significant differences were identified in at least one BA in each biofluid. This implies a potential influence of diet on BA metabolism in vegans and omnivores across the studied biofluids. At the same time, very few correlations were found between dietary data and BAs from each biofluid. This finding might indicate potential inter-individual variations and underscore that factors beyond diet could regulate BA metabolism. These factors could include genetics, gut microbiota, environmental influences, and host metabolism.

5.7.1 BMI and blood pressure differences between vegans and omnivores population

We investigated the differences in anthropometry, biochemical analysis, diet composition, and BAs from serum, urine, and faeces to understand the impact of these dietary patterns on BAs metabolism. As previously mentioned in the discussion in Chapter 3 (Section 3.7.1), there were no significant differences between the studied groups regarding BMI and blood pressure, suggesting that participants were apparently healthy for these parameters.

5.7.2 Differences in diets and biochemical biomarkers between vegans and omnivores and their potential impact on BAs

In recent years, there has been an increasing trend towards vegan diets in developed countries (158-160). A vegan dietary pattern excludes all animal products and their derivatives, is low in saturated fat, high in fibre, and contains carbohydrates with a low glycaemic index, such as resistant starch (22). As previously described in Chapter 3 (see Section 3.7.2), despite the differences in starch and fibre consumption between vegans and omnivores, biochemical parameters related to glucose metabolism, such as fasting glucose, HbA1c and HOMA, were within the normal range. These findings suggest that neither group exhibited an altered glucose metabolism.

The evidence supporting the health benefits of vegan diets over omnivorous ones has shown many positive outcomes (26, 154, 189, 295, 296). A meta-analysis conducted in 2018 concluded that a vegan diet is associated with a more favourable cardiometabolic profile than an omnivorous diet (295). Additionally, a high-fibre diet has been found to confer a protective effect by reducing the incidence of colorectal cancer (CRC). One proposed mechanism is the reduction of secondary BAs through the modulation of the microbiota, which is influenced by dietary fibre (296, 297). Furthermore, fibre possesses absorptive and viscous properties that interact with intestinal cholesterol and regulate plasma cholesterol levels by limiting bile salt reabsorption (298).

In our study of the vegan and omnivore groups, although significant differences were found in total cholesterol and LDL, it can be hypothesised that a healthy vegan dietary pattern might be the primary contributor to the lower levels of these lipid metabolism by-products. However, from a clinical perspective, total cholesterol and LDL levels in both groups fell within the normal range according to the UK reference standards. Despite these differences, the observed levels did not indicate any clinical abnormalities (299).

Chapter 3 (Section 3.7.2) states that other biochemical parameters showed significant differences between vegans and omnivores. Notably, plasma vitamin D, leukocyte count, ferritin, and cobalamin levels were significantly lower in vegans. These findings are consistent with the literature, and despite their lower levels, they are still considered within the normal range (129, 192, 193, 300).

As discussed in Chapter 3 (Section 3.7.2), we observed higher betaine levels in vegans than in omnivores, a finding that aligns with the previous literature on vegans (206). Choline metabolism can be categorised into four distinct pathways: synthesis of acetylcholine, betaine, phospholipids, and trimethylamine (TMA)(301). It has been proposed that the conversion rate of choline to TMA in vegans may differ from that in omnivores, thereby indirectly affecting the proportions of betaine and other metabolites (201).

In the study cohort, serum concentrations of trimethylamine N-oxide (TMAO) were lower in vegans than in omnivores. This outcome is consistent with previous research that has reported similar trends in non-vegetarian dietary patterns (195), as discussed earlier in Chapter 3 (Section 3.7.2).

Trimethylamine N-oxide (TMAO) is a hepatic metabolite formed from trimethylamine (TMA), a microbial by-product of choline and carnitine fermentation (199, 200). TMA is converted to TMAO in the liver by flavin monooxygenase 3 (FMO3). Plant compounds, such as resveratrol, which are common in vegan diets, may inhibit TMAO production. Higher TMAO levels, often linked to regular meat consumption, are associated with an increased risk of cardiovascular disease, chronic kidney disease, and other comorbidities (221, 302). TMAO reduces bile acid (BA) synthesis and liver BA transporters, decreases the BA pool, inhibits reverse cholesterol transport, and reduces cholesterol absorption (303). It also contributes to macrophage foam cell formation, which is a factor in atherosclerosis. The activation of FXR and increased FMO3 and TMAO synthesis underscores the complex interplay between diet, BA metabolism, and cardiovascular risk (200, 303). While vegans in the cohort had lower TMAO levels, this does not necessarily indicate a higher cardiovascular risk for omnivores, as there is no clinically validated TMAO cut-off for cardiovascular risk as screening (304, 305).

Further analysis of the biochemical parameters of both groups suggested that both the vegan and omnivorous participants were metabolically healthy. This is indicated by the markers for BMI, glucose, and lipid metabolism, all of which fell within the normal range for both diet groups, despite the statistically significant differences between them.

5.7.3 Interpreting nutrient intake data for vegans and omnivores with caution

The adoption of a vegan or omnivorous dietary pattern leads to differences in nutrient intake, which can, in turn, influence metabolism (195, 289). As mentioned earlier in this chapter (Section 5.6.1, Table 5.1 and Table 5.2), the differences in nutrient intake between vegans and omnivores primarily include carbohydrates, fibre, fatty acids, and certain fat-soluble vitamins (Chapter 3, Table 3.3, Table 5.1 and Table 5.2). The nutrient intake of the remaining participants in this cohort has been previously reported (129), but considering a total of 1257 participants. In this sub-cohort, 150 subjects were studied, and their nutrient intakes were summarised in the following table.

Table 5. 6. Summary of intake of nutrients associated with BAs.

Nutrient	Higher in Group	Food source	Danish recommendations	Met Recommendation (Vegans)	Met Recommendation (Omnivores)	Reference Numbers
Fibre	Vegans	Plant-based foods	25 g-35 g/day	Yes	Yes	(50)
Saturated Fatty Acids (SFAs)	Omnivores	Animal products, coconut oil, palm oil	< 10% of daily energy intake	Yes	Slightly above	(141, 306)
Monounsaturated Fatty Acids (MUFAs)	Omnivores	Avocados, olives, nuts, seeds, processed meats	< 10% of daily energy intake	Yes	Slightly above	(141, 306-308)
Polyunsaturated Fatty Acids (PUFAs)	Vegans	Soybeans, walnuts, flaxseeds, corn oil	< 10% of daily energy intake	Yes	Yes	(307-309)
Omega-3 Fatty Acids	Omnivores	Walnuts, hemp seeds, flaxseeds, seafood	1.1 to 1.7 g per day	No	Yes	(309-311)
Omega-6 Fatty Acids	Vegans	Soybeans, walnuts, flaxseeds, corn oil	12 to 17 g per day	Yes	No	(312)
Trans Fatty Acids (TFAs)	Omnivores	Processed foods, baked goods, margarine	Below 1 % of total energy intake	Yes	No	(313)
Vitamin A (Retinol)	Omnivores	Animal products	500 ug	Yes	Slightly above	(167, 191)
Vitamin E (Alpha-tocopherol)	Vegans	Nuts, seeds, wheat germ, plant oils	12 mg	Yes	Slightly below	(206)
Vitamin D	Omnivores	Animal products, supplemented intake	10 ug/day	No	No	(167, 206)
Vitamin K	Vegans	Green vegetables, vegetable oils	70 µg/day to 300 µg/day	Yes	Yes	(167, 314)

The dietary intake of both groups was studied using self-reported questionnaires completed over four days. Dietary questionnaires are a useful tool for diet analysis, but it has been reported that dietary misreporting using these methods can vary from 21.5 to 67% in free-living people (212), generating misinterpretation and bias of dietary intake.

The dietary intake was calculated using the Danish Food Composition Databank and Danish data composition (315). It is well known that food composition varies, and the information provided in the food composition databases may not be updated (129, 316). Again, these limitations may influence the results reported for factual dietary intake and should be interpreted carefully. Despite these limitations, our results are consistent with those of previous studies that have evaluated nutrient intake in vegans and omnivores (129).

The data collected from the dietary questionnaires were collected over four different days to improve the precision of the data and reduce the risk of misreporting (212). Biofluid samples (urine and blood) were collected in the fasting state, while patients at their convenience provided faecal samples (129). It is important to note that the reported dietary intake may not reflect the metabolic status of the day the samples were collected. However, the collected dietary information provides an approach to the metabolic responses under specific dietary patterns, such as vegan and omnivore (167).

5.7.4 Multivariate analysis: no separations between BA profiles despite different dietary patterns

After analysing the BAs data across various biofluids using unsupervised and supervised multivariate methods, no noticeable differences were observed, and separation was largely absent from the studied biofluids. This suggests that, regardless of the contrasting dietary patterns, the BAs data do not provide definitive discriminative information or that there is a class distribution overlap. Hence, considering the metabolic health of the volunteers, as indicated by their biochemical parameters, and the absence of distinct separations in BAs data in faeces and serum, it is plausible to suggest that both groups share certain similarities in circulating BAs, independent of their dietary patterns.

5.7.5 Omnivores presented significant elevated serum concentrations of 3,7,12-Dehydrocholic acid and total secondary was significantly higher BAS in vegans.

We compared serum concentrations of BAs between the groups in order to investigate the potential influence of vegan and omnivorous dietary patterns on metabolism. Within this biofluid, we detected a total of 28 BAs, of which only 3,7,12-Dehydrocholic Acid (DHCA) was found to be significantly elevated in omnivores. Additionally, total concentrations of secondary BAs were found to be significantly higher in vegans. DHCA is a synthetic compound that is derived from the oxidation of the 3α, 7α, and 12α-hydroxyl groups of cholic acid, using chromic acid as a reagent (317). This compound is known for its hydrocholeretic properties, as it has been reported to stimulate bile production in both animal and human studies (318-320).

Upon initial detection of this metabolite in our samples, we speculated that it might be an oxidation artefact of cholic acid, potentially produced during LC-MS sample analysis. However, this speculation was discarded after the revision of the recovery data from the PhD thesis of the former student who developed the method (98, 321). During method development, intra-day and inter-day validations of accuracy and precision were conducted for all bile acids, with the intra-day accuracy of cholic acid ranging from 88.6% to 104% and inter-day accuracy oscillating between 92.4% and 101.5%. The obtained intra-day precision was between 1.8 and 11.5%, and the inter-day precision varied from 1.7 to 16.1%. Therefore, it is highly improbable that the detection and quantification of DHCA were derived from the cholic acid present in the sample. Furthermore, the SIM utilised in the DHCA analysis matched the value described in the method publication (m/z 401.23), and the MRM used for bile acid detection and quantification was appropriate according to the RT determined for the metabolite (see Chapter 2).

However, the presence of this metabolite in this set of serum samples from healthy individuals is not well understood. Previously, during the development and validation of the BAs method, this metabolite was detected in serum and plasma samples obtained from obese subjects, as described by Sarafian et al. (98).

Therefore, it could be speculated that the presence of this metabolite is a result of cholic acid oxidation by the gut microbiome. Only a limited number of studies have reported organisms capable of inducing the oxidation of cholic acid under in vitro conditions (322, 323). Microbial degradation of cholic acid has been attributed to the actions of *A. faecalis* (Alcaligenes faecalis) and *E. coli* (Escherichia coli) (317). *A. faecalis* is a Gram-negative, obligate aerobic, oxidase-positive, catalase-positive, and nonfermenting bacterium (324). This commensal microorganism has been isolated from a range of clinical materials such as urine, blood, wound discharge, stool, cerebrospinal fluid, and respiratory secretions (325). On the other hand, *E.coli* is a ubiquitous microorganism in the intestinal microbiome, gram-negative, facultative anaerobe, oxidase-negative, catalase-positive, and fermenter (326, 327). The type of oxidation generated by these microorganisms has been described as the same chemical reaction as that produced by chromic acid (317).

It has been reported that *Ruminococcus sp* obtained from the human intestine is capable of reducing DHCA to 3β-hydroxy-7,12-dioxo-5β-cholanic acid by the action of the 3β-hydroxysteroid dehydrogenase enzyme (328). This finding supports the role of microbiome enzymes in the modification and biotransformation of BAs at the gastrointestinal level. However, it has been described that DHCA reductases can be found in red blood cell cytosol, suggesting that DHCA can be exposed to reductive metabolism in the systemic circulation (329).

Previous research has documented a significantly higher presence of *Ruminococcus torques* in faecal samples from omnivores than in vegans (330). Additionally, the abundance of this microbial species increased in response to a fibre-free diet. In this context, the presence of DHCA could be potentially related to a higher abundance of *Ruminococcus torques* in omnivores. Moreover, unpublished results from faecal metagenomic analysis from the Danish cohort, conducted by collaborators (331) (manuscript in preparation co-jointly with collaborators) reported a higher abundance of *Ruminococcus torques* in omnivores than in vegans.

Remarkably, the median levels of DHCA were nearly five times higher in omnivores compared to vegans (45.4 nM versus 8.36 nM, respectively). However, as mentioned previously, the function of this metabolite in the studied samples remains elusive. Nevertheless, considering its use in animal and human experiments wherein the synthetic form of DHCA is employed as a choleretic agent to stimulate bile production by the liver in omnivores, this metabolite could potentially modulate the production of BAs under fasting conditions. However, further research is needed to comprehend its physiological impact when endogenously produced.

The total secondary BAs were significantly higher in vegans than in omnivores. Previous studies comparing BAs in vegans and omnivores reported an increase in the serum concentrations of glycine-conjugated BAs in vegans, but not in total secondary bile acids (26). A double-blind crossover double-arm study reported a significant increase in plasma secondary BAs after the consumption of 12 g of RS-4 for twelve weeks. The intervention group reported higher concentrations of taurodeoxycholic, glycodeoxycholic, and deoxycholic (332). These BAs were associated with microbiota changes, specifically *Bifidobacterium adolescentis*, in the intervention group.

In this study, serum metabolites were initially compared using the Mann-Whitney test, as stated in Chapter 2, revealing that three secondary conjugated bile acids (BAs), UDCA, GHCA, and GDCA, were significantly higher in vegans. However, these differences were not significant after FDR correction (Table 5.2). Previous studies have shown elevated levels of total glycine-conjugated BAs in healthy populations compared to non-healthy ones (330, 333-336). In this study, the differences in conjugated BAs between the groups were insignificant, noting that both groups were healthy despite their dietary differences. However, it seems plausible that the total secondary BAs resulted in an increase in vegans compared to omnivores.

The conjugation of BAs with glycine or taurine is influenced by diet composition (99). Diets rich in animal products result in higher taurine BA conjugation, whereas diets rich in vegetables, fruits, and carbohydrates promote glycine BA conjugation. The ratio of glycine-conjugates to taurine-conjugates in human bile is typically 3:1 but can vary widely (337). In our study, no differences were found in

glycine- or taurine-conjugated BAs in serum (338, 339), suggesting a similar capacity for BA conjugation in both groups, regardless of the dietary pattern. However, the quantified dietary intake of glycine was higher in vegans (Chapter 3, Table 3.3), which did not correlate with an increase in glycine-conjugated BAs in the serum. Similarly, taurine-conjugated BAs did not differ between groups, and their dietary intake was not quantified. The absence of significant differences between glycine and taurine-conjugated BAs suggests a similar capacity for conjugation in both study groups, irrespective of the dietary pattern.

5.7.6 Conjugated BAs and other BAs were found significantly higher in urine from vegans

In our evaluation of BA concentrations in urine samples from both the vegan and omnivorous groups, we detected 26 different BAs. We identified significant differences in several BAs, including apocholic acid, 12-DCA, GCDCA, GDCA, GHCA, TCDCA, and total glycine-conjugated BAs. All of these metabolites exhibited higher concentrations in vegans except for 12-DHCA.

Apocholic acid is a dehydration product of cholic acid (CA) and a potential carcinogenic BA in mice (340) and elevated concentrations in alcohol-associated liver disease in humans (341). However, this study did not quantify apocholic acid levels, making it difficult to draw comparisons to healthy individuals. Therefore, its relevance between the studied groups in our study is unclear, and it is unknown if these differences may have any biological relevance for either group.

12-DHCA is a secondary BA produced by bacterial oxidation of CA, identified in higher concentrations in omnivores due to Clostridium spp. presence (331) (Manuscript in preparation co-jointly with collaborators). Higher 12-DHCA in omnivores might be linked to increased systemic synthesis rates of this metabolite. Previously, it was reported to be a potential biomarker for primary biliary cholangitis treatment response (342). However, the results for this metabolite should be interpreted with caution, as the p-value was significant but borderline ($P\text{-value}_{adj} = 0.048056$) after FDR adjustment.

GCDCA is produced from chenodeoxycholic acid and glycine and has been proposed as a potential early biomarker of gastric cancer (343). The role of this metabolite in our study remains elusive since its role as a biomarker for cancer cannot be attributed to this cohort of healthy individuals.

Our study found that GDCA, a glycine-conjugated form of deoxycholic acid, was more abundant in vegans than in omnivores. Some research has described high concentrations of this metabolite in urine samples from diabetic type 2 patients with normal BMI and overweight diabetic patients compared to healthy controls (344). In the present study, GDCA levels were lower in omnivores than in vegans, contrary to earlier findings in healthy individuals. However, both the groups were healthy.

GCA is a conjugated form of CA associated with hepatocellular carcinoma (HCC) (345). In the present study, GCA levels were higher in vegans than in omnivores. GCA in urine has been associated with a vegetarian high-fat diet from fast food intake in Indian subjects (346). In the present study, GHCA was higher in vegans than in omnivores. GCHA is a glycine-conjugated form of hyocholic acid. Previously, it was identified in altered samples from patients with cancer undergoing total-body irradiation (347). There is limited literature on GHCA in urine and its role in this biofluid is poorly understood.

High concentrations of TCDCA have been described in the urine of patients with hepatitis and cholestatic conditions (348, 349). Its role in this study remains unclear, with unknown clinical or dietary significance, considering that it was found to be higher in vegans than in omnivores.

Total glycine BAs were higher in vegans, likely because of their dietary intake of glycine-rich foods (Chapter 3, Table 3.3). Glycine-conjugated BAs are excreted in urine, possibly because they escape enterohepatic circulation and kidney metabolism at very low concentrations (350). Under this premise, the presence of FXR and TGR5 receptors in the kidneys may play a key role in the regulation of glucose and lipid metabolism (3, 351).

In this study cohort, some few weak negative correlations were found between glycine-conjugated BAs, GCDCA, and GCYCA, and lipid structure nutrients. A few positive correlations were found between GCYCA, GCA, and PUFAs. These associations could reinforce the potential role of BAs in the metabolism of nutrients with lipidic structures under healthy conditions.

Kidneys are involved in bile acid (BA) metabolism, playing a role in maintaining the pool size of bile acids under physiological conditions. Approximately 10-50% of the bile acids reabsorbed in the terminal ileum bypass portal circulation and enter the systemic circulation (350). Each day, approximately 100 μmol of unbound bile acids are filtered at the glomeruli, nearly all of which are reabsorbed in the proximal tubule through a sodium-dependent mechanism (352, 353). Under physiological conditions, the kidneys excrete 1–2 μmol of bile acids daily through urine. Studies using labelled BAs have shown that the recovery of BAs in urine is below five per cent in healthy subjects (354). This explains the generally low concentration of BAs found in urine samples. Certain conditions, such as cholestasis, can increase urinary bile acid excretion, potentially leading to kidney damage due to the capacity of bile acids to inflict oxidative damage and injure tubular cell membranes, which can impair renal function.

Understanding the biological mechanisms involved in the reabsorption and excretion of BAs in the kidneys is crucial. It turns out to be relevant to continue investigating the effect of BAs at the kidney level, despite the low concentrations that are excreted in urine in healthy subjects (354). However, most

studies on BAs in urine are related to diseases because their concentrations increase in pathological states. Since BA concentrations in urine are low under healthy conditions, the study of dietary patterns affecting BA metabolism should not be evaluated using urine as the sole biofluid to understand metabolic effects. Additionally, low concentrations of BAs in urine should be considered in BA analysis. The method used in this study provided a good coverage for these metabolites in urine (98). For future studies, it is recommended to use more sensitive methods capable of detecting lower concentrations in order to uncover the role of BAs in urine, as their function beyond pathological states remains elusive.

5.7.7 Secondary BAs and other BAs were significantly higher in omnivores in faecal samples

Faecal BAs were quantified in the vegan and omnivore cohorts, and 27 bile acids were consistently detected in our samples. NorDCA, DCA, βMCA, total secondary BAs, unconjugated BAs, and total BAs were significantly higher in the omnivores.

NorDCA is a secondary bile acid derived from DCA and norCA (355). Significantly higher concentrations were observed in omnivores than in vegans. Endogenously, norDCA is rarely produced in the liver under in vitro conditions (356); however, recent studies have detected its presence in various biofluids under different pathological conditions. Elevated serum norDCA levels have been observed in patients experiencing the first episode of major depression, correlating with increased depression severity (357). In a Chinese cohort, norDCA levels were higher in women than in men (336). In patients with alcohol-associated liver disease, urine norDCA concentrations decrease progressively with disease severity (336). NorDCA has primarily been studied in animal faeces. For instance, it has been found in mice undergoing cisplatin-induced chemotherapy injury and treated with C-phycocyanin (358), and in mice fed a low-methionine-choline-deficient diet to induce NASH, supplemented with Salvia-Nelumbinis naturalis (359). However, its role in faeces remains unclear in humans.

Common bile acids have a C24 side chain, whereas nor-bile acids possess a C23 side chain, demonstrating distinct different biotransformation and physiological properties (360, 361). For instance, norcholic acid induces more bile flow and less phospholipid secretion than cholic acid (361). NorDCA appears to have an anti-inflammatory effect starting at the intestinal level, in contrast to the inflammatory and carcinogenic roles of DCA in mice (362-365). Most studies on norDCA have primarily focused on animal models rather than humans.

DCA is a secondary BA, and total bile acid concentrations were found to be higher in omnivores than in vegans. This finding aligns with previous research examining faecal samples from vegans and omnivores. Trefflich et al. studied BAs in a small group consisting of 36 vegans and 36 omnivores, finding that the total concentrations of BAs were higher in omnivores, as observed in our study (289).

However, the DCA results of this study did not show any intergroup differences between the studied groups. Another study explored the effect of the consumption of different dietary patterns, such as Western, lacto-ovo-vegetarian, and vegan, in 12 subjects in a randomised order for 20 days each. The amount of DCA in faeces collected after 4 days was significantly lower in lacto-ovo-vegetarians and vegans than in the Western diet (366).

The changes in bile acid profiles during the consumption of various dietary patterns have been extensively studied in relation to high and low fibre, as well as fat intake, and their impact on faecal BAs (18, 253). For example, a study of African American (AA) and rural African (RA) subjects who switched their diet for two weeks (18). The diet of AA was typically a Western diet high in animal protein and fat, while the diet of AA was rich in fibre and resistant starch. The RA subjects who consumed the AA diet for two weeks presented an increase in faecal BAs such as LCA, CA, and DCA. Conversely, when AA subjects consumed the typical RA diet for two weeks, their faecal secondary BAs were reduced (18). Endogenous BAs such as CDCA, CA, and DCA have been shown to activate FXR (367). An in vitro study demonstrated that a concentration as low as 400 μM/L was sufficient to stimulate metaplasia of normal gastric cells through FXR activation (368). There is extensive evidence of the pro-inflammatory and pro-carcinogenic effects of DCA (362-365). However, there are some relevant aspects to consider in the deleterious role of DCA, such as the time of exposure of this metabolite to the colonic epithelium as well as the synergies or antagonism that could be generated at the intestinal level with other BAs that need to be explored (369).

An important aspect to consider is that BAs metabolism can be modulated by an increase in total fat and saturated fat, while the intake of soluble, viscous dietary fibre increases excretion of fat by faeces (370-374). Insoluble fibre contributes to the reduction of total faecal acid concentrations by increasing faecal weight (375). In our study groups, vegans reported a higher intake of nuts and fibres than omnivores. A clinical trial reported that a high-fibre diet increases faecal weight, and 42 g/day of walnuts, a rich source of fibre, reduced faecal DCA by 29% (376). Another study investigated the effect of daily intake of 15 g of peas and reported that faecal DCA was reduced by 48% (377).

In this cohort, saturated fat and total intake were higher in omnivores, whereas vegans had higher fibre intake. As discussed in Chapter 3, the recruited participants were healthy despite their differences in dietary patterns. However, these differences in faecal BAs could be mostly related to their diet and microbiome composition. Although DCA is normally a predominant colonic BAs (378).

In parallel, levels of βMCA were significantly higher in omnivores compared with vegans. Muricholic BAs and CA are mainly found in mice, whereas cholic and chenodeoxycholic acid are the main BAs in humans (379, 380). Information about these BAs in humans is scarce. Murocholic acids have been

reported as circulating BA in human serum during fasting, specifically tauro-α-muricholic acid, which correlated positively with a distinct gut microbial community composed primarily of *Clostridia spp.* and inversely correlated with adiposity traits and markers of dysmetabolism (381). Nevertheless, the presence of βMCA in faeces is more frequently reported in animal studies rather than in human studies (382).

The abundance of unconjugated BAs was significantly higher in omnivores than in vegans. This increased proportion is mainly due to the concentrations of significantly higher DCA, but also due to the higher proportion of LCA in faecal samples. These findings are in line with previous research describing BAs in omnivores and vegans (289).

The total BAs were higher in omnivores than in vegans. This finding is in line with previous research that explored the effects of vegetarian diets on omnivorous diets. In a small Dutch interventional study, participants were randomised to receive vegan, lacto-ovo vegetarian, or mixed diets for a period of 20 days. They found that total BAs and DCA were lower in vegans and lacto-ovo vegetarians than in mixed vegetarians (290). Another crossover study of 10 subjects reported the impact of a plant- and animal-based diets for five days each. (383). Total faecal bile acids and DCA increased in animal products compared with the plant-based diet (383).Furthermore, a recent study reported that vegans, compared to omnivores, present a lower level of total BAs (289).

Our study is one of the first to report the quantification of BAs in three different biofluids, with a sample size of over 75 subjects per group. However, as mentioned in Chapter 3, the dietary records were completed by participants in four different opportunities, and these records do not necessarily represent the intake on the day before when samples were collected. Therefore, these food registers could be inaccurate in estimating factual food intake and reflect physiological responses to BAs metabolism (212). A recent study on the dynamics and determinants of BAs in plasma profiles reported a remarkable heterogeneity in fasting concentrations due to the diverse pathways that BAs enter into the peripheral blood (384). Therefore, differences in the profile of BAs in the different biofluid area respond to individual factors alongside the consumption of a specific dietary pattern.

5.8 Conclusions

This study explored a novel aspect of BAs metabolism by analysing three biofluids (serum, urine, and faeces) in a cohort of omnivores and vegans who adhered to their respective dietary patterns for a minimum of five years. The examination of BAs across various biofluids enriches the understanding of BA metabolism and its relationship with diet. In fasting serum, only two BAs were significantly different in our study. BAs tend to be lower than postprandial concentrations during fasting (62, 385). In urine, BAs presented very low concentrations during fasting, which is in line with the literature on the elimination of BAs through urine in healthy subjects (354). The concentrations of BAs in the serum and urine were strongly correlated, suggesting a potential direct or indirect systemic relationship in the metabolism or excretion of these compounds in both blood and urine.

In faecal samples, BAs were detected, some of which were the result of different biotransformations by the gut microbiota in the colon. The results of our study were aligned with previous research comparing BAs in vegans and omnivores, confirming that omnivores have higher concentrations of secondary and total BAs (289, 383). The analytical techniques utilised for BA quantification permitted a broad-spectrum analysis of metabolites in the examined biofluids (98).

Future research should explore the interplay between dietary patterns, metabolites, and their interactions at the colonic level through the modulation of the colonic microbiome composition, with dietary fibre increasing SCFA production during fermentation and counteracting the deleterious metabolites (71, 386). In addition, the contribution of polyphenols and their role in modulating the gut microbiota and BAs should be considered in the study of gut metabolites. Moreover, these studies should strive for a more precise recording of food intake, including the type of fibre consumed, considering the solubility, fermentability, and viscosity rather than considering fibre as a homogeneous entity. Thus, the physicochemical and functional properties of the fibre could be associated with the effects of diet (50). Plant-based diets differ from animal-based diets in several microbe-dependent metabolic pathways, including increased metabolism of fibre and polyphenols and decreased metabolism of bile acids and amino acids (387).

The investigation of BAs and their response to food intake has predominantly been reported under fasting or postprandial conditions within brief window periods. To enhance our understanding of BA metabolism, future studies should consider sampling time as a pivotal factor for a more comprehensive elucidation of the influence of diet on metabolism. Another critical aspect for future studies is the incorporation of microbiome data analysis and functionality, including other omics techniques. In this manner, the responses of BAs to diet can be traced and monitored by integrating various aspects of human physiology using omics sciences. Thus, deciphering how different dietary components impact BAs production could indeed offer valuable insights for personalised nutrition strategies.

6. Circulating bile acid concentrations after the intake of two contrasting dietary patterns under controlled conditions

Note: For this chapter, bile acid abbreviations have been used, so please find them in the Abbreviation Section (Page 17).

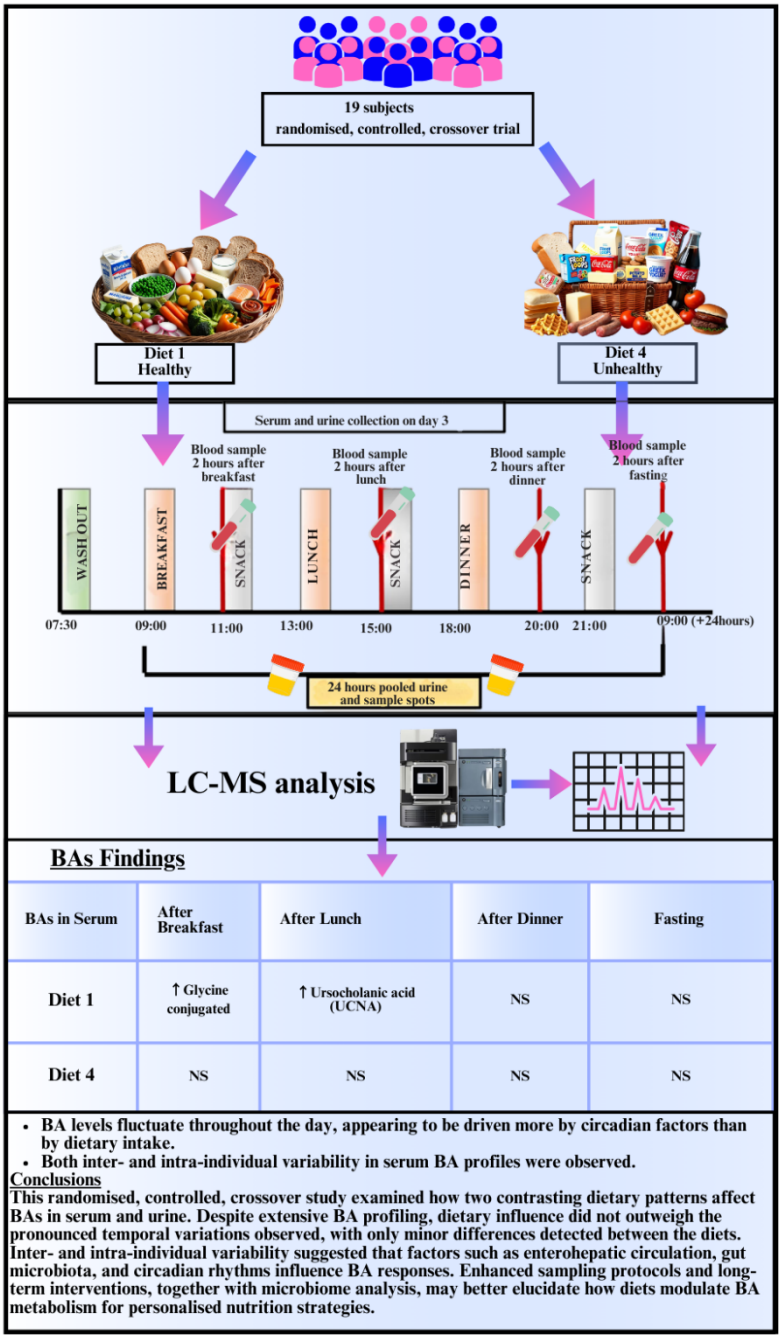

Figure 6. 1 Schematic Summary of Chapter 6.

6.1 Introduction

The consumption of different types of diets and foods is one of the factors that can regulate BAs metabolism and synthesis that impact on health and disease (18, 285). In Chapter 5, the differences between BAs in a cohort of free-living omnivores and vegans were studied. Different biofluids were collected under fasting conditions (serum, faeces, and urine), and some significant differences between the two groups were found in some of the BAs. These differences could be associated with the type of fatty acid consumption, carbohydrates, and fibre previously described as key nutrients in the modulation of BAs (61, 286) as subjects consumed the same dietary pattern but not the same dietary intake.

This chapter studied BAs after the intake of two contrasting dietary patterns provided under inpatient controlled conditions using a novel LC-MS targeted technique developed by Sarafian et al. (5), quantifying BAs in serum and urine. As mentioned in Chapter 2, this is a sub-study from a previously published crossover, randomised dietary trial that assessed the effect of diets with different healthiness levels on metabolism under inpatient controlled conditions (132). Nutritional studies have been reported to be inaccurate in reporting dietary intake (212, 234, 235). It is not well understood whether the intake of two contrasting dietary patterns under controlled conditions can provide a better understanding of BAs synthesis and metabolism.

In this study, four dietary patterns were evaluated based on their alignment with the WHO's healthy eating recommendations, varying stepwise in healthiness (132, 141). In this chapter, the two extreme dietary patterns were studied that presented differences in fatty acid, carbohydrate and fibre composition related to the synthesis and metabolism of BAs (62). Diet 1 (healthy diet) was aligned 100% with WHO nutritional eating recommendations, being high in fruits, vegetables, and fibre and low in saturated fats, salt and sugar. On the other hand, diet 4 (unhealthy diet) was high in saturated fat, sugar, and salt, low in fibre, and aligned 25% with the WHO healthy eating recommendations (Table 4.1 from Chapter 4 for differences in dietary composition).

The provided diets were given to the volunteers in a randomised order, and there was a washout period of at least five days between their consumption. Participants attended the clinical facilities four times, where they were provided the diets under inpatient conditions for 72 hours, maintaining the same diet and timing of food consumption each day.

This sub-study involved targeted metabolomics analysis employing LC-MS techniques to assess serum and urinary samples collected during this clinical trial. We monitored the temporal changes in BAs throughout the day under controlled conditions following the intake of two contrasting dietary patterns to understand the systemic exposure of BAs modulated by dietary pattern intake.

6.2 Hypothesis

The consumption of two omnivorous dietary patterns that differ in the nutritional composition will be reflected in the concentrations of BAs in serum and urine.

6.3 Aims

The central aim of this study was to compare the temporal changes in BA concentrations in response to two omnivorous dietary interventions that differed in nutritional composition.

6.4 Objectives

The specific objectives were as follows

1. To provide baseline sociodemographic and clinical characteristics of participants who consumed both dietary interventions.
2. To compare the serum concentrations between the two contrasting diets at four different time points over a 24-hour period.
3. To describe the variability in the response between participants in serum BA concentrations.
4. To evaluate concentrations of BAs in urine and the potential role of diet in their excretion

6.5 Materials and methods

The methodology for the study design, participant enrolment, collection of samples, LC-MS sample preparation, and LC-MS data analysis was detailed in Chapter 2. The LC-MS analytical method was previously published by Sarafian et al. (98).

6.6 Results

6.6.1 Baseline sociodemographic and clinical characteristics of participants

In Chapter 4, Section 4.6.1, these characteristics were described for this sub-study but in the context of SCFAs. In this case, the same characteristics corresponded to this study, since the same patients were considered. Therefore, 10 male and 9 women participated in the study, and their ages ranged from 29 to 65 years. BMI ranged from 21.1 to 33.3 kg/m^2, which classified participants from normal weight to obese. Glucose metabolism, lipid metabolism, and liver function test parameters were within normal ranges (Table 4.2) (180, 181, 239, 388, 389).

6.6.2 Comparison of serum concentrations of BAs between the two different diets at different time points within a period of 24-hour period

Concentrations of BAs in serum were compared over a 24-hour period, as described in Chapter 2. Samples were collected at three time points on day 3: after breakfast (AB), after lunch (AL), and after dinner (AD) and fasting (FA). This sample was collected on day four (Chapter 2, Figure 2.1). The concentrations of BAs after the intake of both diets showed some significant differences in AB for total glycine conjugated BAs being higher in diet 1 compared to diet 4 ($P_{adj} = 0.021$) (Figure 6.2D). Similarly, UCNA in AL demonstrated a significant difference between the groups, being notably higher in diet 1 than in diet 4 ($P_{adj} = 0.0269$) (Figure 6.1A). No other significant differences were found at the AD and FA (Figure 6.1, 6.2, Appendix Figures 12.1 and 12.2).

Independent of the two significant differences observed between the concentrations of serum BAs, their concentrations fluctuated throughout the day, which followed their own geometric trajectories regardless of the type of diet consumed. For instance, isolithocholic acid (ILCA) maintained steady concentrations after breakfast (AB), after-lunch (AL), and after-dinner (AD) time points. However, its concentration increased by approximately 2.5 times in the fasting (FA) sample (Figure 6.1C). A similar pattern was observed for murocholic acid (MCA) and ursodeoxycholic acid (UDCA), with concentrations at the FA time point increased threefold compared to the other mealtime points (Figure 6.1D and 6.1H). Similarly, taurochenodeoxycholic acid (TCDCA) followed the pattern observed in the aforementioned BAs (Figure 6.2J), but its concentration in the FA sample was five times higher than that in ILCA. These elevated concentrations in the FA samples, observed independently of the diet consumed the previous day, suggested that circadian rhythms may exert a greater influence on these BAs than the diet itself. Primary, secondary unconjugated, taurine-conjugated, and total BAs did not show significant differences (Figure 6.2). Other BAs figures could be found in Appendix 12.

Most postprandial BA concentrations remained stable regardless of dietary intake, which indicated that BA levels were unlikely to be significantly affected when blood samples were collected two hours post-meal, irrespective of the diet consumed. The duration of this time window might not accurately reflected any acute systemic responses of BAs induced by diet within two hours of intake, considering the short half-life of BAs in systemic circulation (354). The trends observed in our study suggest that under the specified conditions of sample collection, the BAs measured were more likely influenced by metabolic regulation linked to circadian rhythms after two hours of eating rather than the diet itself. Another relevant aspect to highlight illustrated in the results (Figure 6.2, Figure 6.3, Appendix 12, Figure 12.1, and 12.2) is that very few peaks of BAs concentrations were observed at AB. Most of the BAs concentration peaks were observed at AD. Additionally, concentrations of BAs presented a wide range of variation in AD compared to the other mealtimes studied.

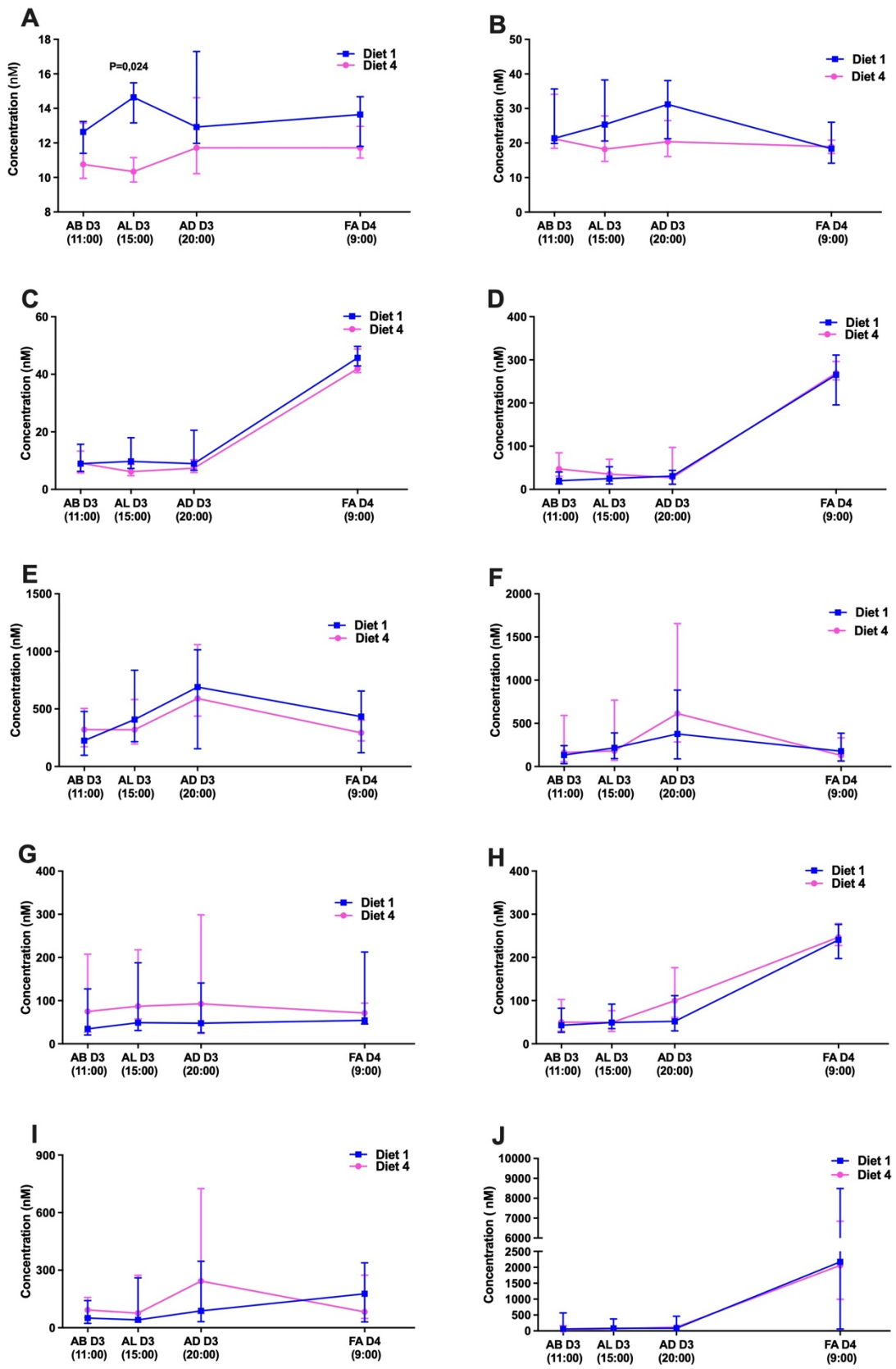

Figure 6. 2 Comparison of serum BA concentrations throughout the day for the same time points for diet 1 and diet 4. (A) Ursocholanic acid, (B) Lithocholic acid, (C) Isolithocholic acid, (D) Murocholic acid, (E) Deoxycholic acid, (F) Chenodeoxyholic acid, (G) 12- Dehydrocholic acid, (H) Ursodeoxycholic acid, (I) Cholic acid and (J) Taurochenodeoxycholic acid. Values are shown in median and interquartile range n=19. Differences between the same time points for different diets were determined by Wilcoxon signed rank test and False Discovery Rate (FDR). Significance was accepted at $P_{adj}<0.05$.

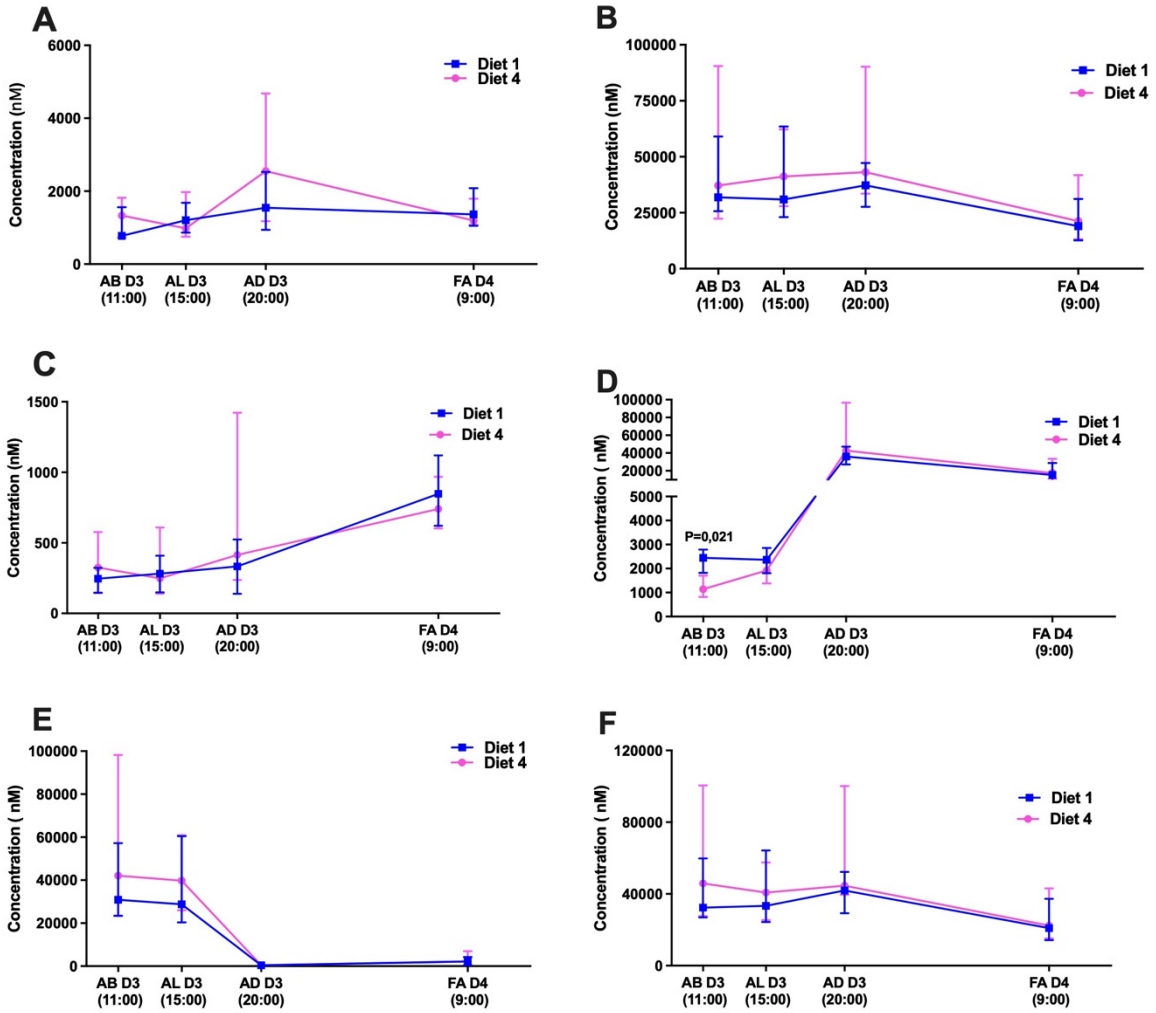

Figure 6. 3 Comparison of serum BA concentrations throughout the day for the same time points for diet 1 and diet 4. (A) Primary BAs, (B) Secondary BAs, (C)Unconjugated BAs, (D) Glycine conjugated BAs, (E) Taurine conjugated BAs and (F) Total BAs. Values are shown in median and interquartile range n=19. Differences between the same time points for different diets were determined by Wilcoxon signed rank test and False Discovery Rate (FDR). Significance was accepted at $P_{adj}<0.05$.

6.6.3 Variations of serum BAs throughout the day within and across diets

BA concentrations were analysed by comparing collection time points (Table 6.1) (Appendix 13, Figure 13.1, Figure 13.2, Figure 13.3, and Figure 13.4). No significant differences were found when these time points were compared across the postprandial study times. However, a few differences were observed when BAs were categorised into unconjugated, total glycine-conjugated, and taurine-conjugated groups and compared across postprandial states (see Table 6.4).

As shown in Table 6.1, most of the BA concentrations from AB did not exhibit higher peaks than other postprandial BA concentrations. However, some BAs had lower concentrations in the FA state than in the AB state. This was observed for 5-Cholenic acid-3β-ol ($P_{adj} < 3.4\times10^{-3}$) in diet 4, lithocholic acid (LCA) ($P_{adj} < 2.32\times10^{-2}$), glycolithocholic acid (GLCA) ($P_{adj} < 1.15\times10^{-3}$) for both diets, glycodeoxycholic acid (GDCA) ($P_{adj} < 1.15\times10^{-3}$) for diet 1, glycoursodeoxycholic acid (GCUDCA) ($P_{adj} < 7.95\times10^{-3}$) for diet 1 and ($P_{adj} < 7.95\times10^{-3}$) for diet 4, and taurodeoxycholic acid (TDCA) ($P_{adj} < 4.5\times10^{-3}$) for diet 1 and ($P_{adj} < 0.017$) for diet 4. Additionally, total taurine conjugated BAs presented a lower concentration in FA compared to AB in diet 1 ($P_{adj} < 4.92\times10^{-4}$) and diet 4 ($P_{adj} < 1.2\times10^{-3}$).

Simultaneously, most postprandial BA concentrations and those in the FA did not present differences; however, there were exceptions where all postprandial BA concentrations were significantly lower than FA for both diets. For instance, isolithocholic acid (ILCA) exhibited significantly higher concentrations than after breakfast (AB) ($P_{adj} < 8.56\times10^{-4}$), after lunch (AL) ($P_{adj} < 1.28\times10^{-3}$), and after dinner (AD) ($P_{adj} < 8.56\times10^{-4}$) for diet 1. The same pattern of results was found for diet 4 in AB ($P_{adj} < 4.38\times10$^-3), AL ($P_{adj} < 4.38\times10^{-3}$), and AD ($P_{adj} < 4.38\times10^{-3}$). Murocholic acid presented similar results in both diets: for AB ($P_{adj} < 8.58\times10^{-4}$ to 1.926×10^{-3}), AL ($P_{adj} < 1.28\times10^{-3}$), and AD ($P_{adj} < 1.926\times10^{-3}$) for diet 1; significant results for diet 4 were observed for AB ($P_{adj} < 1.84\times10^{-3}$), AL ($P_{adj} < 2.9\times10^{-3}$), and AD ($P_{adj} < 8.58\times10^{-3}$). Similarly, ursodeoxycholic acid (UDCA) exhibited higher concentrations in FA than in AB ($P_{adj} < 1.28\times10^{-3}$), AL ($P_{adj} < 0.11$), and AD ($P_{adj} < 0.02$) for diet 1. For diet 4, results were similar in AB ($P_{adj} < 1.8\times10^{-3}$), AL ($P_{adj} < 0.02$), and AD ($P_{adj} < 0.036$).

Similarly, taurochenodeoxycholic acid (TCDCA) presented higher concentrations in the fasting state than in the other postprandial time points studied. For diet 1, significant differences were found at AB ($P_{adj} < 8.58\times10^{-5}$), AL ($P_{adj} < 1.58\times10^{-3}$), and AD ($P_{adj} < 3.68\times10^{-3}$), whereas for diet 4, significant differences were found for AB ($P_{adj} < 1.29\times10^{-3}$), AL ($P_{adj} < 1.9\times10^{-3}$), and AD ($P_{adj} < 2.86\times10^{-3}$).

Additionally, unconjugated BAs were significantly lower in the fasting state (FA) than in AB for diet 1 ($P_{adj} < 8.58\times10^{-4}$) and diet 4 ($P_{adj} < 0.023$). Concentrations exhibited a similar pattern from AL to FA

for diet 1 ($P_{adj} < 0.021$) and diet 4 ($P_{adj} < 0.023$), and the comparison of AD with FA showed a similar trend solely for diet 1 ($P_{adj} < 0.039$).

The concentrations of BAs obtained postprandially, when compared against other postprandial concentrations for the same metabolite and the same diet, did not exhibit differences between them (Table 6.1, Table 6.2, Table 6.3 and Table 6.4). However, a summary of a few BAs presented in Tables 6.1 - Table 6.4, such as total primary BAs, total secondary BAs, total unconjugated BAs, total glycine-conjugated BAs, and total taurine-conjugated BAs, demonstrated some differences when their postprandial outcomes were compared between postprandial results for the same diet.

Table 6. 1. Summary of differences throughout the day and comparison between different time points for each diet (Part 1).

BAs	Type of diet	Differences throughout the day [1]	After breakfast versus After lunch [2,3]	After breakfast versus After dinner [2,3]	After lunch versus After dinner [2,3]	After dinner versus Fasting [2, 3]	After lunch versus Fasting [2,3]	After breakfast versus Fasting [2,3]
BA1. Ursocholanic Acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA3. 5-Cholenic acid-3β-ol	Diet 1	$3.52x10^{-3}$	NS	NS	NS	↑$1.17x10^{-2}$	↓$1.15x10^{-2}$	NS
	Diet 4	$4.6x10^{-4}$	NS	NS	NS	↑$3.68x10^{-3}$	↓$5.89x10^{-3}$	↓$3.4x10^{-3}$
BA5.Lithocholic acid	Diet 1	$1.4x10^{-2}$	NS	NS	NS	NS	↓$3.72x10^{-2}$	↓$2.32x10^{-2}$
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA6. Isolithocholic acid	Diet 1	$3.07x10^{-7}$	NS	NS	NS	↑$8.56x10^{-4}$	↑$1.28x10^{-3}$	↑$8.56x10^{-4}$
	Diet 4	$1.38x10^{-6}$	NS	NS	NS	↑$4.38x10^{-3}$	↑$4.38x10^{-3}$	↑4.38x10-3
BA7. Allolithocholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA18.3α-Hydroxy-7 Ketolithocholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA19. 3α-Hydroxy-12 Ketolithocholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA20. Murocholic acid	Diet 1	$2.85x10^{-7}$	NS	NS	NS	↑$1.926x10^{-3}$	↑$1.28x10^{-3}$	↑$8.58x10^{-4}$
	Diet 4	$6.25x10^{-6}$	NS	NS	NS	↑$1.84x10^{-3}$	↑$2.9x10^{-3}$	↑$1.84x10^{-3}$
BA 22. 5β-Cholanic acid-3β, 12α-diol	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA23. Deoxycholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS

[1] Differences throughout the day were analysed with the Skillings-Mack test. [2] Arrows indicate direction of BA concentrations accordingly to chronological order of time points.
[3] Differences between different time points for same diets were determined by Wilcoxon signed-rank test and Hommel post hoc correction. $P_{adj} < 0.05$ = significant. NS: Not significant.

Table 6. 2. Summary of differences throughout the day and comparison between different time points for each diet (Part 2).

BAs	Type of diet	Differences throughout the day [1]	After breakfast versus After lunch [2,3]	After breakfast versus After dinner [2,3]	After lunch versus After dinner [2,3]	After dinner versus Fasting [2, 3]	After lunch versus Fasting [2,3]	After breakfast versus Fasting [2,3]
BA24.Chenodeoxycholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA31. 12 Dehydrocholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA32.Ursodeoxycholic acid	Diet 1	$2.27x10^{-6}$	NS	NS	NS	↑0.02	↑0.011	$↑1.28x10^{-3}$
	Diet 4	$2.3x10^{-5}$	NS	NS	NS	↑0.036	↑0.02	↑1.8x10-3
BA35.Cholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA36. Hyocholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA39. Glycolithocholic acid	Diet 1	$1.9x10^{-5}$	NS	NS	NS	$↓2.23x10^{-3}$	$↓1.35x10^{-3}$	$↓1.15x10^{-3}$
	Diet 4	$7.9x10^{-5}$	NS	NS	NS	NS	NS	$↓1.15x10^{-3}$
BA41.Glycochenodeoxycholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA42.Glycodeoxycholic acid	Diet 1	$6.65x10^{-3}$	NS	NS	NS	$↓2.23x10^{-3}$	$↓1.32x10^{-3}$	$↓1.15x10^{-3}$
	Diet 4	$1.65x10^{-3}$	NS	NS	NS	↓0.036	↓0.0419	NS
BA43.Glycoursodeoxycholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA46.Glycocholic acid	Diet 1	$9.71x10^{-3}$	NS	NS	NS	↓0.021	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS

[1] Differences throughout the day were analysed with the Skillings-Mack test. [2] Arrows indicate direction of BA concentrations accordingly to chronological order of time points.
[3] Differences between different time points for same diets were determined by Wilcoxon signed-rank test and Hommel post hoc correction. $P_{adj} < 0.05$ = significant. NS: Not significant.

