Effects of pomegranate polyphenols on the gut microbiota-dependent production of proatherogenic trimethylamine from L-carnitine

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A thesis submitted to the University of East Anglia
for the Degree of Doctor of Philosophy

UEA registration number 100358892

September 2025

Abstract

Background: Cardiovascular disease (CVD) is the leading cause of mortality worldwide, with circulating trimethylamine N-oxide (TMAO) levels receiving increasing attention as a potential risk factor. TMAO is formed