Table 6. 3. Summary of differences throughout the day and comparison between different time points for each diet (Part 3).

BAs	Type of diet	Differences throughout the day [1]	After breakfast versus After lunch [2,3]	After breakfast versus After dinner [2,3]	After lunch versus After dinner [2,3]	After dinner versus Fasting [2, 3]	After lunch versus Fasting [2,3]	After breakfast versus Fasting [2,3]
BA48.Taurolithocholic acid	Diet 1	$3.23x10^{-5}$	NS	NS	NS	↓$2.3x10^{-3}$	↓$1.5x10^{-3}$	↓$7.95x10^{-3}$
	Diet 4	$1.166x10^{-6}$	NS	NS	NS	↓$1.6x10^{-3}$	↓$3.4x10^{-3}$	↓$1.29x10^{-3}$
BA49.Glycohyocholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA50. Tauroursodeoxycholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA52.Taurochenodeoxycholic acid	Diet 1	$1.45x10^{-5}$	NS	NS	NS	↑$3.68x10^{-3}$	↑$1.58x10^{-3}$	↑$8.58x10^{-5}$
	Diet 4	$1.31x10^{-6}$	NS	NS	NS	↑$2.86x10^{-3}$	↑$1.9x10^{-3}$	↑$1.29x10^{-3}$
BA53.Taurodeoxycholic acid	Diet 1	$2.85x10^{-3}$	NS	NS	NS	↓0.012	↓$3.36x10^{-3}$	↓$4.5x10^{-3}$
	Diet 4	0.014	NS	NS	NS	NS	NS	↓0.017
BA55.Taurohyocholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
BA59.Taurocholic acid	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
Total Primary BAs	Diet 1	$9.84x10^{-3}$	NS	↑0.044	NS	NS	NS	↑0.026
	Diet 4	NS	NS	NS	NS	NS	NS	NS
Total Secondary BAs	Diet 1	$1.85x10^{-2}$	NS	NS	NS	↓0.041	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS
Total Unconjugated BAs	Diet 1	$1.37x10^{-6}$	NS	↑0.026	NS	↑0.039	↑0.021	↑$8.58x10^{-4}$
	Diet 4	$7.91x10^{-3}$	NS	NS	NS	NS	↑0.038	↑0.023

[1] Differences throughout the day were analysed with the Skillings-Mack test. [2] Arrows indicate direction of BA concentrations accordingly to chronological order of time points.
[3] Differences between different time points for same diets were determined by Wilcoxon signed-rank test and Hommel post hoc correction. $P_{adj} < 0.05$ = significant. NS: Not significant.

Table 6. 4. Summary of differences throughout the day and comparison between different time points for each diet (Part 4).

BAs	Type of diet	Differences throughout the day [1]	After breakfast versus After lunch [2,3]	After breakfast versus After dinner [2,3]	After lunch versus After dinner [2,3]	After dinner versus Fasting [2, 3]	After lunch versus Fasting [2,3]	After breakfast versus Fasting [2,3]
Total Glycine conjugated	Diet 1	$4.61x10^{-7}$	NS	↑$5.04x10^{-4}$	↑$5.04x10^{-4}$	↓$3.32x10^{-2}$	↑$4.29x10^{-4}$	↑$4.29x10^{-4}$
	Diet 4	$1.78x10^{-8}$	NS	↑$7.61x10^{-4}$	↑$6.93x10^{-4}$	↓$5.07x10^{-4}$	NS	↑$5.07x10^{-4}$
Total Taurine conjugated	Diet 1	$3.65x10^{-10}$	NS	↓$4.29x10^{-4}$	↓$4.29x10^{-4}$	↑$5.43x10^{-3}$	↓$4.29x10^{-4}$	↓$4.92x10^{-4}$
	Diet 4	$4.06x10^{-9}$	↓$1.28x10^{-3}$	↓$1.28x10^{-3}$	↓$3.29x10^{-3}$	↑$4.89x10^{-3}$	↓$1.92x10^{-3}$	↓$1.2x10^{-3}$
Total Bile acids	Diet 1	NS	NS	NS	NS	NS	NS	NS
	Diet 4	NS	NS	NS	NS	NS	NS	NS

[1] Differences throughout the day were analysed with the Skillings-Mack test. [2] Arrows indicate direction of BA concentrations accordingly to chronological order of time points.
[3] Differences between different time points for same diets were determined by Wilcoxon signed-rank test and Hommel post hoc correction. $P_{adj} < 0.05$ = significant. NS: Not significant.

6.6.4 Inter-individual variability in serum BAs profile of selected participants

The selected BA concentrations exhibited transient variations throughout the day and at different times (Figures 6.4 and 6.5). The individual trajectories of the selected participants displayed varying responses compared to the median response, which was illustrated in red in both figures alongside their respective identifiers for each metabolite and mealtime.

Concentrations of CA at AB demonstrated a spectrum of responses, ranging from 46.04 nM to 437.88 nM following the consumption of diets 1 and 4, characterised by a higher concentration of CA after the intake of diet 4 compared to diet 1 (Figure 6.4E). The responses of selected individuals varied in their transitions from AB to AL, with some exhibiting a decrease in CA levels, while others showed an increase independent of diet intake. For instance, ID 3 and ID 7, following the intake of diet 4, exhibited a minor reduction in the concentration of CA, whereas ID 15 demonstrated a slight increase with the same diet intake. Similarly, after the consumption of diet 1, the transition from AB to AL revealed consistent concentrations among subjects.

In the transition to AD, an increase in CA concentrations was observed in most participants who consumed diet 4; however, ID 15 exhibited a dramatic increase, approximately 16-fold compared to the concentration FA — from 149.6 nM to 2488.64 nM. Following the consumption of diet 1, the CA concentrations in AD were higher than those in AL. All quantified responses in FA fell within a range of similar concentrations, regardless of the diet consumed. Exceptionally, ID 7, who consumed diet 1, experienced an increase in the CA concentration from 86.52 nM to 1404 nM.

Individual responses to the GCA demonstrated variability among participants, deviating from the median response (Figure 6.4F). At AB, GCA responses did not follow a consistent trend regardless of the participant or diet consumed. For instance, the response of participant ID 3 to diet 4 was five-fold higher than compared to diet 1. At AL, BA concentrations exhibited diverse responses across all selected participants irrespective of diet intake (Figure 6.4J). For example, ID 3, which had consumed diet 1, presented a concentration three times higher than the same individual's response to diet 4. Conversely, responses to diets 1 and diet 4 did not exhibit a uniform pattern at AL, with variations that increased or decreased the responses for each diet.

Responses in AD tended to show similar patterns between diets, where GCA concentrations were consistently higher following the consumption of diet 4 than diet 1 across all individuals. Thirteen hours later, responses at FA indicated higher concentrations of GCA when participants consumed diet 4. Nevertheless, some transitions were characterised by either an increase or decrease in concentrations

compared to the levels in AD. For instance, ID 7, after consuming diet 4, peaked at a concentration of 1404.28 nM, in stark contrast to 81.32 nM following the consumption of diet 1.

Variability in the concentrations of TCA, was depicted in Figure 6.4O, demonstrated diverse responses among individuals and diets. For instance, ID 3 and ID 15 exhibited similar response patterns following the intake of diets 1 and 4. Conversely, ID 7 displayed an inverse pattern relative to other subjects. The transition levels from AB to AL varied, with some instances showing an increase, whereas others showed a decrease in concentrations, independent of diet intake. Notably, ID 3 experienced a sharp increase after consuming diet 1, whereas the same individual's response to diet 4 was characterised by a significant reduction. Some TCA levels remained stable or decreased compared to AL levels, with a tendency towards stabilisation within a range of 2 nM to 12.24 nM for both diets. Similar responses were observed for FA, except for ID 7, following the consumption of diet 4. A recurring pattern was that each subject who consumed diet 4 exhibited higher concentrations of this metabolite than those who consumed diet 1.

Similar patterns between the concentrations of GCA and TCA were shown in Figure 6.3J and 6.3O. However, the proportions of these bile acids differed, with GCA concentrations were higher compared to TCA.

DCA concentrations at AB ranged from 58.2 nM to 1062 nM, covering all diets and subjects (Figure 6.5E). Diverse responses were observed at AL, with DCA levels either increasing or decreasing from the baseline concentrations noted at AB. Notably, the response of participant ID 7, who consumed diet 1, was significantly higher than that of other subjects and diets. For all participants, AD concentrations were higher in those who consumed diet 1 than in diet 4 for the same individual. However, for subject ID 3, this difference was less pronounced as was shown in Figure 6.4E, but the same trend was marginally evident (ID 3 diet 1 at 709.44 nM versus diet 4 at 693.16 nM). Concentrations of AD in these subjects were higher than those of FA. This decline in concentration was observed in all the participants.

GDCA concentrations exhibited variability throughout the day among participants, with each individual displaying distinct trends irrespective of diet consumption and time of day (Figure 6.5J). Levels of GDCA among subjects ranged from 226.31 nM to 1353.8 nM at AB, with these metabolites fluctuating during the postprandial times without a discernible pattern relative to diet intake and demonstrating a broad range of variation. Notably, concentrations of GDCA tended to converge at AD, and ranged from 496.2 nM to 846.9 nM, independent of previous values and diet. Most subjects exhibited a reduction in FA values regardless of diet. However, ID 7, who consumed diet 4, presented a sharp increase in GDCA concentration.

The levels of TDCA demonstrated variability in AB values (Figure 6.5O) without a discernible response to diet. Upon transition to AL, a distinct pattern emerged, with subjects who consumed diet 1 exhibited higher TDCA concentrations. Similarly, during the AD mealtime, individuals consuming diet 1 again displayed elevated levels of TDCA compared to those on diet 4. For instance, ID 7, after consuming diet 4, presented a value very similar to that of ID 3 when consuming diet 1. Individual responses to FA either decreased or increased relative to AD; however, these concentration responses were not associated with the intake of any specific diet. Notably, for ID 3 and ID 15, regardless of the diet, the values were remarkably similar.

The results for GDCA and TDCA indicated differing proportions of BA concentrations, yet their geometrical trajectories across mealtimes bore striking resemblance. However, the concentrations of GDCA were 20 times higher compared to those of TDCA.

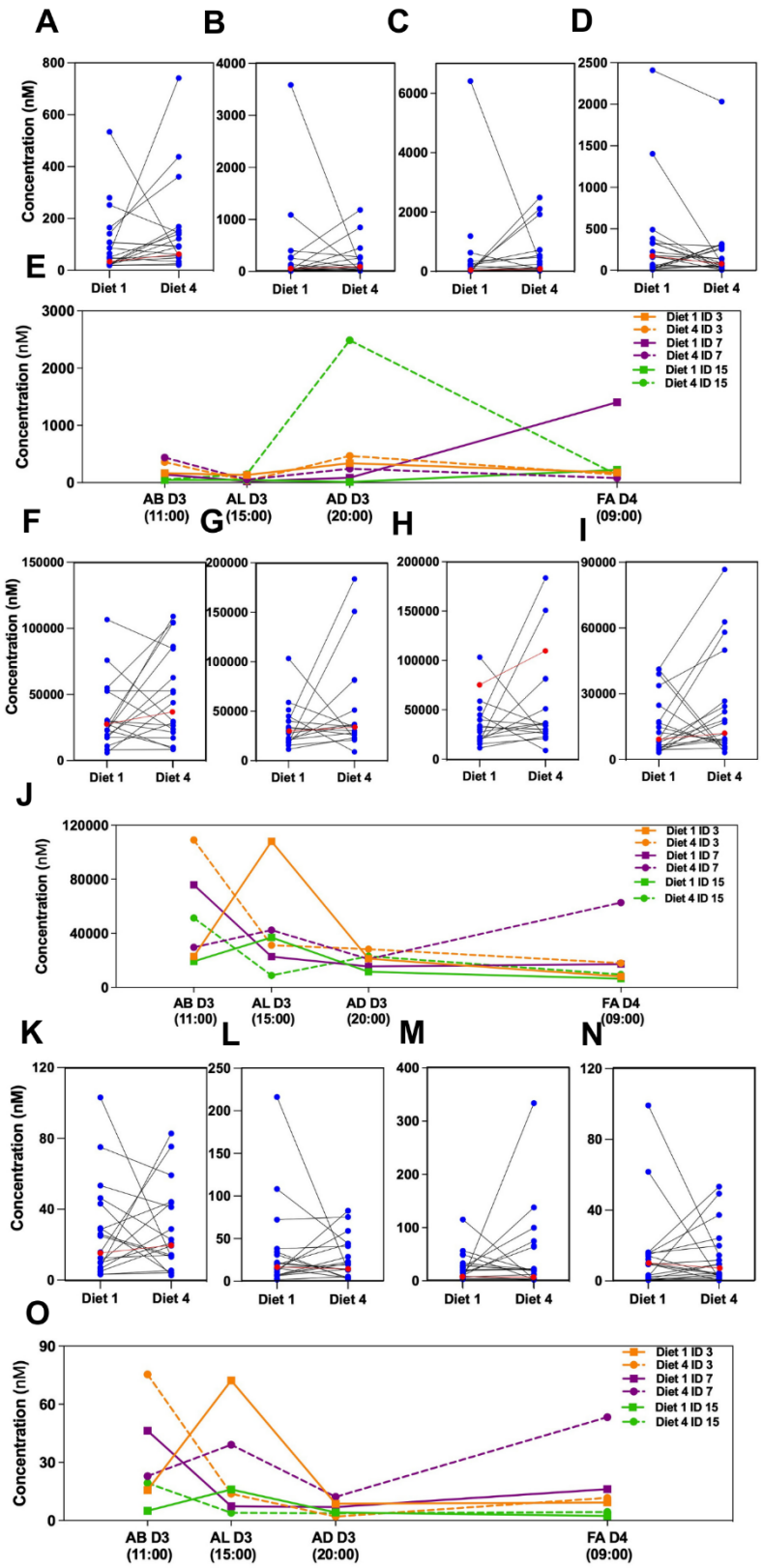

Figure 6.4 Serum variation response in selected BAs between participants.
A)Cholic acid inter- variation after breakfast on day 3.
B) Cholic acid inter-individual variations after lunch on day 3.
C) Cholic acid inter-individual variation after dinner on day 3.
D) Cholic acid inter-individual variation in selected participants.
E)Cholic acid variation in selected participants.
F) Glycocholic acid inter- variation after breakfast on day 3.
G) Glycocholic acid inter-individual variations after lunch on day 3.
H) Glycocholic acid inter-individual variation after dinner on day 3 .
I)Glycocholic acid inter- individual variation in selected participants.
J) Glycocholic acid variation in selected participants.
K) Taurocholic acid inter- variation after breakfast on day 3.
L) Taurocholic acid inter-individual variations after lunch on day 3.
M) Taurocholic acid inter-individual variation after dinner on day 3. N)Taurocholic acid inter-individual variation in selected participants.
O) Taurocholic acid variation in selected participants.

After breakfast on day 3 (AF D3), after lunch on day 3 (AL D3), after dinner on day 3 (AD D3) and fasting on day 4 (FA D3). In inter-individuals variations graphics, blue dots represent results of 19 participants and red dots and lines indicate median.

Differences between the same time points were determined by Wilcoxon signed rank test and False Discovery Ratio (FDR) post-hoc correction. Significant differences are indicated by Padj <0.05.

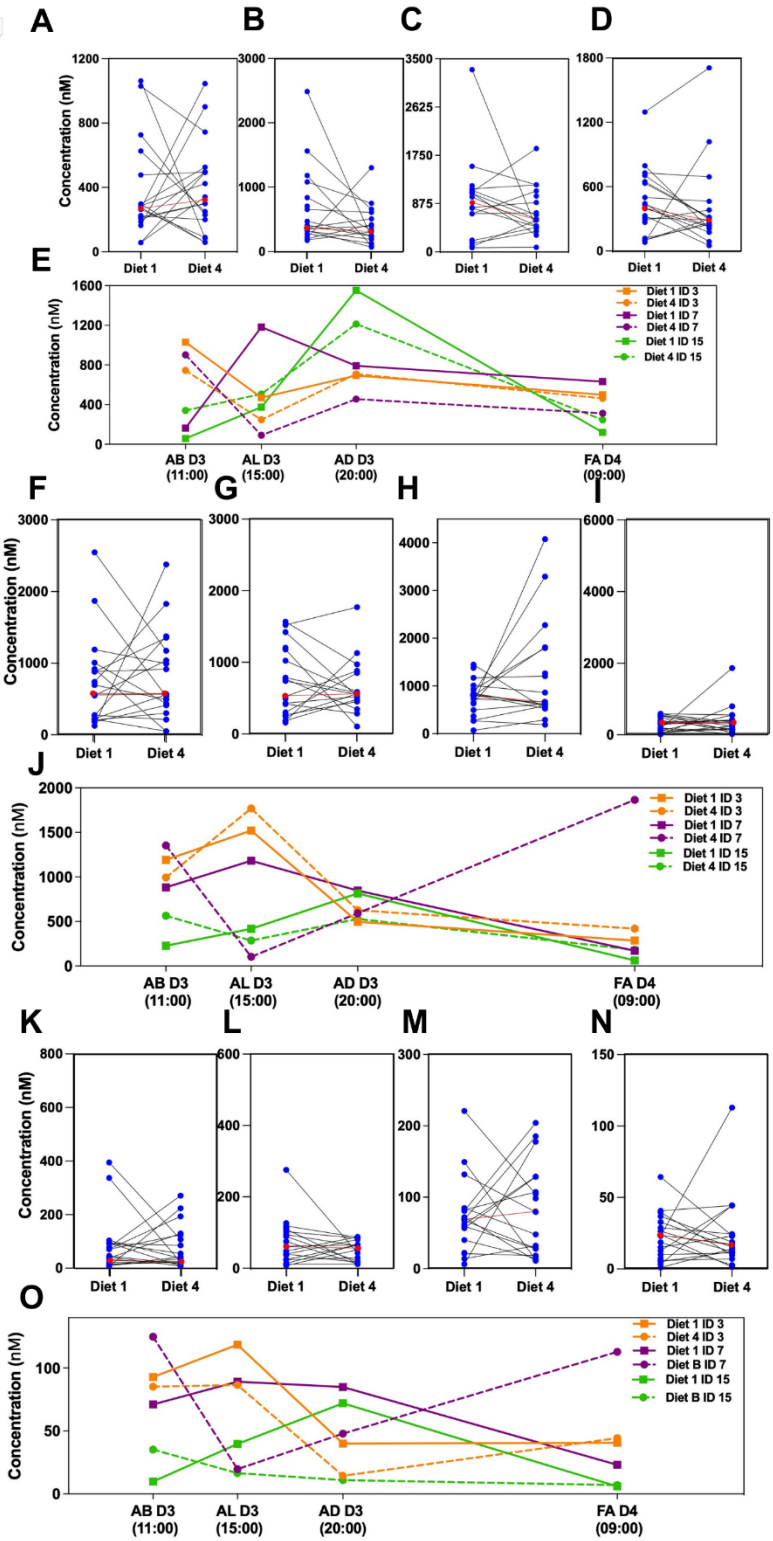

Figure 6.5 Serum variation response in selected BAs between participants.
A)Deoxycholic acid inter- variation after breakfast on day 3.
B) Deoxycholic acid inter-individual variations after lunch on day 3.
C) Deoxycholic acid inter-individual variation after dinner on day 3.
D) Deoxycholic acid inter-individual variation in selected participants.
E)Deoxycholic acid variation in selected participants.
F) Glycodeoxycholic acid inter- variation after breakfast on day 3.
G) Glycodeoxycholic acid inter-individual variations after lunch on day 3.
H) Glycodeoxycocholic acid inter-individual variation after dinner on day 3 .
I) Glycodeoxycholic acid inter- individual variation in selected participants.
J) Glycodeoxycocholic acid variation in selected participants.
K) Taurodeoxycholic acid inter- variation after breakfast on day 3.
L) Taurodeoxycholic acid inter-individual variations after lunch on day 3.
M) Taurodeoxycholic acid inter-individual variation after dinner on day 3 .
N)Taurodeoxycholic acid inter- individual variation in selected participants.
O) Taurodeoxycholic acid variation in selected participants.

After breakfast on day 3 (AF D3), after lunch on day 3 (AL D3), after dinner on day 3 (AD D3) and fasting on day 4 (FA D3). In inter-individuals variations graphics, blue dots represent results of 19 participants and red dots and lines indicate median.

Differences between the same time points were determined by Wilcoxon signed rank test and False Discovery Ratio (FDR) post-hoc correction. Significant differences are indicated by Padj <0.05.

6.6.5 Intra-individual variability in serum BAs profile of selected participants

BA concentrations displayed varied fluctuations throughout the day, suggestd that meal intake might have influence BA concentrations measured two hours post-consumption (Figure 6.4). Specifically, for CA among selected participants, concentrations spanned from 46.04 nM to 437.8 nM for both diets, with higher levels observed following the consumption of diet 4 by the same participants. For instance, participant ID 15 demonstrated similar concentrations of CA, at 55.28 nM, after consuming diet 4, whereas the response from diet 1 diminished to 46.04 nM. Conversely, ID 7 exhibited a CA concentration of 437.8 nM following diet 4 intake, which reduced to 141.7 nM with the consumption of diet 1.

Transitions in CA levels from AB to AL did not exhibit a uniform pattern among individuals; some showed an increase, while others displayed a decrease in response values. For instance, for ID 3 and ID 7, values decreased regardless of diet, but this reduction was notably more pronounced following the intake of diet 4, indicated the potential impact of dietary interventions on these subjects. Conversely, the response from ID 15 was the opposite, with the reduction in concentrations being more marked after the consumption of diet 4 compared to diet 1. However, it was crucial to note that because of the scale in Figure 6.3E, these changes were not readily discernible. Values at AD varied across a broad range, tended towards stabilisation, yet a discernible trend was observed in response to diet 1, which resulted in higher values compared to diet 4. Notably, ID 7 experienced a sharp increase in CA concentration after consuming diet 4 (Figure 6.4E).

Concentrations of GCA demonstrated varied responses across participants, underlining a broad spectrum of variability in the responses at AB (Figure 6.4J). This variability indicated that each participant exhibited a distinct response following breakfast, regardless of the diet consumed. Between AB and AL, a reduction in GCA concentration was observed for ID 3 and ID 15 following the consumption of diet 4. Conversely, when diet 4 was consumed, ID 7 showed a similar response to the baseline. In contrast, a marked increase was noted when ID 3 consumed diet 1, whereas moderate increases were observed for ID 7 and ID 15 upon consuming diets 4 and 1, respectively.

GCA concentrations appeared to stabilise at AD, as depicted in Figure 6.4J. Notably, for all participants, the concentrations were higher after the consumption of diet 4 than diet 1. Following a 12-hour fast, GCA concentrations decreased, with the exception of ID 7, who, after consuming diet 4, did not follow this trend.

Responses to TCA at AB did not exhibit a uniform pattern, with significant variability between individuals at two hours post-meal (Figure 6.4O). Notably, a peak in concentration was observed in participant ID 3 following the consumption of diet 4, in stark contrast to the response elicited by diet 1.

Conversely, at AB, ID 7 displayed a response opposite to that of ID 3, with TCA concentrations were higher after consuming diet 1 and lower after diet 4. The responses at AL for TCA showed a geometrical pattern similar to that described for GCA, albeit with different concentration proportions.

Measurements taken in AD revealed a pattern of convergence for almost all subjects; however, ID 7 exhibited the highest levels of TCA following the consumption of diet 4. The transition in concentrations 12 hours post-last food intake reflected a consistent trend across all subjects, except for ID 7, who, after consuming diet 4, peaked at 53.36 nM. Interestingly, the responses observed by participant ID 3 presented a similar geometric pattern for both GCA and TCA throughout the day, though the proportions varied (Figures 6.4J and 6.4O).

Levels of DCA displayed a wide range of variations, lacking a consistent response pattern irrespective of the diet consumed (Figure 6.5E). For instance, at baseline (AB), ID 7 and ID 15 exhibited higher concentrations when diet 4 was consumed compared with diet 1. In contrast, ID 3 showed a higher DCA concentration in diet 1 than in diet 4. The transition to AL varied among all subjects, with concentrations decreased from AB in ID 3 for both diets. Conversely, ID 15 experienced an increase in DCA concentration, independent of diet. However, ID 7 reached peak concentrations from AB to AL when diet 1 was consumed, whereas a decrease from AB to AL was noted with diet 4. AD concentrations increased for ID 3 and ID 15, but ID 7 showed an increase with diet 4 and a decrease with diet 1. At this time point, AD, all responses were higher in diet 1 than in diet 4. A common trend towards a convergence in concentrations was observed at the FA time point, where all responses decreased, tending towards a similar range regardless of the diet.

The individual responses to GDCA at AB did not demonstrate a uniform pattern, with concentrations varied significantly from 563.71 nM to 1353.81 nM (Figure 6.5J). For instance, ID 7 exhibited a higher concentration following the intake of diet 4 than diet 1 at AB. The selected individuals showed varied responses at AL, characterised by either an increase or decrease that was not dependent on the diet. ID 7 and ID 15 presented a higher concentration of this metabolite after consuming diet 4, while the response from ID 3 was the opposite. The transition from AL to AD showed both reductions and increases in concentrations in the subjects, independent of diet. However, ID 7, after consuming diet 1, exhibited a decrease in concentration, whereas an increase was observed with the intake of diet 4, suggested that BA concentrations tended to converge within a range at AD. Similarly, this convergence was observed in FA, with a decrease in the values for this metabolite. Nonetheless, ID 7, following the consumption of diet 4, displayed a sharp increase, reaching a concentration of 1865.07 nM, contrary to the responses of the other selected participants.

TDCA levels displayed varied responses at AB, differed according to diet and individual (Figure 6.5O). For instance, ID 7 and ID 15 exhibited higher concentrations when consuming diet 4 compared to diet 1. In contrast, ID 3 demonstrated the opposite response at AB. Concentrations of TDCA showed a wide range at AL; however, despite this variation, all concentrations were higher after the intake of diet 1 compared to diet 4. The transition to AD was characterised by varyied fluctuations in values for each ID and diet, but the trend of presenting higher concentrations after the consumption of diet 4, as observed in AL, persisted. The fasting responses for ID 3 and ID 15 showed very similar values, tendd towards a similar range. However, this trend was not observed with ID 7, which exhibited opposite responses, showed a dramatic increase after consuming diet 4, while a decrease was noted after the intake of diet 1.

6.6.6 Urine

Urine samples were collected at different time points, as described in (Chapter 2 Section 2.2.4, Table 2.3). Samples were prepared and analysed for BAs using LC-MS (98). Time spot samples presented very low concentrations of BAs; most of them seemed to be below the limit of detection of the method, but IS concentrations were detectable, and QCs were stable throughout the sample run. After that, the identity of the sample groups was revealed, and a directed, selected, and intentional analysis in Target Lynx was conducted again with urine sample time groups to ensure proper analysis of the samples. Again, concentrations of BAs from samples at time points 1, 2 and 3 (see Table 6.5) were not possible to retrieve with the BAs method of this study. A few samples presented detectable concentrations of BAs (approximately four samples) that were insufficient for statistical comparisons between groups and time points.

Subsequently, urine samples from 24-hour collections were randomised for LC-MS analysis. After sample analysis, data extraction using Target Lynx was acceptable for the first 29 samples of the run that included calibration curves. However, after sample number 9 from the 24-hour urine samples set, the chromatogram peaks became flat, and concentrations of neither bile acids nor internal standards were detected. After checking the sample analysis in the instrument before this urine analysis, BA faecal samples were analysed using the same column. It was highly likely that column blockage generated a carryover effect from the samples analysed previously to urine time spots and 24-hour urine samples. This affected the sensitivity of detection, resulted in poor elution and detection of the studied metabolites. Therefore, the BA concentrations could not be adequately quantified in this subset of samples.

During all the urine sample runs, the sample analysis and instrument parameters were monitored every 8 to 12 hours (solvent levels, temperature, gas pressure, MRM, RT). No apparent disturbances were

present in the checks during the first set of sample analysis (time spots) or the second sample set of 24-hour samples. It was not possible to re-run the samples because the data treatment occurred a couple of months after their analysis by LC-MS, and no samples were left. This column blockage could have been responsible for the low concentration of BAs detected in the urine in the time-spot samples. In contrast, in the detection of urine for 24-hour period, it was not possible to obtain them due to carryover and blockage that occurred during the analysis using the LC-MS instrument.

Table 6. 5 Urine collection times and details.

Urine sample time group	Mealtime spot	Time	Total hours of collections
1	Breakfast to Lunch	9:00 to 13:00	4 hours
2	Lunch to Dinner	13:00 to 18:00	5 hours
3	Dinner to Fasting	18:00 to 9:00 (+24 hours)	15 hours
4	24 hours	Pooling of 3-time spots	24 hours

6.7 Discussion

To my knowledge, this is one of the first studies to explore the production of BAs after the consumption of two dietary patterns that differ significantly in their nutritional composition under controlled conditions. For example, Table 4.1 shows that the amount of total fat consumed per day in diet 1 corresponds to 59 g versus 113 g in diet 4. Despite these differences in diet composition, BA concentrations after 2 hours of each meal and fasting presented very few significant differences. Total glycine-conjugated BAs were higher in diet 1 than in diet 4, whereas UCDA at AL was higher in diet 1 than in diet 4. The BAs fluctuated throughout the day. This suggests that most of the BAs concentrations were not significantly affected when they were compared between diets after 2 hours after meal intake or fasting.

Comparisons of BA concentrations taken for each collection time for each diet did not present differences between postprandial time points, but only for a few BAs categories, such as unconjugated, glycine conjugated, and taurine conjugated. However, when postprandial time points were compared with fasting, some differences were found. This suggests that each BA followed its own geometrical trajectory independent of diet consumption, and fasting seemed to provoke changes in these geometrical trajectories. Simultaneously, the geometric trajectories exhibited unique patterns when different individual responses were considered.

Faecal samples were not collected, and urine BA concentrations could not be obtained from the studied samples. In this study, BAs seemed to be more influenced by time of day than diet. Therefore, it is necessary to understand the impact of time sampling to evaluate the potential effects of food intake on temporal BA concentrations. This would help to understand how specific dietary interventions may modulate circulating BAs to understand their impact on health, which could be translated into personalised nutritional interventions and management of chronic diseases.

6.7.1 Characteristics of the patients as a factor for BAs responses

As mentioned in Chapter 4, individuals presented different classifications according to their BMI, ranging from normal to obese. The inclusion criteria for all these recruited subjects (9 female and 10 men) presented normal glucose metabolism, lipid metabolism, and liver function test parameters (Table 4.2) (180, 181, 239, 388, 389). Previous authors have described that sex is a factor to consider in BAs metabolism that generates differences in their concentration in their postprandial systemic circulation response (333, 384, 390). In addition, the BAs responses of women are influenced by menopausal status. Hormonal variations during the menstrual cycle seem to play a role in BA alterations in women, such as gallstones. Conversely, some authors have reported no differences in BA concentrations during fasting (391). However, differences in sex hormones and sex have been described as factors influencing

BA synthesis (392). Hormonal variations during the menstrual cycle appear to play a role in BA alterations in women as gallstones (393). The analysis of these samples was part of a sub-study; information about the sex of the subjects was not provided for further study.

6.7.2 Diet and time effect on BA concentrations

Concentrations of BAs fluctuated during the time of the day, but these fluctuations seemed to be more dependent on the time of the day than the dietary influence caused by the intake of the studied diets. The fluctuation of BAs during the day has been described previously in human studies, where it has been shown that BAs present a diurnal rhythm and peak in their levels of systemic circulation after food ingestion (333, 394).

In our comparisons of BA concentrations following the intake of diets at each time point, the concentrations of total glycine-conjugated BAs at baseline (AB) were higher in diet 1 than in diet 4. However, considering that breakfast was consumed at 9 am and samples were collected at 11 am, the appearance of BAs in circulation during this period is unlikely to be directly caused by food intake. The presence of BAs in circulation results from bile secretion into the small intestine and their absorption, which escapes from the portal circulation (395). Similarly, given that the oro-cecal transit time takes between 10 to 12 hours, it is very unlikely that the food reached the small intestine within two hours (270). Hence, the influence of food intake on the concentrations of glycine-conjugated BAs seems unlikely. In previous studies, marked responses of glycine-conjugated BAs have been observed in a shorter timeframe; however, in those instances, challenge liquid meals were investigated, and their transit time through the digestive system was shorter than that of whole foods, as observed in our study (333, 384). Therefore, the concentrations of BAs at AB are more likely modulated by the BA pool than by the intake of breakfast itself.

UCNA exhibited a significant difference, being higher at AL and two hours after the consumption of diet 1, which contained half the fat compared to diet 4. Breakfast, consumed at 9 am, had 13 g of fat in diet 1, whereas the breakfast in diet 4 included 16 g of fat (see Table 4.1). Subsequently, a mid-morning snack was consumed at 11 am, containing 1 g of fat in diet 1 and 13 g of fat in diet 4. It appears plausible that blood samples taken at 15 hours (two hours AL) and UCNA concentrations may be influenced by the diet consumed earlier. The role of this metabolite in postprandial contexts remains elusive, yet it has been proposed as a potential therapeutic agent for Parkinson's disease treatment in neural model systems (396).

The different geometric patterns observed in BAs could be related to their intestinal biotransformations, the capacity for clearance of these metabolites at other times of the day, gallbladder emptying during

the day, and other physiological conditions (333, 354, 394, 395, 397, 398). Therefore, this may explain the difference in geometric patterns between BAs and concentrations found in venous blood in this study. It is crucial to acknowledge that the geometric patterns observed in this study are not directly comparable to the findings from other investigations. This discrepancy arises due to variations in sampling times and meal intake schedules, which were conducted on a different temporal scale in this investigation. Such differences in methodology underscore the importance of considering the specific context of each study when interpreting and comparing results.

In the case of ILCA, MCA, UDCA, TCDCA, and unconjugated BAs, their concentrations were higher during fasting after consuming both diets. This suggests that these fasting BAs can have different origins, including spillover from portal circulation, were derived from the pool of BAs sequestered in the liquid content of the small intestine, or these concentrations may originate directly from hepatocyte efflux or were part of unconjugated colonic bile acids or increased microbial transformations of BAs coupled with a lower clearance of BAs from peripheral circulation (384, 394). Simultaneously, it has also been suggested that small quantities of BAs can be released from the gallbladder by the action of the migrating motor complex during the day (MMC) (399). In the case of TCDCA, the high concentration during fasting could be related to the conjugation of CDCA in the liver with taurine during an overnight period coupled with less clearance of BAs from peripheral circulation and potential efflux from hepatocytes after BAs synthesis (384). The reason for this increase under fasting conditions is not fully understood. However, evidence suggests that their elevation could be related to their affinity for or agonist of the TGR5 receptor, which regulates glucose and BA metabolism (400, 401).

Different proportions of CA and CDCA were found during the day; CA concentrations were lower than CDCA in all studied mealtimes, independent of diet (Figure 6.2F and Figure 6.2I). These high levels of CDCA in the serum could be related to less efficient hepatic clearance of CDCA from peripheral venous blood. Still, it has also been reported that CDCA is the dominant BA in some adults (102, 395). This could be explained by a significant influx of CDCA from the portal system towards the systemic circulation due to its higher concentrations compared to CA. However, this suggests that CDCA is highly absorbed at the small intestine level in fasting and mealtime states by different passive mechanisms in the proximal small intestine or its synthesis rate is higher than that of CA. Concentrations of CA and CDCA tended to have some variations during the day, but these fluctuations were stable throughout the time and not statistically significant.

It is important to highlight that the BAs serum results of this study provide information about the fluctuation in levels of BAs at specific postprandial mealtimes or fasting. However, these results do not allow for conclusions regarding the intestinal transport of BAs. It is known that 95% of BAs excreted

into the small intestine are reabsorbed through passive diffusion, uniporters, or Na+-coupled symporters, and then returned to the liver via the portal blood (402).

The presence of BAs in peripheral blood is characterised by their short circulation time, and their concentration in circulation depends on whether they are continuously absorbed at the intestinal level (354). As mentioned previously, postprandial blood samples were taken after two hours of food intake. Previous studies examining the changes of BAs after postprandial food challenges have shown that BAs peaks in circulation can be reached after 30 to 60 minutes, and significant reductions occur after 90 minutes (333). Therefore, the BA responses observed after 120 minutes will not necessarily represent the effects of diet consumption two hours before systemic circulation, since the half-life of BAs is very short. Studies of labelled BAs with intravenous injections have reported the half-life of these biomarkers for a period ranging between 4 and 36 minutes in patients with normal hepatic function (102, 354).

However, this research studied BA responses from regular meals rather than a single nutrient or liquid meal with a faster digestive transit time. The previously mentioned studies explored isolated nutrients, liquid meals, or labelled biomarkers that provide insights under controlled physiological conditions that may not reflect the response after normal meal consumption (62, 333, 384, 397). In this study, the impact of meals within the context of healthy and unhealthy diets and their effects on BA concentrations were investigated. However, blood samples were collected only 2 hours after meal intake, which may not accurately reflect the influence of BAs in the serum after dietary intake due to their transient presence. However, it was possible to observe different geometrical patterns for each studied BAs, which provided important insights that suggested that these metabolites possess their own daily fluctuations. For a better understanding of the dynamics of BAs in response to diets considering their brief half-life, additional time points for blood sample collection within 2 hours and after would have been necessary. Therefore, a better representation of BAs dynamics in serum could have been illustrated by considering the digestion times associated with food consumption and times through the digestive system (270).

6.7.3 Diurnal variations in BA Levels: comparing fasting states with mealtime peaks

Most of the BAs presented their highest concentrations between the periods of AB and AD, compared to FA. Simultaneously, most of the BAs did not show a peak at AB, which supports the evidence that food reaches the duodenum after 5 hours or more to have a potential effect that would be reflected in the BAs that bypass the portal system into the systemic circulation (270). It is unknown if the BAs concentrations were higher or lower within the window period of 2 hours in this study. Postprandial studies of BAs have reported peak concentrations of circulating BAs in a window of time of 30 to 120 minutes, studying liquid meals as food challenges composed of fat, glucose, or both that can reach the duodenum faster than complete meals (62, 270).

BAs are continuously secreted, absorbed, and re-secreted by the enterohepatic circulation. The process begins with the absorption of bile acids from the distal part of the intestine, which then accumulate to form a significant quantity known as the bile acid pool (102). This pool typically amounts to about 2 to 3 grams in adults. Because this pool is recycled multiple times during each meal, the amount of bile acid secreted can reach 4 to 6 grams per meal, totalling 12 to 18 grams daily (102). The synthesis of bile acids is relatively low, averaging around 0.3 grams per day, but is significantly increased due to their recycling through enterohepatic circulation.

Essentially, BAs are recycled to digest meals, complementing them with a minor portion of bile acids from the peripheral circulation that bypass liver extraction. Following meal consumption, bile acids, as components of bile, are discharged into the duodenum triggered by various gut hormones such as cholecystokinin (CKK) and motilin (403, 404). These bile acids are then absorbed towards the end of the small intestine, transported back to the liver, and secreted again into the bile (102).

Food digestion is affected by different aspects such as food mastication, digestion processes and colonic transit time (244, 248, 270, 274, 275). When chime reaches the small intestine after approximately 2 to 5 hours, activating the release of CKK and BAs from the gallbladder and emulsion processes are initiated for the fat digestion processes (107). Transit time in the small intestine can vary between 1 to 5 hours. During this time, reabsorption of BAs is taken into the portal circulation; subsequently, a small portion of BAs can reach the large intestine after 6 to 8 hours (102). Here, again BAs can be deconjugated and converted into secondary BAs for being transported back to portal vein (105). Considering these aspects, BAs concentrations in serum AB are more likely to be derived from the pool of BA, considering that the samples were taken after two hours of food intake. Moreover, it seems reasonable to find high concentrations of BAs between AL and AD owing to digestive processes. This is consistent with previous results reporting that BA peaks after midday or evening meals (394, 398).

Most of the BAs, predominantly conjugated, presented higher concentrations during the period between AL and AD. These higher concentrations seem plausible, considering the period of hours after the first food intake in the morning. In the AD sampling, 11 hours had passed since breakfast intake and 7 hours after lunch intake, which contains the higher fat content of the day for both diets. Therefore, most BAs at AD time are expected to have peaked, as in the case of primary, secondary, and total BAs. Samples taken during fasting, with a window of food intake of 12 hours, presented variable responses compared with postprandial meal points.

6.7.4 Comparative analysis of inter- and intra-individual variability of selected subjects in serum BAs profiles following identical dietary interventions

Differences in BAs responses were observed between the selected subjects when they were exposed to the same diet. Variability in the response of BAs in subjects exposed to the same dietary challenges as OGTT or OLTT has been previously reported (384). These interindividual differences can be associated with the physical and chemical digestive processes initiated in the mouth and finished in the large intestine (270), but also with the effect of the gut microbiota in the digestive and metabolic processes (277, 279).

The microbiome is unique for each individual, and it has been identified as a contributor to the variability of responses to triglycerides, glucose, and insulin when people consume identical meals (405). Different concentrations of BAs between individuals can be influenced by the composition of the meals (61, 333), and which could explain the differences found between the selected subjects that consumed diet 1 and diet 4. Notably, the selected subjects, GCA and TCA, presented similar geometrical patterns in BAs fluctuations during the day. Similarly, GDCA and TDCA presented similar geometrical patterns. Despite the similarities between the conjugated versions of CA and DCA, these patterns were predominantly characterised by a higher concentration of glycine-conjugated BAs than taurine-conjugated BAs. These has been described previously, that in normal subjects, the total glycine to taurine ratios of serum bile acids showed higher values compared to patients with ileal Crohn's disease, with mean ratios of 1.9 and 2.6 in controls fasting and 2.9 fasting and 4.8 postprandial in patients, respectively (406). Another significant finding was the shift in the ratio from 2.5 in the fasting state to a maximum of 3.3 at one to 1 1/2 hours postprandially after the consumption of a high fat breakfast consisting in egg yolk, cream and sugar, indicating a relative increase in glycine conjugates surpassing that of taurine conjugates early after a meal in healthy subjects (407). In this study, the proportions of CA, DCA, and conjugated BAs were higher than these values. However, these metabolite ratios consider BAs as individual metabolites and not the sum of glycine and taurine conjugates.

However, these differences in the geometrical patterns seem to be more influenced by gallbladder contraction, which has been reported previously in the literature as one of the significant contributors to the inter- and intra-postprandial variability between subjects (384, 408). The concentration of BAs in peripheral circulation is the result of different biological factors, such as the release of CKK, which provokes contraction of the gallbladder for the release of bile and BAs (102). Subsequently, BAs are taken up by transporters, crossing the basolateral membrane to export BAs into the portal circulation (402). The process of liver flow-through indicates that the uptake of BAs by hepatocyte basolateral membranes varies in specificity and kinetics depending on the concentration and pattern of BAs presented (402). Consequently, when venous blood is analysed, only a minor fraction of BAs that bypass the liver will be detected in the bloodstream, and their profile is likely to differ from that

observed in the bile and subsequently discharged from the gallbladder and all subsequent physiological processes involved in BAs metabolism (395).

An important aspect is that some of the evaluated BAs tended to peak at AD time points. This could be attributed to the fact that lunch was consumed 7 hours earlier and that food had reached the small intestine (270). Diet 1 comprised 30 g of fat, which was predominantly unsaturated, whereas diet 4 contained 36 g of fat, which was mostly saturated. Regardless of the fat quantity in the diet, it seems that there was no significant influence on the modulation of BAs concentrations in the serum, and it appears that the BAs followed a diurnal pattern rather than a response regulated by dietary intake.

Several aspects should be carefully considered when interpreting the results. The importance of considering that food was consumed as a whole suggests that interpreting these results based solely on dietary fat intake could be misleading. Other factors such as the type of fat, type of fibre, type of carbohydrate, energetic density, food structure, and food matrix could affect the absorptive, digestive, and metabolic processes that are ultimately reflected in the serum concentrations of BAs in subjects (62). Overall, faecal samples were not collected in this study. Thus, it is unknown how these subjects responded to dietary interventions regarding BAs excretion in faeces and explored microbial taxonomy or metagenomic data.

6.7.5 BA concentrations in urine and their relevance

It is essential to highlight that the concentrations of BAs in urine are very low compared to other biofluids, such as blood from the portal and systemic circulation (395). Their presence in the urine has been described as an element of minor importance, because of the very low concentrations found in this biofluid (17, 27). However, when these very small amounts of BAs in urine increase, they are associated with pathological conditions (350, 352, 353, 409). A study of labelled BAs reported that the recovery of BAs in urine was below five per cent in healthy subjects (354). Therefore, this can explain the low concentrations of BAs generally found in urine samples.

The urine samples analysed as time spots presented three different time points, representing collection intervals from 4 to 15 hours. As previously mentioned, low concentrations were detected in this subset of samples. However, it is unclear whether these concentrations were related to the nature of the sample or the poor analytical conditions. On the other hand, the other set of urine samples was pooled for 24-hour period. Unfortunately, the concentration of BAs in urine samples could not be accurately measured. Therefore, given the high urine volumes associated with the consumption of the healthy diet discussed in Chapter 4, it is plausible to suggest that subjects consuming diet 1 might exhibit lower bile acid concentrations due to increased water intake and, consequently, reduced bile acid circulation,

compared to those who consumed diet 4. However, it is important to consider that the subjects were healthy, and therefore, bile acids in the urine would likely fall within normal ranges.

6.8 Conclusions

This research studied the effects of the consumption of two different patterns that were contrasting in their level of healthiness under randomised, controlled, and crossover conditions that studied the changes of BAs in serum and urine. The method employed in this study provided a broad coverage of BAs, allowing us to explore more about these metabolites compared to other nutritional studies that have studied BAs in serum, urine, or both, but not under the same controlled and inpatient conditions.

In this study, the impact of the diet seemed not enough to influence the temporal variations that were detected during the studied period. It would have been expected that BAs would have higher concentrations in the unhealthy diet than in the healthy diet due to the almost double content of fat, but only two significant differences were found between the concentrations of BAs. However, inter- and intra-individual differences were present among individuals when some selected responses were studied individually, indicating that the differences in the serum levels of BAs where conjugated and unconjugated varied in their proportions after the intake of the two diets.

These inter- and intra-differences may originate from the secretion of conjugated BAs from the liver across the basolateral hepatocyte membrane by diverse transporters from this membrane and the release from the gallbladder that influences the postprandial and fasting responses of BAs after food intake. Individual responses after food intake were diverse, showing a range of variability, although the conditions were controlled for the study. These different response patterns between individuals are related to the absorption processes initiated in the small intestine. Moreover, considering the two postprandial hours for sample collection, it seems that BAs are absorbed independently of the amount of fat intake via uniporter transport and continue moving down through the intestine mixed with micelles, suggesting that AD would have the highest level of BAs in the postprandial state for most of the BAs. Simultaneously, enterohepatic circulation and the individual signatures of the microbiome can contribute to these individual differences.

However, it seems plausible to suggest better protocols for the timing of blood samples that can reflect the biological responses of BAs under postprandial circumstances, considering that BAs possess their own circulation patterns that circadian rhythms may regulate. To better understand how dietary patterns can modulate BAs, studies exploring their long-term consumption and their effects on the modulation of BAs in biofluids alongside the inclusion of microbiome analysis would be required. In this way, an understanding of how diets can modulate BAs metabolism could reveal insights with potential uses for

the prevention and management of chronic diseases with potential applications in a personalised nutrition context.

7. General Discussion

Over the last seven decades, the study of specific nutrients and dietary patterns related to chronic diseases has become an important topic in nutritional research (11). Efforts to elucidate the best diet for disease prevention and health maintenance have been investigated, resulting in different dietetic approaches and population recommendations (13-16).

Several dietary guidelines have been published addressing public health challenges for specific populations, and these guidelines highlight the characteristics of dietary patterns such as Mediterranean, Nordic, Healthy, Vegetarian, Vegan and Dash, among others (17). Vegan and omnivore diets have been investigated for a long time (26-28). Although the evidence from epidemiological studies is robust, most are attributing the health benefits of vegetarian diets to cardiovascular health over other dietary patterns as omnivores (29, 30)

A pitfall encountered in the study of dietary patterns is the inaccurate registration of dietary intake. These epidemiological studies provide characteristics of the dietary intake of food, but the inaccuracy in energy reporting in the 24-hour recall can vary from 21.5 to 67% in free-living people (212). The accurate reporting of dietary intake information plays a pivotal role in the adequacy of nutritional recommendations and, subsequently, in making a correct diet assessment of diet on health.

State-of-the-art analytical techniques for analysing diet-related metabolites have emerged as promising tools for understanding the effects of dietary patterns on metabolism (39). Among these diet-related metabolites, SCFAs and BAs were studied. SCFA are microbial metabolites that have gained increasing interest because of their potential roles in health and disease (410, 411). These metabolites are the end products of fermentation of nondigestible carbohydrates, undigested proteins, and amino acids (69, 412). In contrast, BAs are amphipathic molecules with a steroid backbone synthesised from cholesterol, and they play a key role in intestinal lipid digestion (96). BAs are modulated and transformed by the intestinal microbiota, and food intake stimulates their release and influences their concentrations (62, 382). Nonetheless, it is not understood whether the misreporting of dietary intake in the study of dietary patterns could influence the understanding of metabolism through the analysis of SCFAs and BAs.

The overall hypothesis of this thesis was that specific dietary patterns will be reflected in blood, urine and faecal gut metabolites. Metabolic differences related to these dietary intakes will be assessed by understanding the differences in chemical and nutritional compositions.

This thesis primarily investigated the effect of different dietary patterns on the metabolism of SCFA and BAs in two clinical studies, aiming to gain insights into how dietary patterns can regulate these gut metabolites under different dietary settings by analysing different biofluids. The first clinical study was a Danish cohort, a free-living cohort of vegans and omnivores. In this cohort, vegans followed their dietary patterns for an average of five years (129). Blood, urine, and faecal samples were analysed in this cohort. In parallel, samples from a second study were included to fill the gap in the understanding of misreporting of dietary patterns and the effects of sample time on the metabolism of gut metabolites. A randomised controlled crossover clinical trial under inpatient conditions was conducted, in which two contrasting dietary patterns that aligned 100 and 25 per cent to the WHO healthy eating recommendations were given for 3 days. Blood and urine samples were collected on the third day and fasted on the fourth day (78, 132).

Metabolite differences were observed between biofluids and dietary patterns in omnivores and vegans, although not consistently across all metabolites. In the controlled diet study, some differences in serum and urine metabolites were also detected, regardless of dietary conditions. These findings suggest that the proposed hypothesis is only partially supported, highlighting the complexity of diet–metabolite associations.

This Chapter summarises the key findings of this research and discusses the strengths and limitations of each clinical study. Potential alternatives to improve the current state of the conducted research will also be proposed, exploring possible directions for future work on the study of SCFAs, BAs, and diet.

7.1 Metabolic insights from SCFAs in vegans and omnivores from the Danish cohort: serum, urine, and faecal analysis

In our study of vegans and omnivores, the concentration of SCFAs differed significantly in serum and urine, which seems to be related to the individuals' dietary patterns. Previous studies have reported that 2-hydroxybutyric acid in serum is a metabolite associated with insulin resistance in fasting individuals (172, 262). In the present study, this metabolite was present at a higher concentration in omnivores during fasting. However, independent of this finding, the biochemical parameters for glucose metabolism were within the normal range for both groups. Another aspect to consider that could influence the differences between SCFAs in both groups is the different proportions of amino acids consumed, which are higher in omnivores. The metabolite 2 hydroxybutyric is produced in the liver from 2-ketobutyrate due to threonine catabolism or methionine and serine metabolism (220). Nonetheless, despite the higher amount of AAs consumed by omnivores, which can be a substrate for the production of BSCFAs and SCFAs, other compounds can be produced using AA as a substrate by the resident microbiome of omnivores (70, 413). For example, cadaverine is a metabolite of lysine

decarboxylation, and lysine can be simultaneously converted into butyrate in the gut (414). This indicates that the precursors of the studied metabolites could have another metabolic fate, given that they can be regulated by the microbiome (415).

Recently, some studies have highlighted the potential benefits of an increase in serum SCFA levels in the reduction of inflammatory biomarkers related to CKD in patients (226). In this study, SCFAs in urine were significantly lower in vegans than in omnivores, and a different pattern of SCFA in serum was observed. The clinical relevance of SCFAs in the urine of healthy individuals is not well understood. Nevertheless, it could be speculated from the results of this study that a higher concentration of SCFAs in serum would result in lower excretion in urine from the same individuals and vice versa. Interestingly, the average fibre content for vegans and omnivores was above the population recommendation of 25 g daily (48) and was higher in vegans. However, it is unknown which type of fibre was consumed and its fermentation capacity for SCFA production since the final data for the quantification of nutrients were available only from the food records and not from the full description of the consumed food.

SCFAs in faeces were not significantly different between the two groups despite the differences in fibre, protein, and AA intake. Previous studies have reported similar findings, but no significant differences were found in faecal SCFAs. One study used ^{1}H-NMR (167), while another used GC-MS (231), but both reported no differences between groups. It has been proposed that the potential reasons why differences are not found in faecal samples could be attributed to a restrictive profile of the westernised microbiome that does not increase the production of SCFAs if fibre intake is higher (167) or a potential adaptation of the microbiome to produce SCFAs (231). However, it is important to consider that most SCFAs are absorbed by colonocytes, not necessarily reflecting the production of SCFA in the gut (233).

The faecal samples from the Danish cohort were frozen and previously homogenised; therefore, changes associated with continuous handling and thawed cycles were unlikely in this set of samples. Therefore, changing their metabolite composition after freezing and exposure to continuous thawing cycles (416) was reduced to the minimum in the sample handling for SCFAs and BAs analysis. A recent study that compared faecal samples taken within 25 hours apart reported shifts in microbial composition and metabolites (417). This is an important aspect because faecal samples are normally collected when volunteers can provide them, and there is no specific schedule for sample collection. This is how they were collected from the Danish cohort. Therefore, faecal samples should be treated carefully after collection, considering their time of collection or suggesting collection in the morning, when possible. Moreover, the samples should follow a proper freezing protocol to avoid changes associated with the handling of the sample and time of collection that might affect metabolite quantification analysis.

7.2 Metabolic insights from SCFAs in a healthy and unhealthy dietary pattern from a randomised crossover dietary trial: serum and urine analysis

Serum SCFA samples obtained at different times of the day presented significant results at mealtimes throughout the day. The concentration of 2-methylbutyric acid was higher in diet 1 compared to diet 4 in AL and AD. This can be expected since diet 1 contains a higher protein content than diet 4. This metabolite is categorised as a BSCFA and is produced by protein breakdown by the gut microbiota through fermentation in the gut lumen (162). This metabolite is derived from branched AAs, and elevated serum concentrations have been associated with metabolic syndrome (250-252). An increased concentration of this metabolite can be expected because diet 1 contains a higher protein content than diet 4.

Lactic acid was increased in AL and AD after the consumption of diet 4. This diet contained 25 g of sugar, low fibre, and was high in energy density. Similar findings have been described after the intake of Westernised dietary patterns (255, 256). In the case of 2-hydroxybutyric acid, the concentrations were higher in diet 1. Similar to the results obtained in the Danish cohort, the concentrations of this metabolite were higher during fasting, as observed in the omnivorous diet. As mentioned previously, this metabolite is produced in the liver from 2-ketobutyrate, which is derived from leucine or methionine, but it is unclear whether its production derives exclusively from dietary sources (261, 262). Acetic acid presented higher levels in fasting after the intake of diet 1. The levels in fed states are mainly derived from colonic fermentation by bacteria. However, during periods of starvation, these levels are regulated by the liver along with ketone bodies to serve as energy sources (217).

SCFA in urine presented higher concentrations after the intake of diet 4 for propionic acid, butyric acid, isobutyric acid, isovaleric acid, valeric acid, caproic acid, and 2-hydroxybutyric acid. These results should be interpreted carefully because different urine spots were pooled to create 24-hour urine samples. As mentioned in Section 7.1, the clinical relevance of the presence of SCFA in urine is not well understood, but it could be speculated that a lower concentration of SCFAs in serum would result in a higher concentration of SCFAs in urine from the same individuals and vice versa. However, when subjects consumed diet 1, they produced a higher urine volume than diet 4. During this study, water intake was not recorded, but due to less energy-dense food, it is plausible to suggest that diet 1 contained more water than diet 4. The impact of SCFA concentrations in urine remains unclear, but recent research has emphasised that there may be advantages of higher levels of SCFA in the bloodstream, which could potentially help lower the inflammation markers associated with CKD in patients (226).

7.3 Metabolic insights from BAs in a healthy and unhealthy dietary pattern from a randomised crossover dietary trial: serum and urine analysis

The analysis of BAs from this randomised crossover dietary clinical trial showed few differences between BAs concentrations in both diets. The AB samples from diet 1 had a higher total glycine-conjugated BAs content. Similarly, UCNA was present in higher concentrations in diet 1. It is important to highlight that samples were taken 2 hours after food intake, but these elevated concentrations of glycine-conjugated BAs in diet 1 are unlikely to be in response to food intake, considering that the food transit time in the gastrointestinal tract until reaching the small intestine is more than 2 hours (270). Therefore, it is more likely that this response was regulated by the BA pool rather than by the dietary influence of breakfast consumption. UCNA levels were significantly higher in the AL group after the intake of diet 1. Considering previous food intake, it seems plausible to assert that this metabolite was potentially influenced by diet. However, the role of these metabolites remains unclear.

Due to analytical constraints during sample analysis, urine BAs were not possible to obtain. However, the presence of BAs in healthy subjects is described as an element of minor relevance since very low concentrations are found in urine. A study using labelled BAs as biomarkers reported that the recovery of BAs was below five percent in healthy subjects (354).

7.4 Metabolic insights from BAs in vegans and omnivores from the Danish cohort: serum, urine, and faecal analysis

The results from the analysis of BAs from the Danish cohort showed differences between vegans and omnivores that could be associated with the consumption of the studied dietary patterns. Significant differences were found in the intake of different nutrients related to BAs metabolism, such as total fat and saturated fat, which were higher in omnivores, and monosaturated fats, which were higher in vegans. Despite these dietary differences, lipid metabolism biomarkers were within normal clinical ranges in both groups (190).

Fasting serum samples showed two metabolites that differed significantly between groups. DHCA was found higher in omnivores, being 5 times higher compared to vegans. Although the role of this metabolite remains elusive, it has been reported as a synthetic product of CA oxidation (317). Therefore, it could be speculated that the presence of DHCA may be a derived microbiome oxidation product. Under in vitro conditions, *E coli* and *A. Faecalis* have been shown to be capable of oxidising DHCA (317). Therefore, it could be speculated that DCHA is a metabolite produced by the oxidation of CA. On the other hand, total secondary BAs were higher in vegans, which is similar to other studies that have reported an increase in glycine and tauro-conjugated BAs in groups that consumed diets rich in fibre, which are considered secondary BAs (40, 41).

In urine, apocholic acid, 12-DCA, GCDCA, GDCA, GCA, GHCA, TCDCA, and total glycine-conjugated BAs were detected. All of these metabolites exhibited higher concentrations in vegans. The levels of these metabolites were higher in vegans except for 12- DHCA. It is important to highlight the significant differences detected in our samples, which illustrates the biological differences between healthy subjects following vegan or omnivorous dietary patterns. In this context, the presence of these BAs does not necessarily indicate a clinical feature for the development of a medical condition, despite many of the mentioned BAs being reported in urine. This study focused on the concentrations of metabolites detected in our set of samples for each dietary pattern intake group. However, the presence of different BA receptors in the kidney suggests a potential role for these metabolites, but their implications are not yet fully understood. Concentrations of BAs in urine are very low, and previous research on labelled BAs has reported that less than five per cent of BAs can be recovered from urine in healthy subjects (354).

Faecal BAs presented significant differences for the following BAs: NorDCA, DCA, ßMCA, secondary BAs, unconjugated BAs, and total BAs, which were higher in omnivores. These BAs, excluding total and unconjugated BAs, are secondary BAs associated with unhealthy dietary patterns and a higher risk of developing colorectal cancer (18). However, it is important to acknowledge that the results from omnivores in this study were compared with those of a vegan group, but both were characterised by healthy dietary patterns despite the differences in diet composition. Additionally, a higher level of secondary BAs in our cohort does not necessarily imply a risk of colorectal cancer because many factors are involved in its physiopathology.

7.5 The complexity of diet and health: reevaluating vegan and omnivorous dietary patterns

In the Danish cohort study, it is important to highlight that the subjects who consumed an omnivorous or vegan diet were healthy individuals without reporting clinical significance in different biomarkers related to lipid and glucose metabolism. In terms of the healthiness of dietary intake, subject diets were evaluated using DASH scores in both groups and were categorised in the higher quintile, which classifies it as a healthy diet despite the intrinsic differences between vegans and omnivores (210).

As discussed previously, some differences were observed between the biofluids in the two groups. The concentrations of SCFAs and BAs in each biofluid were explored using multivariate analysis. Separations between the groups were not found, and several overlapping points were found, suggesting some similarities in metabolite production independent of the diet consumed.

Some studies have indicated the fact that being vegan does not necessarily imply better health outcomes, considering the basic essence of this dietary pattern, which relies on the absence of animal products in the diet (211). This means that a vegan diet can also be high in saturated and sugar intake and low in fibre consumption, along with a high intake of processed food (211). This is an important factor to consider, because vegan diets do not imply a healthy diet itself due to the consumption of ultra-processed food, and dietary recommendations for healthy eating need to be followed, regardless of the type of dietary pattern.

7.6 Limitations of cross-sectional studies in exploring dietary patterns and gut-related metabolites

It is important to emphasise that the Danish cohort study had a cross-sectional design, collecting four dietary records during the clinical trial and using single time points for biological measurements, including biofluid samples. From that point of view, this represents one biological snapshot associated with dietary intake from self-reported dietary records that are not necessarily aligned with the time of sample collection. This misalignment may interfere with the precision of the reported findings and preclude causal inferences. For a better understanding of the metabolic effects of these dietary patterns, cross-sectional repeated measures of samples after a long term that includes assessing concordance discordance statistical testing (418). These analyses should consider aligning dietary records with sample collection (urine, blood, and faeces) as the timing of their collection. Thus, it would be possible to understand and provide more accurate answers to the biology of these metabolites in the context of specific dietary patterns and food intake.

The information obtained from a cross-sectional study is relevant for determining the conditions of the targeted groups to study at one specific time to measure a range of variables (419). Cross-sectional studies are generally less expensive and require less time to complete than randomised controlled clinical trials (RCT). On the other hand, it is important to acknowledge that a cross-sectional study provides a snapshot of the situation, causality cannot be determined, and there is a susceptibility to bias due to their observational nature (419).

Therefore, the findings from this specific Danish cohort need to be interpreted with caution to avoid generalisation of the results obtained from this thesis regarding the consumption of a vegan and omnivorous dietary pattern and its production of gut-related metabolites.

7.7 Assessing the impact of controlled dietary interventions on gut-derived metabolites and diverse BMI categories

To close the gap associated with the pitfalls in dietary records from nutritional studies in free-living people, samples from this unique inpatient controlled randomised crossover clinical trial were analysed. As discussed previously, different dietary patterns can influence gut-derived metabolites; however, there are relevant aspects that should be acknowledged. In this clinical study, subjects consumed two opposite healthy and unhealthy diets for three days. Serum and urine samples were collected on the third day and again after fasting the morning of the following day (Chapter 2, Figure 2.1). Since food intake was controlled, it is presumed that biofluid samples would represent metabolites associated with the consumed diets. It has been described that transit time differs between subjects, with fast transit below 14 hours, normal transit from 14 to 58 hours, and slow transit over 59 hours (420). Therefore, it is plausible to expect that the measured metabolites reflected the intake of each studied diet to different degrees, considering the process of transient adaptation of the gut microbiome after this three-day intervention (136, 421). However, in this study, faecal samples were not collected; therefore, further investigation of faecal metabolites, microbial taxonomy, and metagenomics studies was not possible.

Recruited subjects were classified according to BMI from normal weight to obese (21.1 to 33.3 kg/m^2), but their metabolic parameters were normal (132). However, the broad range of BMI in the group may result in confounding of the metabolic signature, which affects SCFA and BA metabolism.

Recently, a cross-sectional study reported that plasma propionate was higher in subjects with metabolically healthy normal weight than in those with metabolically healthy and unhealthy obesity (422). Therefore, propionate was inversely associated with total and visceral adiposity in subjects. In this study, propionate did not show differences between the groups, suggesting that the phenotype did not influence the SCFA responses in this study.

In the case of BAs, a meta-analysis reported alterations in the BAs pathways in obese subjects, but this has not yet been explored in metabolically healthy and unhealthy normal-weight and obese subjects (423). In this study, very few differences were found in BAs, but it is more likely that these differences are related to individual differences and diet than to a specific phenotype based on BMI. Further studies are needed to delineate the influence of metabolic health status on BA metabolism across BMI categories.

7.8 The importance of time sampling for nutritional studies

As in this thesis, the main hypothesis was to understand how different dietary patterns can modulate gut-related metabolite metabolism. After the study of both studies, the results highlighted the

importance of time sampling in understanding the relationship between food intake and metabolic response during the day. For example, in the Danish cohort, faecal samples were collected at the convenience of the volunteers. The time of collection of faecal samples may influence the content of metabolites and microbial composition, as has been previously reported when two samples were collected in a window of less than 25 hours (417). Conversely, samples analysed from the Danish cohort were taken in fasting in the case of serum or first-void urine in the case of urine. These samples provided a picture of the last food consumed in the previous meals, assuming that gastrointestinal transit was not fast in the subjects (less than 14 hours).

However, after analysing the randomised crossover dietary trial study, some differences were found between diets. For SCFA and BAs, both diets presented their own geometrical patterns that circadian rhythms may influence. Therefore, four blood samples were taken at different time points over a 24-hour period (Chapter 2, Figure 2.1). The temporal biology of the metabolism may not have been fully captured from these gut metabolites, and it is possible that different meals may influence them differently throughout the day. Therefore, collecting samples with more frequency during the different postprandial windows during the whole day would have been helpful. This would help to understand the biology of these metabolites postprandially more accurately, considering their short half-life in circulation and the potential role of diet intake. Most of the studies that have evaluated these metabolites have considered fasting and postprandial responses for a couple of hours, generally, in the morning hours, undermining how metabolites may change during the afternoon, evening, and night (62). Therefore, evaluating SCFAs and BAs more frequently during the postprandial window of the entire day would be helpful. This would support the understanding of when these metabolites are circulating in low or high concentrations, how these concentrations can be associated with food intake, or whether they only correspond to fluctuations of metabolites that have their own circulation pattern independent of food intake. This would be relevant to consider in future studies aimed at understanding the biology of metabolites influenced by dietary intake. Additionally, it would help to develop a better understanding of specific dietary substrates that could significantly impact metabolite circulation and improve health outcomes through dietary interventions.

7.9 Individual differences rather than diet for each metabolite

The results obtained from the Danish cohort provided only one measurement, and individual differences at different time points could not be evaluated because of the nature of the study design. However, it would be interesting to study these subjects for a period of time in which they could be monitored to see how these dietary patterns can affect the metabolism of SCFAs and BAs along with other health biomarkers over a longer period of time and explore their relationships.

To answer one of the initial research questions related to the inaccuracy of dietary records. This randomised study used a crossover design under controlled conditions to ensure that the same diets would have the same mealtimes consumed by the same individuals. Concentrations of SCFAs and BAs showed different responses from subjects to the same diets during the day, as seen in Chapters 4 and 6. These differences show that, independent of consuming exactly at the same time each day, the same portions, food, and diets, subjects presented inter-individual variations related to their own biology despite the short intervention time of 3 days.

Differences between individuals can be expected because it has been reported that biological and analytical variability together can be greater than 50% for some clinical outcomes (424). However, the variations found in our study were more strongly associated with biological responses than variations in analytical techniques. Both techniques employed for SCFAs and BAs have been validated and have shown robustness for their analysis (78, 98). The samples were analysed under the same analytical conditions for each metabolite.

The temporal differences in SCFAs and BAs between individuals were similar to those reported in previous studies where these metabolites were studied (78, 333). In both sets of metabolites, genetics (273, 333, 425), physiological processes (for example, food mastication, digestion processes, and gastrointestinal transit time) (270, 274) contribute to these differences. However, these differences can be explained by microbiota composition and their functionality in interacting with the substrates for SCFAs and BAs modifications, which then impacts the systemic level.

7.10 The role of SCFAs and BAs in gut metabolism: research limitations and future directions in nutrition

The biology of SCFAs has evolved enormously over the last 30 years, where their understanding has not solely been reduced to the metabolites generated in the gut after the fermentation of dietary fibres (65, 411). In this sense, their role extends beyond the gut and has extra-intestinal effects. For example, in the gut-liver axis, SCFAs influence liver function, provide energy, and modulate liver responses (426). The gut-brain axis, where SCFAs can affect microglial activation and neuroinflammation, potentially impacts conditions such as multiple sclerosis and Parkinson's disease (70-73) and the gut-lung axis, which modulates lung immunity and may influence respiratory health and responses to infections (427). All these gut-related axes open a new understanding of the impact of SCFAs and open possibilities for using dietary interventions, probiotics, prebiotics, or SCFA supplementation as therapeutic strategies for managing diseases through dietary and microbial modulation.

Recent research published last year has discovered new aspects of BAs biology. Bile salt hydrolase (BSH) plays a dual functional role beyond the deconjugation of BAs in the intestine (428). This new function of BSH is as an amine N-acyltransferase capable of conjugating unconjugated BAs, which allows it to conjugate AAs such as phenylalanine, leucine, and tyrosine to bile acids, forming bacterial bile acid amides (BBAAs). These compounds have been described in inflammatory bowel diseases, suggesting that changes in BAs profiles due to microbial metabolism might influence gut inflammation and disease states (429). Therefore, this opens new possibilities in the research field of BAs by understanding these new BBAAs as potential targets for IBD, probiotics, and/or prebiotics for the modulation of these metabolites in the gut, the new role of the AAs that are conjugated to BAs, and how different diets can modulate their metabolism. Simultaneously, these metabolites can be used as biomarkers of intestinal disease progression using analytical techniques, such as LC-MS/MS with a targeted or untargeted approach (429, 430).

The study of dietary patterns and their impact on metabolite levels offers valuable insights despite some challenges. As a trained dietitian, I acknowledge that the research on the two contrasting diets followed the WHO recommendations for healthy eating to varying degrees, with diet 4 being more aligned with unhealthy characteristics. While diet 1 was generally healthy, there were opportunities for improvement in reducing UFP intake by considering the intake of ultra-processed food (UPF) using the NOVA classification of food (431). For instance, replacing Weetabix with oat flakes and margarine with healthier fats such as avocado, nuts, or olive oil could enhance the nutritional quality of diet 1. Additionally, incorporating raw vegetables, fruits, pulses, or beans would have provided more balanced nutrients and fibre.

The innovative aspect of this study lies in its design, as it is the first to compare the intake of two contrasting diets under inpatient conditions, using a crossover and randomised approach. This design serves as a proof of concept, illustrating individual differences in metabolite levels regardless of dietary intake. While SCFAs were significantly affected by diet, BAs showed less variation, highlighting the complexity of the impact of diet on metabolism. Overall, the study successfully demonstrates the potential of dietary intake to influence biofluid metabolites, offering a foundation for future research to refine dietary patterns for better health outcomes by understanding gut metabolites.

The analytical techniques employed in this research for gut-related metabolites were targeted methods for quantifying SCFAs and BAs. These techniques are very straightforward for identifying metabolites, allowing the measurement of defined groups of metabolites with similar chemical characteristics using generally isotope-labelled internal standards (432). In the case of the SCFA method developed by Dr. Sophia Fountana (79), despite the good stability of the internal standard used for method validation (22), it could have been replaced by an isotope-labelled compound to improve the robustness of the

assay. Therefore, all the metabolites studied in this work were predefined beforehand based on the above-mentioned targeted methods. However, it would be worthwhile to detect and quantify this new type of BBAAs in future studies of BAs to understand their biology and metabolism. Recently, it has been described that from the consumption of a Westernised American diet to a Mediterranean diet in a crossover study, levels of faecal amidates and polyamide bile amidates were reduced when this diet transition occurred. The role of these metabolites is not clear, but it is the first bile amidates that are described to be modified by diet (430).

In this study, the analysis of acylcarnitines could have been included because this would have allowed us to explore the effects of diets on these metabolites and their relationship with BAs and lipid metabolism (433), particularly in the dietary intervention study. Simultaneously, for that study, it would have been useful to have access to biochemical parameters, such as glucose levels and lipid metabolism parameters, for each time point when blood samples were collected. This would have provided more insights into how these biomarkers were related to the metabolites studied in this study and the diet intake.

In my view, the roles of SCFAs and BAs in modulating various biological processes, either by regulating gut axes or acting as second messengers, need to be explored further, especially considering the influence of diet. BAs, especially secondary BAs, could exert an effect on SCFA regulation, considering the antimicrobial effect of BAs that can modulate the growth of certain types of gut microorganisms (96). This can influence the inhibition of some microorganisms, affecting the overall composition of the gut microbiota and indirectly modulating SCFA production (386). For this, diet influences levels of secondary bile acids and SCFAs in the gut. High-fat diets, for example, are linked to increased production of secondary bile acids, which can alter the microbial composition and reduce SCFA production (386). Conversely, fibre-rich diets enhance SCFA production and can modify bile acid metabolism by affecting the gut microbiota (18).

In that sense, dietary interventions that are trying to understand the effect on metabolism need to acknowledge the important role of dietary records, sample collection time, type of biofluids, duration of the intervention, metabolites to measure, and which omics integration techniques are relevant to answer the research questions proposed knowing the limitations that may pose. On the other hand, it is relevant to recognise that biological responses are not the same for everyone where the individual biology might explain the differences in the utilisation or depletion of substrates to modulate or generate the production of metabolites in the gut, impacting systemically and influencing the host physiology.

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Appendix 1 Study design

*Information provided by Gary Frost's Team and available at (130)

Study Design

Participants fulfilled the following criteria

Pre randomization criteria
Potential participants will first have a short telephone interview to assess their suitability for the study. Potential participants will then be interviewed and examined by one of the research doctors.

They will have blood tests and height, weight, hip and waist measurements. They will have an electrocardiogram (ECG). All women of childbearing age will have a pregnancy test.

Inclusion criteria:
Healthy volunteers ((body mass index (BMI) of 20-35kg/m^2)
Age between 21 and 65 years old.
Exclusion criteria:
Weight changes ≥ 3 kg in the preceding 3 months.
Current smokers.
Substance abuse.
Excess of alcohol intake.
Pregnancy.
Diabetes.
Cardiovascular diseases.
Cancer.
Gastrointestinal diseases, e.g., inflammatory bowel disease or irritable bowel syndrome
Kidney disease.
Liver disease.
Pancreatitis.
Use of medications likely to interfere with energy metabolism, appetite regulation and hormonal balance, including: anti-inflammatory drugs or steroids, antibiotics, androgens, phenytoin, erythromycin or thyroid hormones.

Individuals with the above conditions would have an altered pattern of hormones and inflammatory molecules because of their disease process and would therefore give us confounding or misleading results.

This randomization was carried out by an external researcher, using randomized sealed envelopes for each type of diet. Envelopes were opened according to the order of recruited patients. Diets delivered at the CRF could not be masked from researchers neither participants, but data analysis was carried out in a blinded manner. Food adherence was monitored closely, served food was weighted, and participants were asked to consume all the food provided during the study. Water consumption was allowed freely.

Health Screening Visit
Participants were clerked and examined by a research doctor. Measurements of blood pressure, an electrocardiogram (ECG) and blood samples (for full blood count, urea and electrolytes, liver function tests and lipid profile) were taken. Height, weight, hip and waist measurements were recorded. All women of childbearing age had a pregnancy test.

Study visits
Participants underwent a 2 weeks study period. Participants were asked to attend the NIHR/Wellcome Trust Imperial CRF at Hammersmith Hospital for 3-days, during 2 consecutive weeks. Participants stayed at the Clinical Investigations Unit for three nights and each week, in a randomized order, participants received a diet with different amounts of specific test foods deemed to have high public health importance (oily fish, wholegrain foods, fruits and vegetables):

Diet 1, healthy diet accordingly to WHO healthy eating recommendations
Diet 4, Westernized diet

Volunteers arrived at approximately 8.30 am on the first morning having fasted overnight. Volunteers were given set menus for breakfast, lunch and evening meals. Urine (18 x 40 ml) and blood samples (6 x 10 ml) were collected each 3-day study period. Blood samples were processed as serum, aliquoted and stored at -80°C . Urine samples collected, aliquoted and stored at -80°C.

Statistics and data analysis
A formal power calculation is not possible as this will be the first study of its type. However, recent studies have demonstrated significant changes in metabolomics profiles in cohorts of 20 volunteers (434, 435). Allowing for a dropout rate of 33%, we intend to recruit 30 volunteers for this research.
An independent researcher (i.e., not linked to the study) was given the task of randomization using randomized sealed envelopes.

<u>Regulatory issues</u>

Ethics approval
The London Brent Research Ethics Committee approved this study. The study was conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

Consent
Consent to enter the study was sought from each participant only after a full explanation was given, an information leaflet offered, and time allowed for consideration. Signed participant consent was obtained. The right of the participant to refuse to participate without giving reasons must be respected. After the participant has entered the study the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so should be recorded. In such cases, the participants remain within the study for the purposes of follow-up and data analyses. All participants are free to withdraw at any time from the study without giving reasons and without prejudicing further treatment.

Confidentiality
The Chief Investigator preserved the confidentiality of participants in the study and is registered under the Data Protection Act.

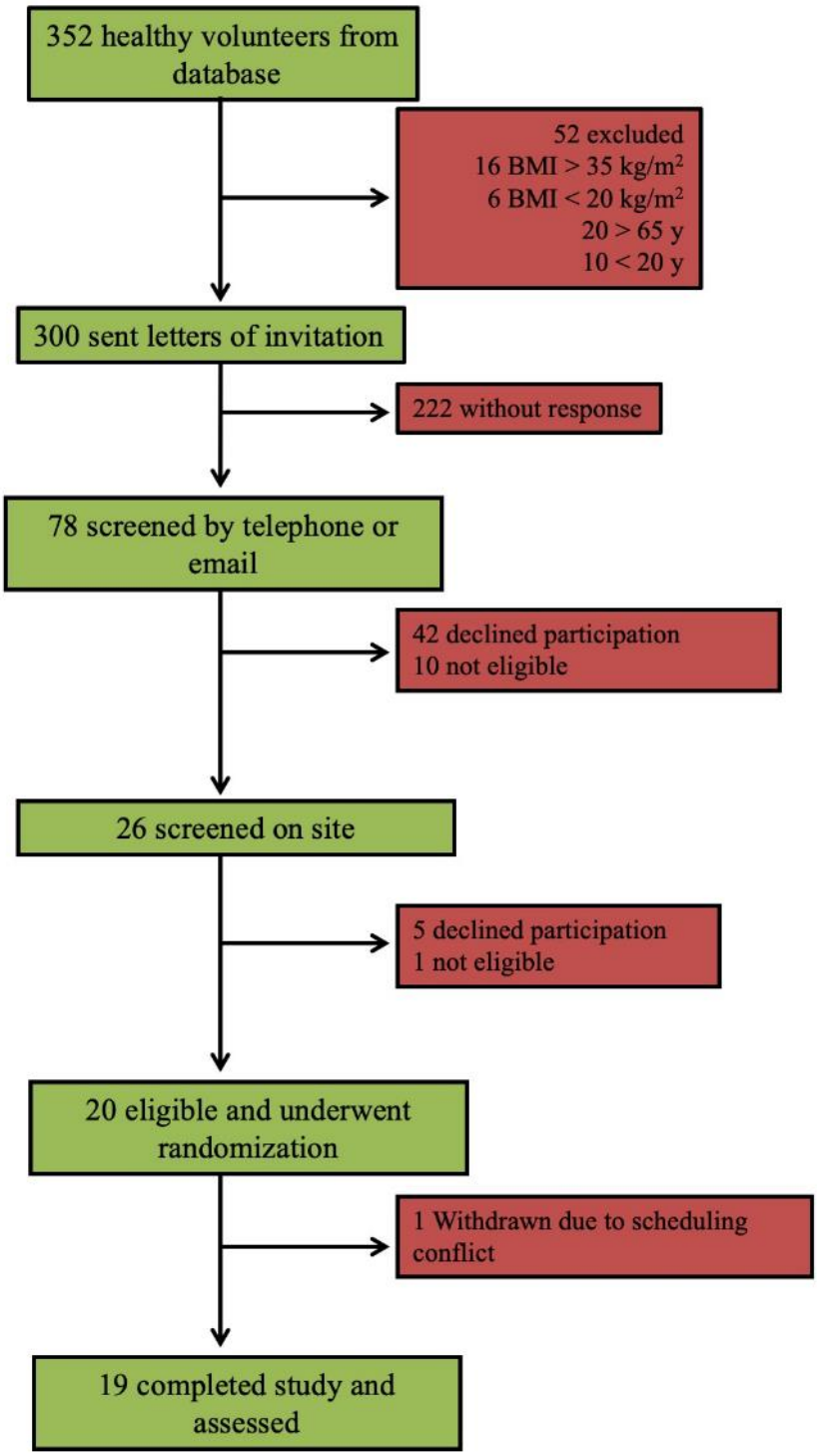
Indemnity
Imperial College holds negligent harm and non-negligent harm insurance policies, which apply to this study.

Sponsor
Imperial College London will act as the main sponsor for this study. Delegated responsibilities were assigned to the NHS trusts taking part in this study.

Audits and inspections
The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

Appendix 2. Participant enrollment chart

Participant enrollment chart . Modified from (78)



Appendix 3. SCFA Consumables organised by CAS Number and provider

Name of Consumable	CAS Number	Experiment
Acetic acid	6283	SCFA
Propionic acid	402907	SCFA
Isobutyric acid	I1754	SCFA
Butyric acid	103500	SCFA
2-methylbutyric acid	193070	SCFA
Isovaleric acid	129542	SCFA
Valeric acid	240370	SCFA
Caproic acid	153745	SCFA
L-lactic acid	L1750	SCFA
(S)-2-Hydroxybutyric acid	54918	SCFA
Methyl Stearate as Internal Standard (IS)	85769	SCFA
Water (ChromasolV)	7732-18-5	SCFA
Hydrochloric acid (HCl)	258148	SCFA
Tert-Butyl Methyl Ether (MTBE)	650560	SCFA
N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA + 1%TBDMCS)	942	SCFA
Liner	5062-3587	SCFA
Blue screw caps with red silicone	5182-0717	SCFA
Regular amber vials	5183-2072	SCFA
Silanized amber vials	5183-2072	SCFA
Deactivated glass inserts	5183-2072	SCFA
Water (CHROMASOL)	7732-18-5	SCFA
Phosphoric acid	7664-38-2	SCFA
Ethyl acetate	141-78-6	SCFA
4-methylvaleric acid as IS	646-07-1	SCFA
DB-WAXetr capillary column (30m, 0.25mm, 0.25 ¼m)	19091S-433UI	SCFA
HP-5MS capillary column (30m, 0.25mm, 0.25 ¼m)	19091S-433UI	SCFA

Appendix 4. BAs Consumables organised by CAS Number and provider

Name of Consumable	CAS Number	Experiment
Acetic acid	6283	SCFA
Propionic acid	402907	SCFA
Isobutyric acid	I1754	SCFA
Butyric acid	103500	SCFA
2-methylbutyric acid	193070	SCFA
Isovaleric acid	129542	SCFA
Valeric acid	240370	SCFA
Caproic acid	153745	SCFA
L-lactic acid	L1750	SCFA
(S)-2-Hydroxybutyric acid	54918	SCFA
Methyl Stearate as Internal Standard (IS)	85769	SCFA
Water (ChromasolV)	7732-18-5	SCFA
Hydrochloric acid (HCl)	258148	SCFA
Tert-Butyl Methyl Ether (MTBE)	650560	SCFA
N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA + 1%TBDMCS)	942	SCFA
Liner	5062-3587	SCFA
Blue screw caps with red silicone	5182-0717	SCFA
Regular amber vials	5183-2072	SCFA
Silanized amber vials	5183-2072	SCFA
Deactivated glass inserts	5183-2072	SCFA
Water (CHROMASOL)	7732-18-5	SCFA
Phosphoric acid	7664-38-2	SCFA
Ethyl acetate	141-78-6	SCFA
4-methylvaleric acid as IS	646-07-1	SCFA
DB-WAXetr capillary column (30m, 0.25mm, 0.25 ¼m)	19091S-433UI	SCFA
HP-5MS capillary column (30m, 0.25mm, 0.25 ¼m)	19091S-433UI	SCFA

Name of Consumable	CAS Number or Provider	Experiment
Acetonitrile (ACN)	75-05-08	BAs
2-propanol (IPA)	67-63-0	BAs
Water (UHPLC-UV grade)	7732-18-5	BAs
Ammonium acetate	631-61-8	BAs
Acetic acid	6283	BAs
Allolithocholic acid	Makaira Ltd	BAs
3,7-diketocholanic acid	Makaira Ltd	BAs
3,7,12-dehydrocholic acid	Makaira Ltd	BAs
3,7,12-glycodehydrocholic acid	Makaira Ltd	BAs
3,7,12-taurodehydrocholic acid	Makaira Ltd	BAs
3 alpha-hydroxy-7,12-diketocholanic acid	Makaira Ltd	BAs
3 alpha-hydroxy-7-ketolithocholic acid	Makaira Ltd	BAs
3 alpha-hydroxy-12-ketolithocholic acid	Makaira Ltd	BAs

3-ketocholanic acid	Makaira Ltd	BAs
5 beta-cholanic acid-3,6-dione	Makaira Ltd	BAs
5 beta-cholanic acid-3 beta±-ol-6-one	Makaira Ltd	BAs
5-cholenic acid-3-ol	Makaira Ltd	BAs
8(14),(5beta)-cholenic acid-3alpha±,12alpha±-diol	Makaira Ltd	BAs
9(11),(5beta)-cholenic acid-3alpha-ol-12-one	Makaira Ltd	BAs
Glycocholic acid	Makaira Ltd	BAs
Glycohyodeoxycholic acid	Makaira Ltd	BAs
Glycoursodeoxycholic acid	Makaira Ltd	BAs
Glycoursocholanic acid	Makaira Ltd	BAs
Isolithocholic acid	Makaira Ltd	BAs
Lithocholenic acid	Makaira Ltd	BAs
Taurolithocholic acid	Makaira Ltd	BAs

Name of Consumable	CAS number or Provider	BAs
Taurohyodeoxycholic acid	Makaira Ltd	BAs
Tauroursocholanic acid	Makaira Ltd	BAs
Tauroursodeoxycholic acid	Makaira Ltd	BAs
alpha-muricholic acid	Steraloids Inc	BAs
beta-muricholic acid	Steraloids Inc	BAs
omega-muricholic acid	Steraloids Inc	BAs
3,6-diketocholanic acid	Steraloids Inc	BAs
3,12-diketocholanic acid	Steraloids Inc	BAs
3 alpha-hydroxy-6,7-diketocholanic acid	Steraloids Inc	BAs
3 alpha,12 alpha,23-nordeoxycholic acid	Steraloids Inc	BAs
3-dehydrocholic acid	Steraloids Inc	BAs
5beta-cholanic acid-3 alpha,12 alpha±-diol	Steraloids Inc	BAs
5 beta-cholanic acid-3 alpha±,6 alpha±-diol-7-one	Steraloids Inc	BAs
5 beta-cholenic acid-7 alpha-ol-3-one	Steraloids Inc	BAs
Cholic acid	Steraloids Inc	BAs
Deoxycholic acid	Steraloids Inc	BAs
Glycochenodeoxycholic acid	Steraloids Inc	BAs
Glycodeoxycholic acid	Steraloids Inc	BAs
Glycolithocholic acid	Steraloids Inc	BAs
Glycohyocholic acid	Steraloids Inc	BAs
Hyocholic acid	Steraloids Inc	BAs
Hyodeoxycholic acid	Steraloids Inc	BAs
Isodeoxycholic acid	Steraloids Inc	BAs
Lithocholic acid	Steraloids Inc	BAs
Murocholic acid	Steraloids Inc	BAs
Taurocholic acid	Steraloids Inc	BAs
Taurochenodeoxycholic acid	Steraloids Inc	BAs

Name of the consumable	CAS number or provider	Experiment

Taurodeoxycholic acid	Steraloids Inc	BAs
Taurohyocholic acid	Steraloids Inc	BAs
Tauro-alpha-muricholic acid	Steraloids Inc	BAs
Tauro-beta-muricholic acid	Steraloids Inc	BAs
Tauro-omega-muricholic acid	Steraloids Inc	BAs
Ursodeoxycholic acid	Steraloids Inc	BAs
Ursocholanic acid	Steraloids Inc	BAs
Ursodeoxycholic acid-d4	Steraloids Inc	BAs
Beta-hyodeoxycholic acid-d4	Steraloids Inc	BAs
Chenodeoxycholic acid-d5	Steraloids Inc	BAs
Glycolithocholic acid-d4	Steraloids Inc	BAs
Glycodeoxycholic acid-d4	Steraloids Inc	BAs
Glycoursodeoxycholic acid-d4	Steraloids Inc	BAs
Taurolithocholic acid-d4	Steraloids Inc	BAs
Taurochenodeoxycholic acid-d4	Steraloids Inc	BAs
Tauroursodeoxycholic acid-d5	Steraloids Inc	BAs
Taurodeoxycholic acid-d4	Steraloids Inc	BAs
Taurocholic acid-d5	Steraloids Inc	BAs
Lithocholic acid-d4	Steraloids Inc	BAs
Cholic acid-d4	Steraloids Inc	BAs
Glycocholic acid-d4	Steraloids Inc	BAs
Deoxycholic acid-d4	Steraloids Inc	BAs
Glycochenodeoxycholic acid-d4	Steraloids Inc	BAs
Clear white vials waters with cap	600000751CV	BAs
Deactivated inserts	WAT094170DV	BAs

Appendix 5. SOP for SCFA protocol for urine, plasma and serum samples (triple quad)

1. Wear lab coat and gloves.
2. Prepare MTBE + IS (100 ppm, using as internal standard methyl stearate) and keep it stored at 5°C.
3. Defrost samples at 5°C and vortex them before to prepare aliquots.
4. Take 100 uL in an Eppendorf of 2 mL. Add 500 uL of MTBE+ IS and 2 uL of hydrochloric acid **. Vortex each sample after this step**.
5. Complete your first batch (Ideally 30 samples, because the centrifuge in the lab has that capacity).
6. Shake for 20 minutes (speed 5).
7. Centrifuge all the samples at 10000 rcf for 5 minutes at 4 °C.
8. Take 90 uL of the polar phase carefully from Eppendorf into silanized vial *(vials with big numbers compared to the regular ones*). Add 60 uL of derivatiser MTBSTF + 1% TBDMSCI (N-tert- Butyldimethylsilyl- N methyltrifluoroacetamide with 1 % tert Butyldimethylchloro-silane), put the **blue cap immediately** and vortex .
9. Incubate for 45 min at 60 °C
10. Label and put the insert in regular vials **(*non silanised, small numbers written*)**
11. Take 70 uL from the silanised vial into a vial with insert (*check the volume capacity of your insert*) and put the blue cap immediately
12. Keep the remaining samples from silanised vials stored at - 20 °C (as backup just in case)

Note: For QCs are recommended to take 20uL of each sample and use 1 QC each 10 samples from the beginning of the run until the end.

Appendix 6. Calibration curve preparation

Document developed by Jerusa Brignardello and Sofia Fountana

Consider the next compounds for CC preparation in the following concentrations. Take each compound to a final **volume of 1 ml.**

	Compound	mM	Volume (L)	MW	mmol/mL	Density (g/ml)	mg	(uL) to be pippete
1	Acetic acid	160	0.001	60.05	0.16	1.049		9.2
2	Propionic acid	20	0.001	74.08	0.02	0.993		1.5
3	Butyric acid	20	0.001	88.11	0.02	0.964		1.8
4	Valeric acid	16	0.001	102.13	0.016	0.939		1.7
5	Isovaleric acid	16	0.001	102.13	0.016	0.925		1.8
6	Hexanoic acid	10	0.001	116.16	0.01	0.927		1.3
7	Isobutyric acid	20	0.001	88.11	0.02	0.95		1.9
8	Lactic acid	8000	0.001	90.08	8		720.6	
9	2-methyl-butyric acid	20	0.001	102.13	0.02	0.936		2.2
10	2-hydroxybutyric acid	800	0.001	104.1	0.8		83.3	

Once each compound dilution is done take carefully the specified volume for each compound and put all of them in one Eppendorf to prepare the **mother stock**.

	Compound	Stock1 (uM)	Dilution	(uL) to be pippete for Mother stock
1	Acetic acid	8000	20	50
2	Propionic acid	400	50	20
3	Butyric acid	400	50	20
4	Valeric acid	80	200	5
5	Isovaleric acid	800	20	50
6	Hexanoic acid	200	50	20
7	Isobutyric acid	400	50	20
8	Lactic acid	800000	10	100
9	2-methyl-butyric acid	400	50	20
10	2-hydroxybutyric acid	40000	20	50
	Total volume from each compound dilution(uL)			**355**
	Total volume H20 (uL)			**645**
	Total volume of mother stock (uL)			**1000**

From the **mother stock** prepare these dilution series carefully, especially from series 7 to 11 (lowest concentrations).

	Dilution Series			Mother Stock to pippete (uL)	Water to be pippete (uL)
Serie 1	Use **mother stock** as Serie 1				
Serie 2	1.6	1.6	From Serie 1	100	60
Serie 3	1.25	2		100	100
Serie 4	1.6	3.2		100	220
Serie 5	1.5625	5		100	400
Serie 6	2	10		100	900
Serie 7	2	2	From Serie 6	100	100
Serie 8	2	4		100	300
Serie 9	2.5	10		100	900
Serie 10	2	2.5	From Serie 9	100	150
Serie 11	2	5		100	400

Finally from each serie, take 5 uL of each stock and add 95 uL of water. **(Serie1: 5 ul mother stock + 95 ul of water).** Include a water sample as blank and prepare all these dilution series (including blank) in the same manner like SCFA samples.

Ideally for each set of samples have to be run 2 calibrations curves. One in the beginning and other at the end. If the samples to analyse are from different fluids, it is suggested to include one calibration curve in the middle of the run. For example, Calibration Curve 1- Serum Samples- Calibration Curve 2- Urine Samples – Calibration Curve 3. Also, if the number of sample to analyse is a large number it is recommended to run another calibration curve in the middle of the run.

Note: Dilution and concentrations of calibration curve are the following.

	Concentration (uM)	2	3	4	5	6	7	8	9	10	11
Compound	Dilution Factor	1.6	1.25	1.6	1.56	2	2	2	2.5	2.5	5
Acetic acid	400	250	200	125	80	40	20	10	4	1.6	0.8
Propionic acid	20	12.5	10	6.25	4	2	1	0.5	0.2	0.08	0.04
Butyric acid	20	12.5	10	6.25	4	2	1	0.5	0.2	0.08	0.04
Valeric acid	4	2.5	2	1.25	0.8	0.4	0.2	0.1	0	0.02	0.01
Isovaleric acid	40	25	20	12.5	8	4	2	1	0.4	0.16	0.08
Hexanoic acid (Caproic)	10	6.25	5	3.125	2	1	0.5	0.25	0.1	0.04	0.02
Isobutyric acid	20	12.5	10	6.25	4	2	1	0.5	0.2	0.08	0.04
Lactic acid	40000	25000	20000	12500	8000	4000	2000	1000	400	160	80
2-methyl-butyric acid	20	12.5	10	6.25	4	2	1	0.5	0.2	0.08	0.04
2-hydroxy butyric acid	2000	1250	1000	625	400	200	100	50	20	8	4

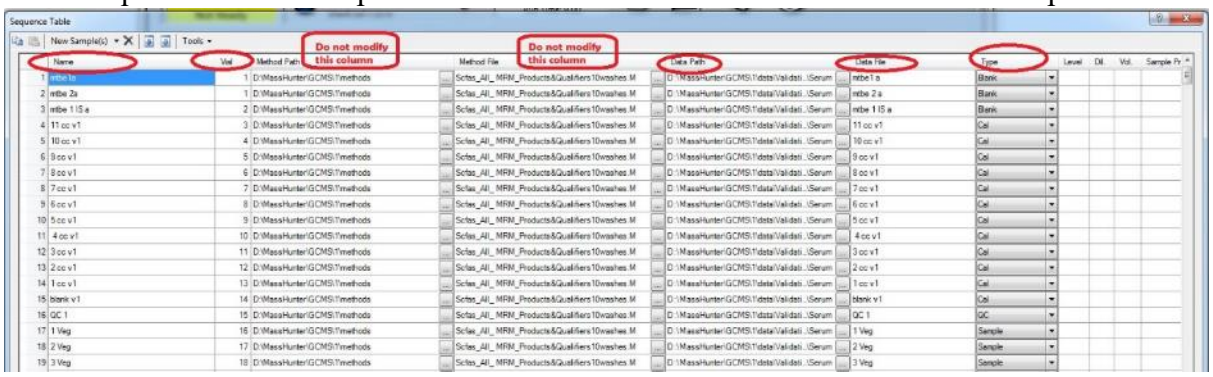
Appendix 7. How to setup a run in GCMS 3Q?

Document developed by Jerusa Brignardello

Go to GC MC – TOQ Enhanced Mass Hunter program which is the software to use with the GCMS machine. (Note: be sure that the GCMS was previously tuned by the personnel in charge.

Use the sequence which is loaded in the software and edit it with your run of samples.

In the sequence table some parameters have to be modified to initiate the run setup.



Name: Initiate each sequence with some vials of clean solvents in the first positions. In the case of the SCFAs method it is recommended to use non silanised vials full of MTBE. Calibration curve has to be loaded from the lowest to highest concentration. That means from 11 to 1 and followed by a blank. Start the sample sequence with a QC and then name each sample by the number of your randomisation list.

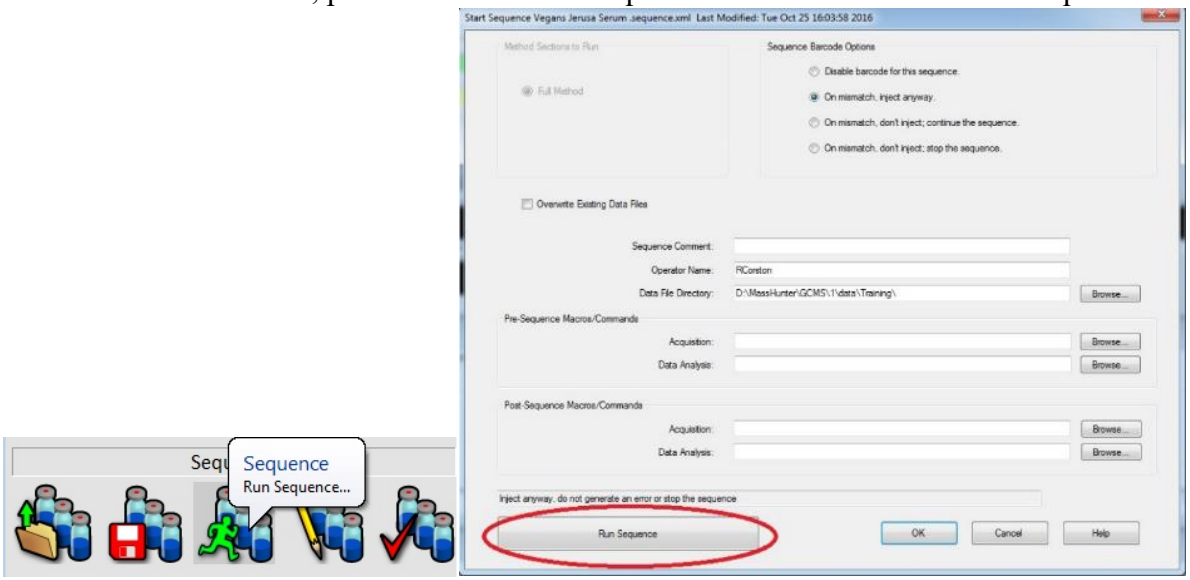
Vial: This indicates the position of each vial to run in the 3Q racks. For MTBE running, this can be repeated from the same vial but must be named differently for the same vial position (see image above). This part of the setup running is essential, each vial must be labelled properly and have the same name/number written in the vial column.

Method Path: Do not modify this column. This information is important for GCMS to find the file with the method to use.

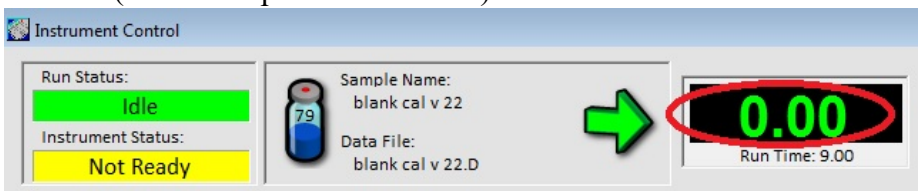
Method File: Do not modify this column. This method is located in the previous path and it has the same name as in the image (see image above).

Data Path: This column is to setup the location of each data file of analysed samples. It is important to create a new file for this and do not mix with folders from previous runs.

Data file: This is the name for each data file after its analysis. To copy and paste from name column. Type column must be defined previously.
Type : This section defines each calibration sample as “Cal” and the rest of the samples as “Samples”
To add samples click in “new samples”. Fill first name column, vial column and the rest. To fill with the same name use “Fill down” and to fill an increment of number use “fill increment” (both right click and press that option)
Once the entire run is set, press OK. Go to the sequence menu and select run the sequence.



Do not forget that the GCMS runs 4 samples per hour. Prepare enough samples to have a run without stops.
To top up the solvents, you need to fill from bottle A1 to A6 with methanol and from bottle B1 and B4 with MTBE. This has to be done after GCMS injection, ideally between 2 to 8.50 minutes (Check the panel of run time).

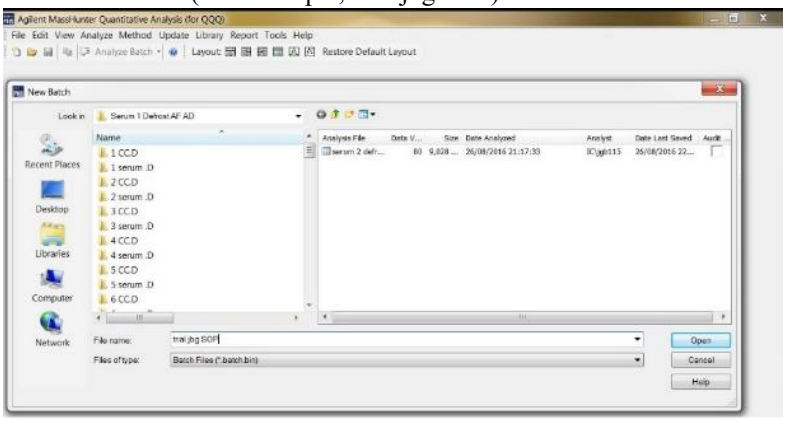


Do not forget that the run time per sample is 15 minutes, so you need to prepare enough samples to load the GCMS and have a continuous

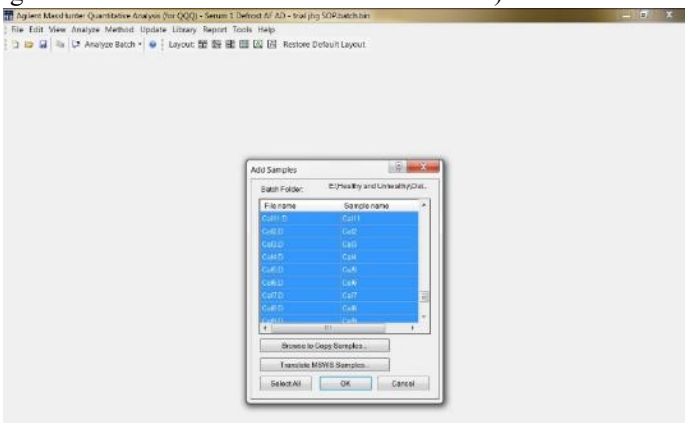
Appendix 8. SCFA method validation for 3Q

Document developed by Jerusa Brignardello

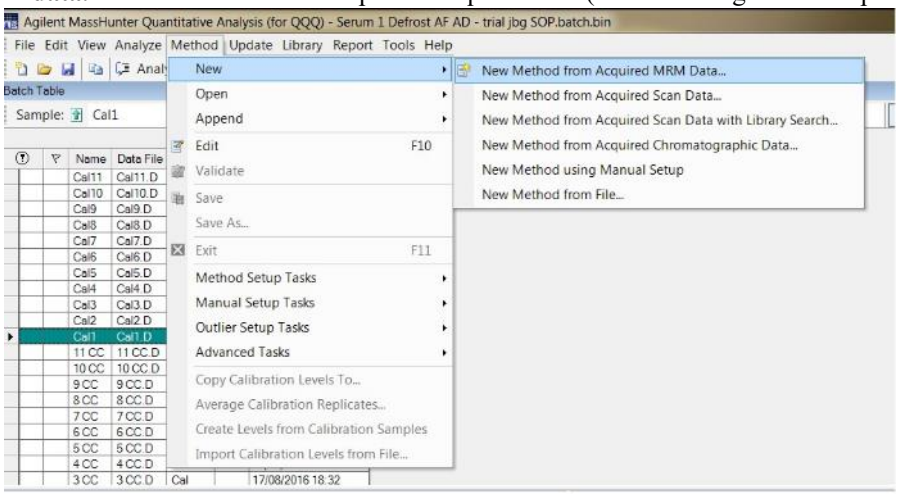
Open Agilent Mass Hunter Quantitative Analysis (QQQ) program which is installed on the 3Q computer. Create a new batch in the folder data (For Example, trial jbg SOP).

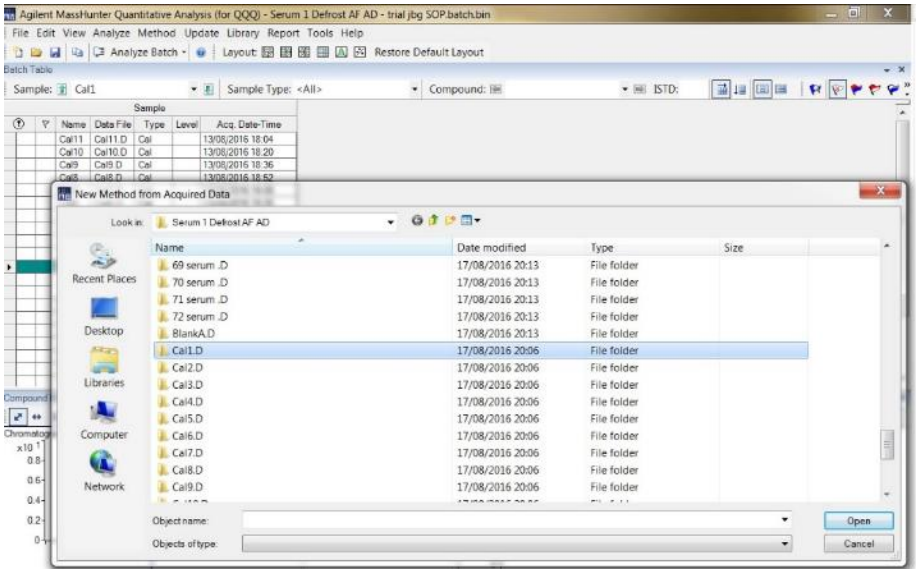


Select calibration curve points from 1 to 11 and include all the calibration curve repetitions. Set up each level of the calibration curve according to its concentration. (For example, Cal 11 has level 11), select "type column" and replace for "Cal" (Right Click>Select Cal> Left Click >Fill Down) and save.

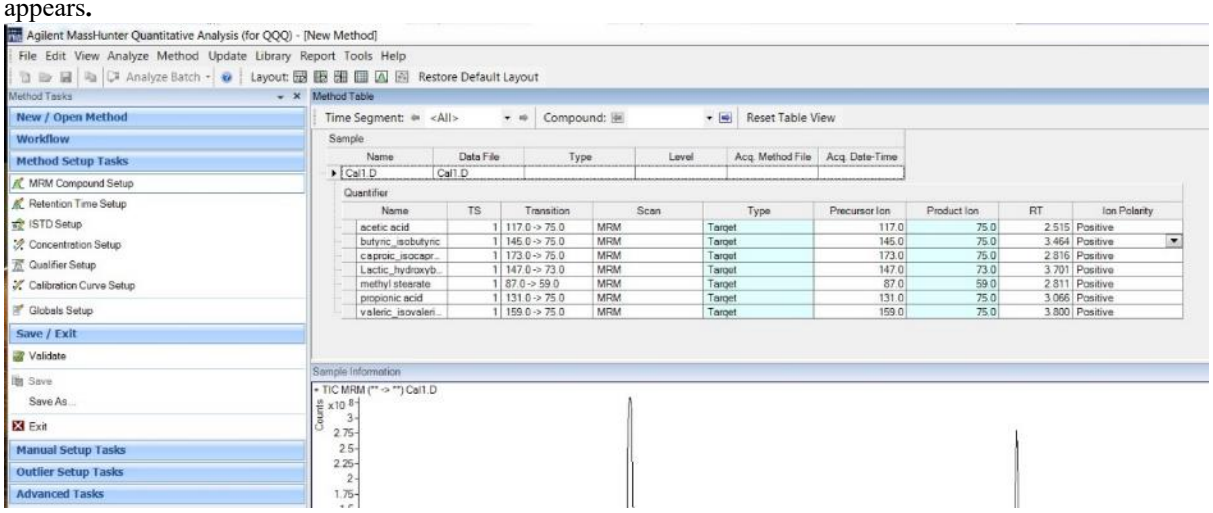


Select the highest concentration point of one calibration curve, click **Method > New > New method from acquired MRM data.** Then click the same sample for acquired data (see second figure of this point).

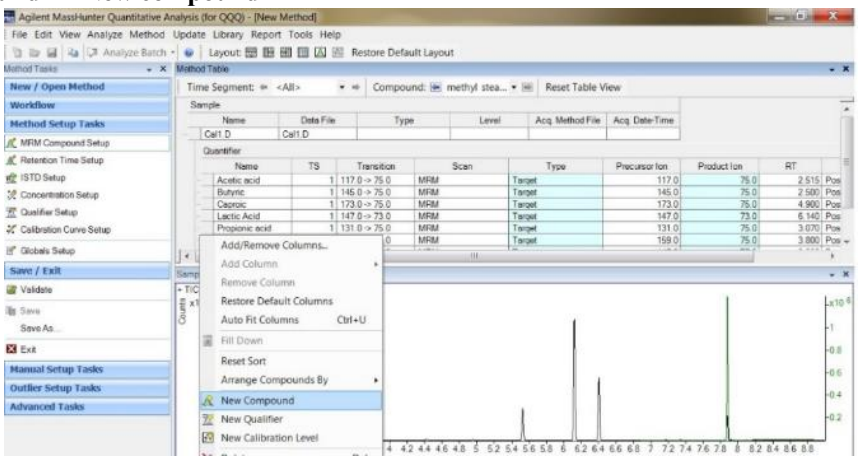




The Agilent Mass Hunter Quantitative Analysis (QQQ) program displays a dialog window for method configuration according to each compound. In the window of **Method Setup Task**, the default data for **Cal 1D** appears**.**



Click **Method> Method Setup Task >MRM Compound Setup**. The compounds to setup for this method are: 1. Acetic, 2. Propionic, 3. Butyric**, 4. Isobutyric, 5. 2 Methyl Butyric**, 6. Isovaleric, **7. Valeric,** 8. Caproic, 9. Lactic Acid, 10. 2 Hydroxy butyric and 11.Methyl Stereate. To add more compounds in the table press right click on "**one compound" > New compound**



Retention times (RT) have to be edited at "Retention Time Setup" for each compound. Click **Method Setup Task> Retention Time Setup**. Compounds can be arranged by retention time (**right click > Arrange Compounds By >Retention Time**)

SCFA	RT	SCFA	RT
Acetic Acid	2.5	Propionic Acid	3.07
Isobutyric Acid	3.2	Butyric Acid	3.4
2 Methyl Butyric Acid	3.7	Isovaleric Acid	3.8
Valeric Acid	4.2	Caproic Acid	4.9
Lactic Acid	6.14	2 Hydroxy butyric Acid	6.4
Methyl Stereate	7.88 (IS)		

In this part it is important to set up the column **Left RT Delta** and **Right RT Delta** with 0.2 minutes. This reduces the range of integration in order to properly apply the RT for integration and get better results for automatic integration results.

Method Table

Time Segment: <All> Compound: Reset Table View

Sample

Name	Data File	Type	Level	Acq. Method File	Acq. Date-Time
11 cc	11 cc.D	Cal	11	Scfas_All_ MRM...	03/10/2016 17:...

Quantifier

Name	TS	Transition	Scan	Type	RT	Left RT Delta	Right RT Delta	RT Delta
Acetic Acid	1	117.0 -> 75.0	MRM	Target	2.500	0.200	0.200	Minutes
Isobutyric	1	145.0 -> 75.0	MRM	Target	3.200	0.200	0.200	Minutes
Caproic	1	173.0 -> 75.0	MRM	Target	4.900	0.200	0.200	Minutes
Lactic	1	147.0 -> 73.0	MRM	Target	6.140	0.200	0.200	Minutes
Methyl Stearate	1	87.0 -> 55.0	MRM	ISTD	7.889	0.200	0.200	Minutes
Propionic Acid	1	131.0 -> 75.0	MRM	Target	3.070	0.200	0.200	Minutes
Butyric	1	145.0 -> 75.0	MRM	Target	3.400	0.200	0.200	Minutes
2 Methyl Butyric	1	159.0 -> 75.0	MRM	Target	3.700	0.200	0.200	Minutes
Valeric	1	159.0 -> 75.0	MRM	Target	4.200	0.200	0.200	Minutes
2 Hydroxy butyric	1	147.0 -> 73.0	MRM	Target	6.400	0.200	0.200	Minutes
Isovaleric	1	159.0 -> 75.0	MRM	Target	3.800	0.200	0.200	Minutes

Click **Method> Method Setup Task > ISTD Setup** (Internal standard). Click on the **ISTD Flag column** for Methyl Stearate, then in the column of **ISTD compound name** methyl stearate will appear as an option to complete for each compound. Select the first one, right click and select **"Fill Down"** and leave IS with **"None**". ISTD concentration is 100 ppm for all the compounds. Fill **Type** Column with target for each compound and ISTD for Methyl stearate. Complete the Time Segment column **TS** with 1.

Method Table

Time Segment: <All> Compound: Reset Table View

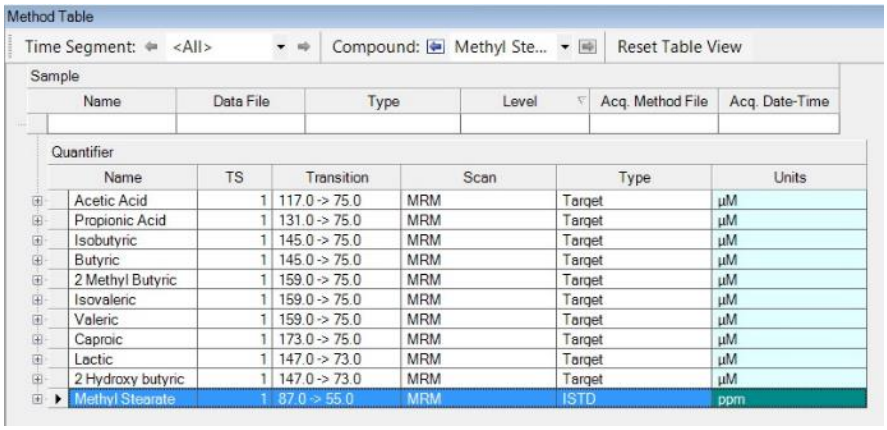
Sample

Name	Data File	Type	Level	Acq. Method File	Acq. Date-Time

Quantifier

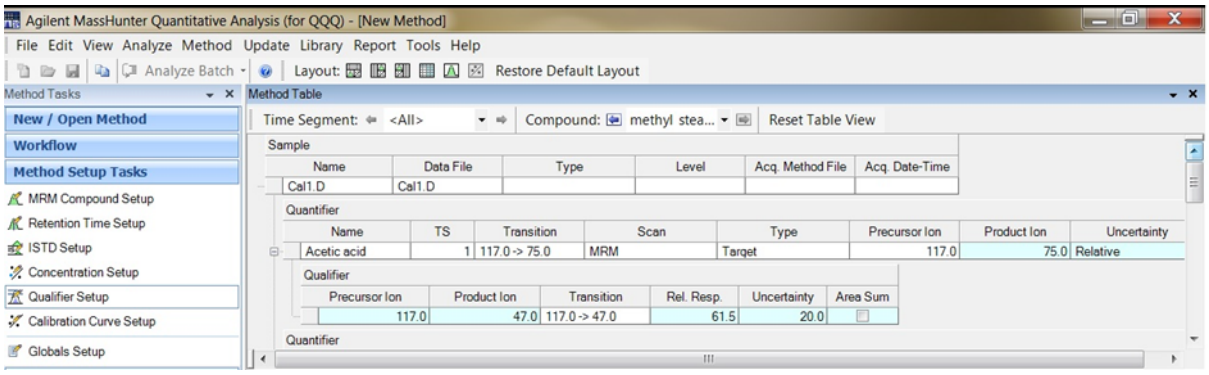
Name	TS	Transition	Scan	Type	ISTD Compound Name	ISTD Flag	ISTD Conc.	Time F
Acetic Acid	1	117.0 -> 75.0	MRM	Target	Methyl Stearate	☐		
Propionic Acid	1	131.0 -> 75.0	MRM	Target	Methyl Stearate	☐		
Isobutyric	1	145.0 -> 75.0	MRM	Target	Methyl Stearate	☐		
Butyric	1	145.0 -> 75.0	MRM	Target	Methyl Stearate	☐		
2 Methyl Butyric	1	159.0 -> 75.0	MRM	Target	Methyl Stearate	☐		
Isovaleric	1	159.0 -> 75.0	MRM	Target	Methyl Stearate	☐		
Valeric	1	159.0 -> 75.0	MRM	Target	Methyl Stearate	☐		
Caproic	1	173.0 -> 75.0	MRM	Target	Methyl Stearate	☐		
Lactic	1	147.0 -> 73.0	MRM	Target	Methyl Stearate	☐		
2 Hydroxy butyric	1	147.0 -> 73.0	MRM	Target	Methyl Stearate	☐		
Methyl Stearate	1	87.0 -> 55.0	MRM	ISTD	<None>	☑	100.0000	

Click **Method> Method Setup Task> Concentration Setup.** Complete the column of unit with uM for SCFAs and ppm methyl stearate.

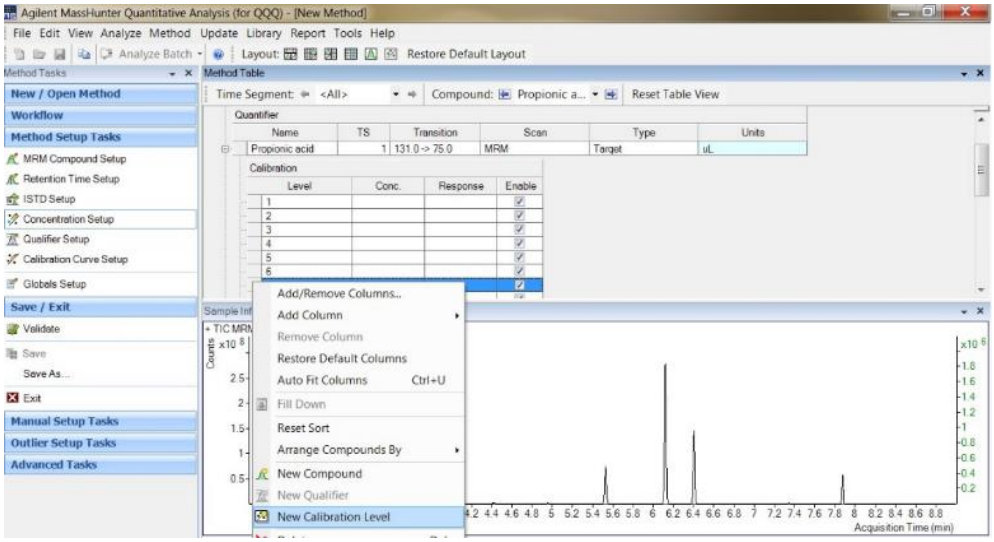


To set up quantifiers and qualifiers ions click **Method> Method Setup Task > Qualifier Setup** according to these values:

Compound	**Quantifier** Precursor Ion	**Quantifier** Product Ion	**Qualifier** Precursor Ion	Qualifier Product Ion
Acetic Acid	117	75	117	47
Propionic Acid	131	75	131	47
Isobutyric Acid	145	75	145	43
Butyric Acid	145	75	145	43
2-methyl-butyric Acid	159	75	159	57
Isovaleric Acid	159	75	159	57
Valeric Acid	159	75	159	57
Caproic Acid	173	75	173	81
Lactic Acid	147	73	147	45
2-hydroxybutyric acid	147	73	147	45
Methyl Stearate	87	55	87	59



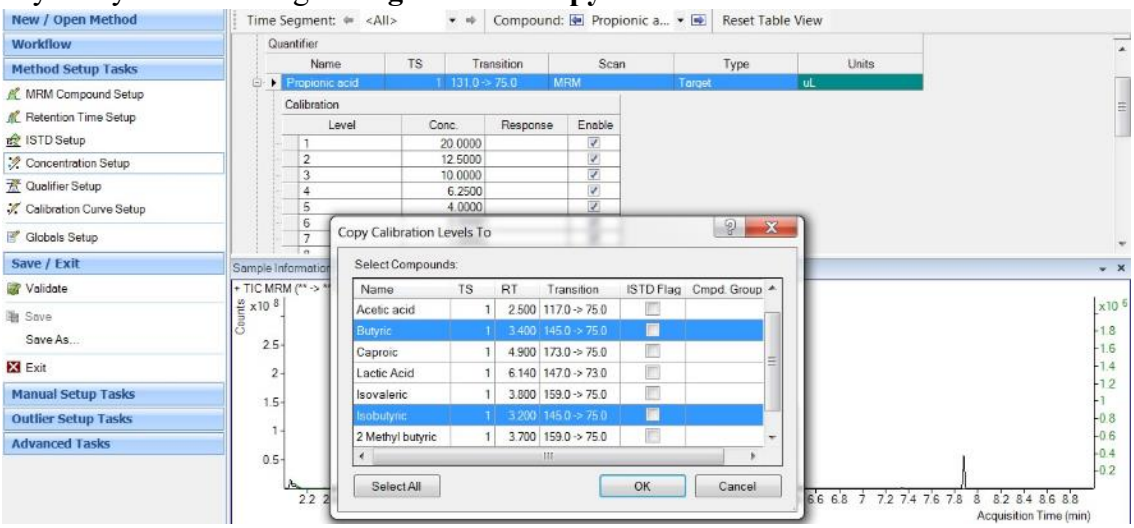
To add the calibration curves levels click **Method> Method Setup Task > Calibration Curve Setup.** To include calibrations levels **(right click > Add New Calibration Levels)** and include 11 levels.



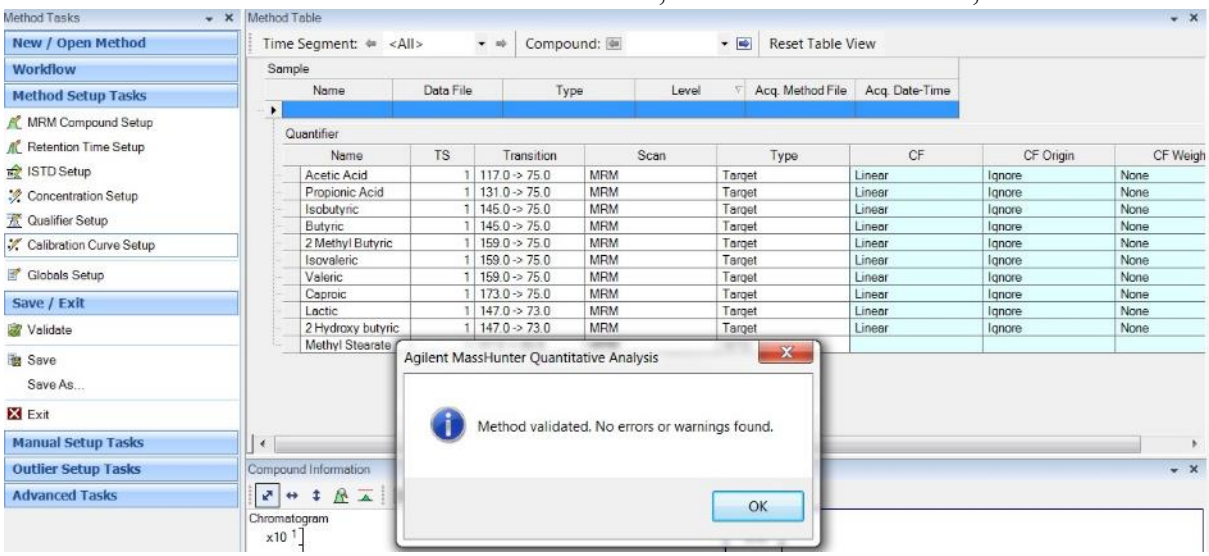
Complete calibration levels for each compound according to these concentrations. (Light blue dilutions are the same).

	Concentration (uM)										
Compounds	Dilution Factor	1.6	1.25	1.6	1.5625	2	2	2	2.5	2.5	5
Acetic acid	400	250	200	125	80	40	20	10	4	1.6	0.8
Propionic acid	20	12.5	10	6.25	4	2	1	0.5	0.2	0.08	0.04
Butyric acid	20	12.5	10	6.25	4	2	1	0.5	0.2	0.08	0.04
Valeric acid	4	2.5	2	1.25	0.8	0.4	0.2	0.1	0.04	0.016	0.008
Isovaleric acid	40	25	20	12.5	8	4	2	1	0.4	0.16	0.08
Hexanoic acid (Caproic)	10	6.25	5	3.125	2	1	0.5	0.25	0.1	0.04	0.02
Isobutyric acid	20	12.5	10	6.25	4	2	1	0.5	0.2	0.08	0.04
Lactic acid	40000	25000	20000	12500	8000	4000	2000	1000	400	160	80
2-methyl-butyric acid	20	12.5	10	6.25	4	2	1	0.5	0.2	0.08	0.04
2-hydroxybutyric acid	2000	1250	1000	625	400	200	100	50	20	8	4
Dilution numbers (levels)	1	2	3	4	5	6	7	8	9	10	11

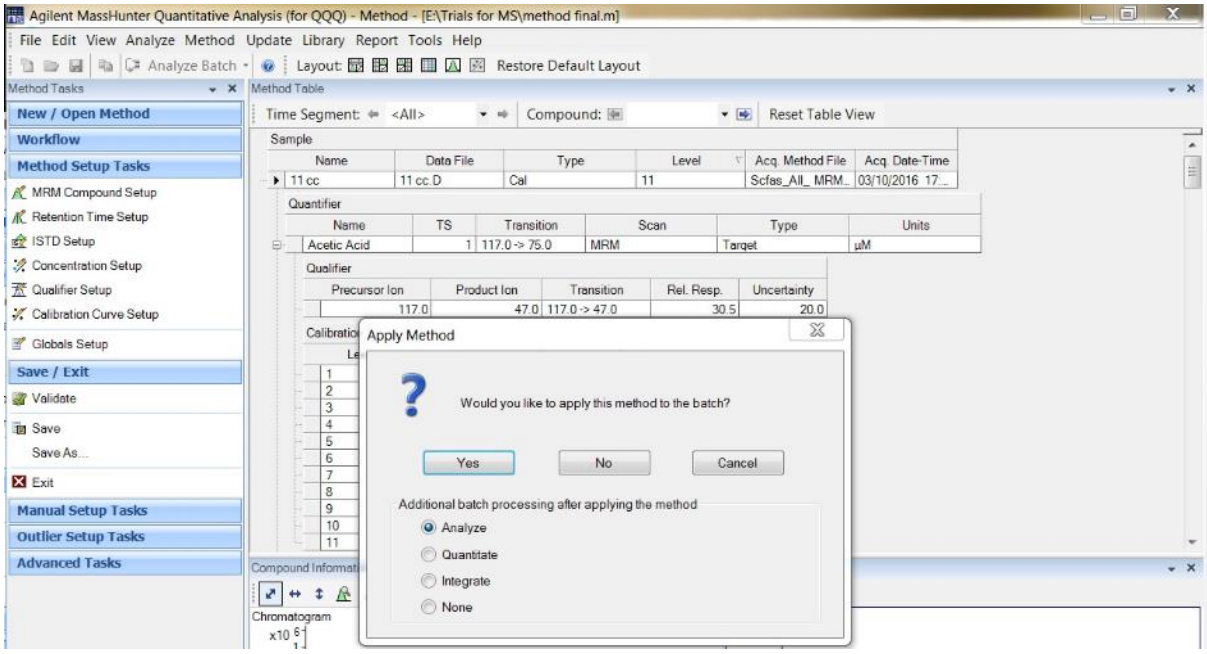
Concentrations of Propionic acid can be copied for Butyric acid, Isobutyric Acid and 2 Methyl Butyric Acid using the **right click> Copy Calibrations Levels To** .



Once the entire concentrations levels are set, validate the method, save and exit.



Open your previous batch created for this method and go to **Method> Open Method from Existing File** and select the saved method. Validate the method again, select exit and apply the method to the batch. (see the pic below)

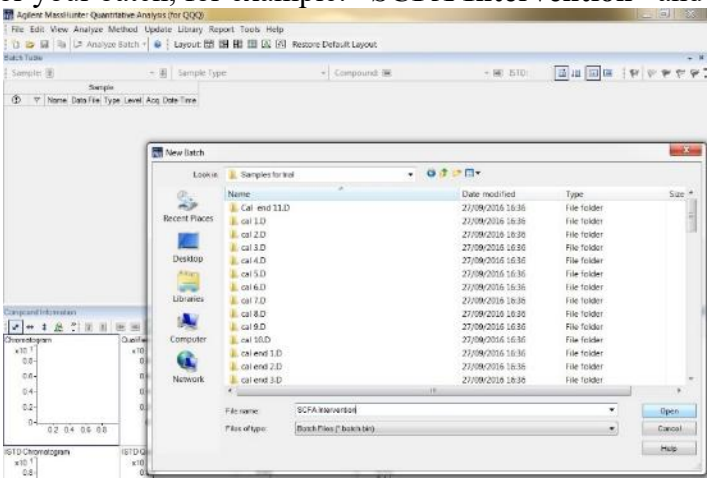


Then continue with the document named **"Use of a previous validated method for SCFA in GCMS 3Q"** from point 9.

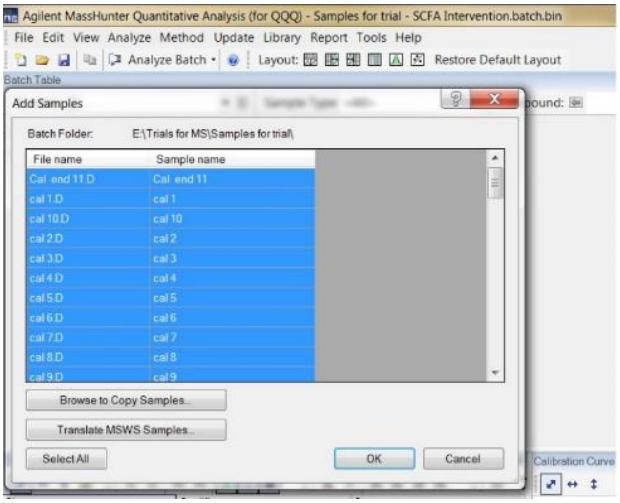
Appendix 9. Use of a previously validated method for SCFA in GCMS 3Q and obtain data

Document developed by Jerusa Brignardello

Open Agilent Mass Hunter Quantitative Analysis (QQQ) which is installed in the GCMS-3Q computer.
Creating a batch in MassHunter begins by defining the batch name and the folder in which it will be saved. Use your name or project to call your batch folder. Select **File> New Batch**
It is most convenient if the batch folder is in the same folder that contains the data files which you will put into the batch. In this example, we are going to use the folder called **"Samples for trial"**.
Navigate to the folder you want to use as the batch folder. In this case, it is the folder that contains the translated data files from the MS Workstation (those identified in the Sample List). The folders contained in "samples for trial" have **".D"**extension.
Enter a **file name** for your batch, for example: **"SCFA Intervention"** and then click **Open.**

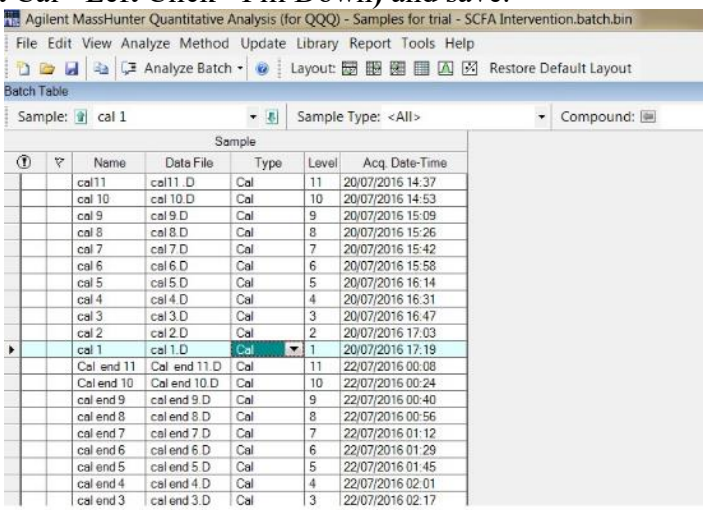


The program displays the batch table with the name of your sample folder (for this case, Samples for trial). It will also:
Create a batch file with the name you specified and the extension .**batch.bin**
Save the batchname**.batch.bin** file in the defined folder. Select <u>your **calibration curve files**</u> and press continue. Now each translated file is included in the new batch

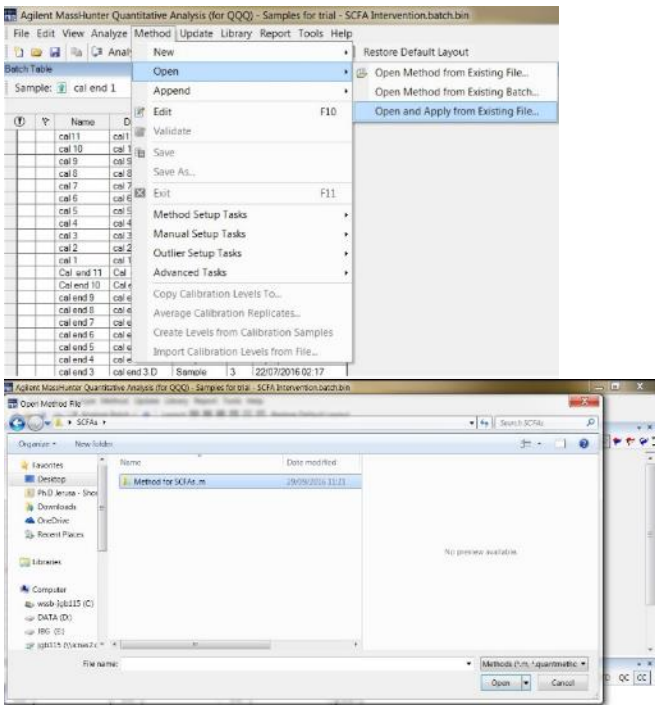


Each file is included in the new batch **"SCFA Intervention"** (File name **SCFA intervention.batch.bin**). Set up each level of the calibration curve according to its

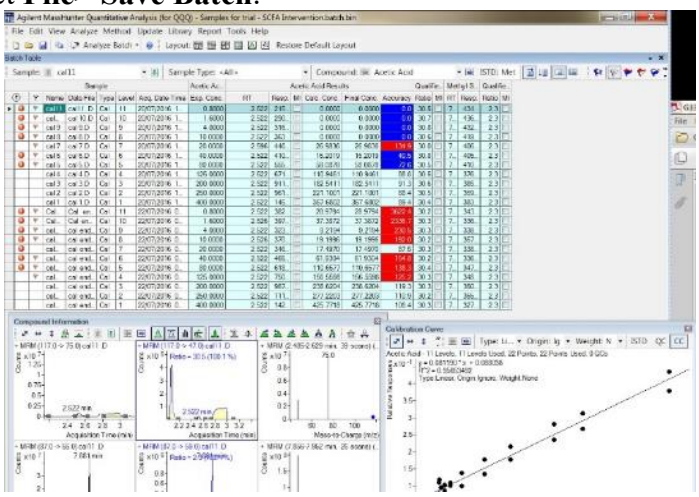
concentration. (For example, Cal 11 has level 11), select “type column” and replace for **“Cal”** (Right Click>Select Cal> Left Click >Fill Down**)** and save.



Select the **Method> Open> Open and Apply from Existing File**. Select from the folder called “SCFAs” from the desktop, then select method file previously validated called “**Method for SCFAs.m**”



A new screen will appear. Select **"Analize batch"** in order to obtain the concentrations from your calibration curves. Select **File> Save Batch**.



Then, it is necessary to **manually check each chromatogram** (essential step) from the calibration curve points according to its retention time, transitions and qualifiers ions:

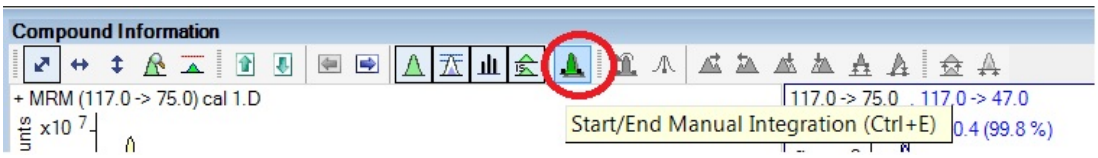
Table 1. SCFAs retention time

SCFA	RT (min)	SCFA	RT (min)
Acetic Acid	2.5	Propionic Acid	3.07
Isobutyric Acid	3.2	Butyric Acid	3.4
2 Methyl Butyric Acid	3.7	Isovaleric Acid	3.8
Valeric Acid	4.2	Caproic Acid	4.9
Lactic Acid	6.14	2 Hydroxy butyric Acid	6.4
Methyl Stereate	7.88 (IS)		

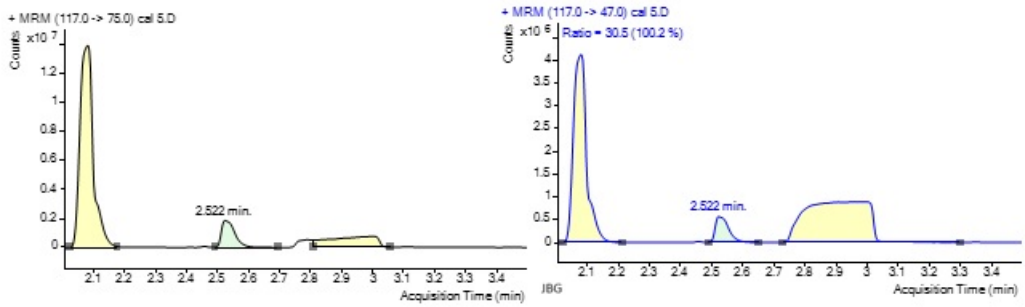
Table 2. Quantifiers and qualifiers ions

Compound	Quantifier		Qualifier	
	Precursor Ion	Product Ion	Precursor Ion	Product Ion
Acetic Acid	117	75	117	47
Propionic Acid	131	75	131	47
Isobutyric Acid	145	75	145	43
Butyric Acid	145	75	145	43
2-methyl-butyric Acid	159	75	159	57
Isovaleric Acid	159	75	159	57
Valeric Acid	159	75	159	57
Caproic Acid	173	75	173	81
Lactic Acid	147	73	147	45
2-hydroxybutyric acid	147	73	147	45
Methyl Stearate	87	59	87	55

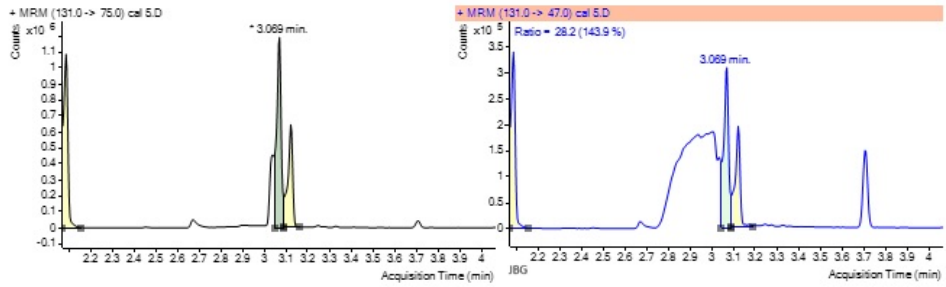
To manually edit the integration done by MassHunter QQQ for each sample, select the tool **"Start/ End Manual Integration"** from the **"Compound Information"** window and check each chromatogram.



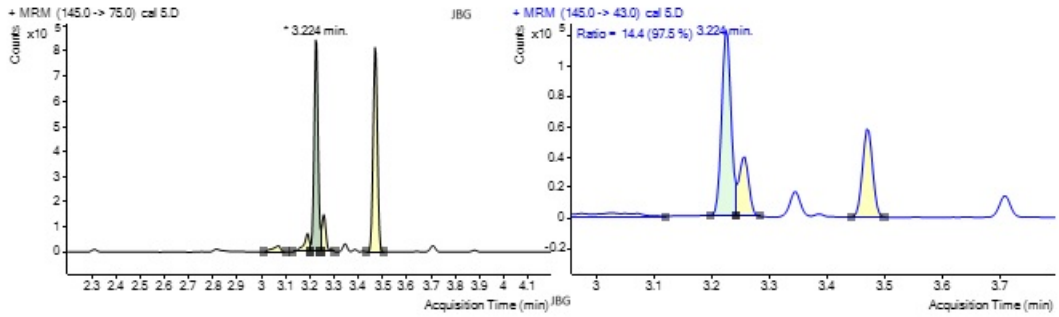
Chromatograms for each SCFAs have the following appearance:
Acetic acid:



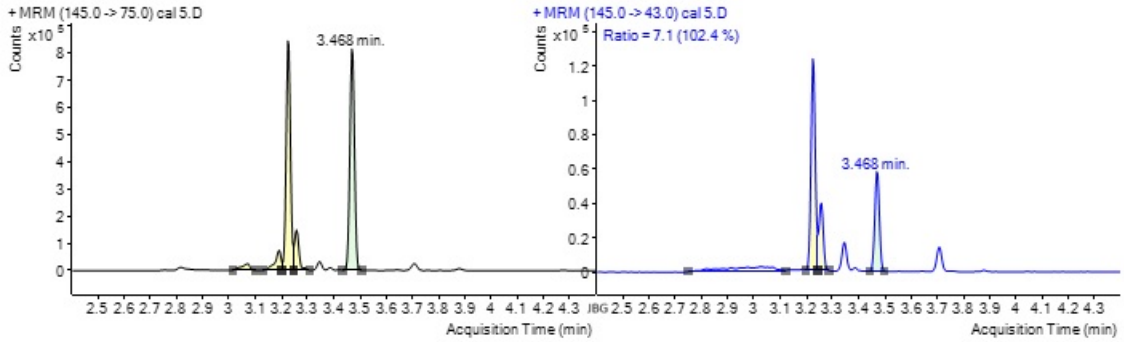
Propionic acid:



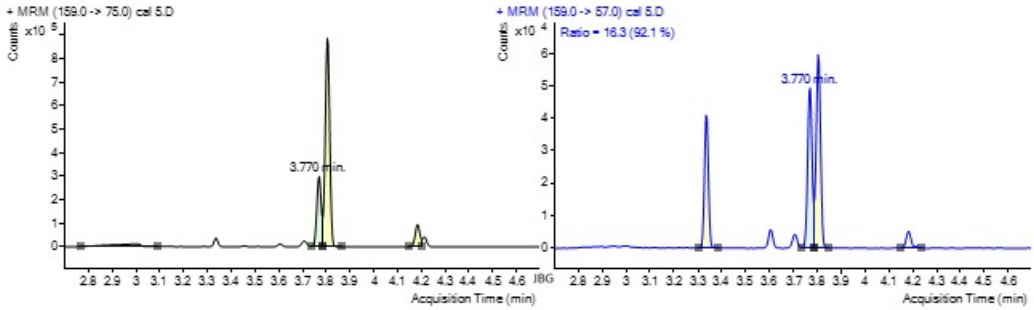
Isobutyric acid:

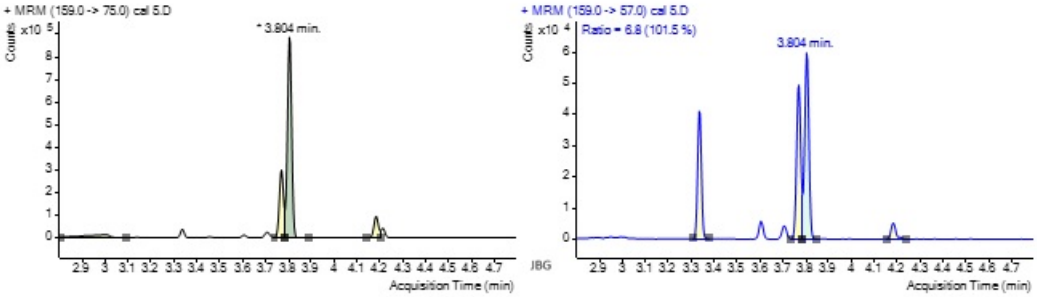


Butyric acid:



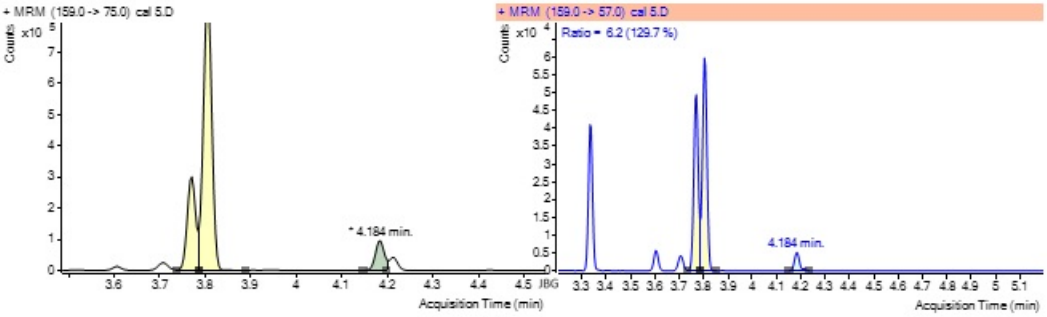
2 Methyl butyric acid:



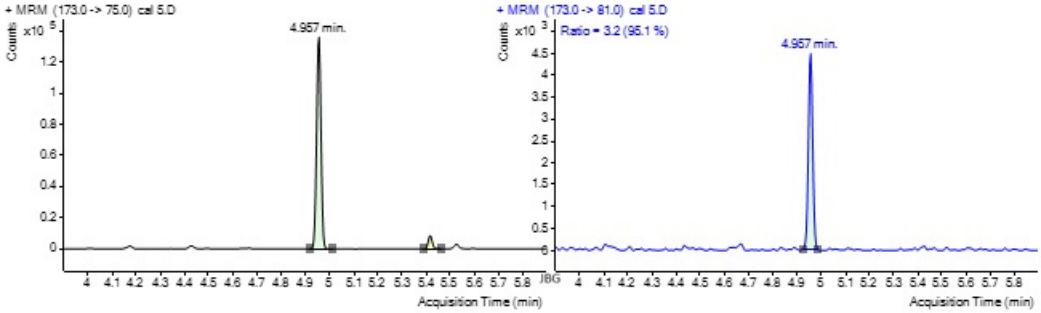


Isovaleric acid:

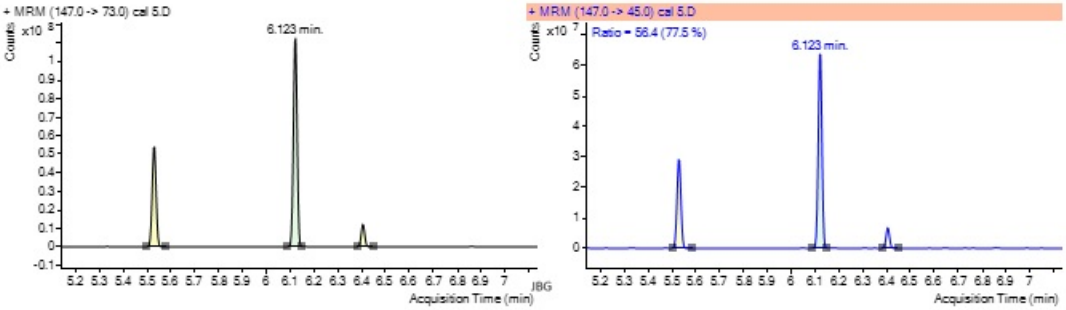
Valeric acid:



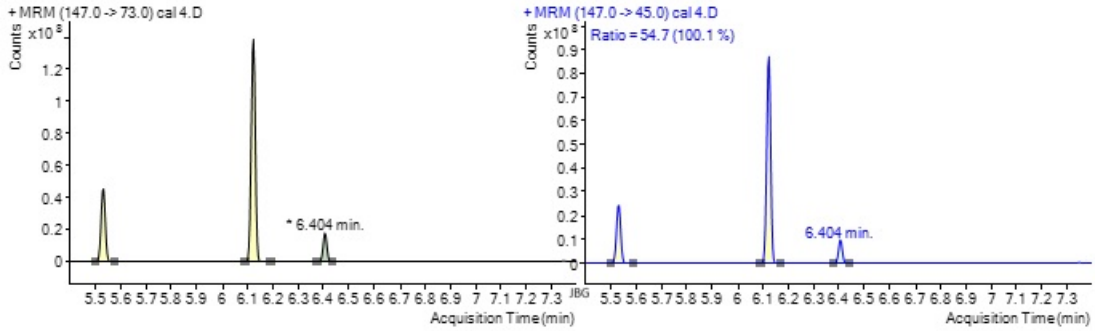
Caproic acid:



Lactic acid:



2 Hydroxy Butyric acid:



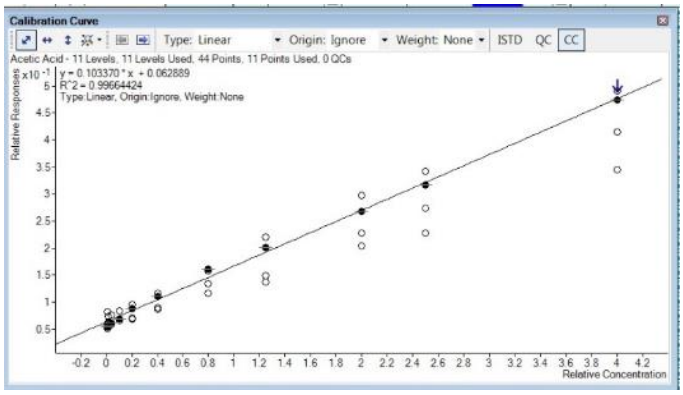
Once each chromatogram peak from the calibration curve was checked for its retention time, calibration curves will be ready to use to calculate the concentration of each compound. Then select **Analyze> Analyze Batch** in order to obtain the new order of calibration curve points.

To choose the best points for the definitive calibration curve to use, consider the expected concentration as a reference (see calibration curve protocol) and the calculated concentration of each calibration curve point. In order to get the best R^2, it is recommendable to run 2 calibration curves or 3 in the case of the analysis of different biofluids or a large number of samples. For example, Calibration Curve 1- Serum Samples- Calibration Curve 2- Urine Samples – Calibration Curve 3.) Also, in the calibration curve the settings used are:

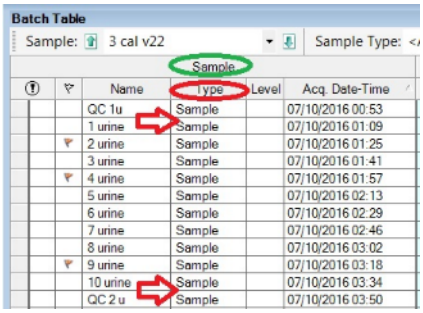
Type of curve: linear

Origin: Ignore

Weight: none



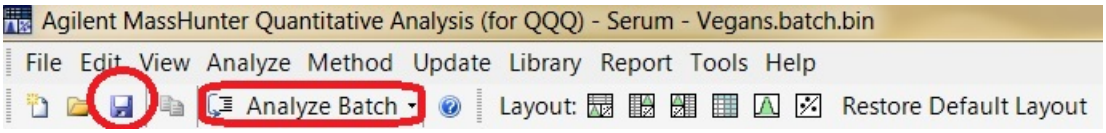
Add the remaining samples **File> Add Samples**. It is highly recommendable to configure in the batch table **"Sample"/ "Type"** the QC and samples as samples. (Right Click>Select samples> Left Click >Fill Down).



Add the remaining samples **File> Add Samples**, **"Analyze Batch"** and save. In order to obtain a suitable **final concentration result** after the calculation of each analysed sample it is necessary to use a number of calibration levels according to the distribution of the samples results through the calibration

curve points. Some small concentrations of SCFAs are found in biofluids, so this approach could be useful. For example: propionic acid, isobutyric acid, butyric acid, valeric acid, caproic acid, lactic acid are normally found in small concentrations and one calibration curve with 11 points probably will not allow to calculate them properly. Additionally, it is recommended for some cases to set up different number of calibration curves points for different matrix samples. Urine samples are prone to have lower levels of SCFAs compared to serum or plasma samples. (The minimal number of points to use is 5)

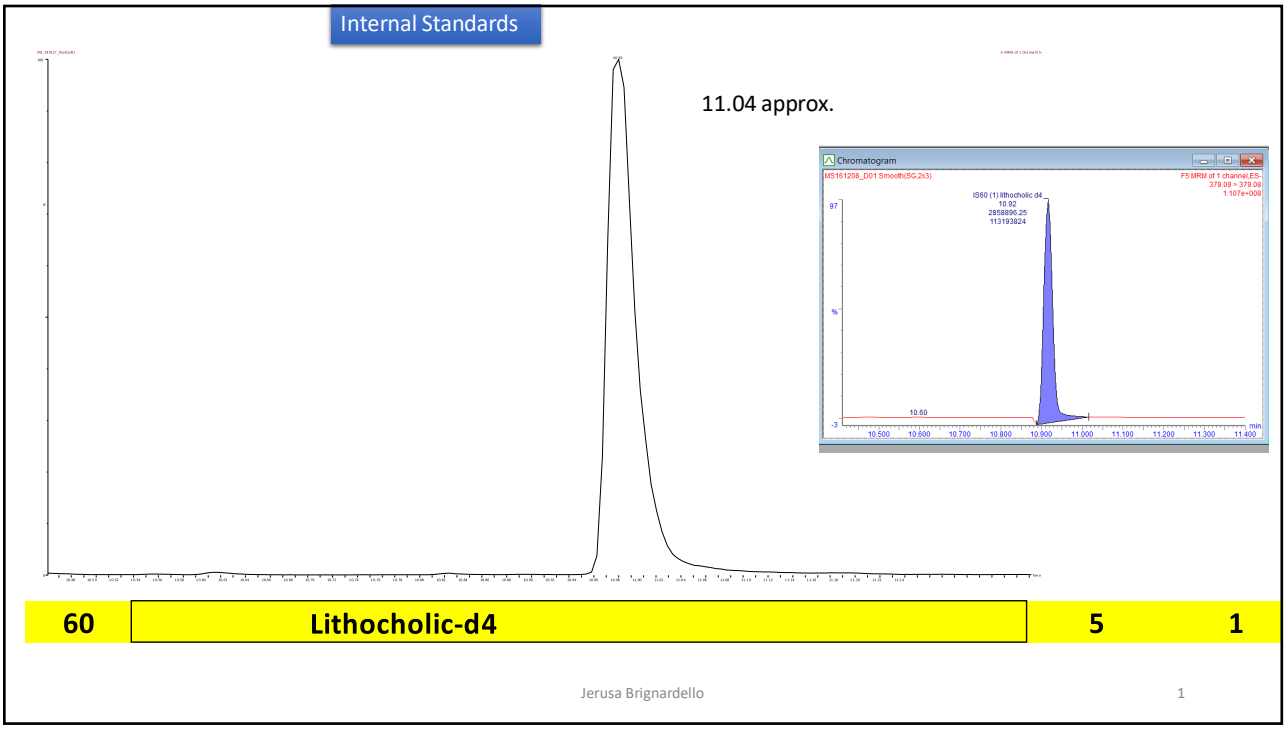
Once the calibration curve is ready, save your work and check each sample chromatogram in order to confirm if the automatic integration of the software was done adequately and manually check each integration (The program does the automatic integration, but it is important to check manually). Then, select **"Analize Batch"** again and save.



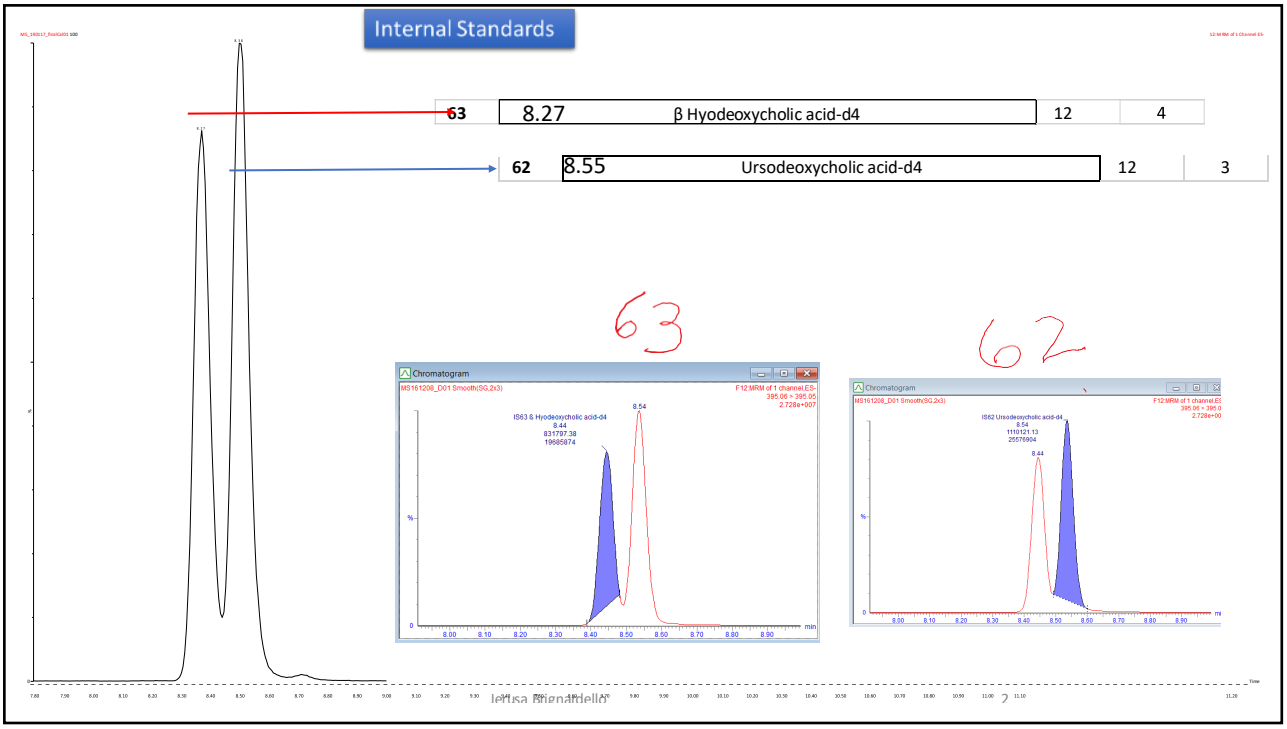
To export the calculated results selected one row (it will turn blue) from **Batch Table** and then select from the main menu **File> Export> Export Table**. It is important to have an established order of samples at the moment to export in the batch table, for example organize the samples by "Acq. Date time" Use the same order for each data compound exportation data, that makes the data organization in excel easy).

Appendix 10. BAs chromatograms guidance

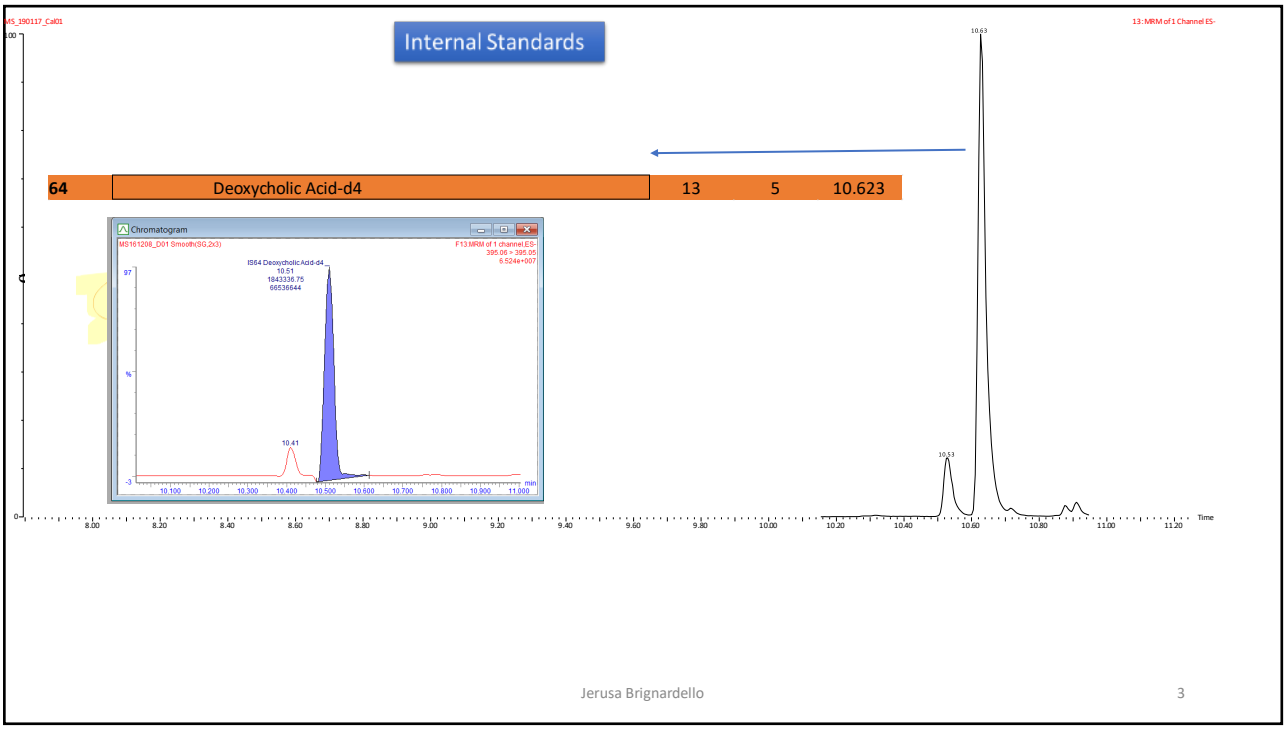
Document developed by Jerusa Brignardello



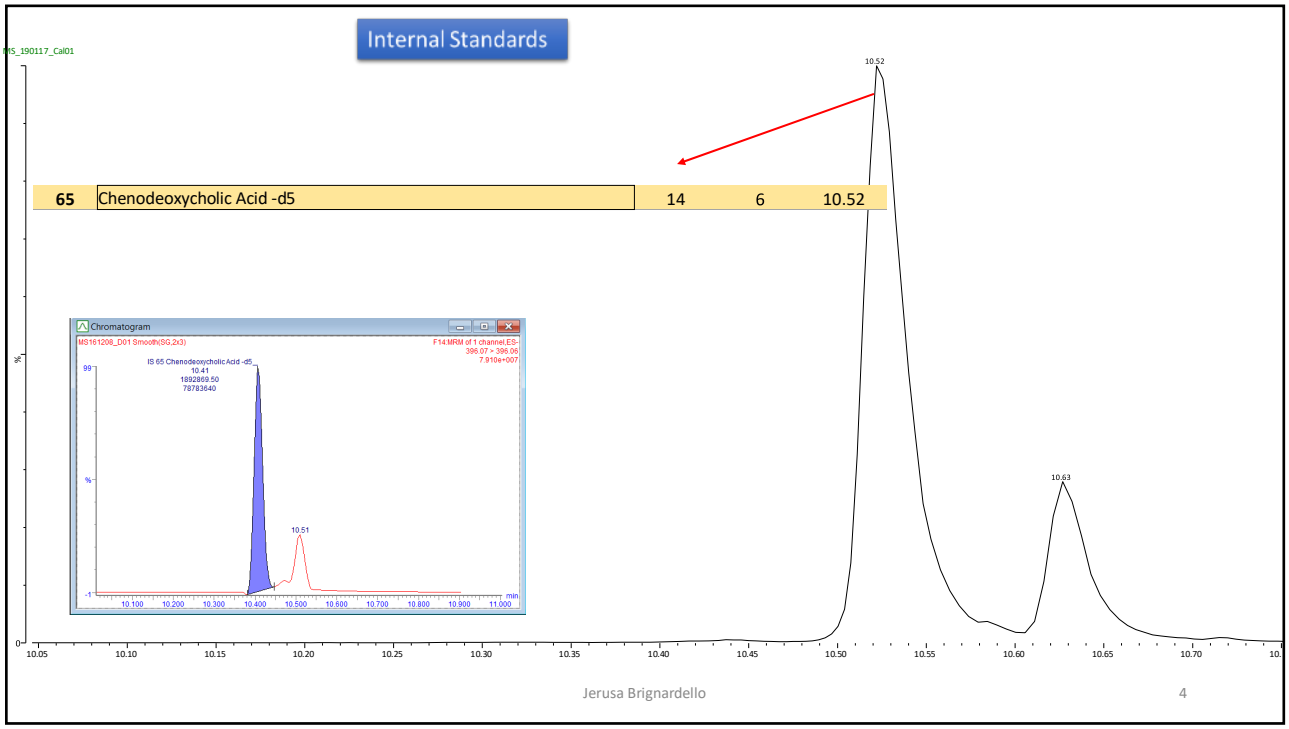
1



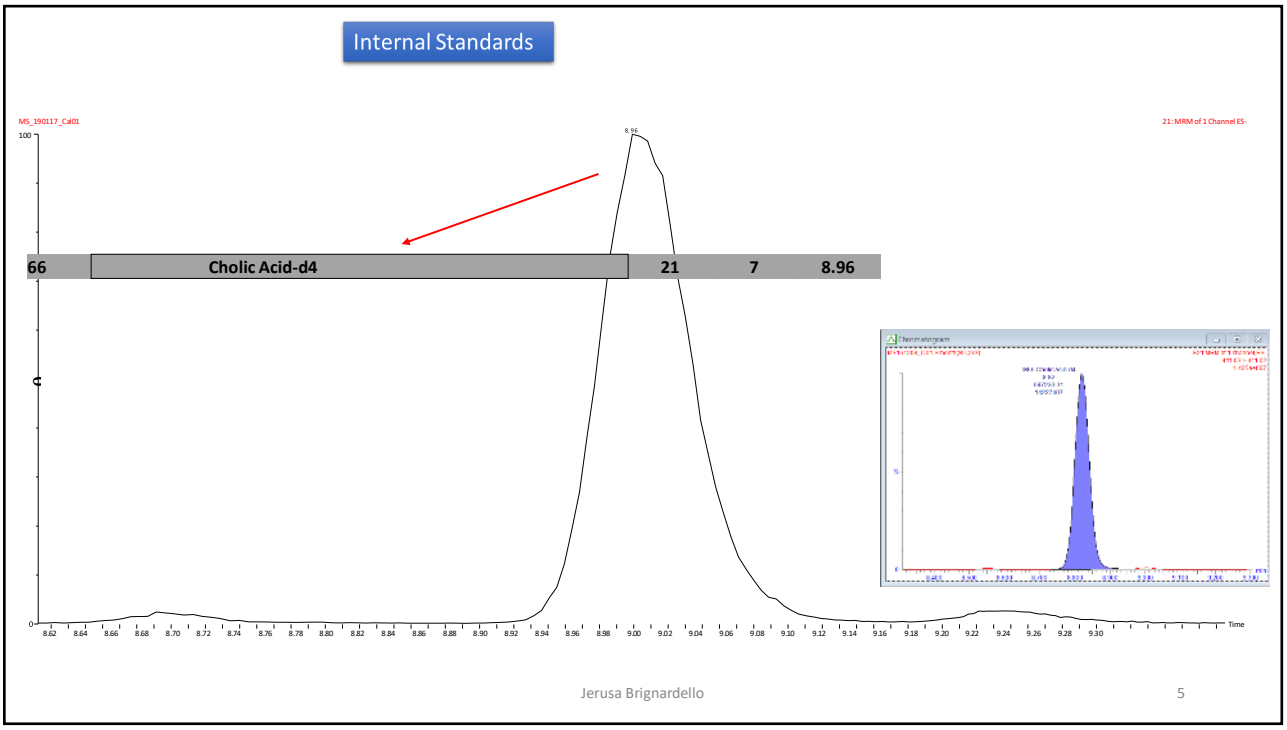
2


Internal Standards
64 Deoxycholic Acid-d4 13 5 10.623
Chromatogram
10.63
10.41
Time
Jerusa Brignardello
3

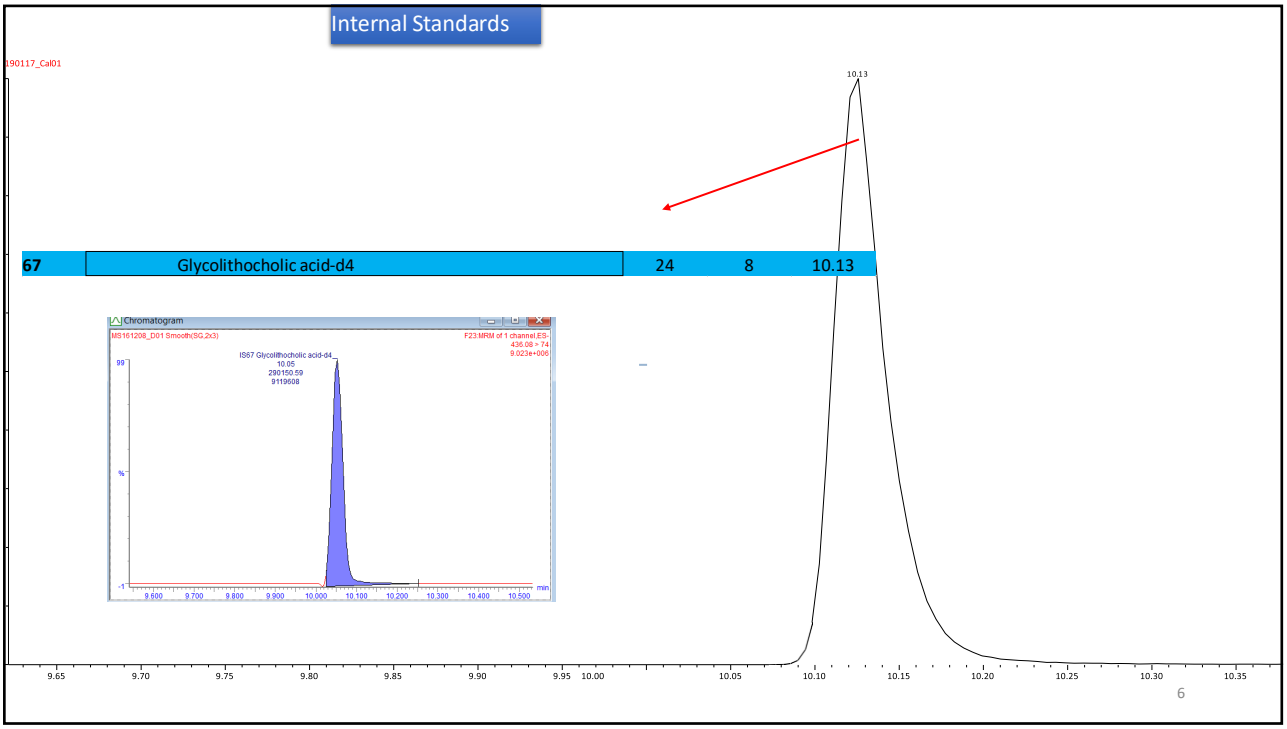
3


Internal Standards
65 Chenodeoxycholic Acid -d5 14 6 10.52
Chromatogram
10.52
10.63
10.51
Jerusa Brignardello
4

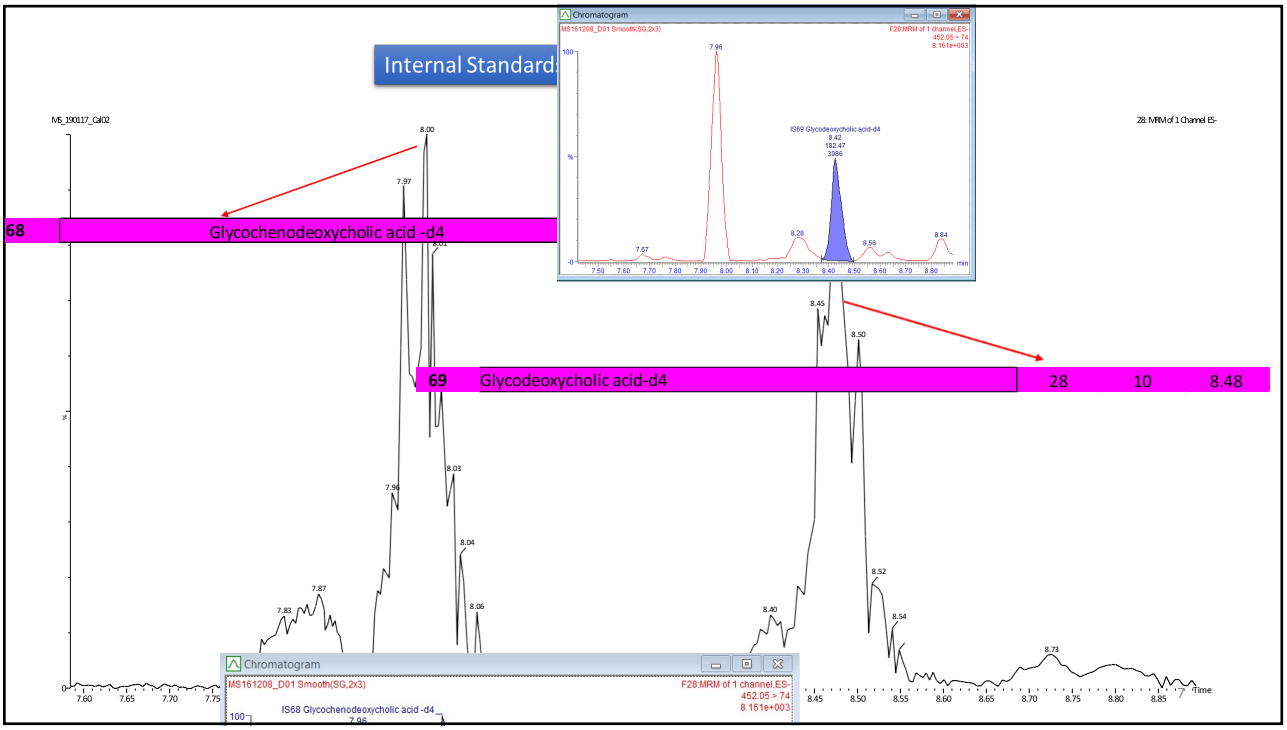
4


Internal Standards
MS_190117_Cal01
21: MRM of 1 Channel ES-
8.96
66
Cholic Acid-d4
21
7
8.96
Time
Jerusa Brignardello
5

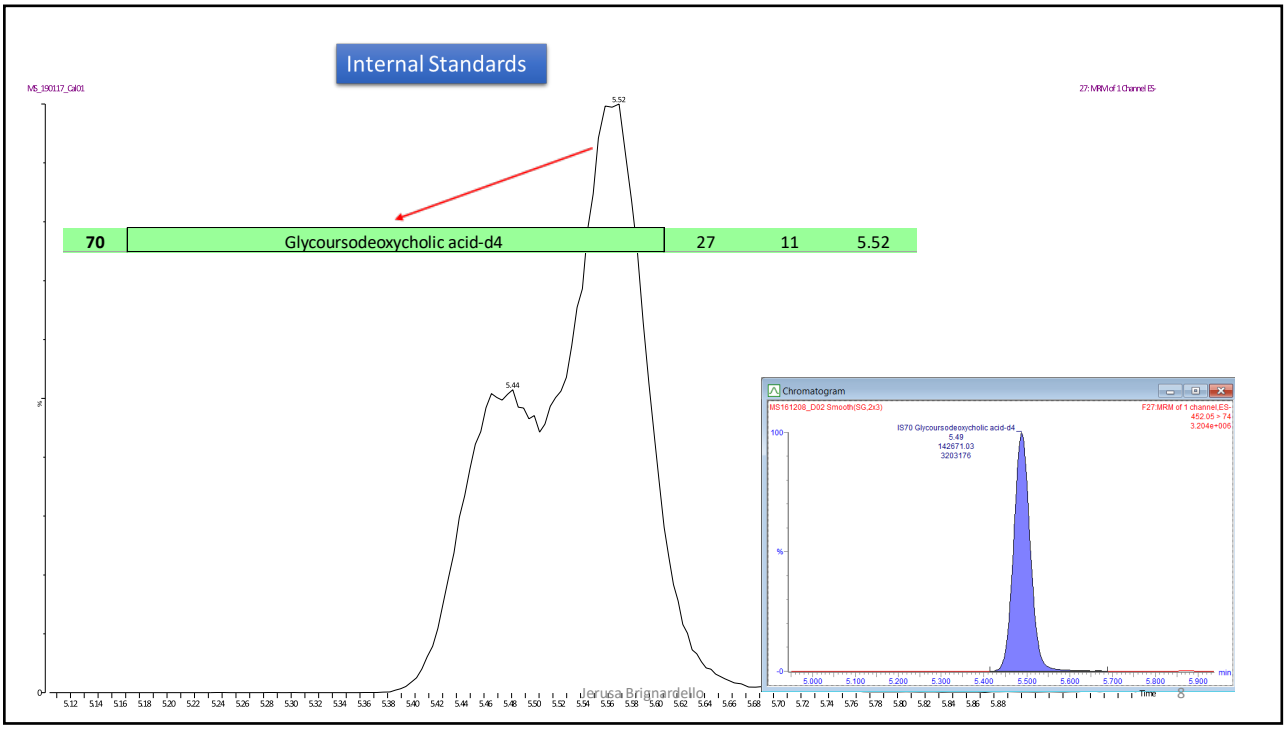
5


Internal Standards
190117_Cal01
10.13
67
Glycolithocholic acid-d4
24
8
10.13
Chromatogram
6

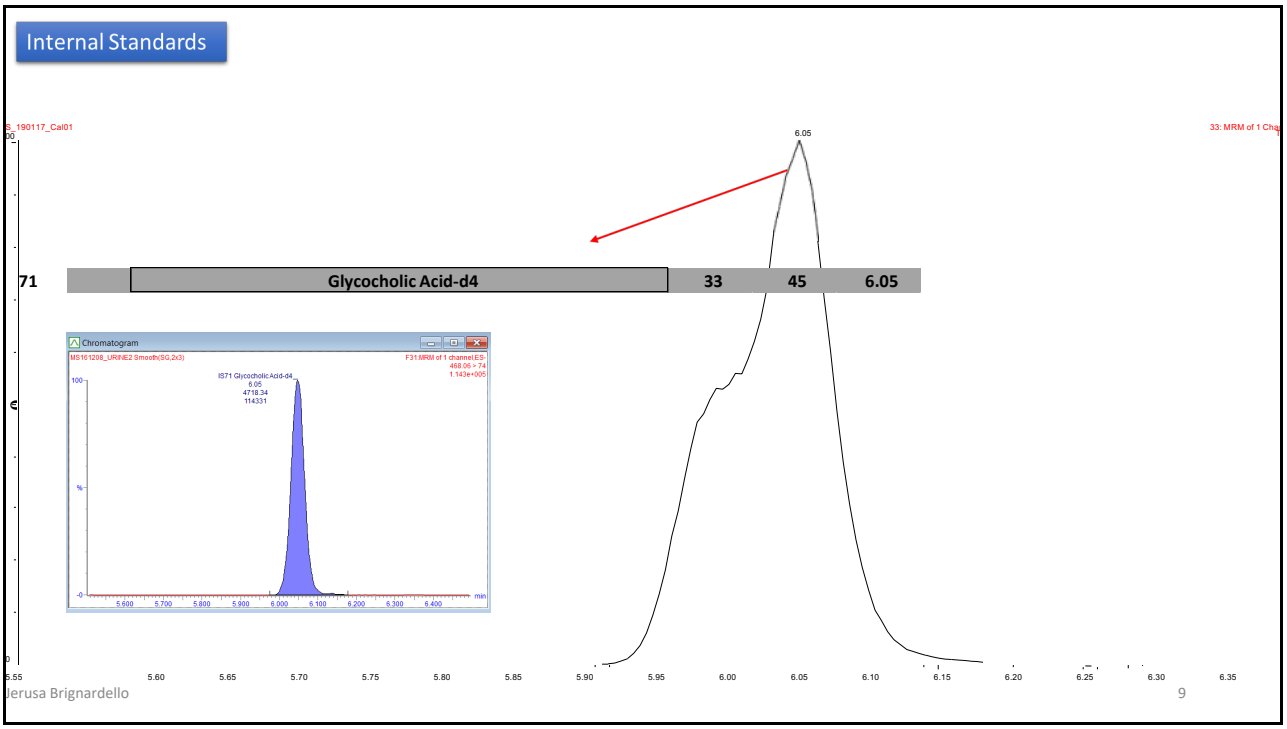
6


Internal Standards
68 Glycochenodeoxycholic acid -d4
69 Glycodeoxycholic acid-d4 28 10 8.48
Chromatogram
Time

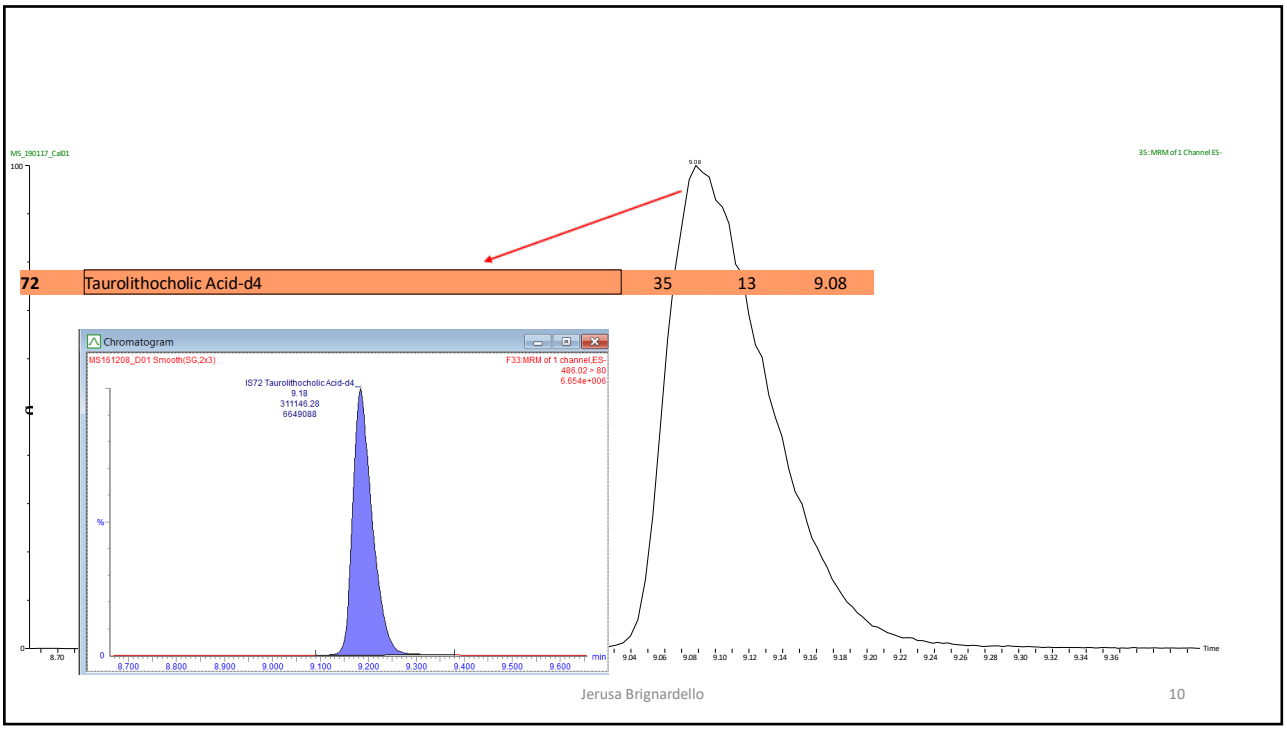
7


Internal Standards
70 Glycoursodeoxycholic acid-d4 27 11 5.52
Chromatogram
Time

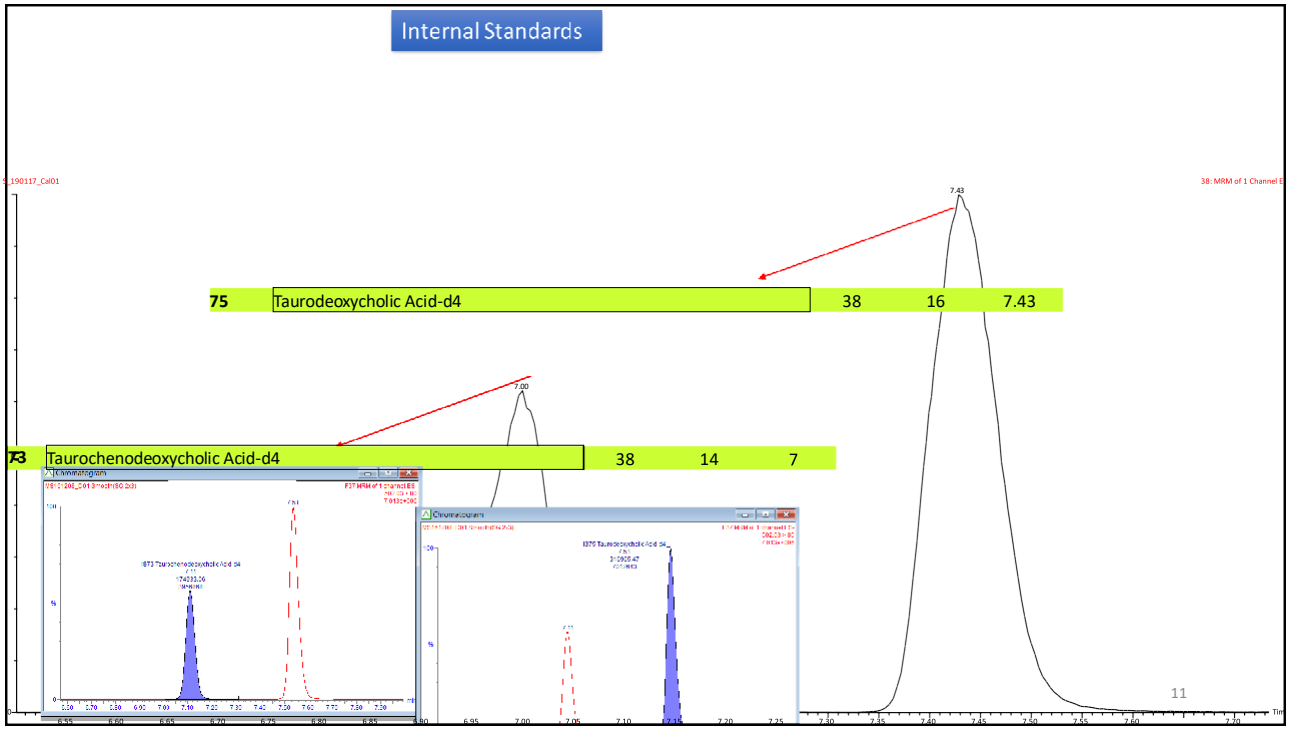
8


Internal Standards
71
Glycocholic Acid-d4
33
45
6.05
Chromatogram
Jerusa Brignardello
9

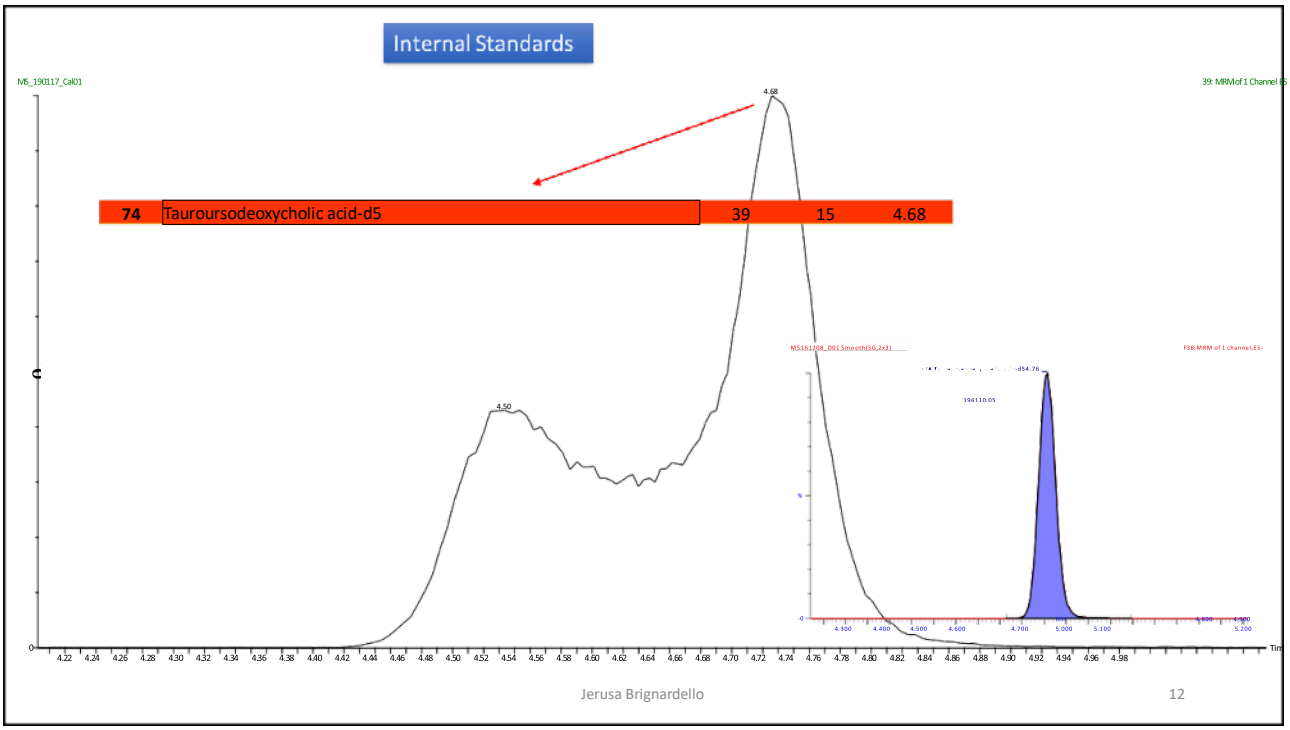
9


72
Taurolithocholic Acid-d4
35
13
9.08
Chromatogram
Jerusa Brignardello
10

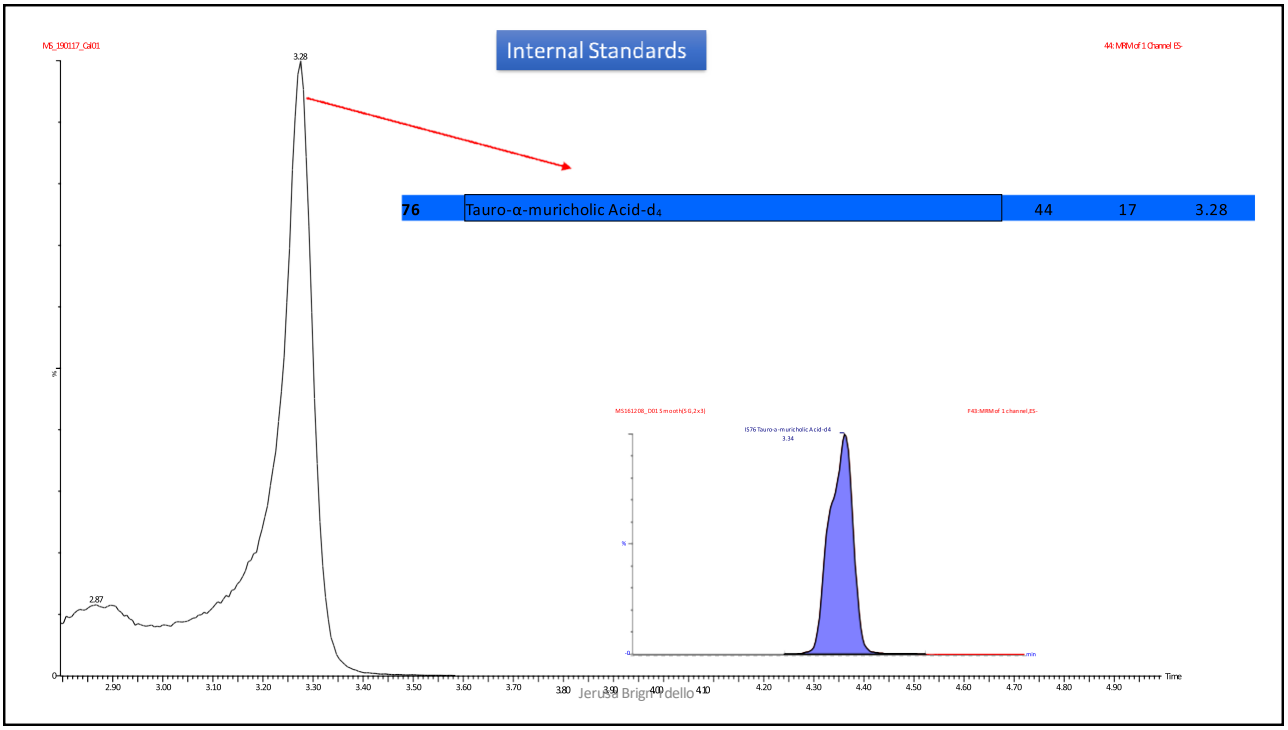
10


Internal Standards
75 Taurodeoxycholic Acid-d4 38 16 7.43
73 Taurochenodeoxycholic Acid-d4 38 14 7
7.43
7.00
11

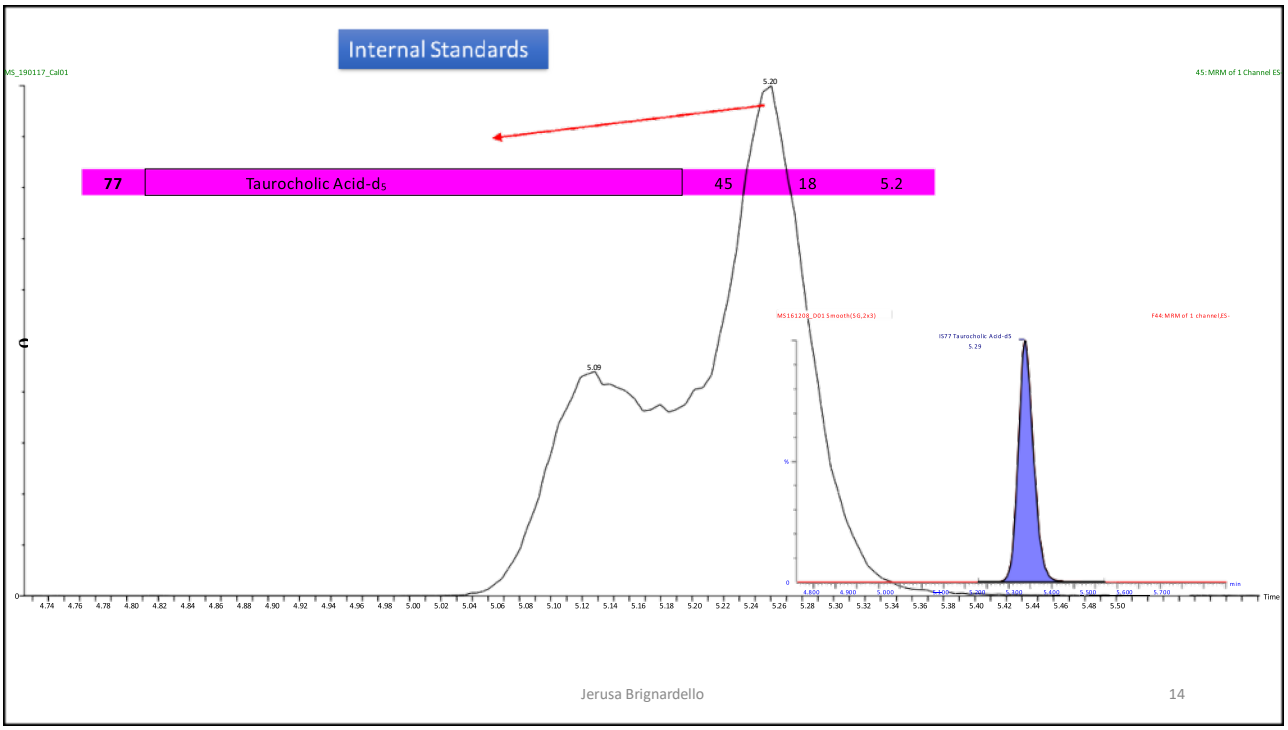
11


Internal Standards
74 Tauroursodeoxycholic acid-d5 39 15 4.68
4.68
4.50
Jerusa Brignardello
12

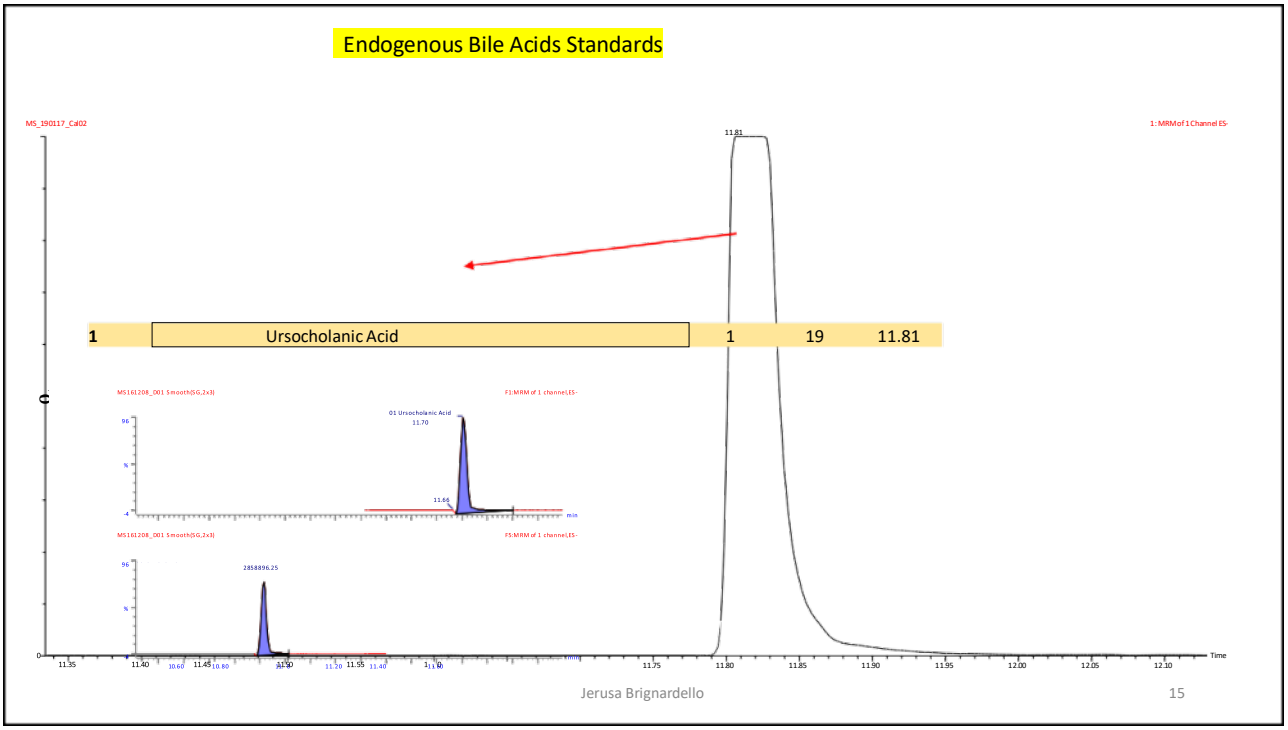
12


Internal Standards
MS_190117_Cal01
44: MRM of 1 Channel ES-
3.28
2.87
76 Tauro-α-muricholic Acid-d4 44 17 3.28
IS76 Tauro-α-muricholic Acid-d4
3.34
Time
Jerusa Brignardello

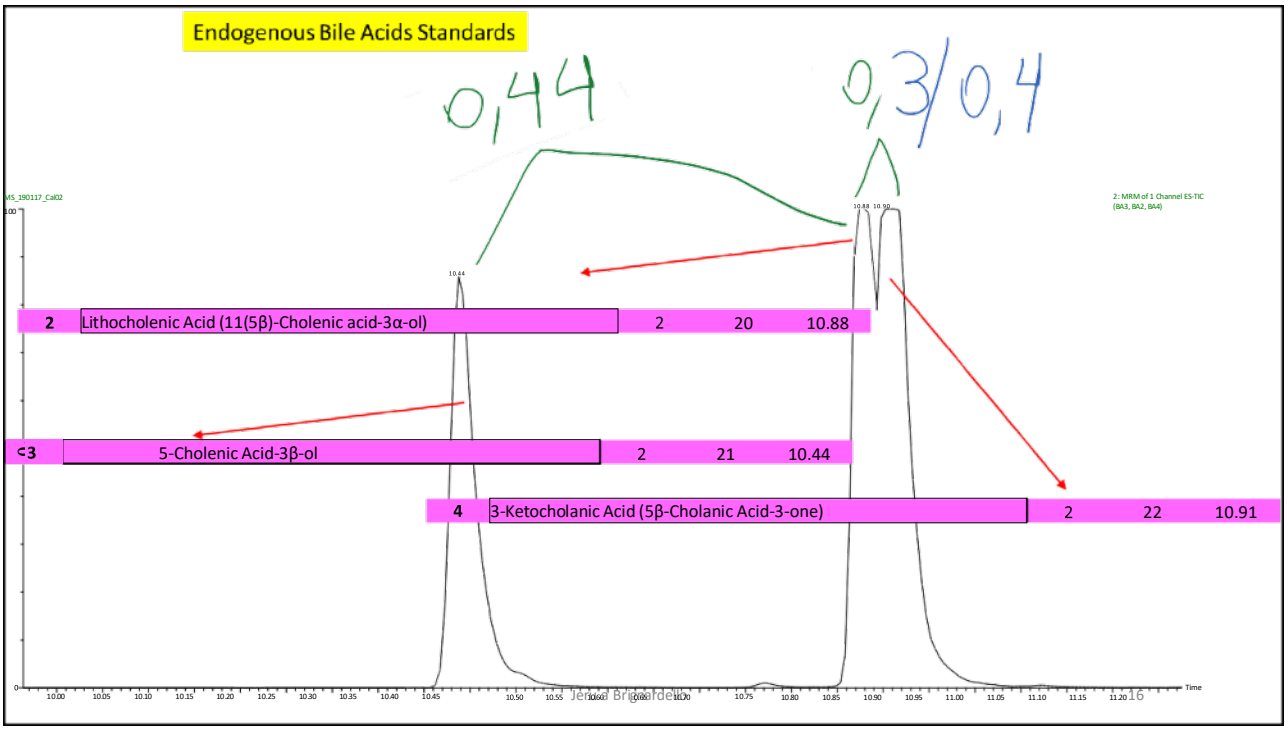
13


Internal Standards
MS_190117_Cal01
45: MRM of 1 Channel ES-
5.20
5.09
77 Taurocholic Acid-d5 45 18 5.2
IS77 Taurocholic Acid-d5
5.29
Time
Jerusa Brignardello
14

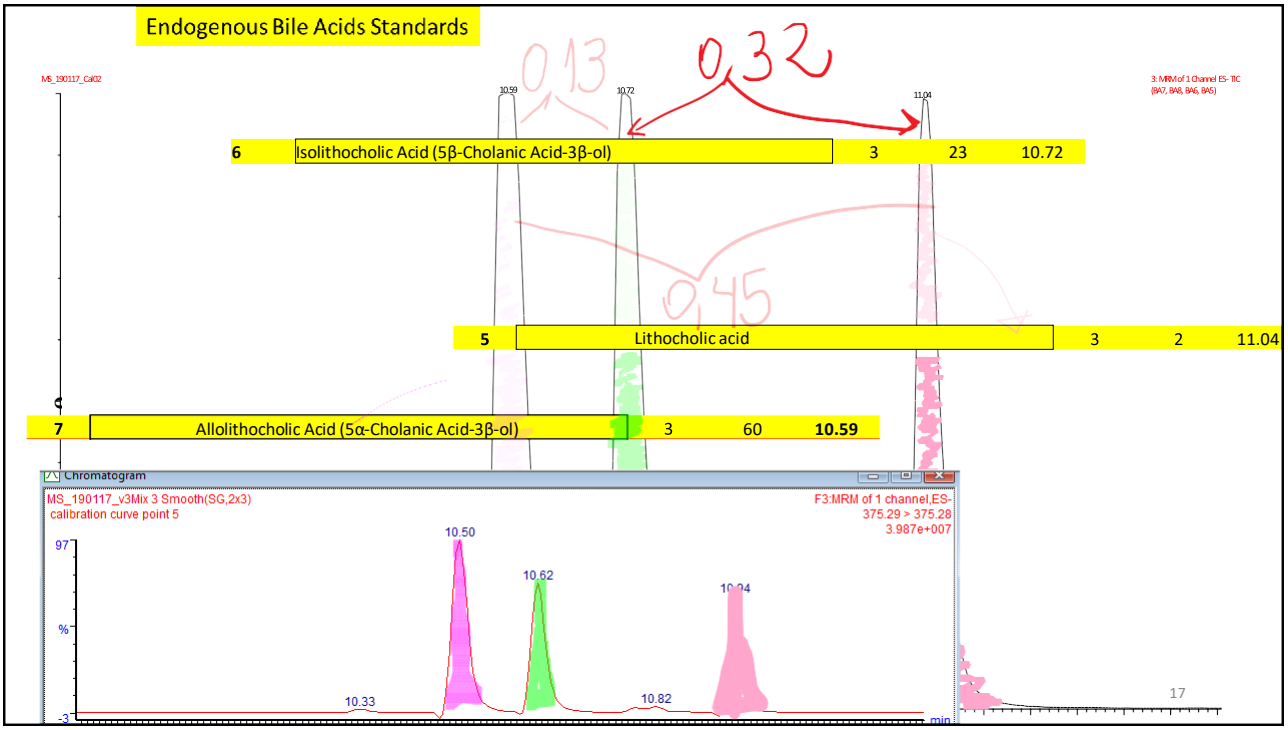
14


Endogenous Bile Acids Standards
1 Ursocholanic Acid 1 19 11.81
Jerusa Brignardello
15

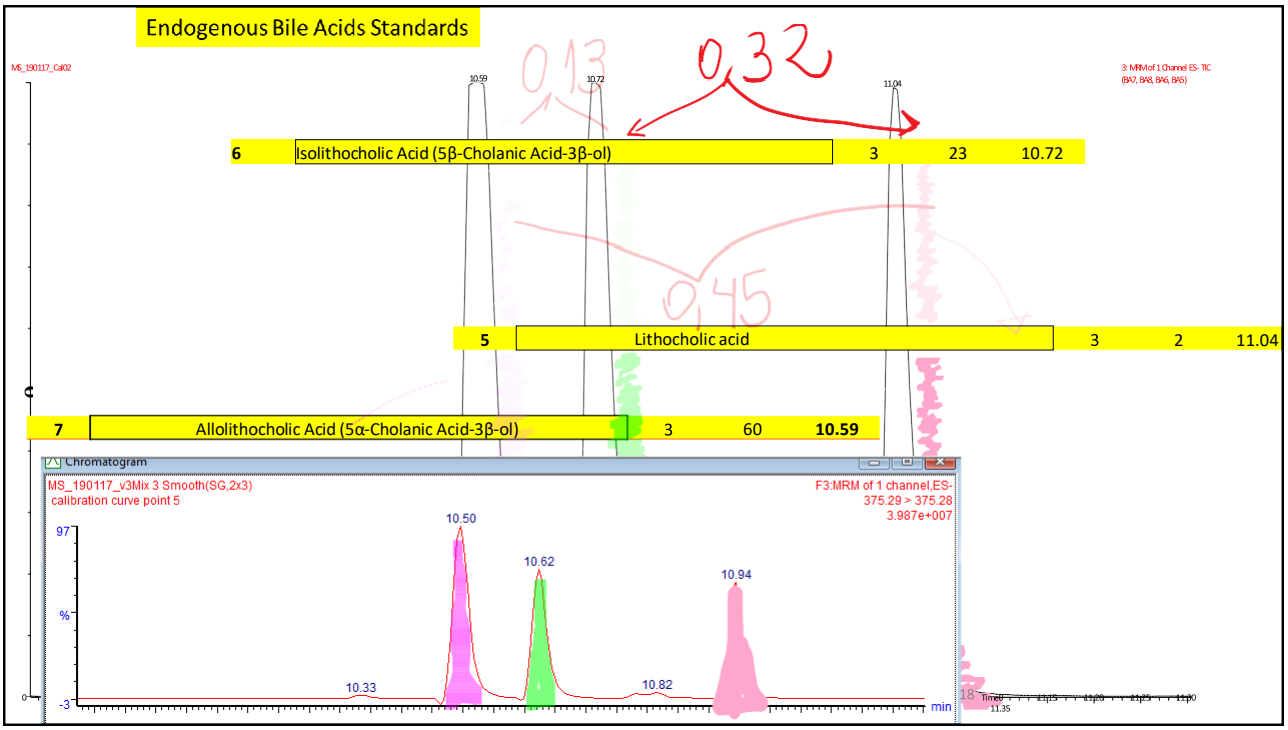
15


Endogenous Bile Acids Standards
0,44
0,3/0,4
2 Lithocholenic Acid (11(5β)-Cholenic acid-3α-ol) 2 20 10.88
3 5-Cholenic Acid-3β-ol 2 21 10.44
4 3-Ketocholanic Acid (5β-Cholanic Acid-3-one) 2 22 10.91
Jerusa Brignardello
16

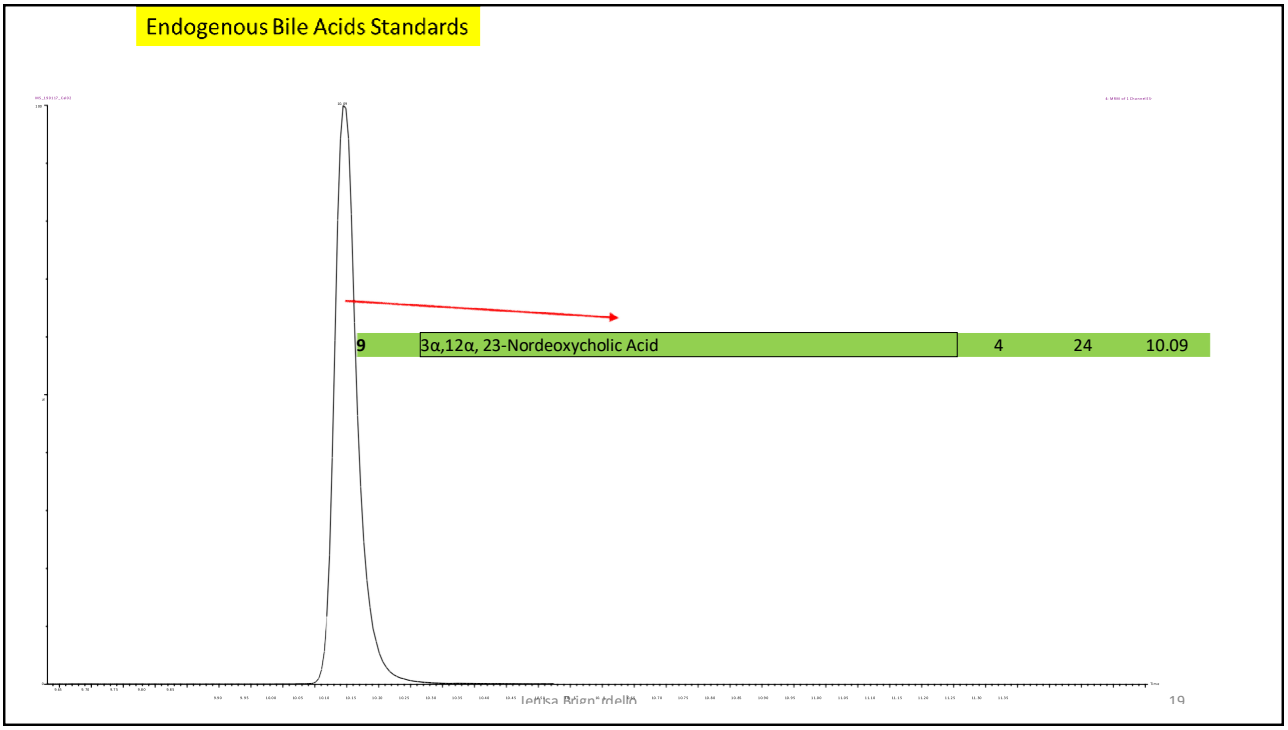
16


Endogenous Bile Acids Standards
0,13
0,32
0,45
6 Isolithocholic Acid (5β-Cholanic Acid-3β-ol) 3 23 10.72
5 Lithocholic acid 3 2 11.04
7 Allolithocholic Acid (5α-Cholanic Acid-3β-ol) 3 60 10.59
Chromatogram
MS_190117_v3Mix 3 Smooth(SG,2x3)
calibration curve point 5
F3:MRM of 1 channel,ES-
375.29 > 375.28
3.987e+007
10.50
10.62
10.94
10.33
10.82
17

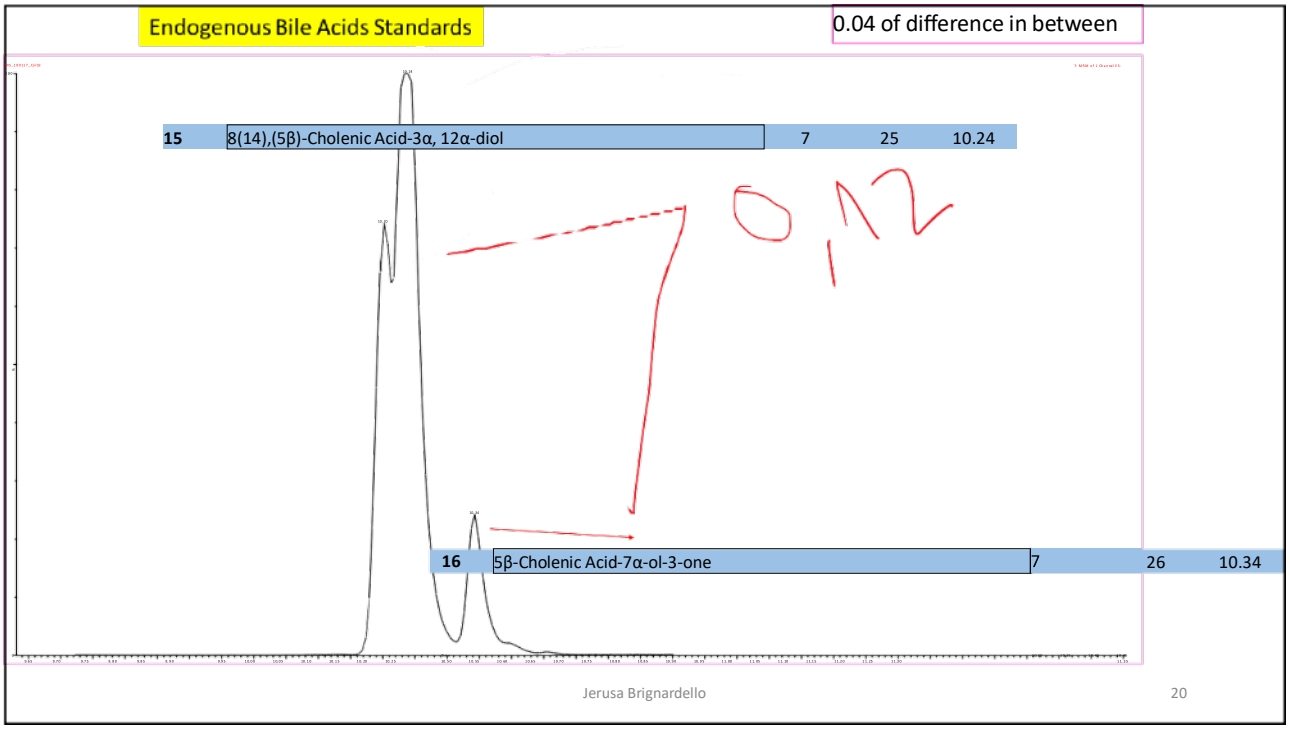
17


Endogenous Bile Acids Standards
0,13
0,32
0,45
6 Isolithocholic Acid (5β-Cholanic Acid-3β-ol) 3 23 10.72
5 Lithocholic acid 3 2 11.04
7 Allolithocholic Acid (5α-Cholanic Acid-3β-ol) 3 60 10.59
Chromatogram
MS_190117_v3Mix 3 Smooth(SG,2x3)
calibration curve point 5
F3:MRM of 1 channel,ES-
375.29 > 375.28
3.987e+007
10.50
10.62
10.94
10.33
10.82
18

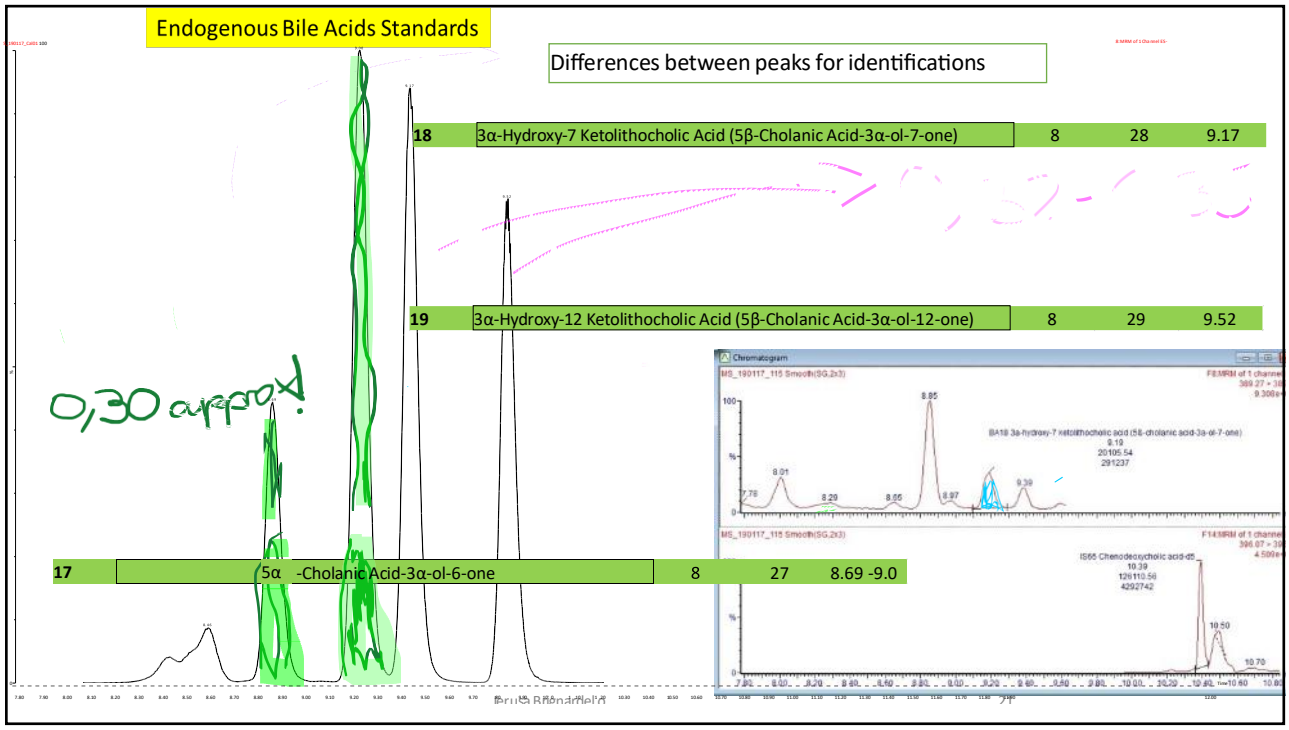
18


Endogenous Bile Acids Standards
9
3α,12α, 23-Nordeoxycholic Acid
4
24
10.09
19

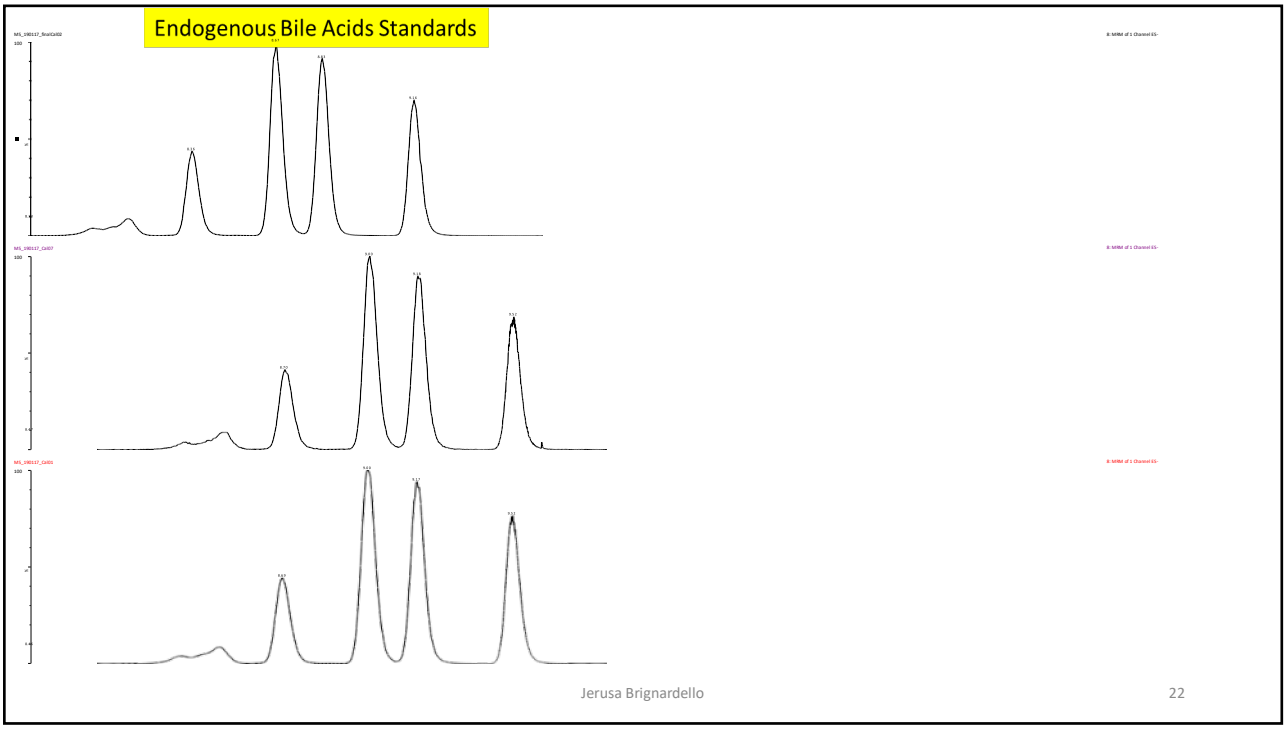
19


Endogenous Bile Acids Standards
0.04 of difference in between
15
8(14),(5β)-Cholenic Acid-3α, 12α-diol
7
25
10.24
0,12
16
5β-Cholenic Acid-7α-ol-3-one
7
26
10.34
Jerusa Brignardello
20

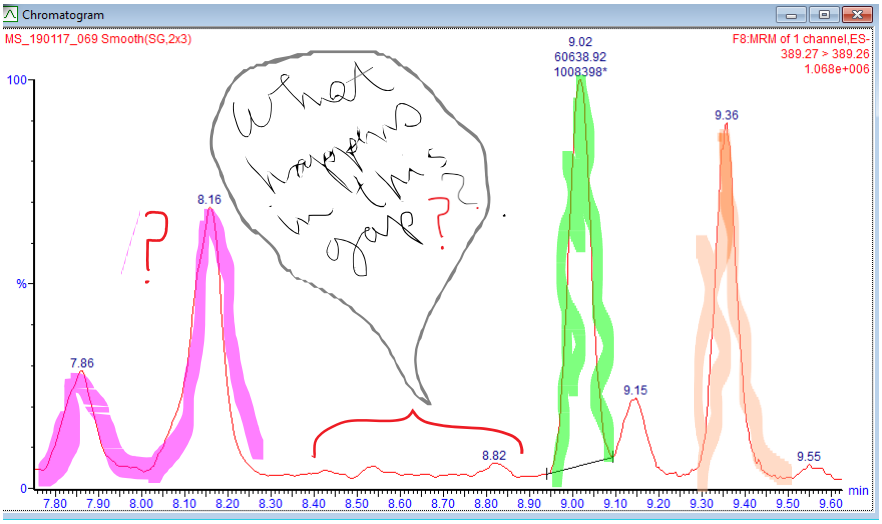
20


Endogenous Bile Acids Standards
Differences between peaks for identifications
18 3α-Hydroxy-7 Ketolithocholic Acid (5β-Cholanic Acid-3α-ol-7-one) 8 28 9.17
19 3α-Hydroxy-12 Ketolithocholic Acid (5β-Cholanic Acid-3α-ol-12-one) 8 29 9.52
0,30 approx!
17 5α -Cholanic Acid-3α-ol-6-one 8 27 8.69 -9.0
Chromatogram

21


Endogenous Bile Acids Standards
Jerusa Brignardello
22

22



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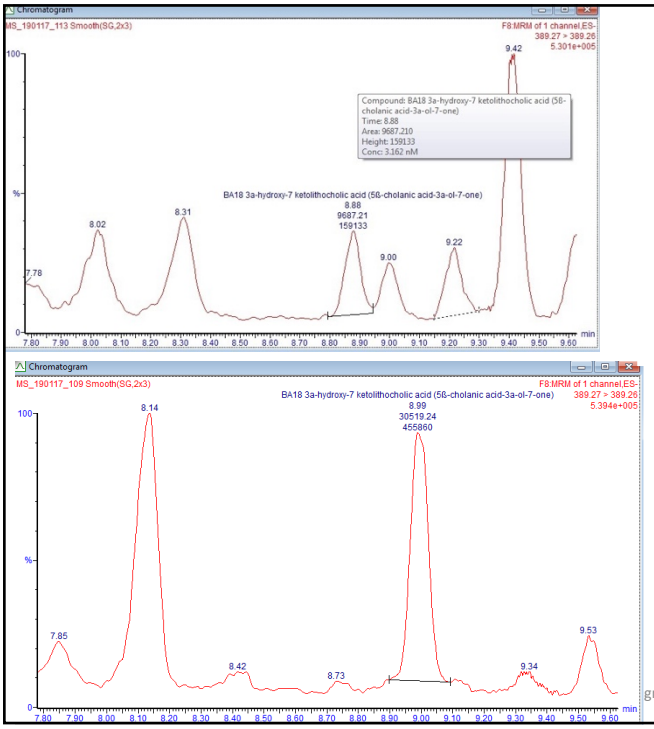
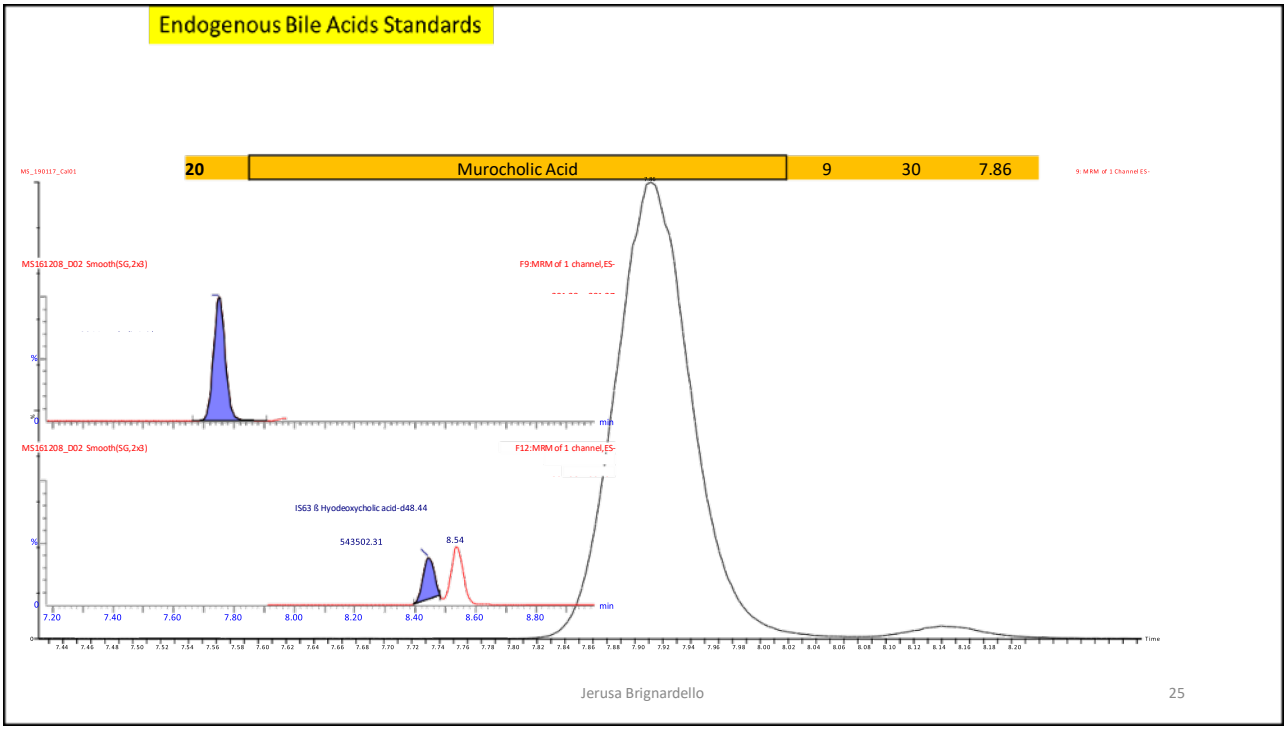
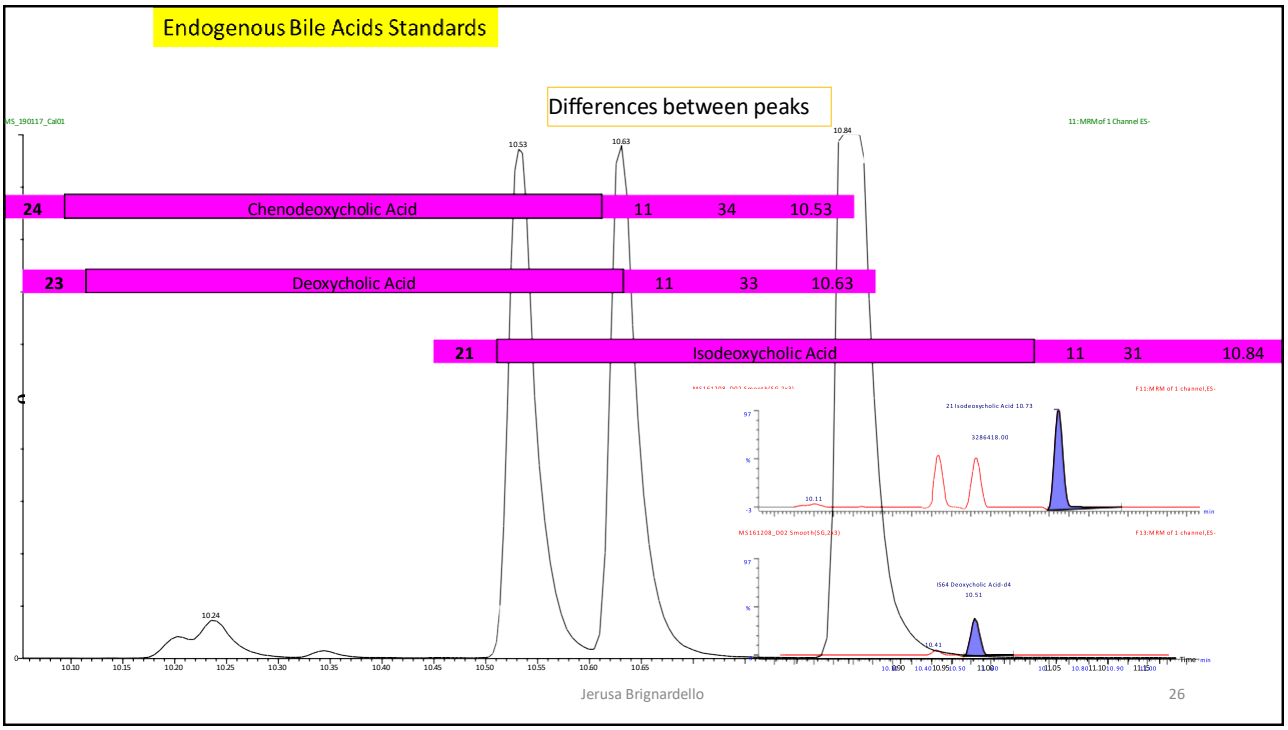


gnardello 24

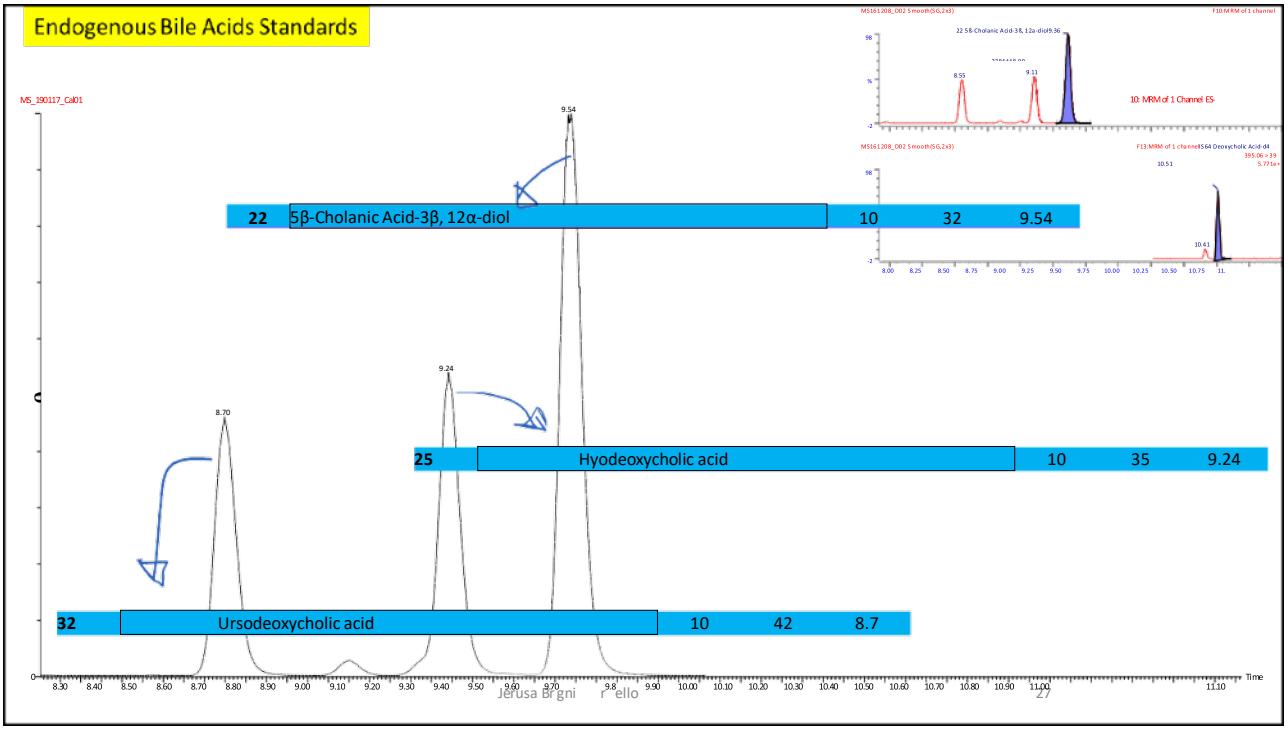
24


Endogenous Bile Acids Standards
20
Murocholic Acid
9
30
7.86
MS161208_D02 Smooth(SG,2x3)
F9:MRM of 1 channel,ES-
F12:MRM of 1 channel,ES-
IS63 ß Hyodeoxycholic acid-d48.44
543502.31
8.54
Jerusa Brignardello
25

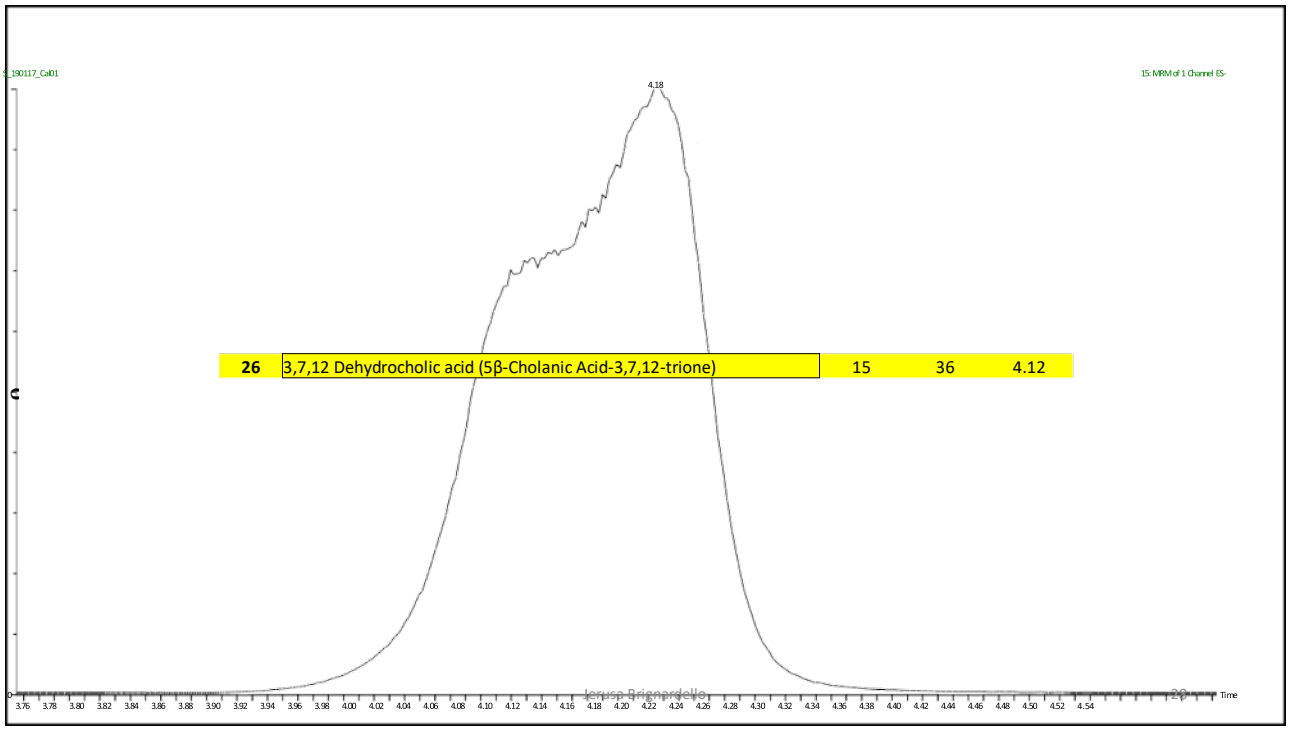
25


Endogenous Bile Acids Standards
Differences between peaks
24
Chenodeoxycholic Acid
11
34
10.53
23
Deoxycholic Acid
11
33
10.63
21
Isodeoxycholic Acid
11
31
10.84
21 Isodeoxycholic Acid 10.73
3286418.00
IS64 Deoxycholic Acid-d4
10.51
10.24
Jerusa Brignardello
26

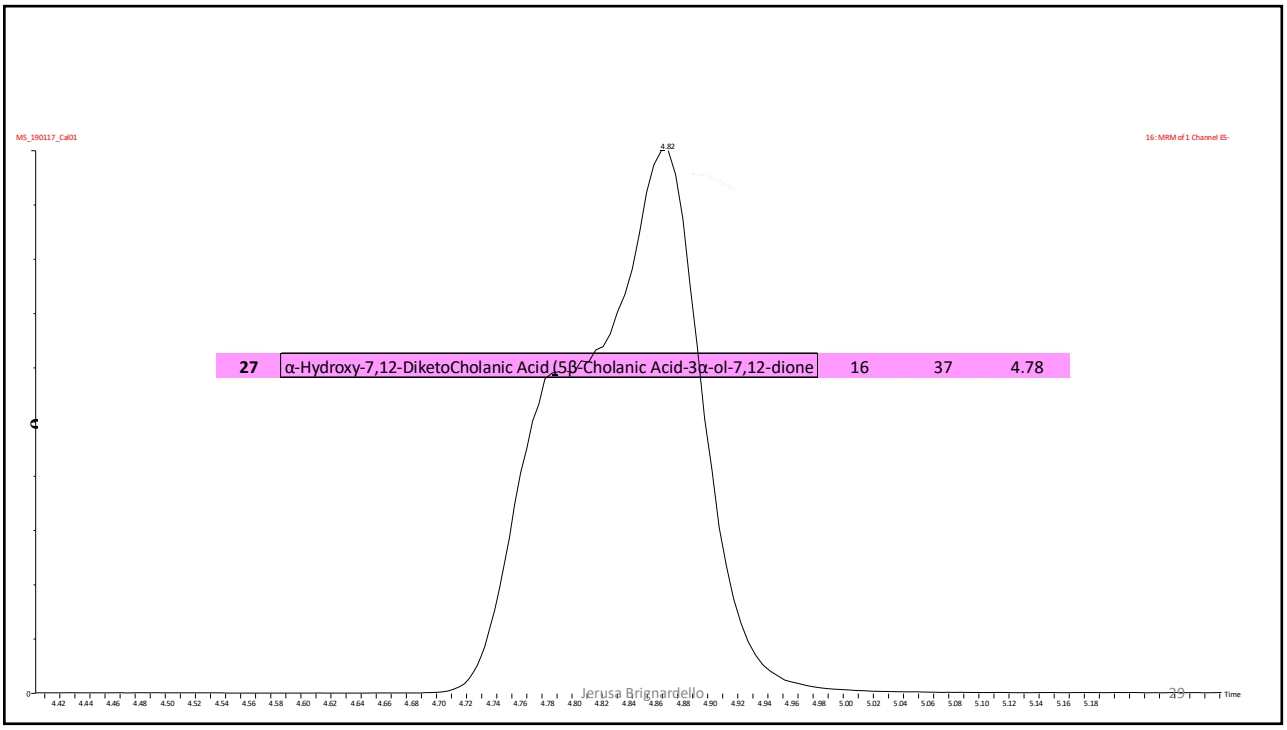
26


Endogenous Bile Acids Standards
MS_190117_Cal01
9.54
22 5β-Cholanic Acid-3β, 12α-diol 10 32 9.54
9.24
25 Hyodeoxycholic acid 10 35 9.24
8.70
32 Ursodeoxycholic acid 10 42 8.7
Time
Jerusa Brigni r ello
27

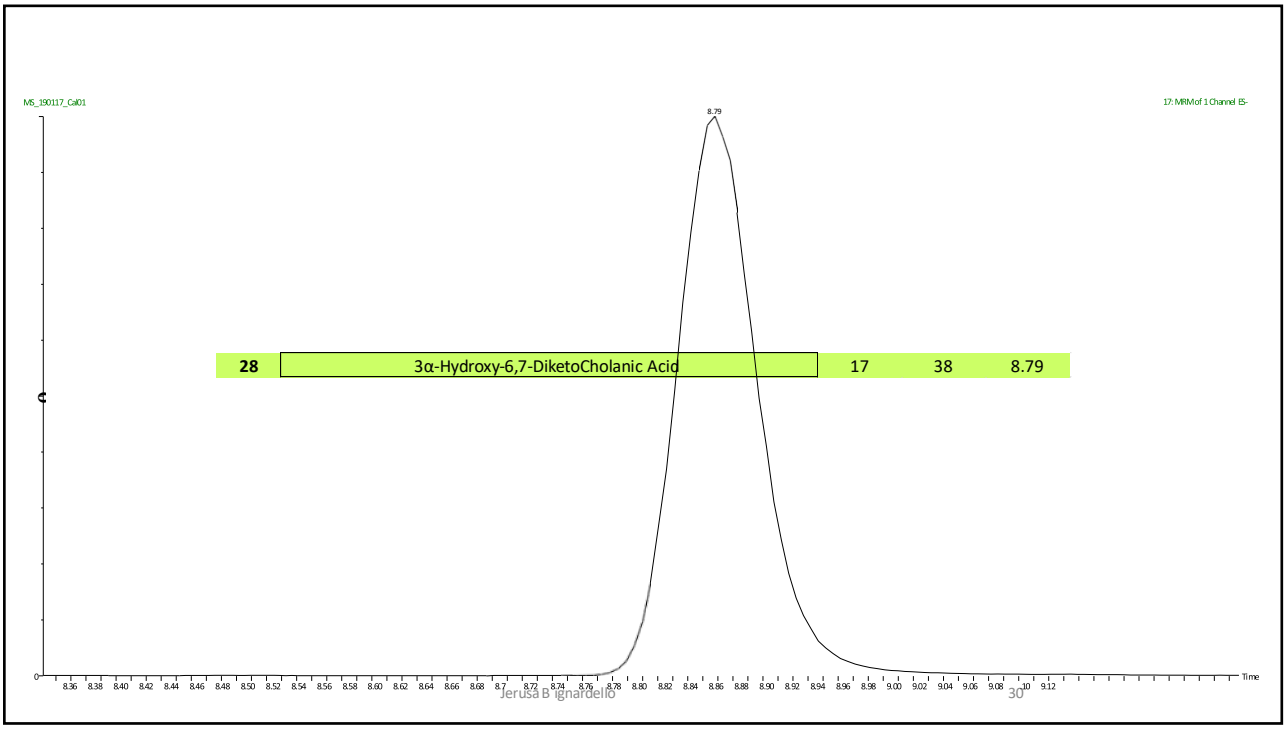
27


4.18
26 3,7,12 Dehydrocholic acid (5β-Cholanic Acid-3,7,12-trione) 15 36 4.12
Time
Jerusa Brignardello
28

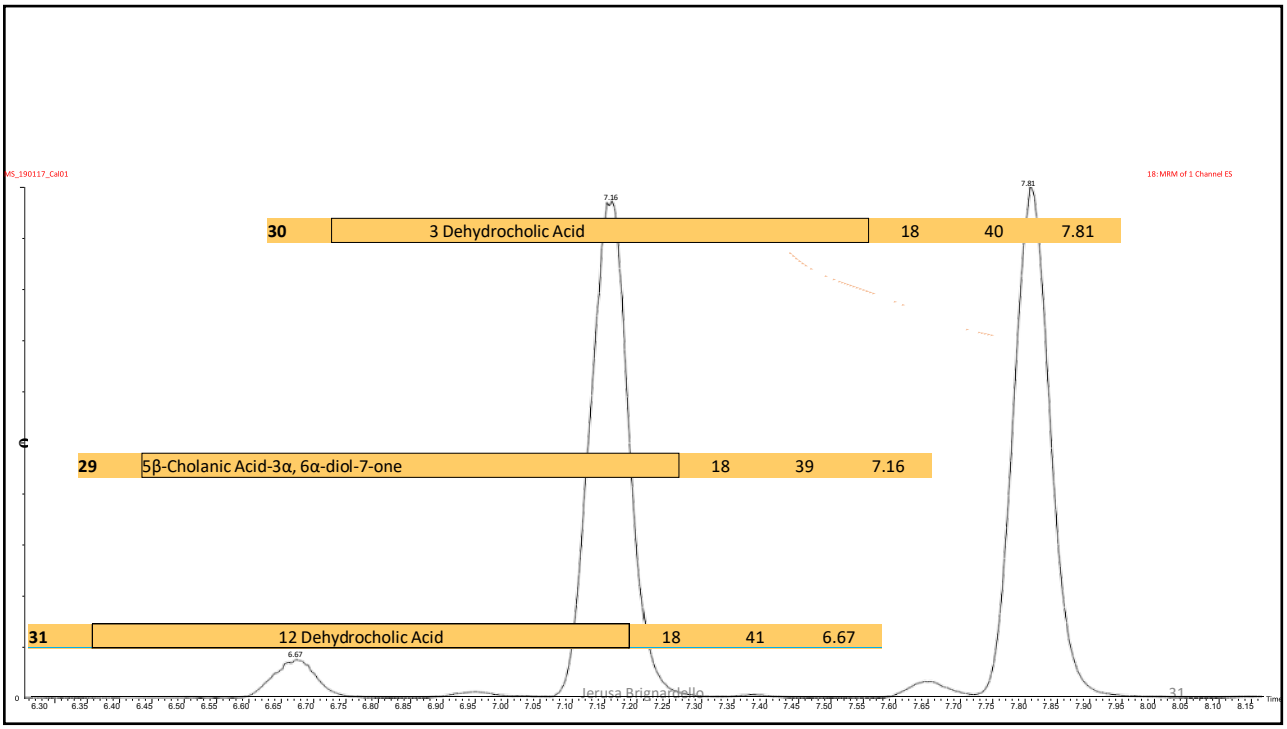
28


MS_190117_Cal01
16: MRM of 1 Channel ES-
4.82
27 α-Hydroxy-7,12-DiketoCholanic Acid (5β-Cholanic Acid-3α-ol-7,12-dione 16 37 4.78
Jerusa Brignardello
29
Time

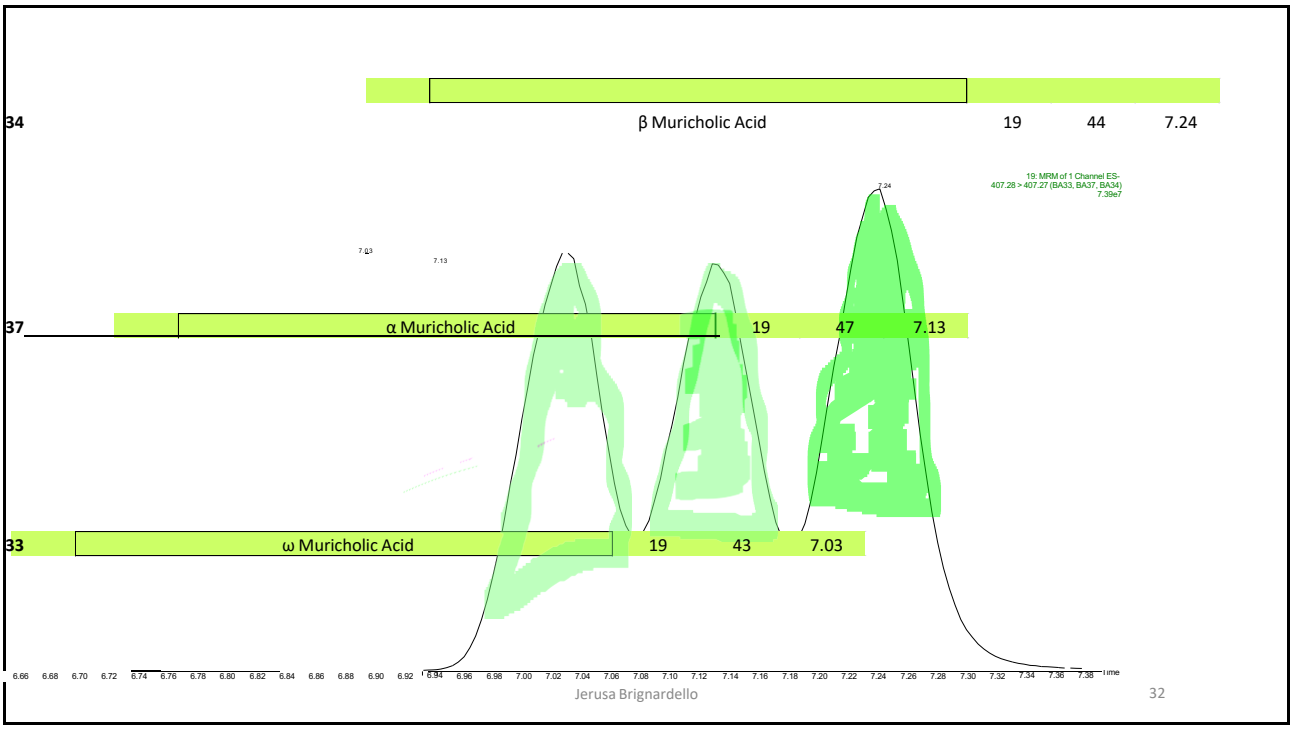
29


MS_190117_Cal01
17: MRM of 1 Channel ES-
8.79
28 3α-Hydroxy-6,7-DiketoCholanic Acid 17 38 8.79
Jerusa Brignardello
30
Time

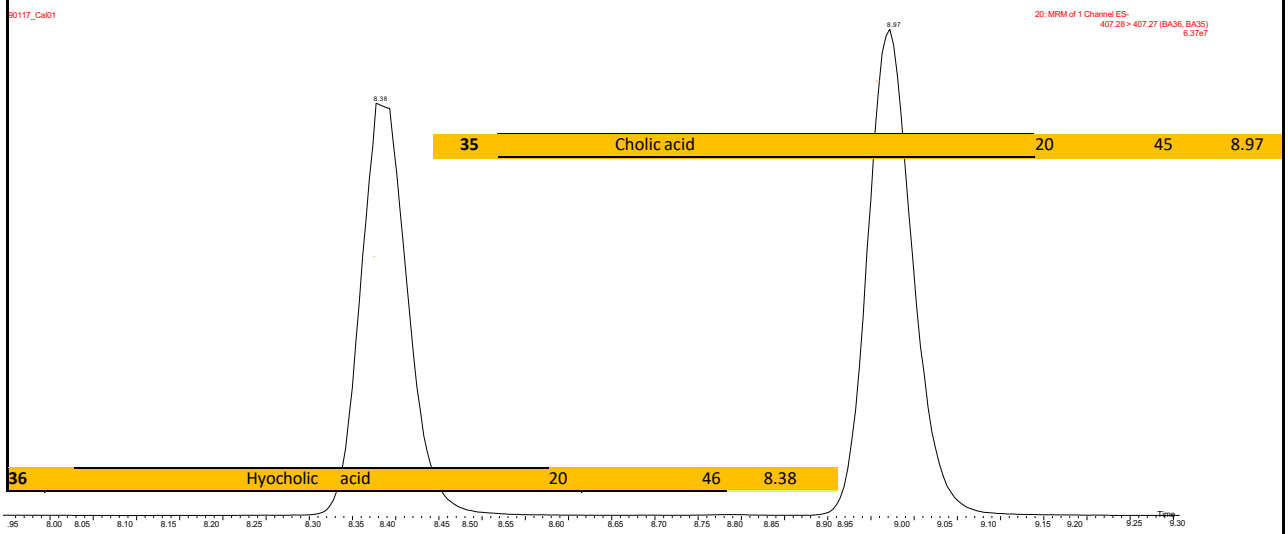
30


MS_190117_Cal01
18: MRM of 1 Channel ES
30 3 Dehydrocholic Acid 18 40 7.81
29 5β-Cholanic Acid-3α, 6α-diol-7-one 18 39 7.16
31 12 Dehydrocholic Acid 18 41 6.67
Jerusa Brignardello
31

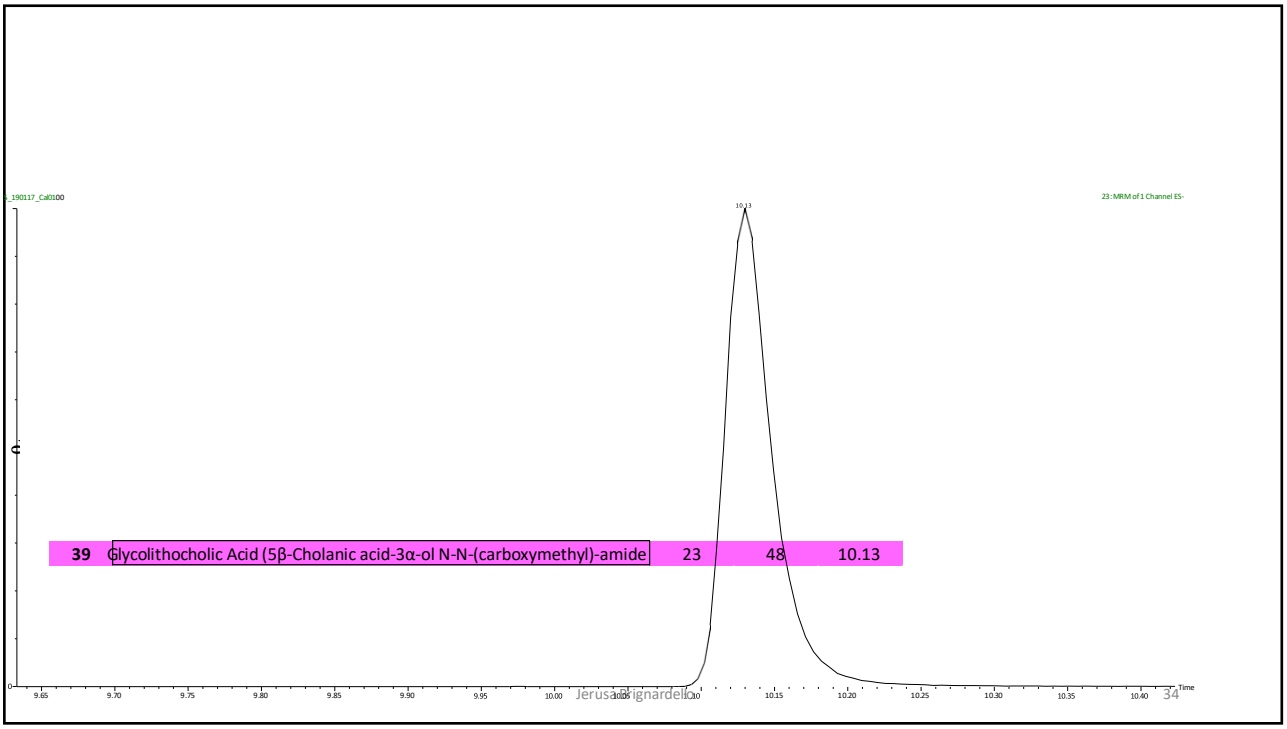
3
1


34 β Muricholic Acid 19 44 7.24
19: MRM of 1 Channel ES-
407.28 > 407.27 (BA33, BA37, BA34)
7.39e7
37 α Muricholic Acid 19 47 7.13
33 ω Muricholic Acid 19 43 7.03
Jerusa Brignardello
32

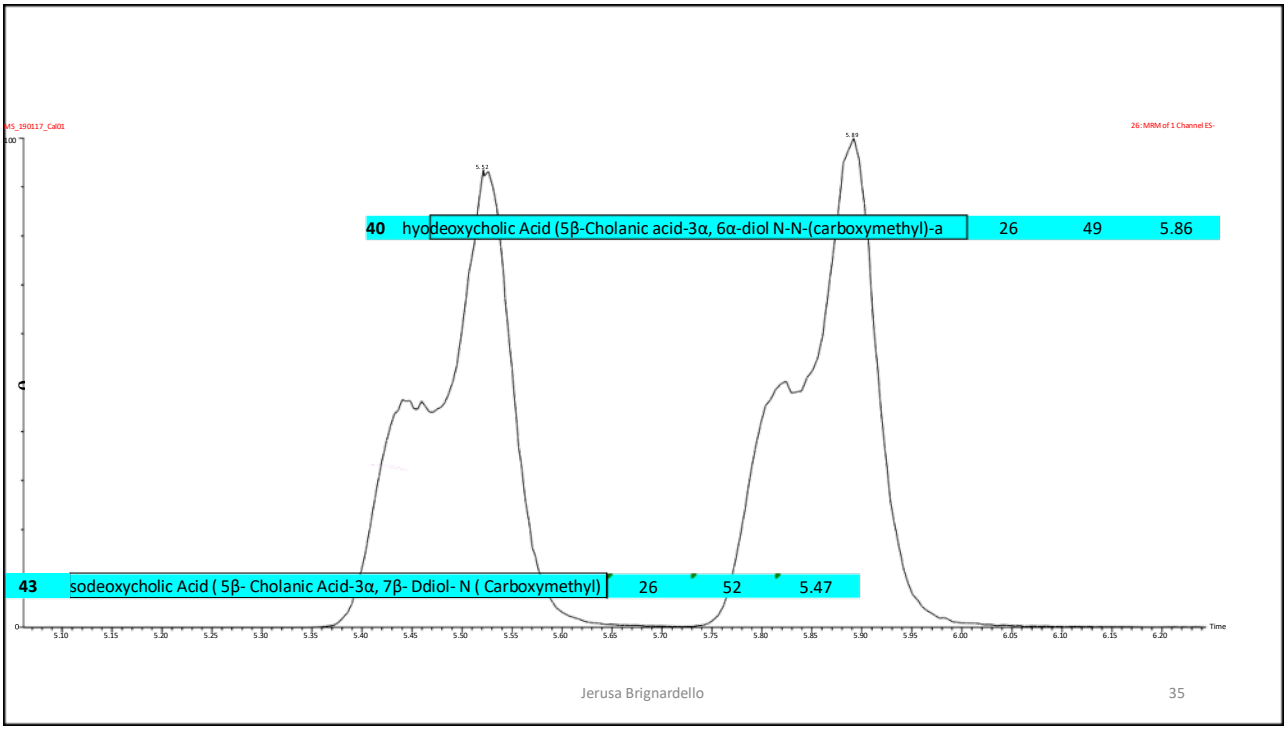
32


90117_Cal01
20: MRM of 1 Channel ES-
407.28 > 407.27 (BA36, BA35)
6.37e7
8.38
8.97
35 Cholic acid 20 45 8.97
36 Hyocholic acid 20 46 8.38
Time

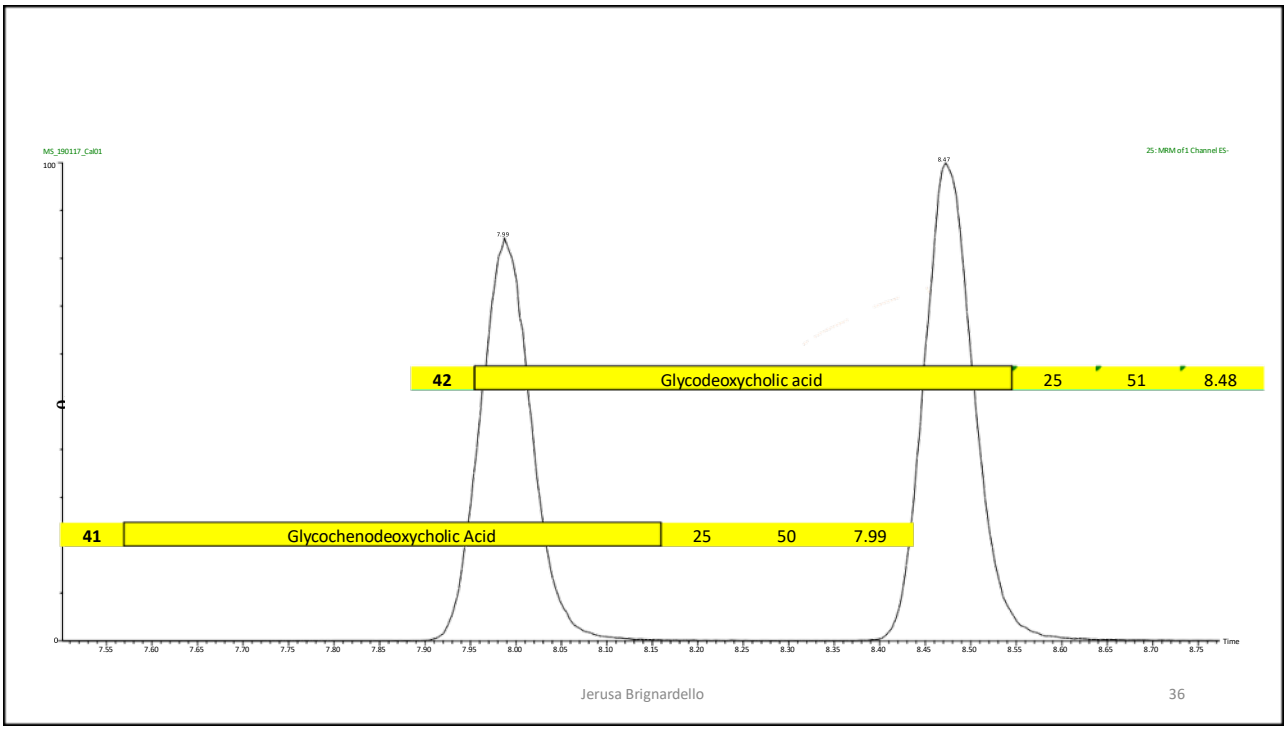
33


_190117_Cal01
23: MRM of 1 Channel ES-
10.13
39 Glycolithocholic Acid (5β-Cholanic acid-3α-ol N-N-(carboxymethyl)-amide 23 48 10.13
Time

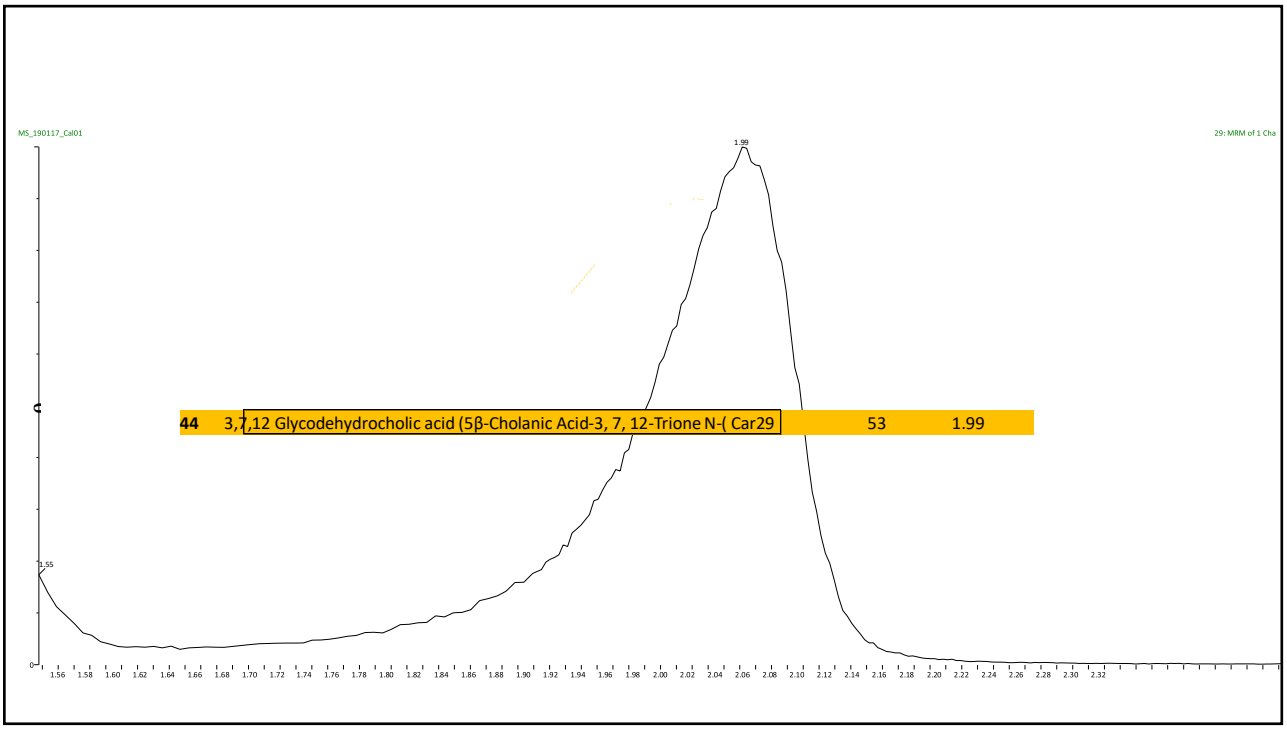
34


MS_190117_Cal01
26: MRM of 1 Channel ES-
40 hyodeoxycholic Acid (5β-Cholanic acid-3α, 6α-diol N-N-(carboxymethyl)-a 26 49 5.86
43 sodeoxycholic Acid (5β- Cholanic Acid-3α, 7β- Ddiol- N (Carboxymethyl) 26 52 5.47
Time
Jerusa Brignardello
35

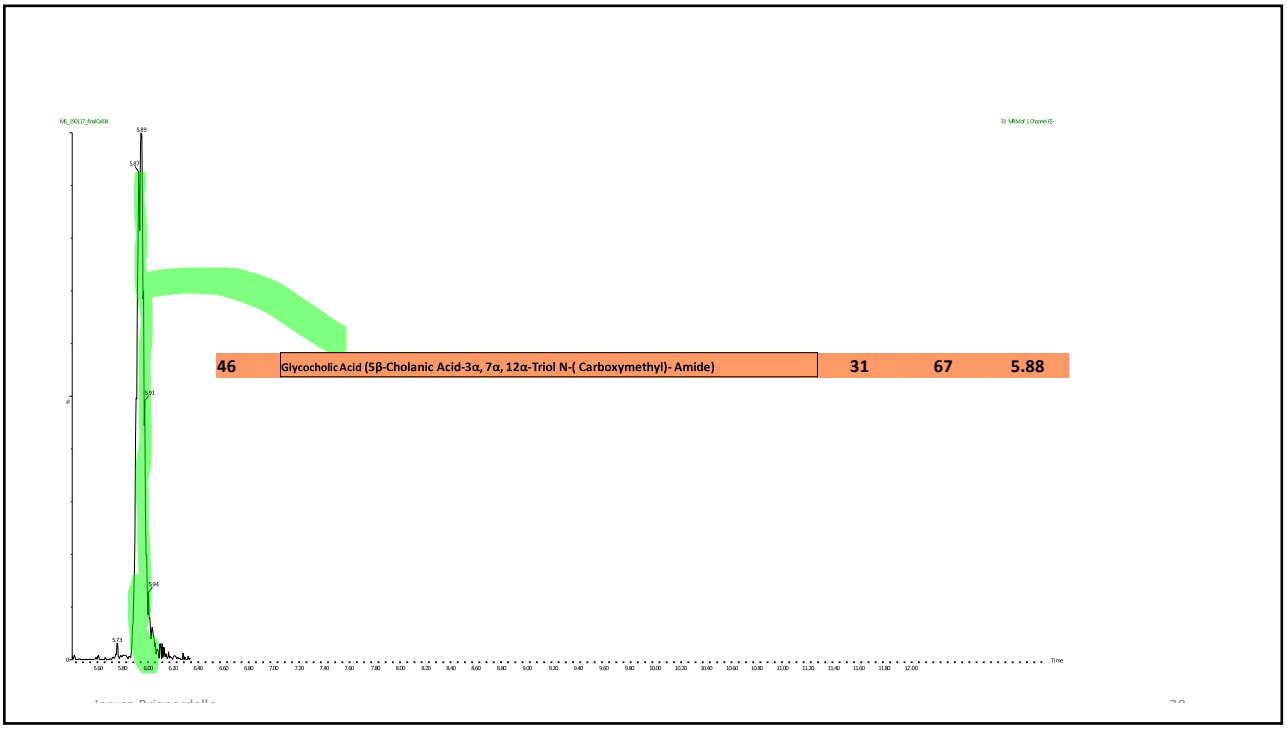
35


MS_190117_Cal01
25: MRM of 1 Channel ES-
42 Glycodeoxycholic acid 25 51 8.48
41 Glycochenodeoxycholic Acid 25 50 7.99
Time
Jerusa Brignardello
36

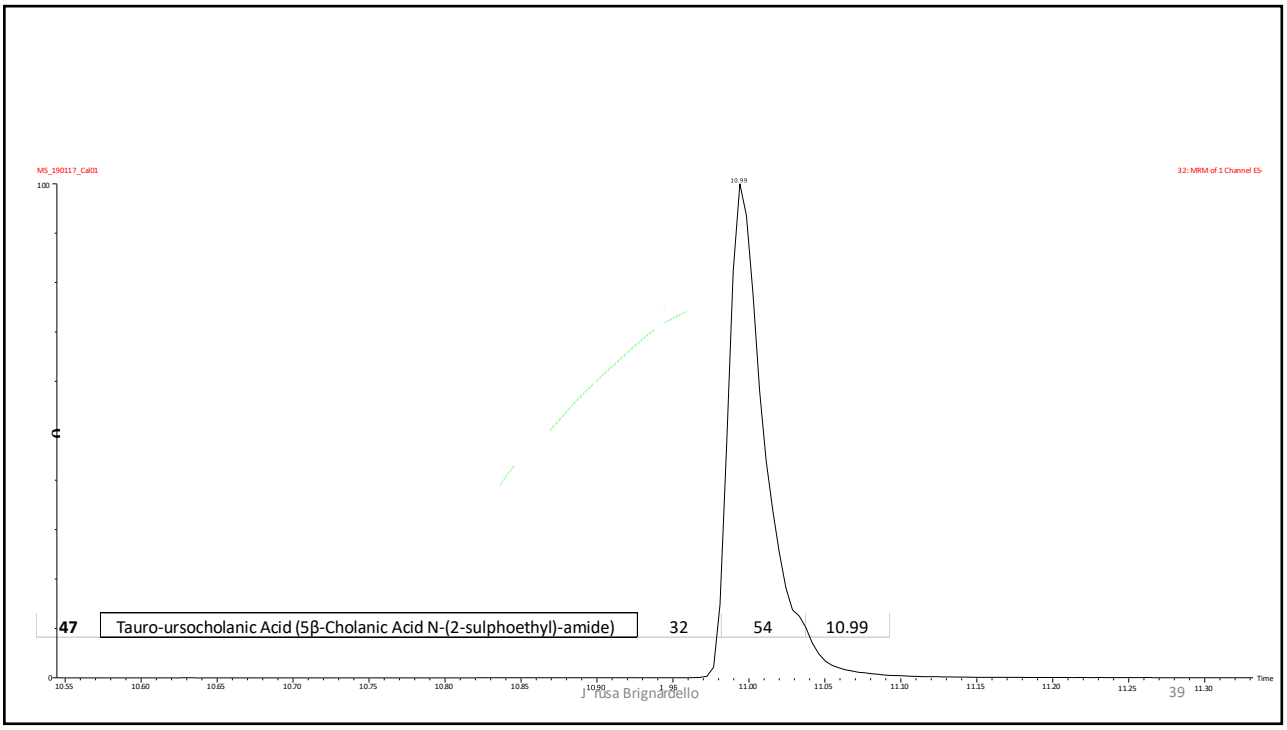
36


MS_190117_Cal01
29: MRM of 1 Cha
1.99
1.55
44 3,7,12 Glycodehydrocholic acid (5β-Cholanic Acid-3, 7, 12-Trione N-(Car29 53 1.99

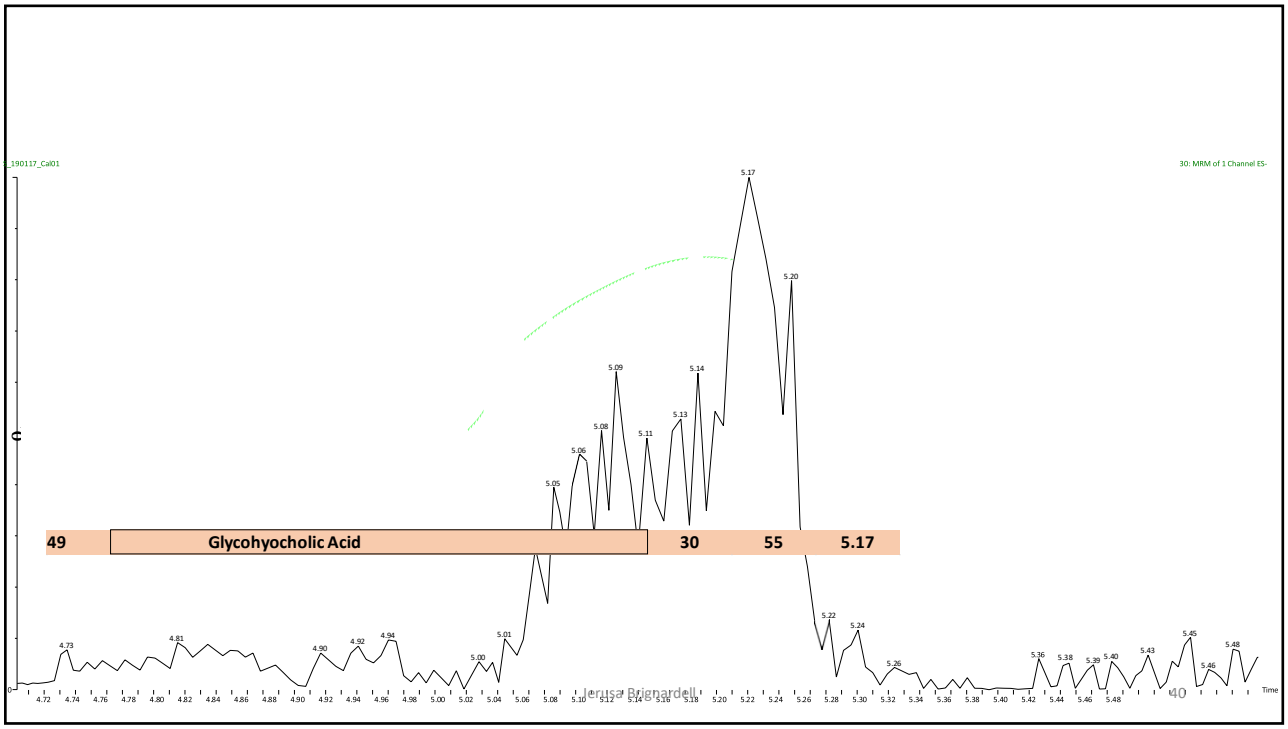
37


5.89
5.87
5.91
5.84
5.73
46 Glycocholic Acid (5β-Cholanic Acid-3α, 7α, 12α-Triol N-(Carboxymethyl)- Amide) 31 67 5.88
Time

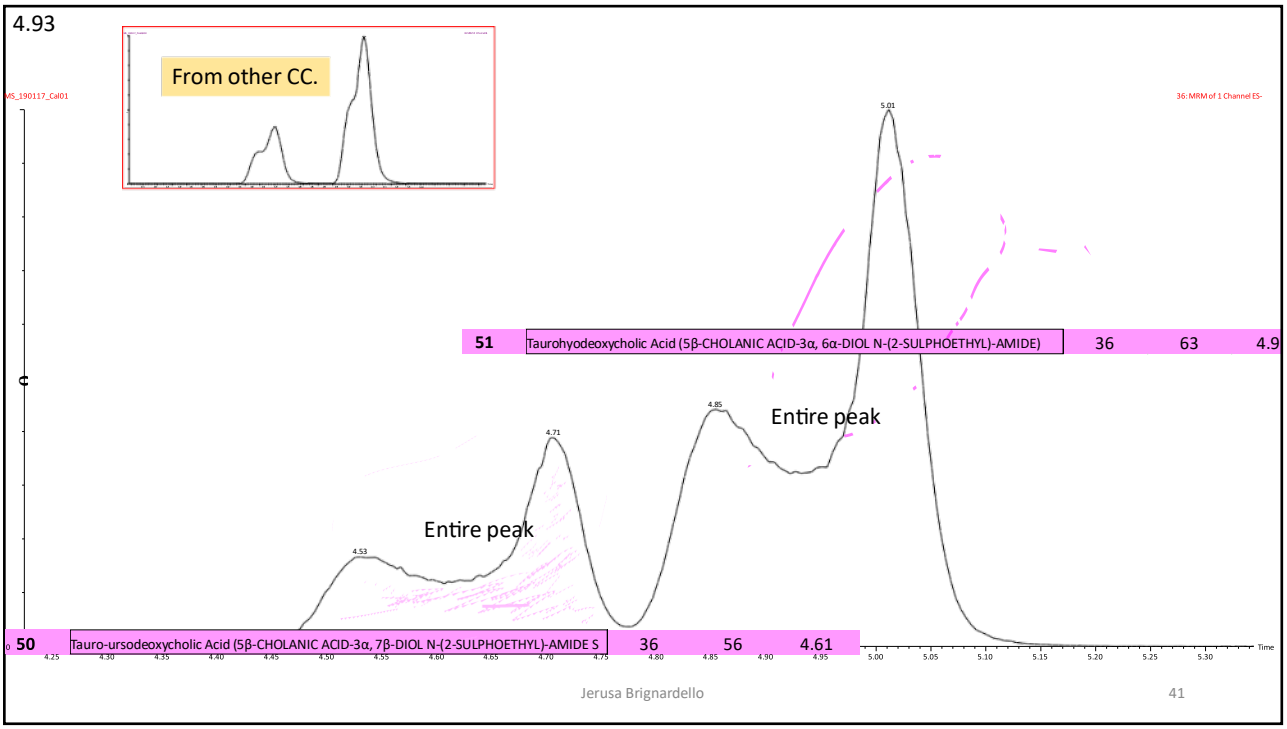
38


MS_190117_Cal01
32: MRM of 1 Channel ES-
10.99
47 Tauro-ursocholanic Acid (5β-Cholanic Acid N-(2-sulphoethyl)-amide) 32 54 10.99
Time
39

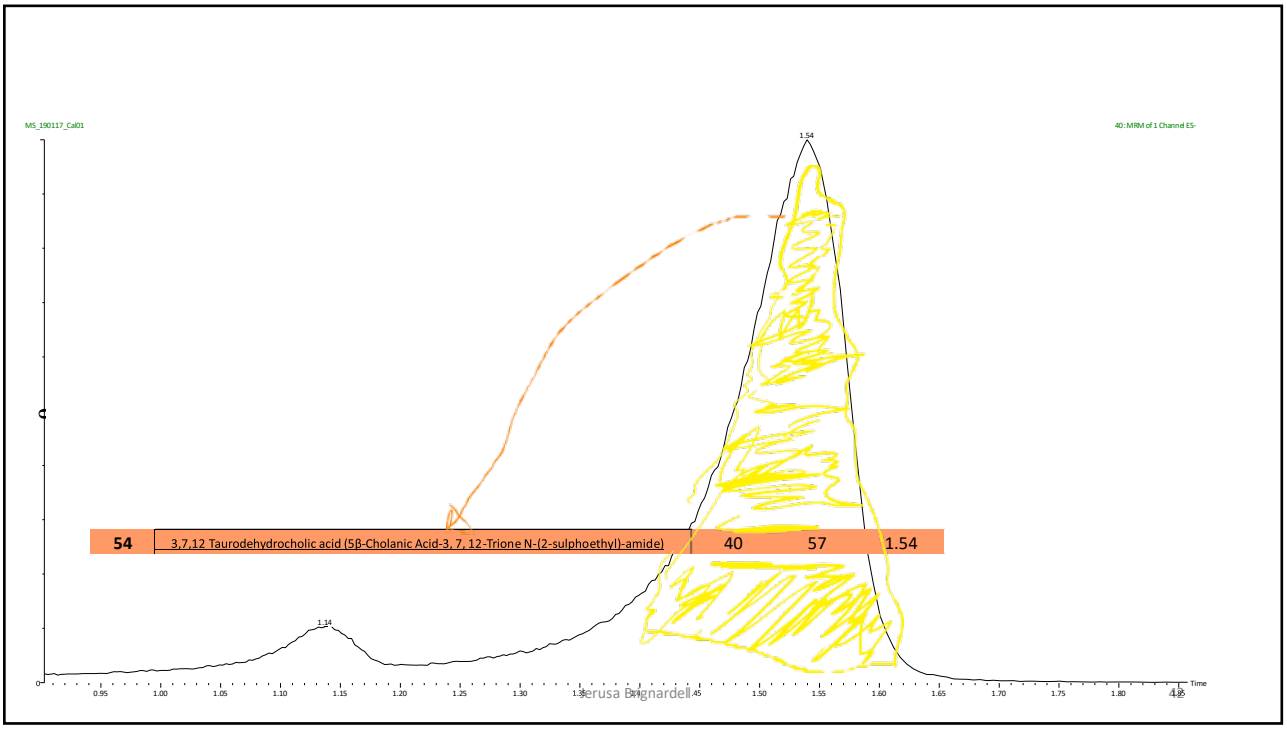
39


30: MRM of 1 Channel ES-
5.17
5.20
49 Glycohyocholic Acid 30 55 5.17
Time
40

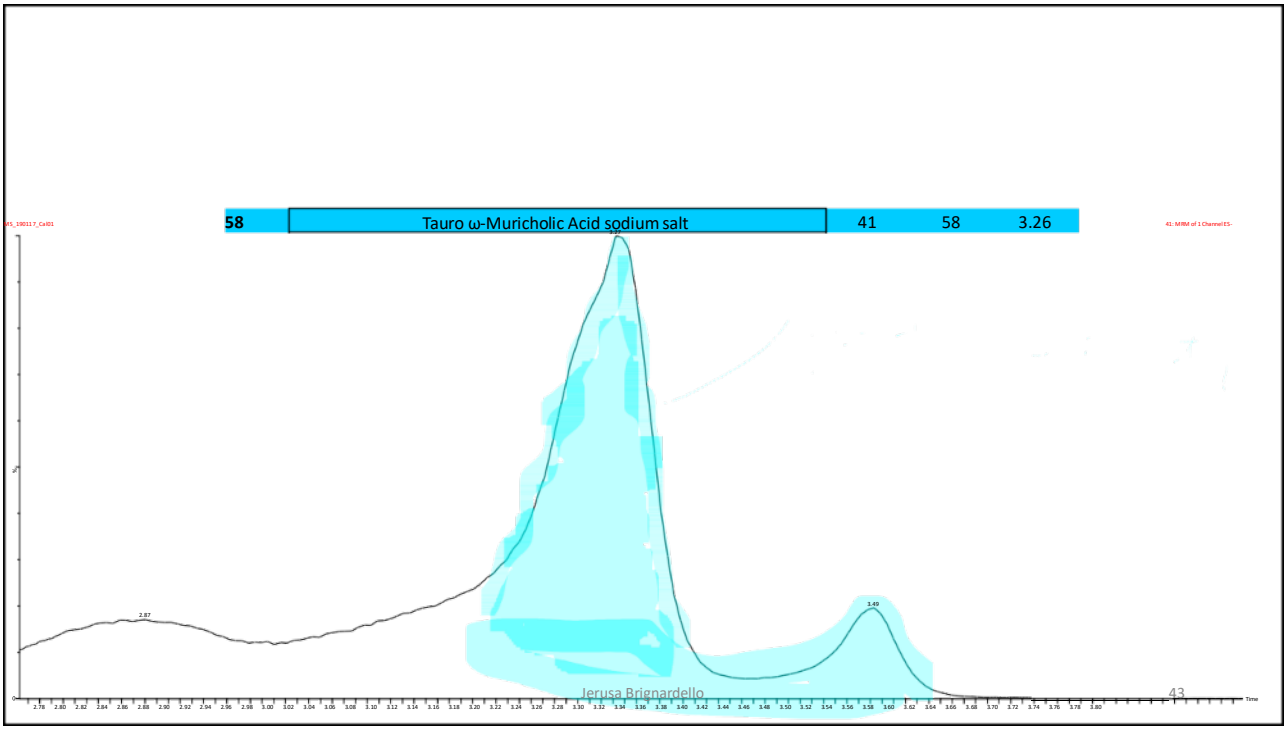
40


4.93
From other CC.
MS_190117_Cal01
36: MRM of 1 Channel ES-
51 Taurohyodeoxycholic Acid (5β-CHOLANIC ACID-3α, 6α-DIOL N-(2-SULPHOETHYL)-AMIDE) 36 63 4.9
Entire peak
Entire peak
50 Tauro-ursodeoxycholic Acid (5β-CHOLANIC ACID-3α, 7β-DIOL N-(2-SULPHOETHYL)-AMIDE S 36 56 4.61
Time
Jerusa Brignardello
41

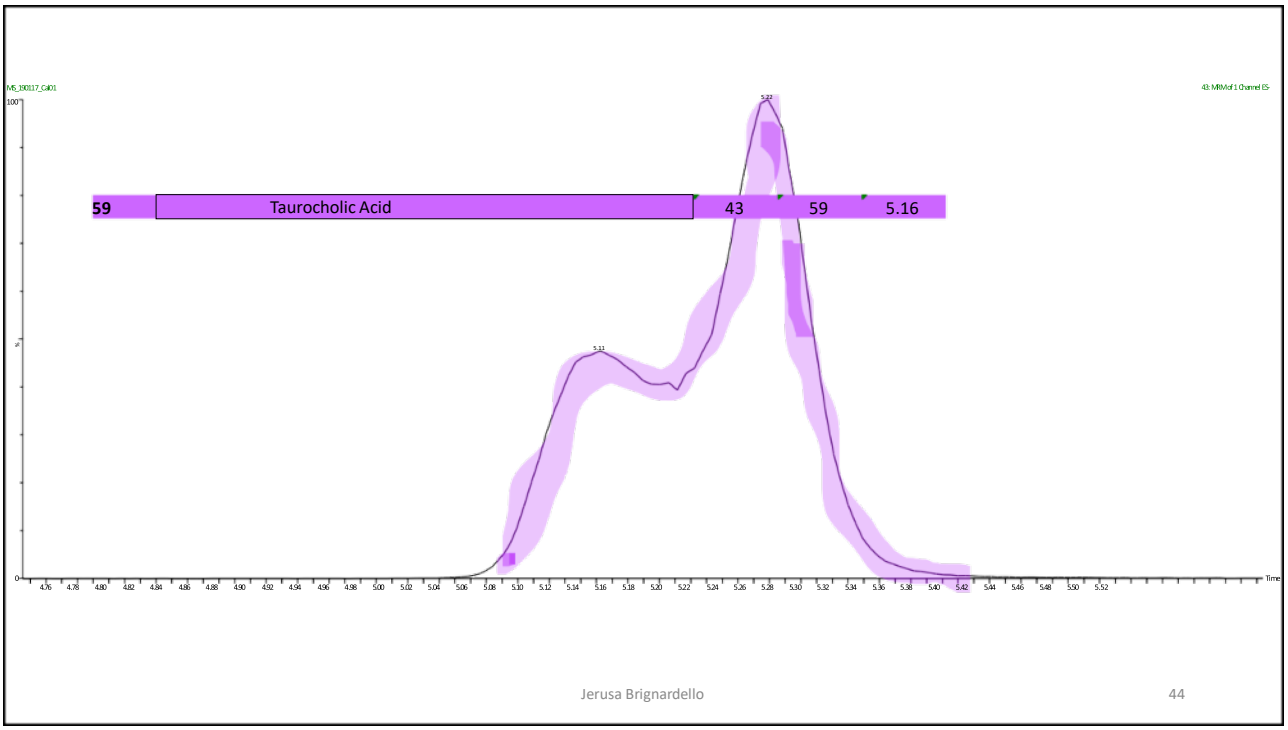
41


MS_190117_Cal01
40: MRM of 1 Channel ES-
54 3,7,12 Taurodehydrocholic acid (5β-Cholanic Acid-3, 7, 12-Trione N-(2-sulphoethyl)-amide) 40 57 1.54
Time
Jerusa Brignardello

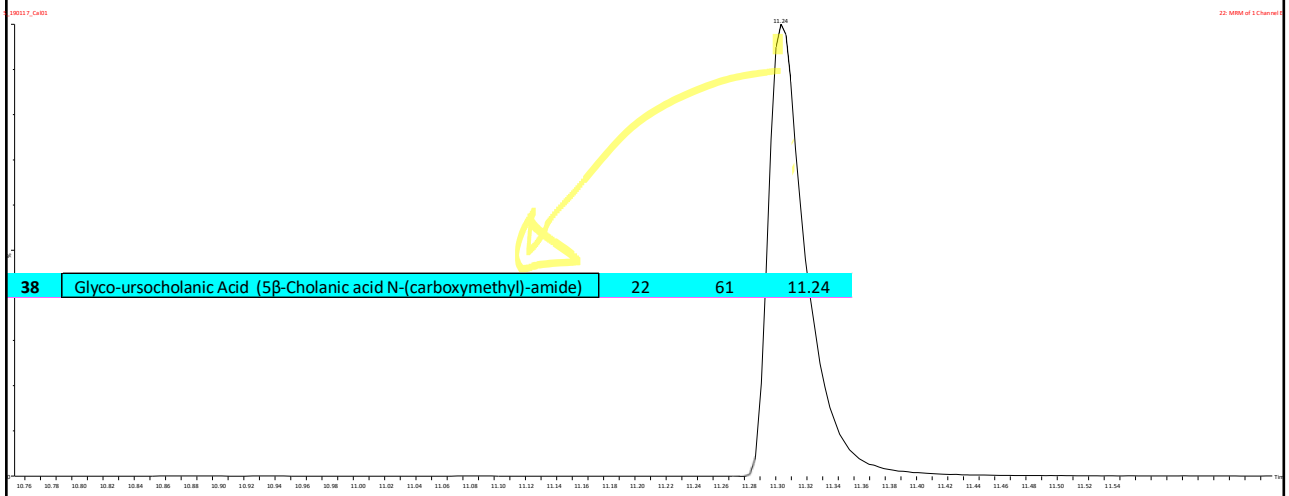
42


58
Tauro ω-Muricholic Acid sodium salt
41
58
3.26
2.87
3.49
Jerusa Brignardello
43

43

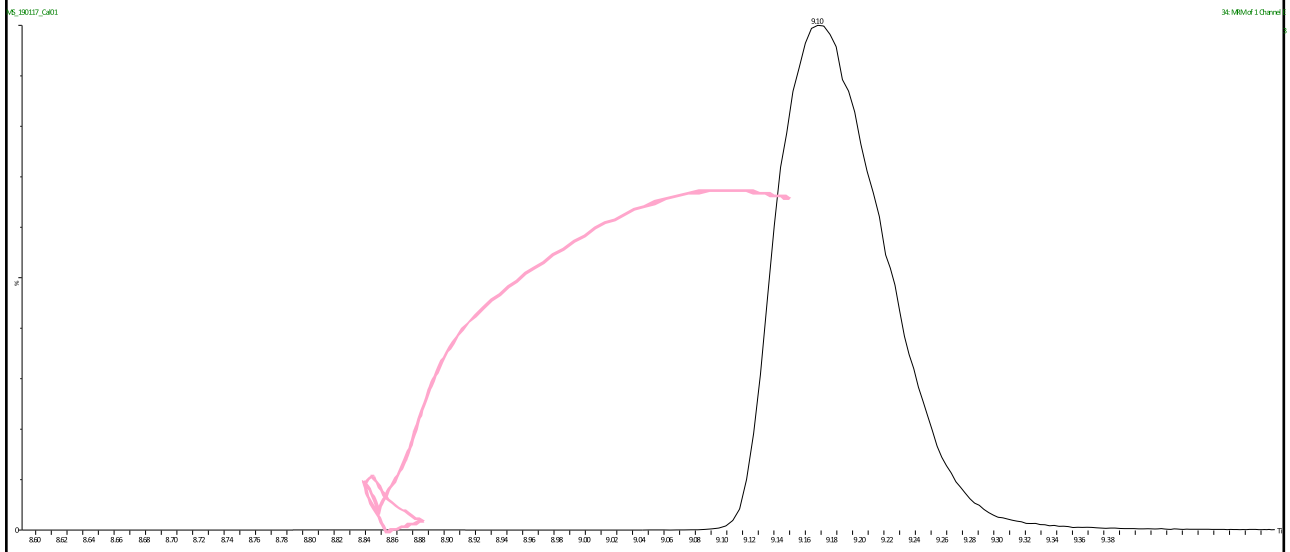

59
Taurocholic Acid
43
59
5.16
5.11
5.22
Jerusa Brignardello
44

44



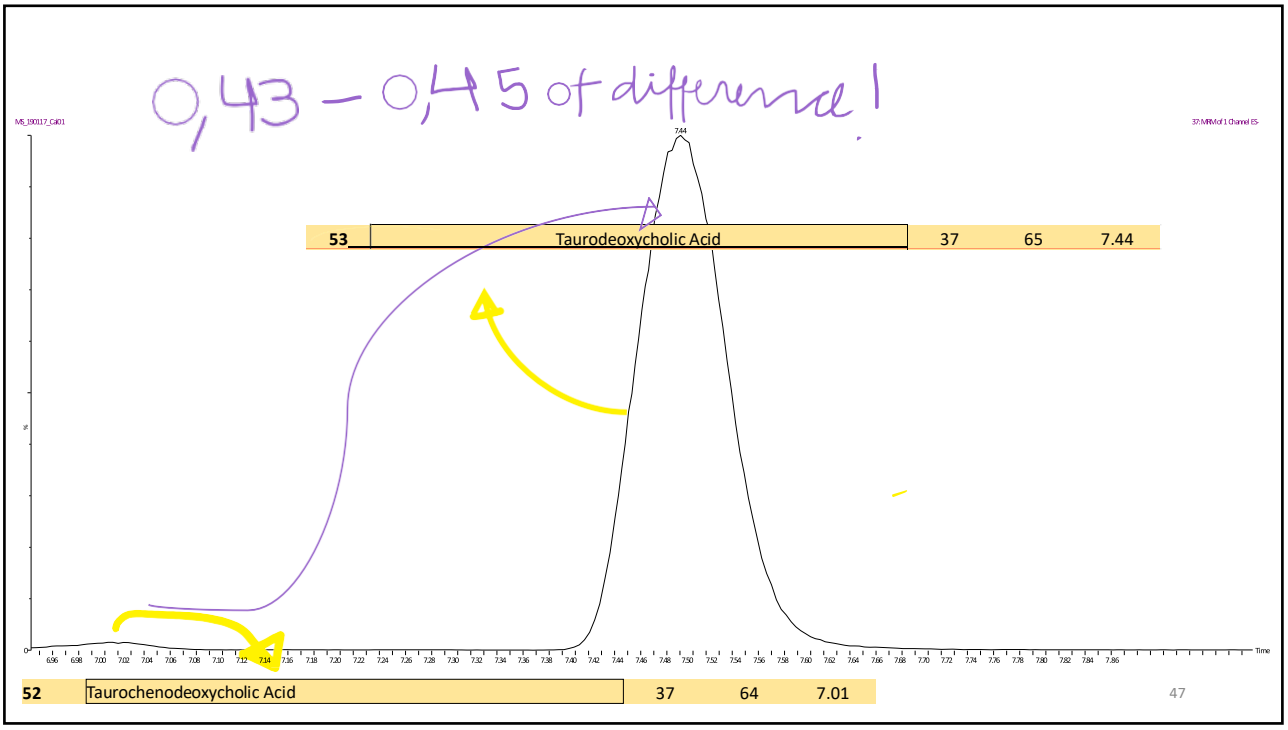
38	Glyco-ursocholanic Acid (5β-Cholanic acid N-(carboxymethyl)-amide)	22	61	11.24

45

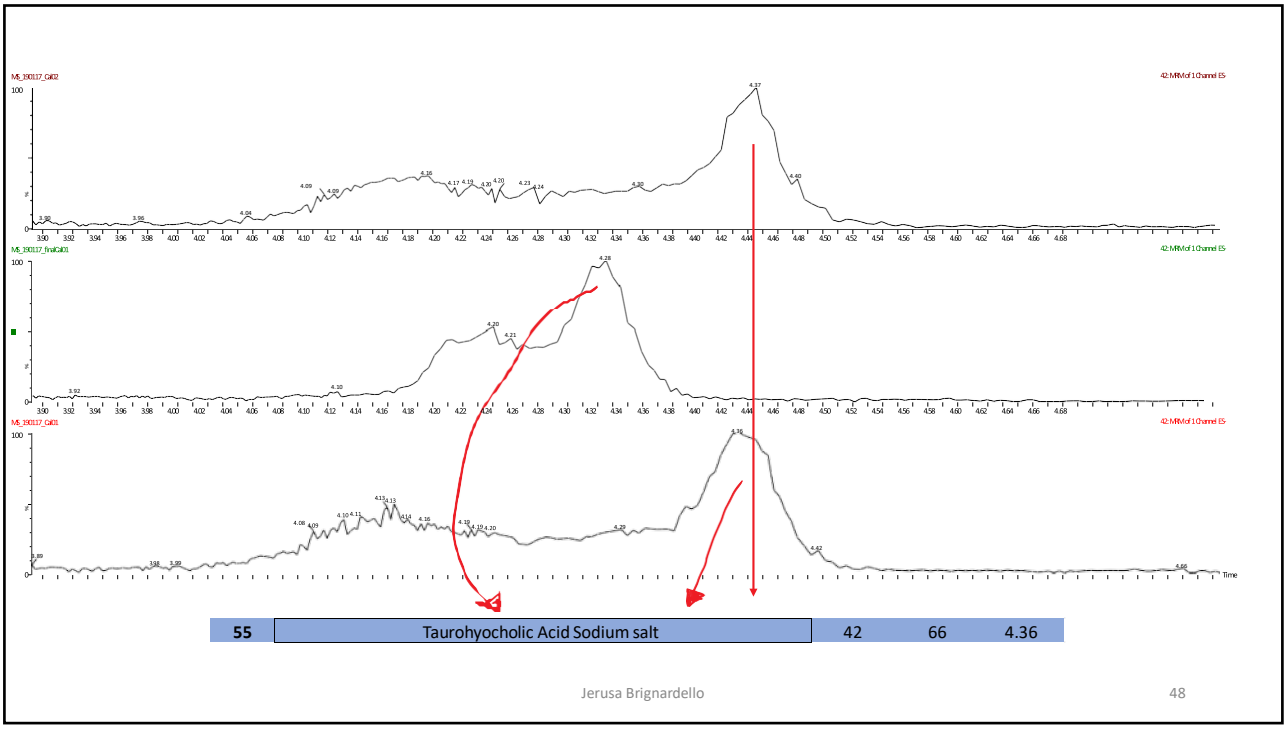


48	Taurolithocholic Acid (5β-Cholanic Acid-3α-ol N-(2-sulphoethyl)-amide)	34	62	9.1

46


0,43 – 0,45 of difference!
53 Taurodeoxycholic Acid 37 65 7.44
52 Taurochenodeoxycholic Acid 37 64 7.01
47

47


55 Taurohyocholic Acid Sodium salt 42 66 4.36
Jerusa Brignardello
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Appendix 11. Additional figures from Chapter 5

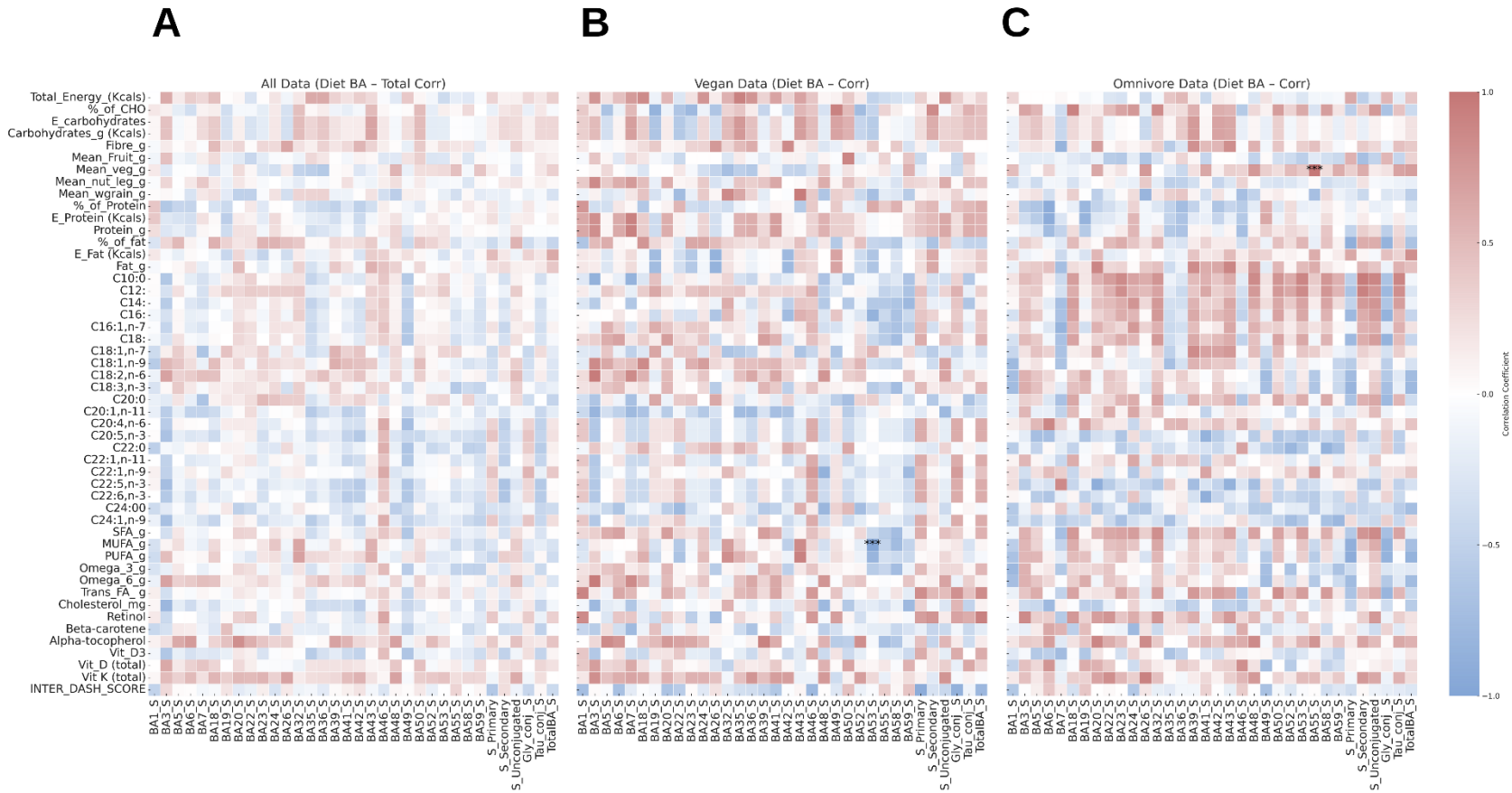

Figure 11. 1 Correlations between dietary nutrients and serum BAs of omnivores and vegans. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively. (A) vegan and omnivore data, (B) vegan data, and (C) omnivore data.

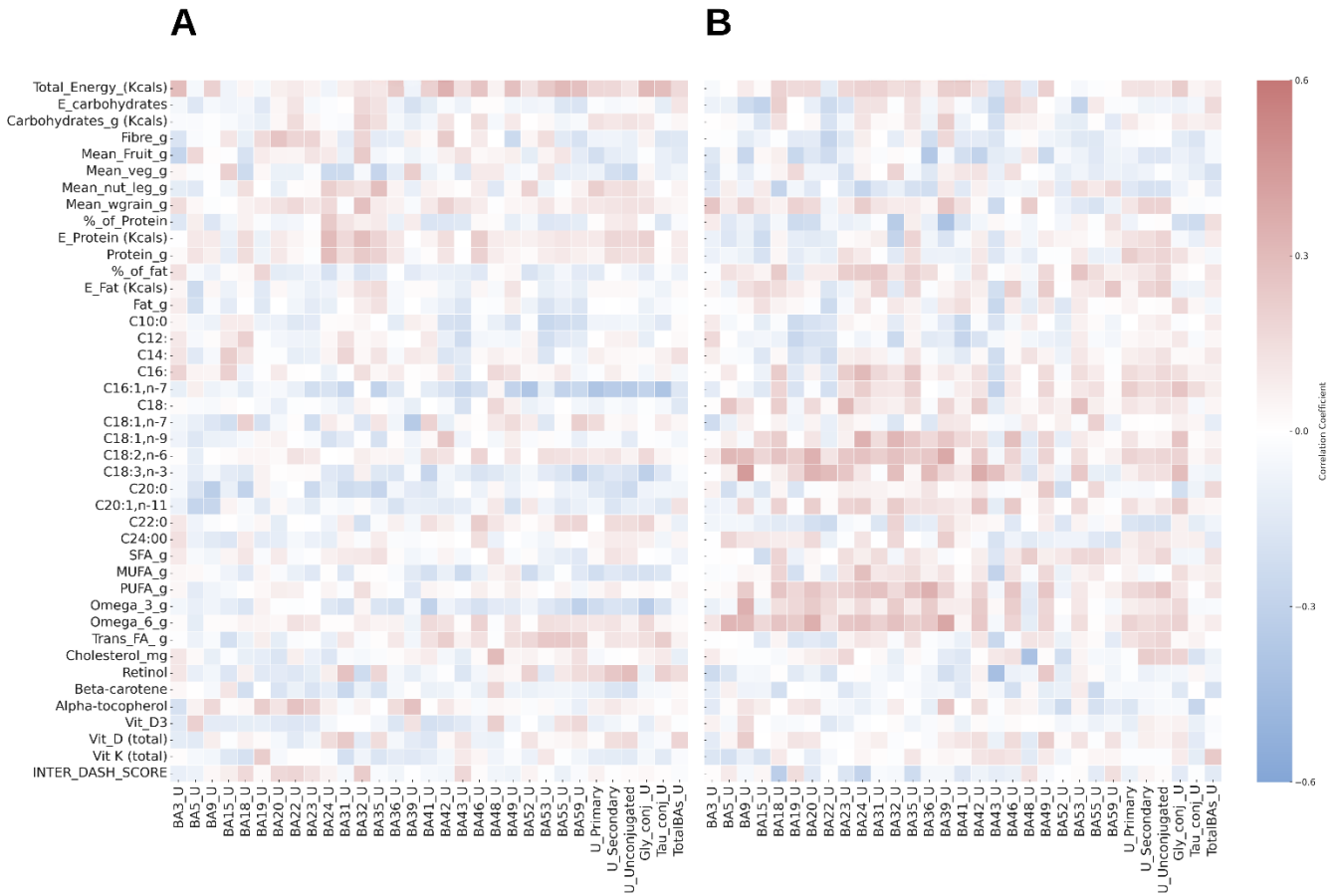

Figure 11. 2 Correlations between dietary nutrients and urine BAs of omnivores and vegans. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively. (A) vegan data, and (B) omnivore data.

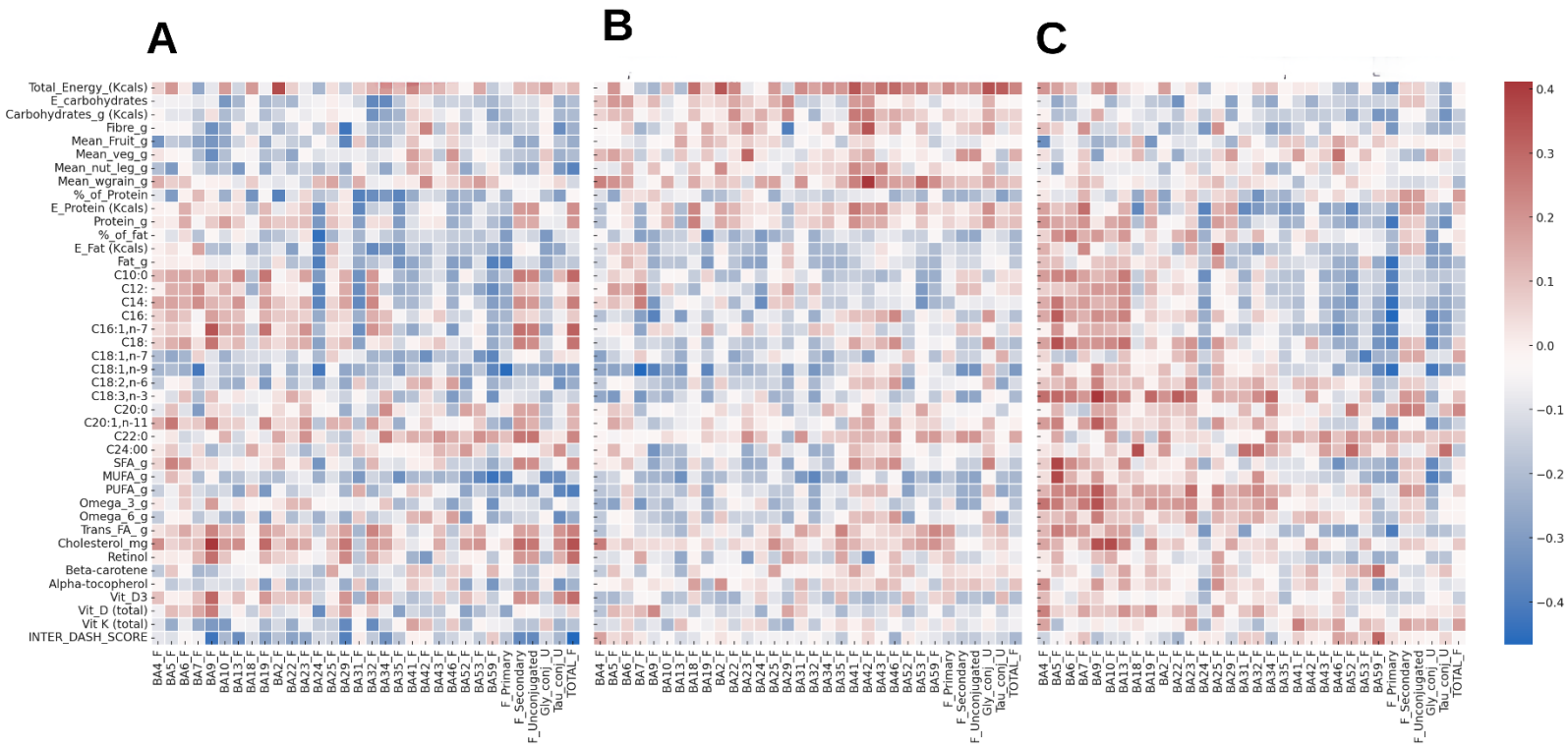

Figure 11. 3 Correlations between dietary nutrients and faecal BAs of omnivores and vegans. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively. (A) vegan and omnivore data, (B) vegan data, and (C) omnivore data.

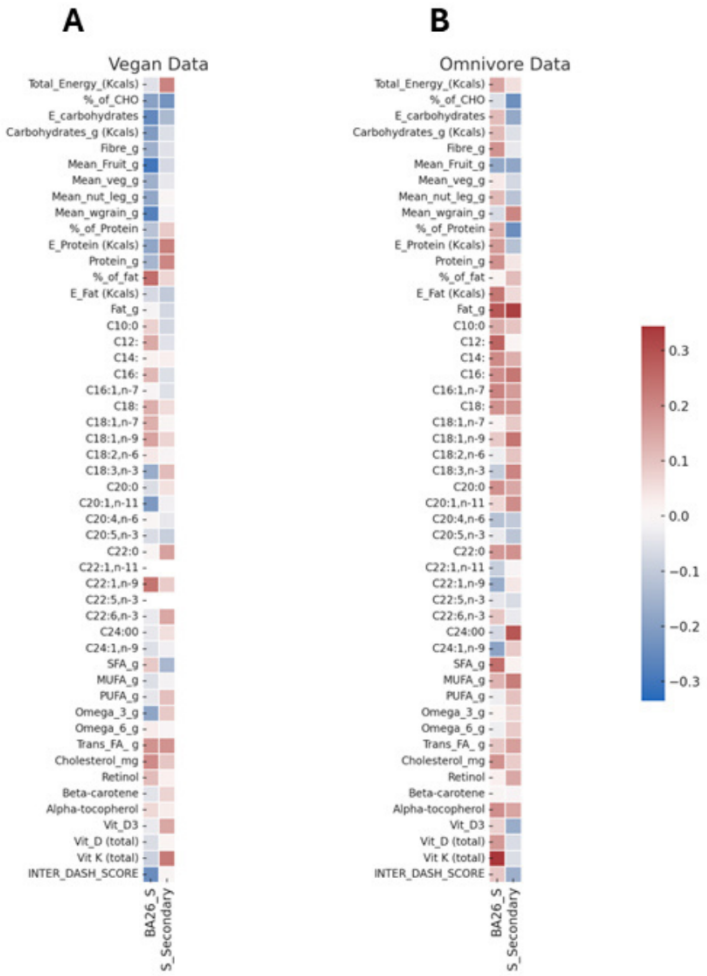

Figure 11. 4 Correlations between dietary nutrients and significant serum BAs of omnivores and vegans. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at Padj<0.001, Padj<0.01, Padj< 0.05. Significances are labelled as ***, ** and * respectively. (A) vegan data, (B) omnivore data.

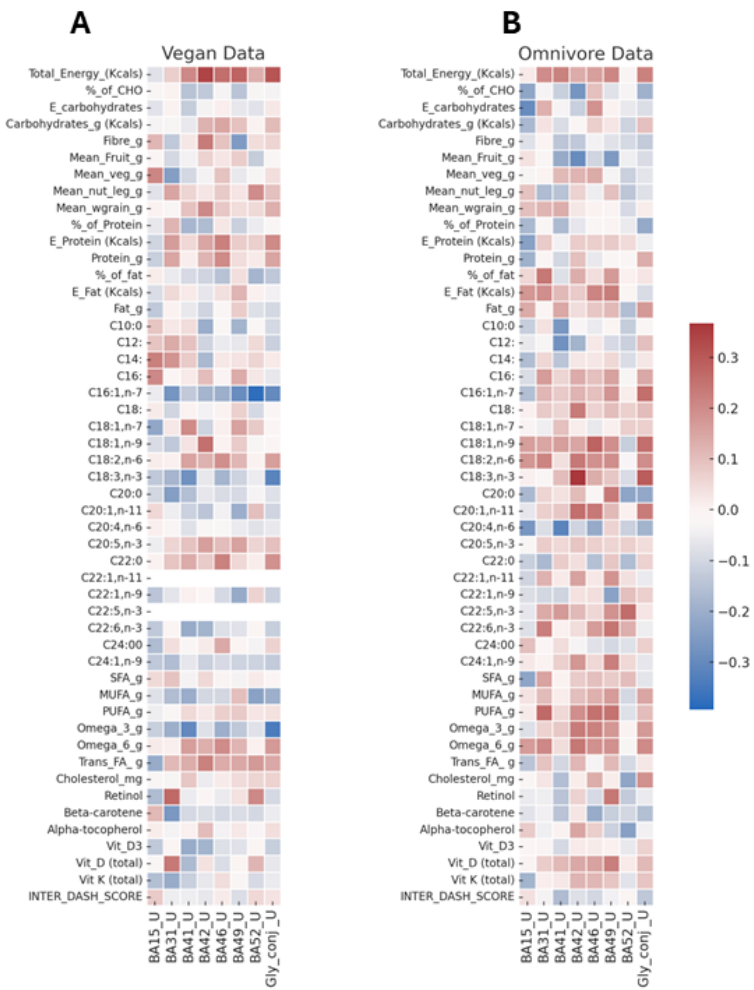

Figure 11. 5 Correlations between dietary nutrients and significant urine BAs of omnivores and vegans. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at Padj<0.001, Padj<0.01, Padj< 0.05. Significances are labelled as ***, ** and * respectively. (A) vegan data, (B) omnivore data.

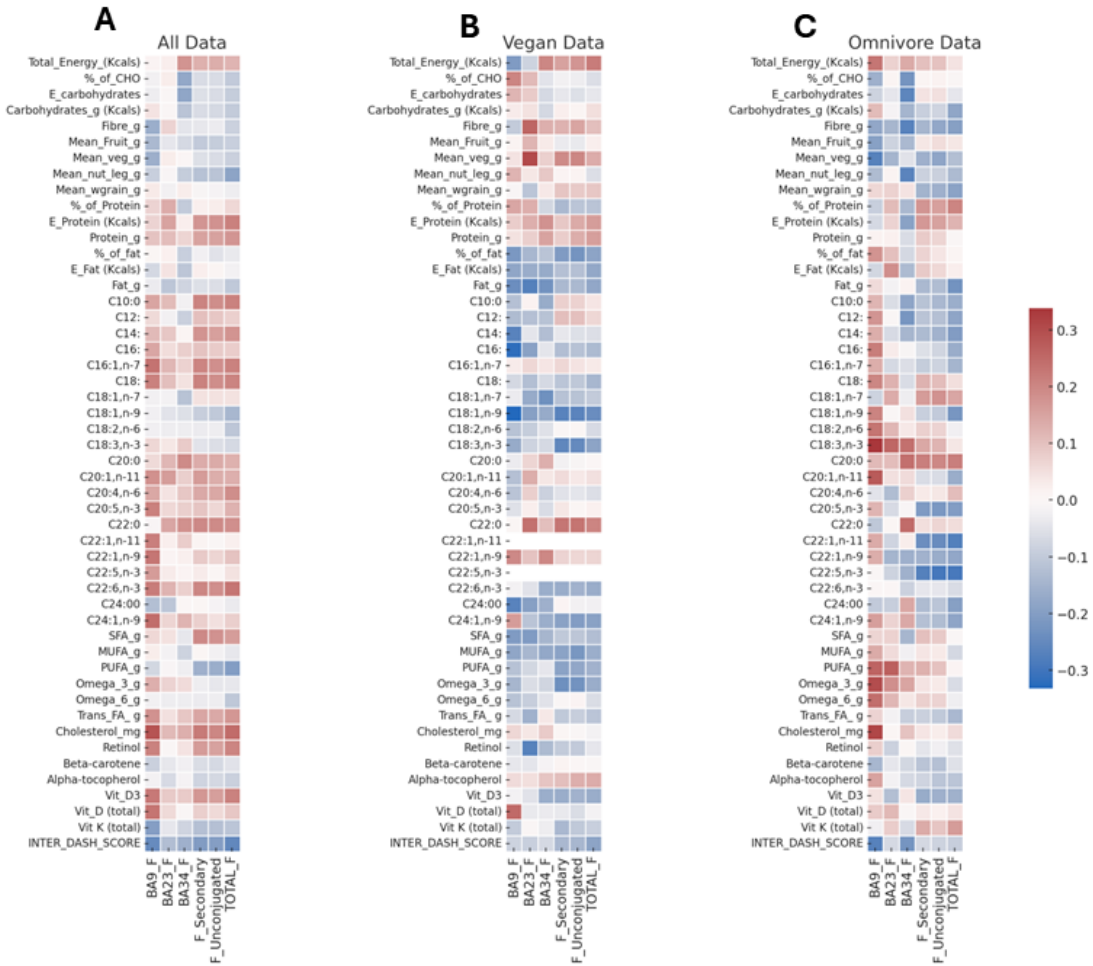

Figure 11. 6 Correlations between dietary nutrients and faecal BAs of omnivores and vegans. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively. (A) All data, (B) vegan data, (C) omnivore data

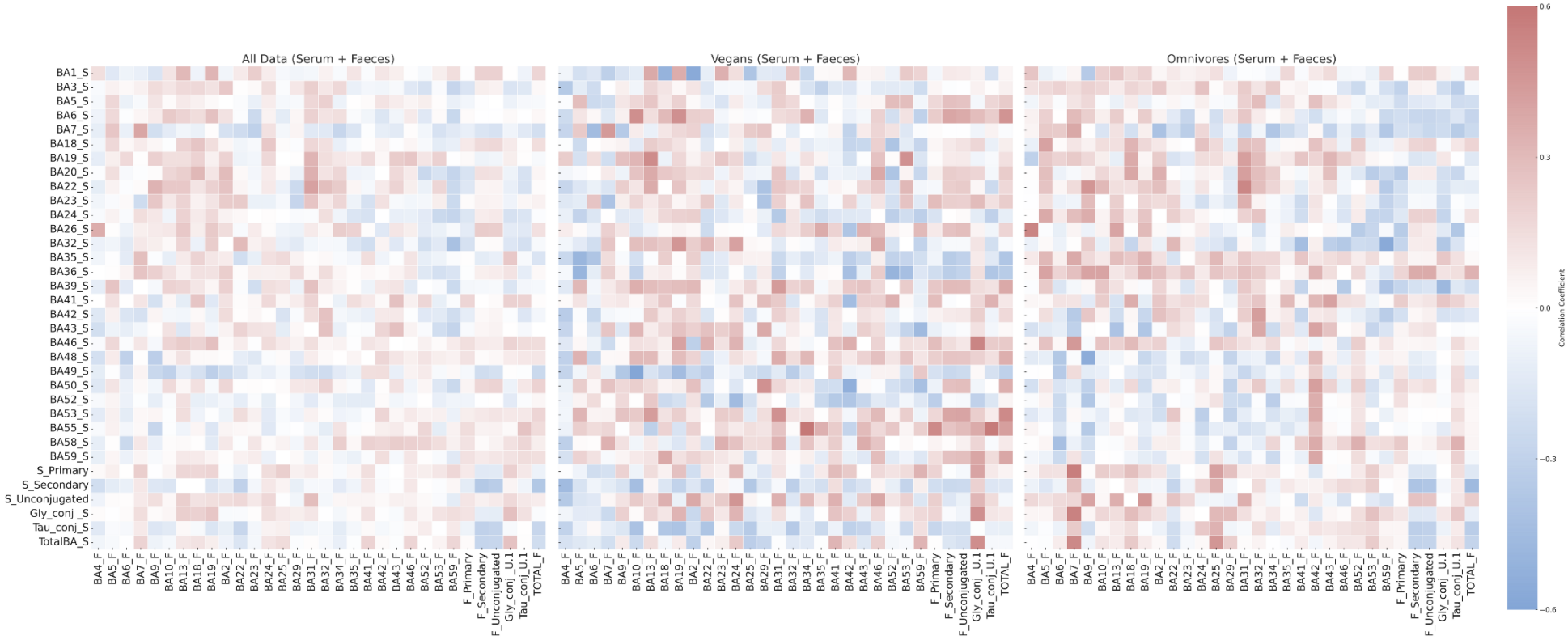

Figure 11. 7 Correlations between serum and faecal BAs from omnivores and vegans. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively. .(A) All data, (B) vegan data, (C) omnivore data.

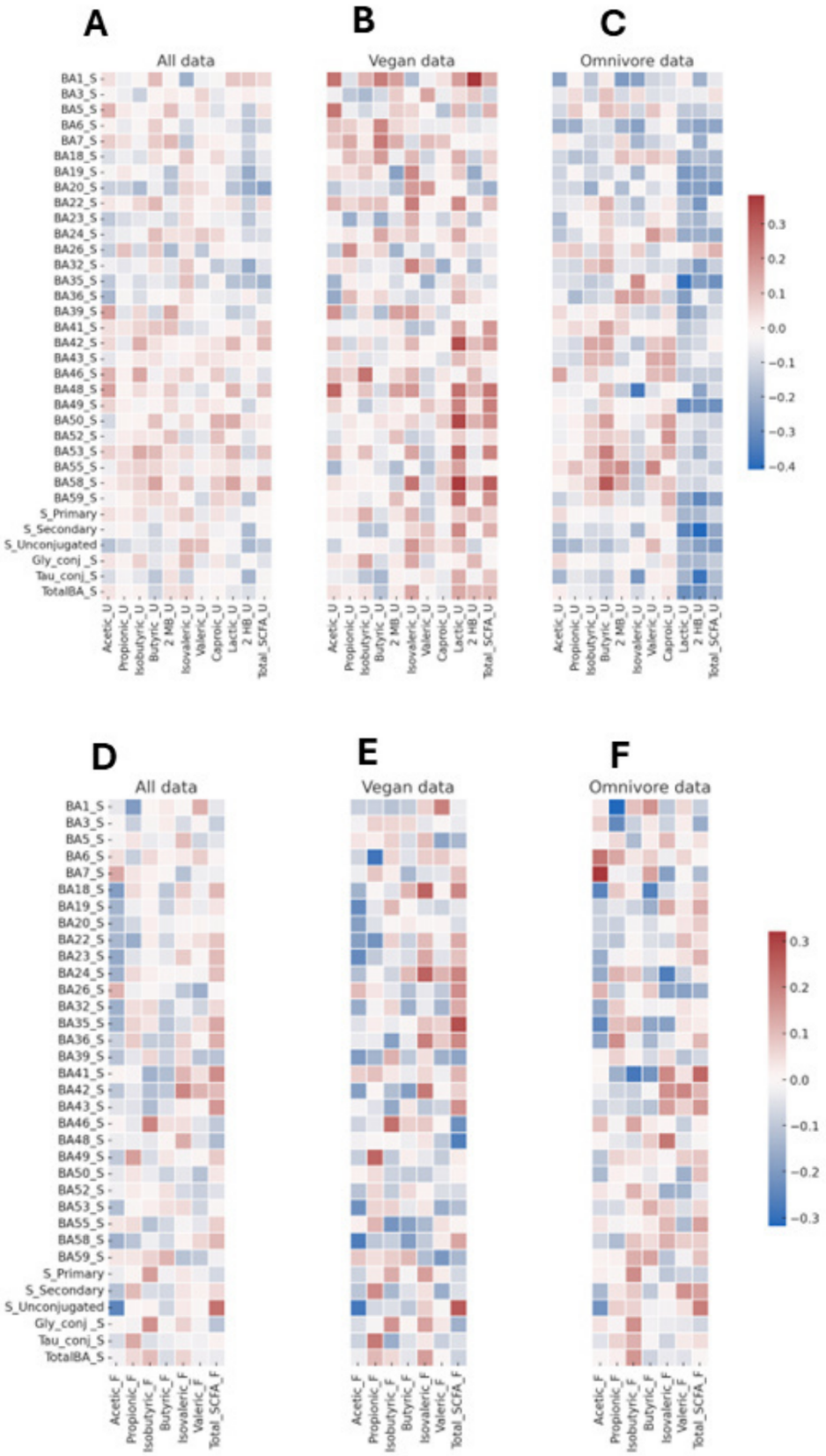

Figure 11. 8 Correlations between serum BAs and urine SCFAs (A) All data, (B) vegan data, (C) omnivore data. Correlations between serum BAs and faecal SCFAs (D) All data, (E) vegan data, (F) omnivore data. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively.

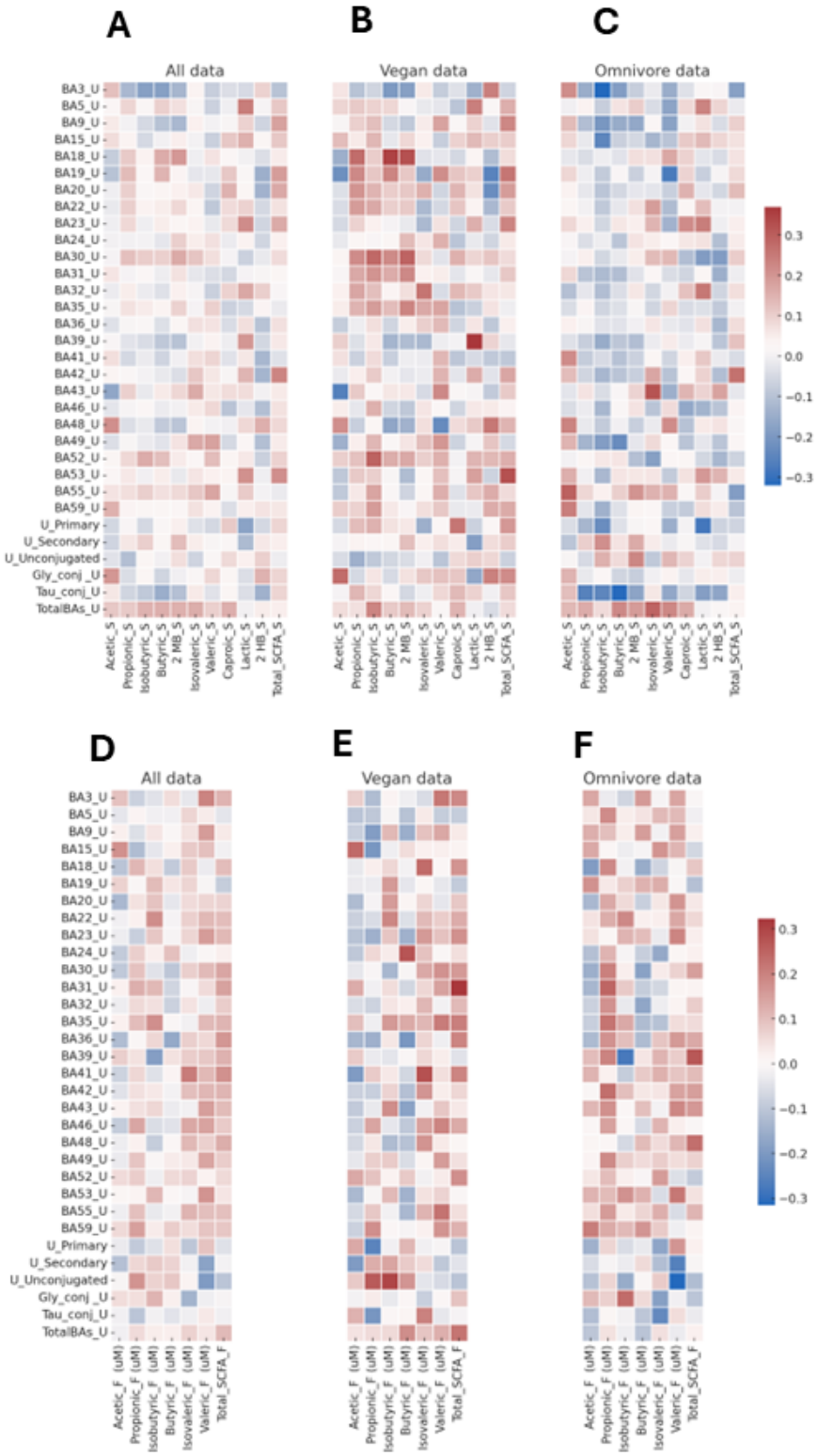

Figure 11. 9 Correlations between urine BAs and serum SCFAs (A) All data, (B) vegan data, (C) omnivore data. Correlations between urine BAs and faecal SCFAs (D) All data, (E) vegan data, (F) omnivore data. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively.

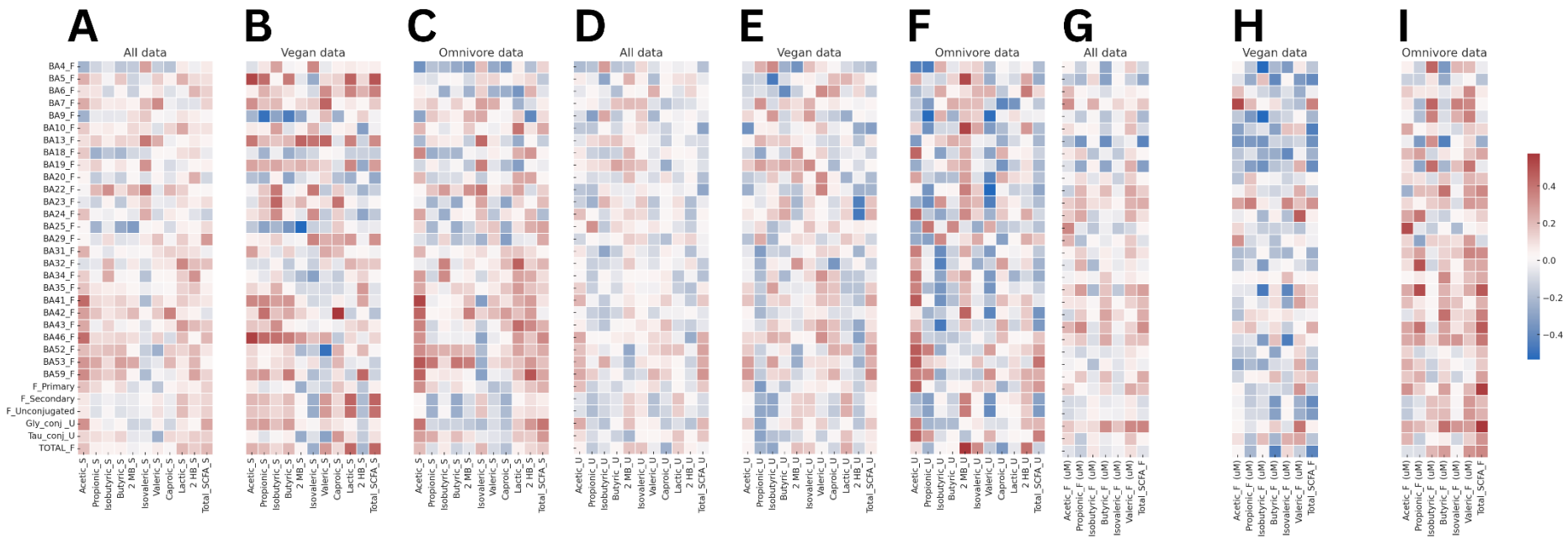

Figure 11. 10 Correlations between faecal BAs and serum SCFAs (A) All data, (B) vegan data, (C) omnivore data. Correlations between faecal BAs and urine SCFAs (D) All data, (E) vegan data, (F) omnivore data. Correlations between faecal BAs and faecal SCFAs (G) All data, (H) vegan data, (I) omnivore data. Correlations were calculated using Spearman correlation and adjusted using False Discovery Rate (FDR). Heatmap shows correlations significance accepted at $P_{adj}<0.001$, $P_{adj}<0.01$, $P_{adj}< 0.05$. Significances are labelled as ***, ** and * respectively.

Appendix 12. Additional figures from Chapter 6

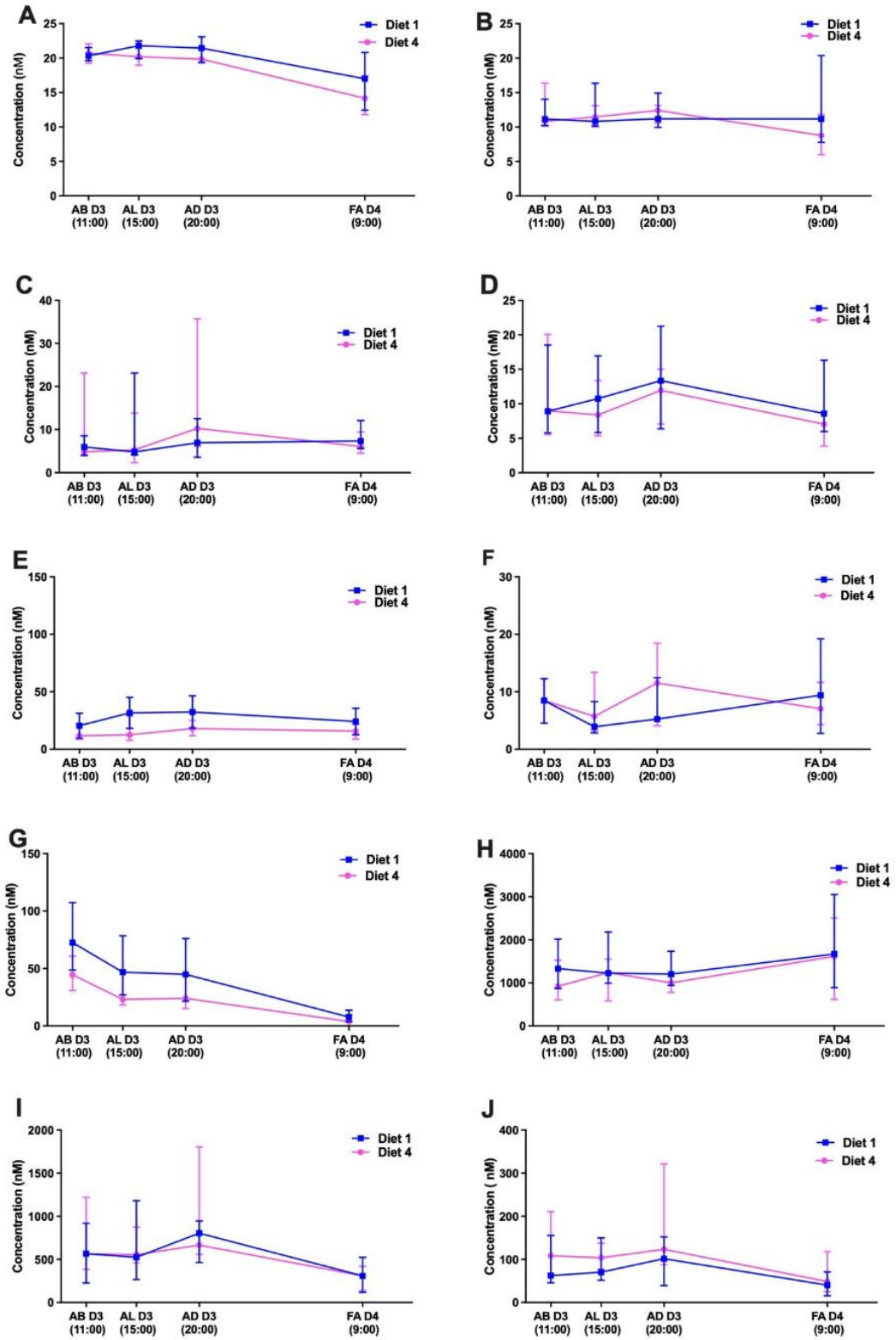

Figure 12. 1 Comparison of serum BA concentrations throughout the day for the same time points for diet 1 and diet 4. (A) 5-Cholenic acid-3β-ol, (B) Allolicholic acid, (C) 3α-Hydroxy-7 Ketolithocholic acid, (D) 3α-Hydroxy-12 Ketolithocholic acid, (E) 5β-Cholanic acid-3β, 12α-diol, (F) Hyocholic acid, (G) Glycolithocholic acid, (H) Glycochenodeoxycholic acid, (I) Glycodeoxycholic acid and (J) Glycoursodeoxycholic acid. Values are shown in median and interquartile range n=19. Differences between the same time points for different diets were determined by Wilcoxon signed rank test and False Discovery Rate (FDR). Significance was accepted at $P_{adj}<0.05$

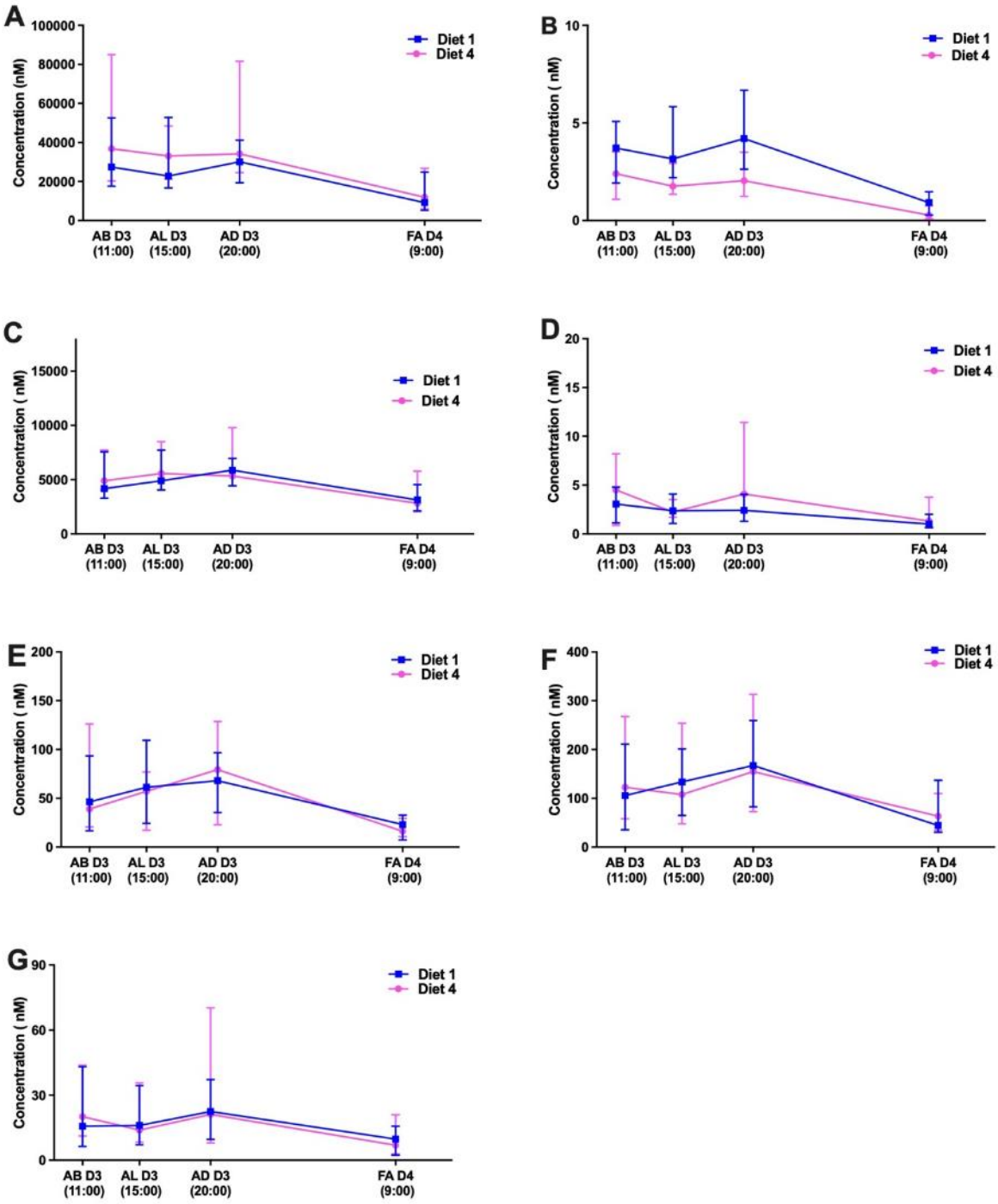

Figure 12. 2 Comparison of serum BA concentrations throughout the day for the same time points for diet 1 and diet 4. (A) Glycocholic, (B) Taurolitocholic acid, (C) Glycohyocholic acid, (D) Tauroursodeoxycholic acid, (E) Taurodeoxycholic acid, (F) Taurohyocholic acid, (G) Taurocholic acid. Values are shown in median and interquartile range n=19. Differences between the same time points for different diets were determined by Wilcoxon signed rank test and False Discovery Rate (FDR). Significance was accepted at $P_{adj}<0.05$.

Appendix 13. Additional figures from Chapter 6 (Second part)

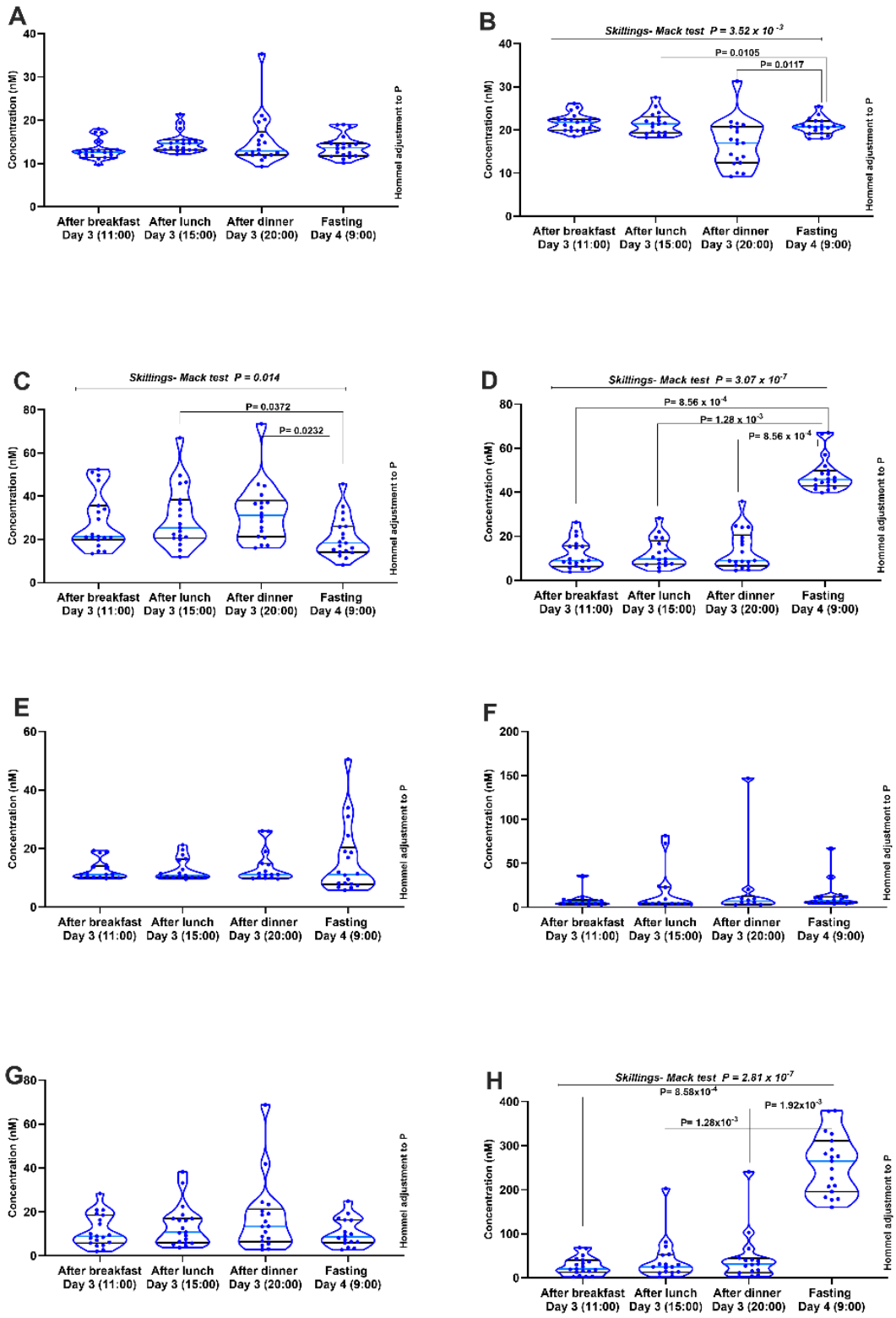

Figure 13. 1 Variations of BAs throughout the day within diet 1. Differences between the same time points for different diets were determined by Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method Significance was accepted at P_{adj} <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) Ursocholanic acid, (B) 5-Cholenic acid-3β-ol acid, (C) Lithocholic acid, (D) Isolithocholic acid, (E) Allolithocholic acid, (F) 3α-Hydroxy-7 Ketolithocholic acid, (G) 3α-Hydroxy-12 Ketolithocholic acid, (H) Murocholic acid, (I) 5β-Cholanic acid-3β, 12α-diol and (J) Murocholic acid.

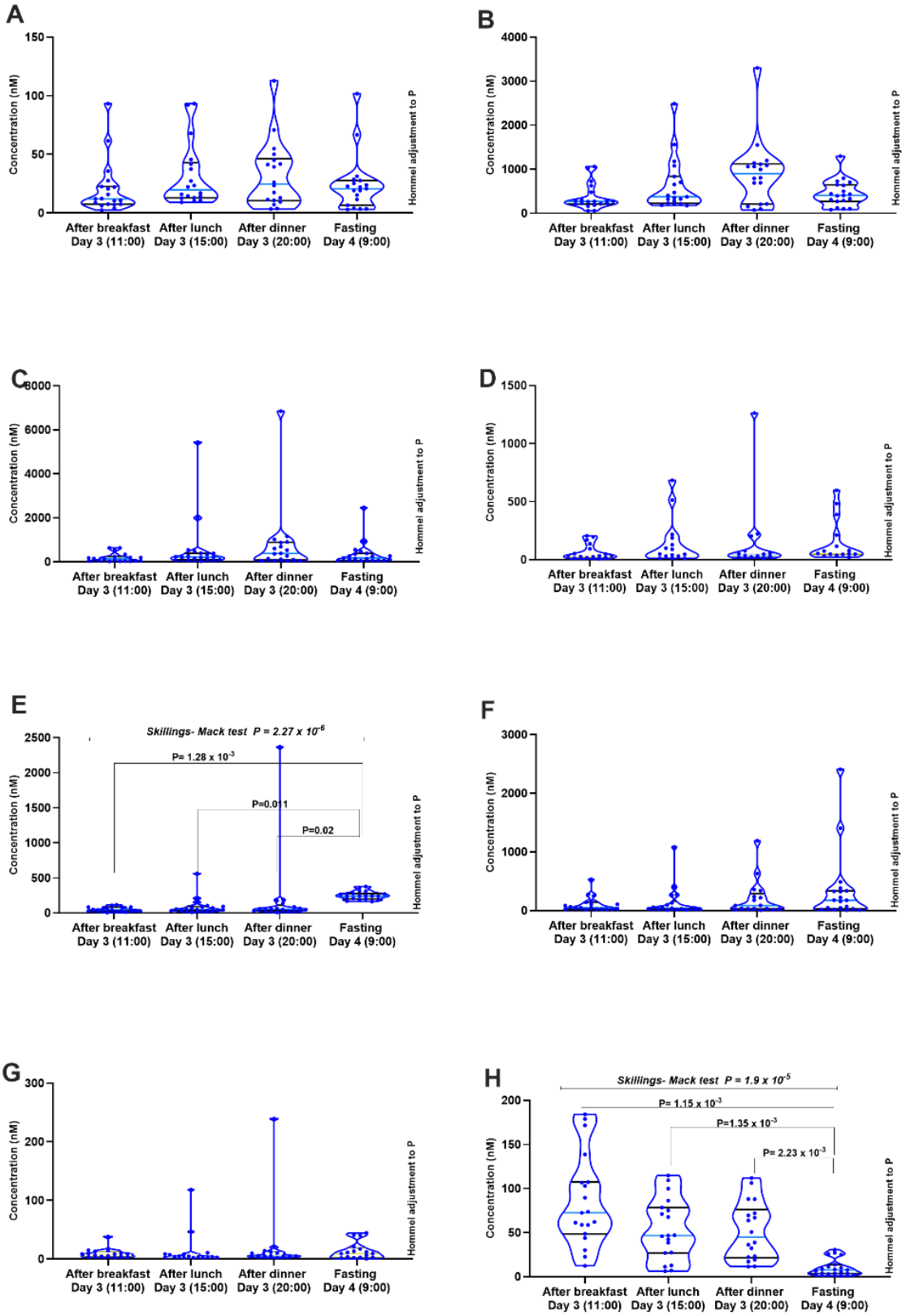

Figure 13. 2 Variations of BAs throughout the day within diet 1. Differences between the same time points for different diets were determined by Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method Significance was accepted at P_{adj} <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) 5β-Cholanic acid-3β, 12α-diol acid, (B) Deoxycholic acid, (C) Chenodeoxycholic acid, (D) 12 Dehydrocholic acid, (E) Ursodeoxycholic acid, (F) Cholic acid, (G) Hyocholic acid and (H) Glycolithocholic.

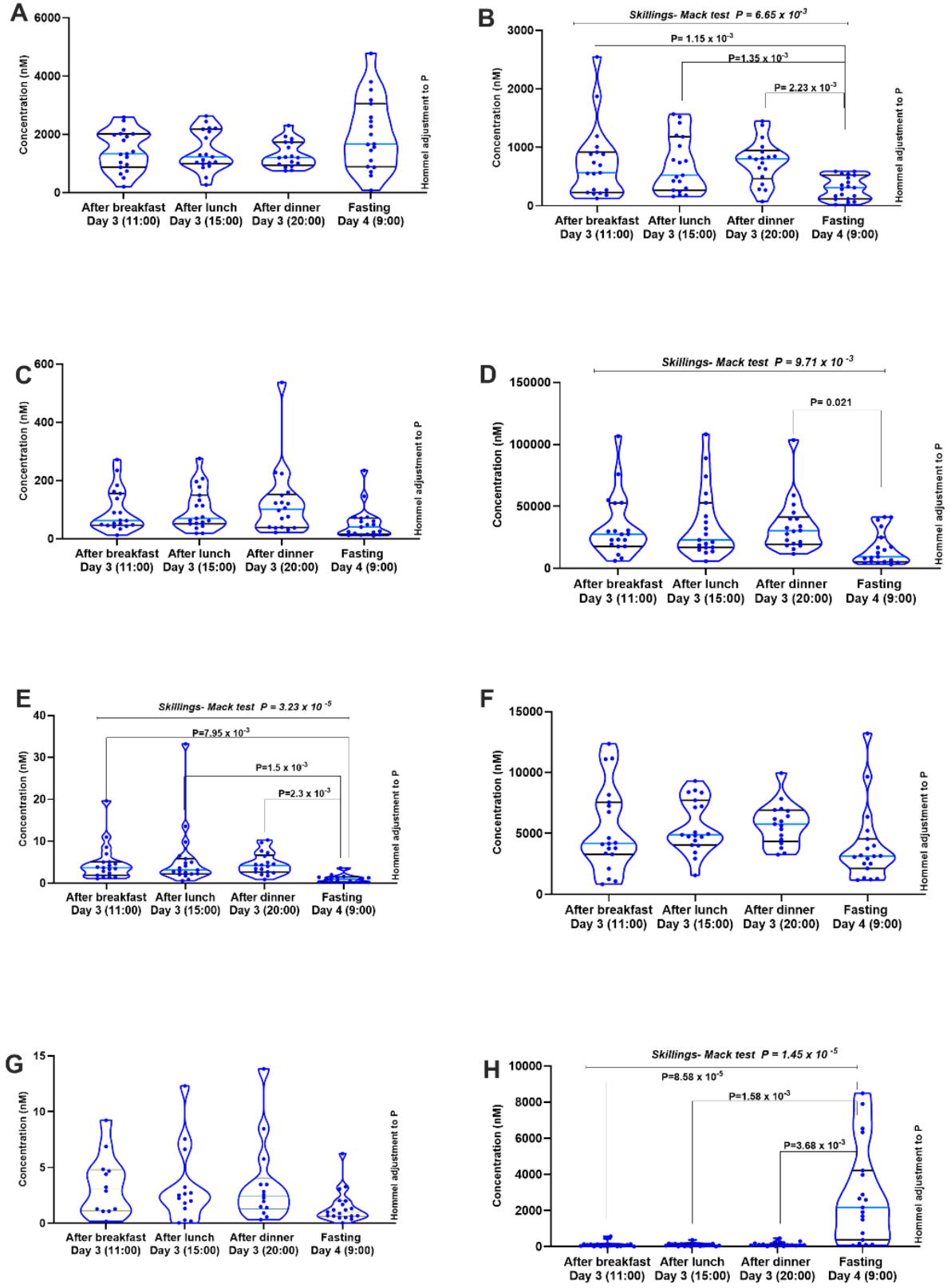

Figure 13. 3 Variations of BAs throughout the day within diet 1. Differences between the same time points for different diets were determined by Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method Significance was accepted at P_{adj} <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) Glycochenodeoxycholic acid, (B) Glycodeoxycholic acid, (C) Glycoursodeoxycholic acid, (D) Glycocholic acid, (E) Taurolithocholic acid, (F) Glycohyocholic acid, (G) Tauroursodeoxycholic acid and (H) Taurochenodeoxycholic.

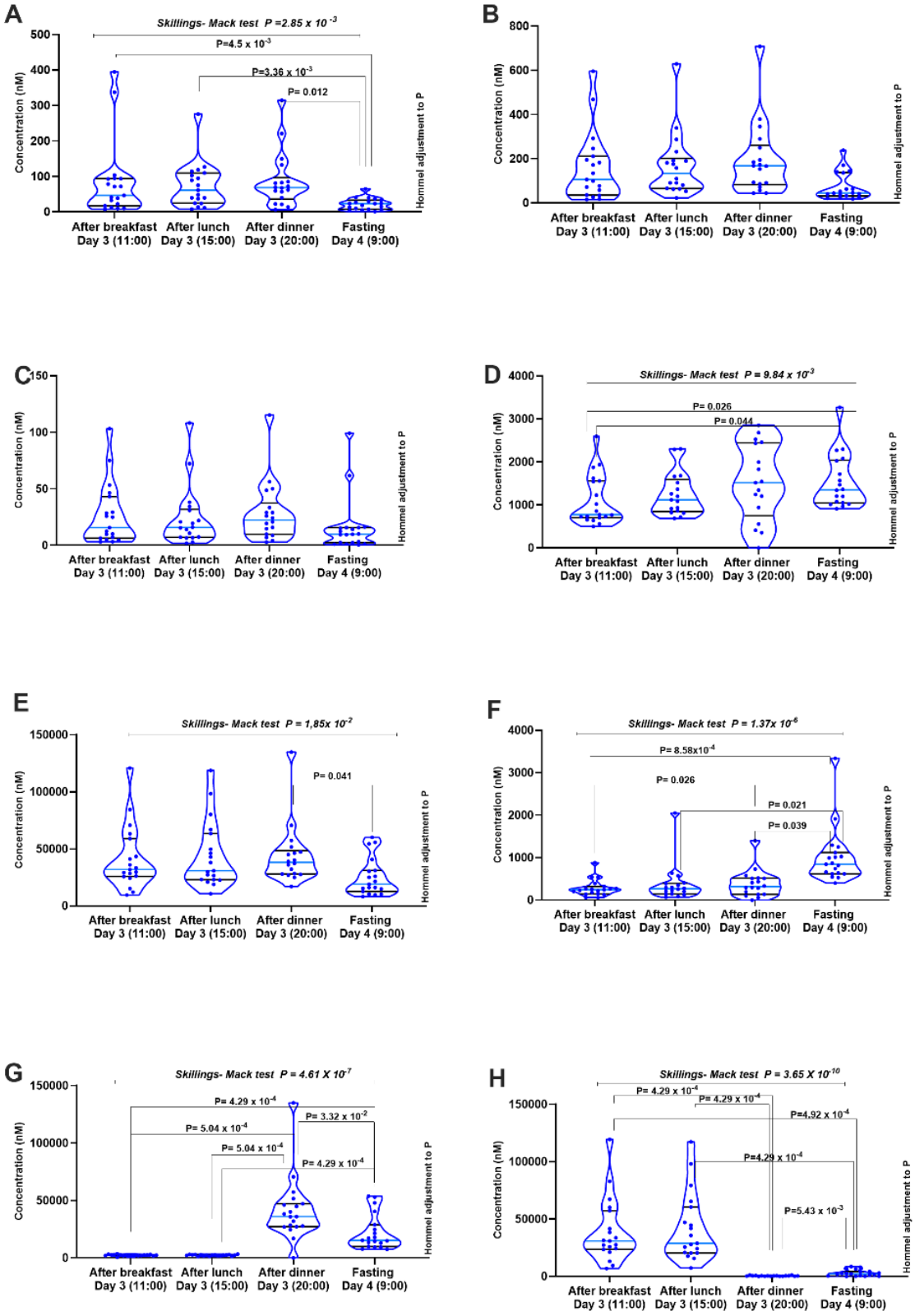

Figure 13. 4 Variations of BAs throughout the day within diet 1. Differences between the same time points for different diets were determined by Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method Significance was accepted at P_{adj} <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) Taurodeoxycholic acid, (B) Taurohyocholic acid, (C) Taurocholic acid, (D) Primary BAs, (E) Secondary BAs, (F) Unconjugated BAs, (G) Glycine conjugated BAs and (H) Taurine conjugated BAs.

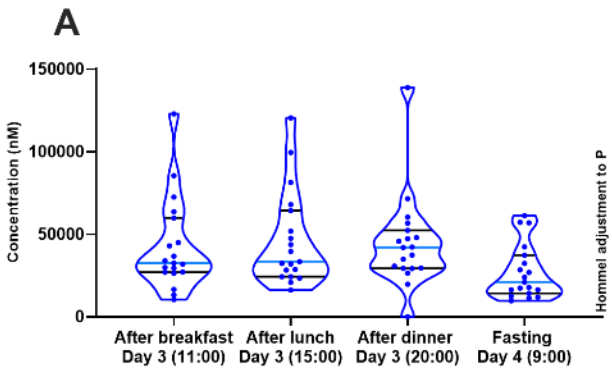

Figure 13. 5 Variations of BAs throughout the day within diet 1. Differences between the same time points for different diets were determined by Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method. Significance was accepted at P_{adj} <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) Total BAs.

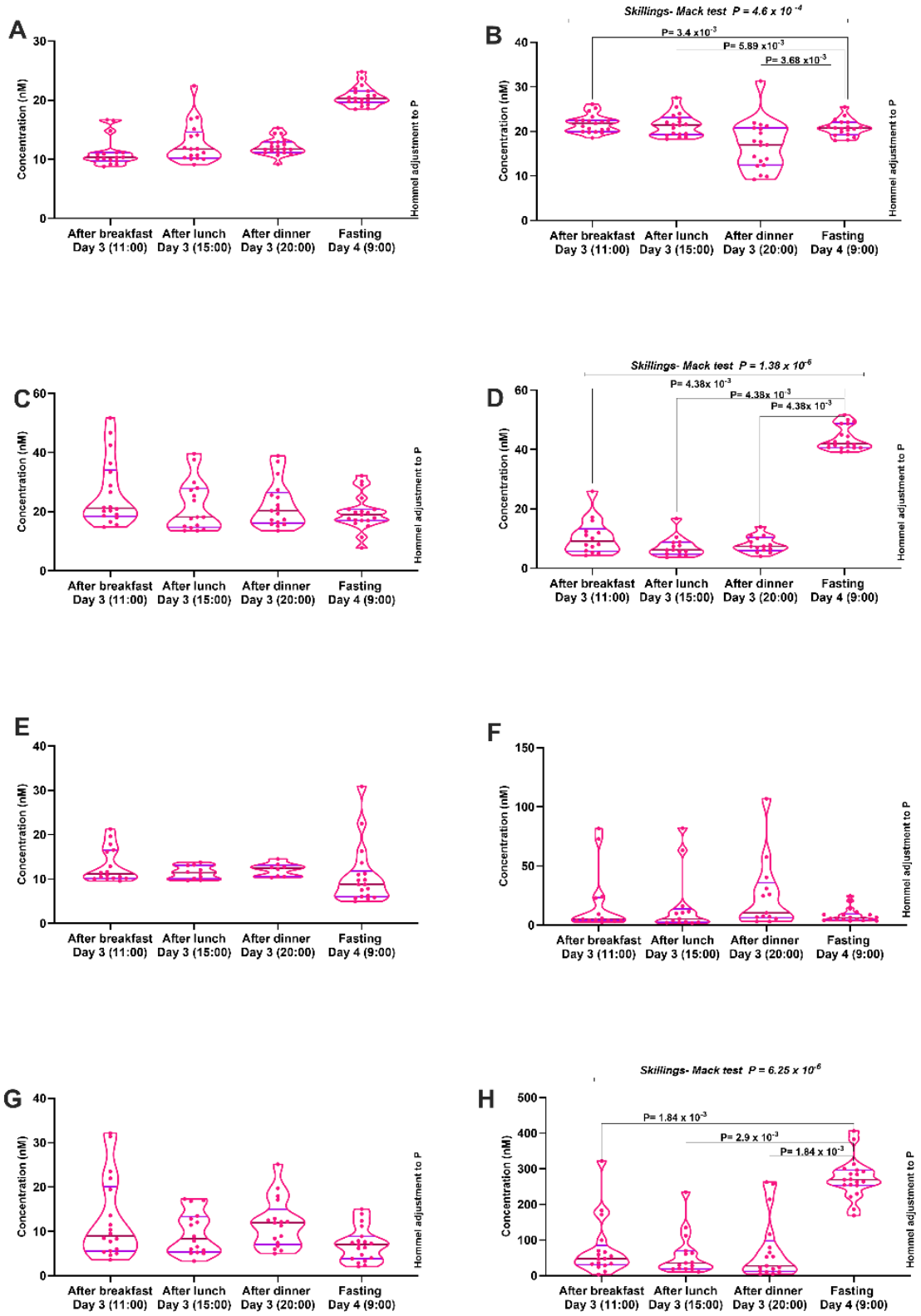

Figure 13. 6 Variations of BAs throughout the day within diet 4. Differences between the same time points for different diets were determined by Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method Significance was accepted at P_{adj} <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) Ursocholanic acid, (B) 5-Cholenic acid-3β-ol acid, (C) Lithocholic acid, (D) Isolithocholic acid, (E) Allolithocholic acid, (F) 3α-Hydroxy-7 Ketolithocholic acid, (G) 3α-Hydroxy-12 Ketolithocholic acid, (H) Murocholic acid, (I) 5β-Cholanic acid-3β, 12α-diol and (J) Murocholic acid.

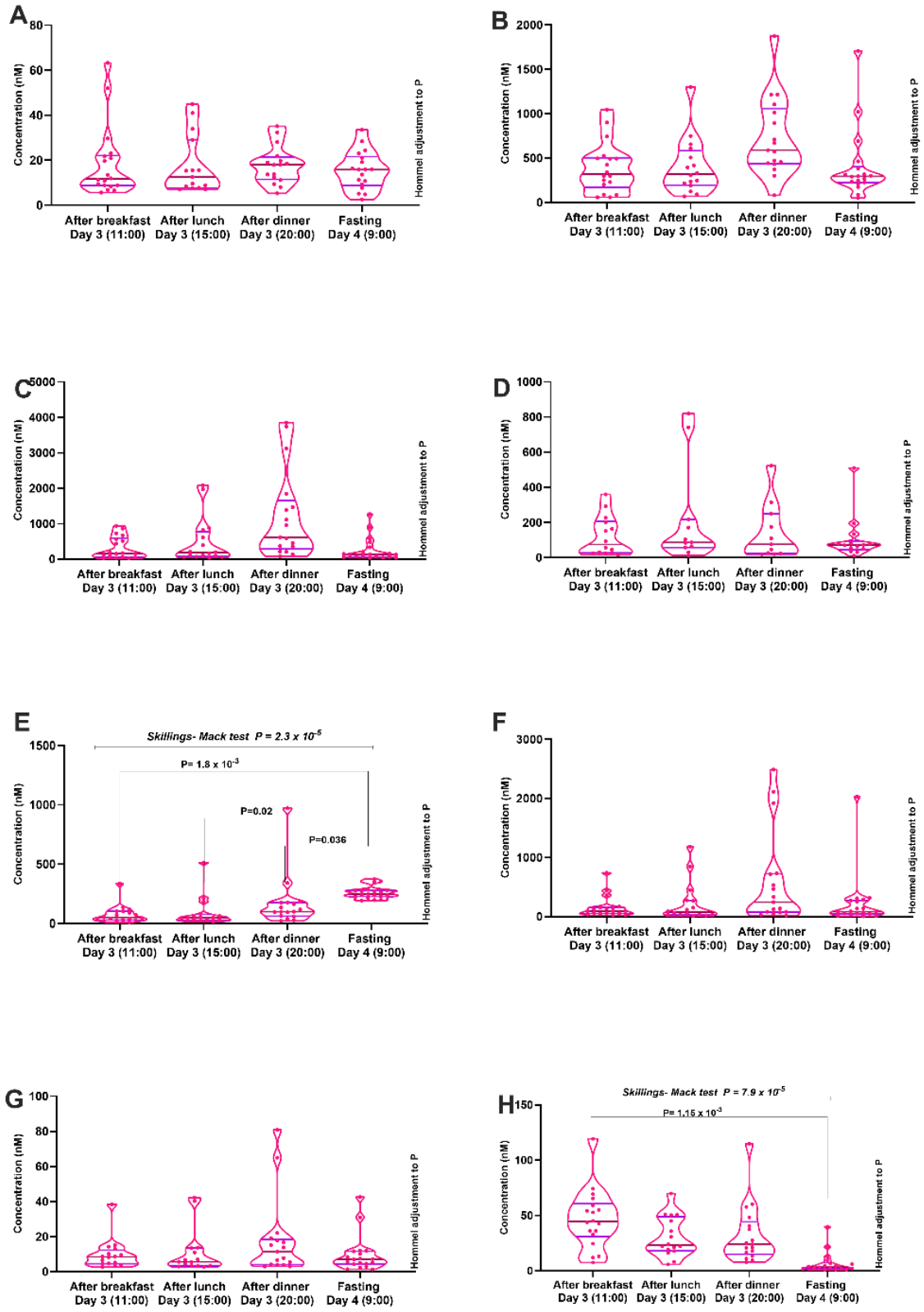

Figure 13. 7 Variations of BAs throughout the day within diet 4 Differences between the same time points for different diets were determined by Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method Significance was accepted at P_{adj} <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) 5β-Cholanic acid-3β, 12α-diol acid, (B) Deoxycholic acid, (C) Chenodeoxycholic acid, (D) 12 Dehydrocholic acid, (E) Ursodeoxycholic acid, (F) Cholic acid, (G) Hyocholic acid and (H) Glycolithocholic.

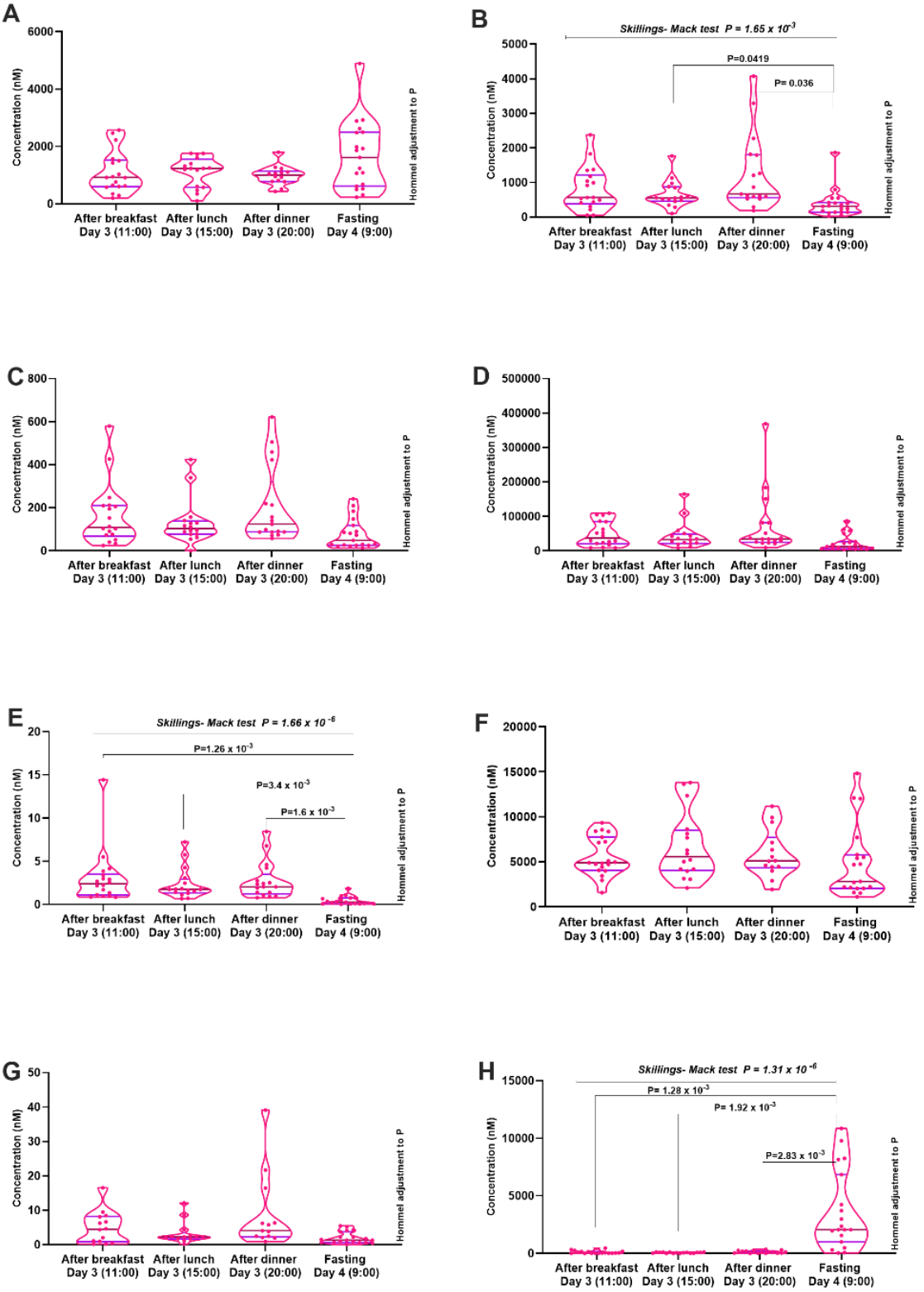

Figure 13. 8 Variations of BAs throughout the day within diet 4. Differences between the same time points for different diets were determined by Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method Significance was accepted at P_{adj} <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) Glycochenodeoxycholic acid, (B) Glycodeoxycholic acid, (C) Glycoursodeoxycholic acid, (D) Glycocholic acid, (E) Taurolithocholic acid, (F) Glycohyocholic acid, (G) Tauroursodeoxycholic acid and (H) Taurochenodeoxycholic.

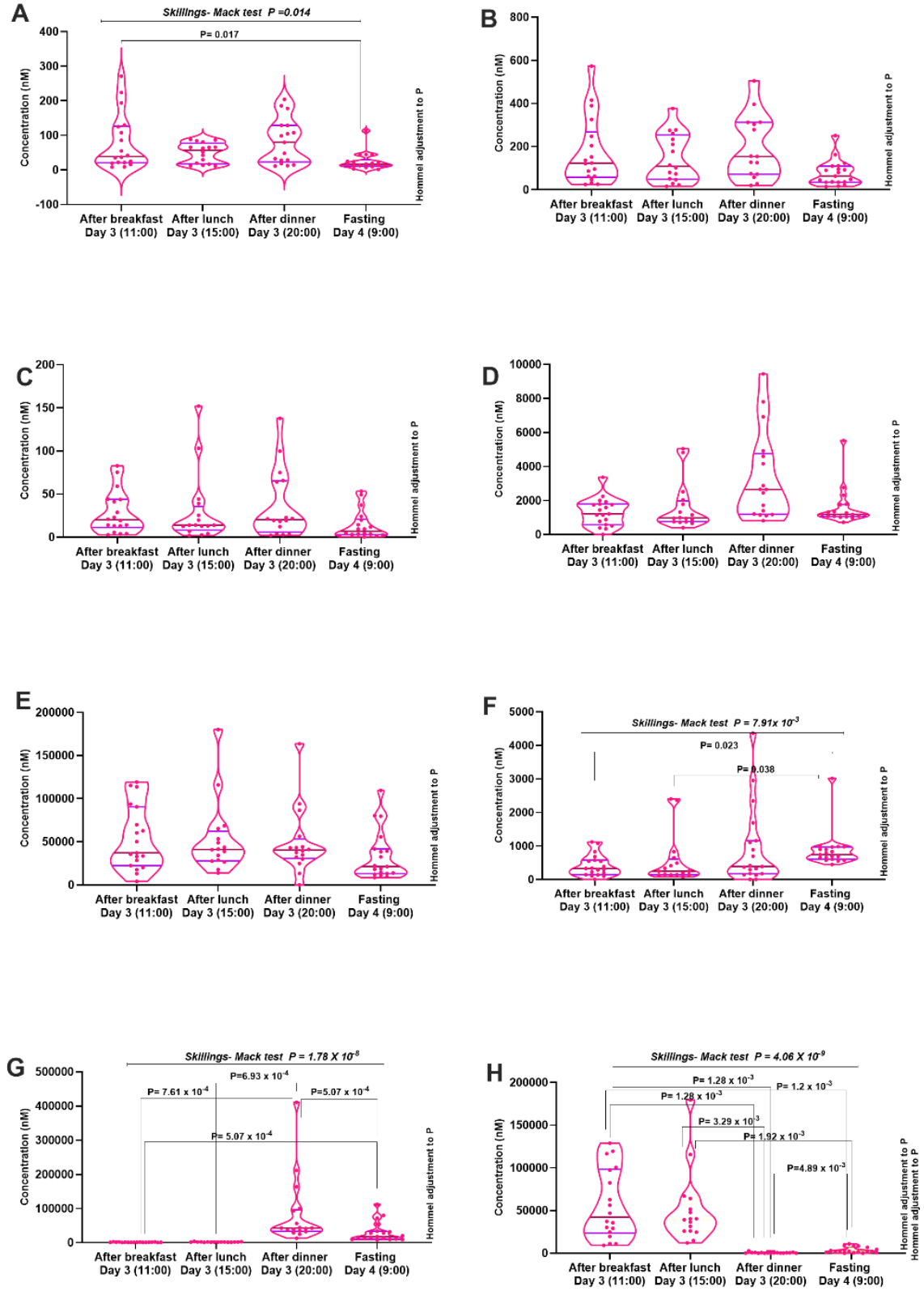

Figure 13. 9 Variations of BAs throughout the day within diet 4. Differences between the same time points for different diets were determined by Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method Significance was accepted at P_{adj} <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) Taurodeoxycholic acid, (B) Taurohyocholic acid, (C) Taurocholic acid, (D) Primary BAs, (E) Secondary BAs, (F) Unconjugated BAs, (G) Glycine conjugated BAs and (H) Taurine conjugated BAs.

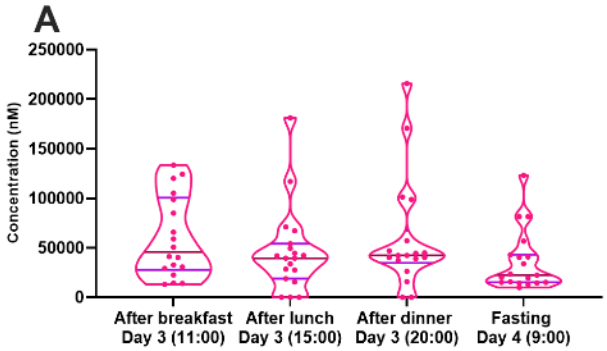

Figure 13. 10 Variations of BAs throughout the day within diet 4. Differences between the same time points for different diets were determined by Skillings-Mack test and subsequent Wilcoxon signed-rank tests corrected by Hommel's method Significance was accepted at P_{adj} <0.05. Values are shown in median and interquartile range; n = 19. Key: (A) Total BAs.

Appendix 14. Publications, conference presentations, awards, dissemination and talks

Publications related with PhD research

- **Brignardello J**, Fountana S, Garcia-Perez I, Posma M J, et al. 2021. "Characterization of diet-dependent temporal changes in circulating short-chain fatty acid concentrations: A randomized crossover dietary trial". Am J Clin Nutr. 2022. Nov; 116(5): 1368–1378. PMID: 36137188 Link here.
- Calder N, Walsh K, Olupot-Olupot P, Ssenyondo T, Muhindo R, Mpoya R, **Brignardello J,** et al."Modifying gut integrity and microbiome in children with severe acute malnutrition using legume-based feeds (MIMBLE): A pilot trial" . Cell Rep Med . 2021 May 18;2(5):100280. PMID: 34095882 Link here.
- Byrne C, Chambers E, **Brignardello J,** et al., 2019, "Effects of inulin propionate ester incorporated into palatable food products on appetite and resting energy expenditure: a randomised crossover study", Nutrients, Vol:11 . PMID: 30995824 Link here.
- Byrne C, Preston T, **Brignardello J,** et al., 2018, "The effect of L-rhamnose on gastrointestinal transit rates, short chain fatty acids and appetite regulation". Proc Nutr Soc. 77(OCE4) . Link here
- McDonald JAK, Mullish BH, Pechlivanis A, **Brignardello J,** et al., 2018, ¨Inhibiting growth of clostridium difficile by restoring valerate, produced by the intestinal microbiota¨, Gastroenterology, Vol:155, ISSN:0016-5085, Pages:1495-1507.e15 . PMID: 30025704 Link here.
- Byrne C, Preston T, **Brignardello J,** et al., 2018, "The effect of L-rhamnose on intestinal transit time, short chain fatty acids and appetite regulation: a pilot human study using combined 13CO2 / H2 breath tests", Journal of Breath Research, Vol:12, ISSN:1752-7155. PMID: 30015629 Link here.

Book Chapter

- **Brignardello J,** Holmes E, Garcia-Perez I."Metabolic Phenotyping of Diet and Dietary Intake". Adv Food Nutr Res. 2017;81:231-270 (Book Chapter) . PMID: 28317606 Link here .

Conference Presentations

- Gonzalez G, **Brignardello J,** Laponogov I, Chio D, Reyna-Jeldes M, Rita L, Qiu J, Gallota M, Wang Y, Zhang E, Zhong Y, Wang X, Bronstein M , Veselkov K. Uncovering New Food-Drug Interactions Using a Novel Graph Deep Learning Model. Nugo Week 2023. Senegallia, Italy. (Poster Presentation).
- **Brignardello J**, Fountana S, Garcia-Perez I, Posma M J, et al "Characterization of diet-dependent temporal changes in circulating short chain fatty acid concentrations: A randomized crossover dietary trial" European Nutrition Conference**,** FENS 2019.Dublin. Ireland. (Poster Presentation).
- **Brignardello J**, Fountana J, Posma JM, Frost G, Holmes E, Garcia-Perez I. Temporal changes of short chain fatty acids concentrations throughout the day: A randomized crossover dietary trial**.** Nutrition Futures Conference 2019. Nutrition Society, Nottingham, UK. (Oral Presentation).
- Segal J, Sarafian M, Pechlivanis A, Serrano-Contreras I, **Brignardello J**.et al. "Mucosal tissue short chain fatty acids contribute to prediction of pouchitis in restorative proctocolectomy" Gut 68(Suppl 1): A1.1-A1. DOI: DOI: 10.1136/gutjnl-2019-BSGAbstracts.177 .2019. (Poster Presentation).
- Segal J, Sarafian M, Serrano-Contreras I, Pechlivanis A, **Brignardello J**. et al. "Urinary formate and glycine are associated with treatment response in patients treated with antibiotics for pouchitis" Gut 68(Suppl 1):A9.2-A10.2019. (Poster Presentation).
- Segal J, Sarafian M, Pechlivanis A, Jose Serrano Contreras I, **Brignardello J**, et al., 2019, IDDF2019-ABS-0028 Mucosal tissue short chain fatty acids contribute to prediction of pouchitis in restorative proctocolectomy, International Digestive Disease Forum (IDDF) 2019, Hong Kong, 8–9 June 2019, Publisher: BMJ Publishing Group Ltd and British Society of Gastroenterology. (Poster Presentation).
- Byrne C, Preston T, **Brignardello J,** et al., 2018, "The effect of L-rhamnose on gastrointestinal transit rates, short chain fatty acids and appetite regulation". Proc Nutr Soc. 77(OCE4) (Oral Presentation).

Awards and Honours

2015 - 2021	Chilean Government "Becas Chile" Fellowship for postgraduate studies ($250K).
2018 - 2020	Young investigator award. Egg Nutrition Center- USA. "Discovery of potential egg biomarkers by the use of metabolic phenotyping techniques" Young investigator award for graduate students. Egg Nutrition Center- USA ($20K).
2019	Travel grant from Chilean Government "Becas Chile" for congress attendance ($2000).
2017	Highly Commended MPHrp Presentation Award at Eighth NIHR Infrastructure Doctoral Research Training Camp organised by National Institute for Health Research (NIHR) . Leeds- UK. Link here .
2016	Fellow at 2nd European Summer School on Nutrigenomics. Camerino University- Italy (€500).
2016	Global Health Fellow, Imperial College London- University Cape Town. Cape Town. South Africa.

Public Engagement

2024	Presentation for Chilean secondary students from the "Fembiobio" organization to empower Chilean girls in STEM, visiting Imperial College London.
2023	Cancer-busting Hyperfoods, Imperial Lates, Future Food. Activity for community engagement. Imperial College London.
2022	The digestive system. Science month celebration at "Early Years Nursery". Imperial College London. Link here
2020	Food and Coronavirus. Recommendations for Food during the Pandemic. Imperial College London Link here
2017-2018	"Gut Health Science Club". Activity about gut health for primary school students at Prime Rose Primary. London. This activity was designed in different 4 sessions. MRC funded. Link here.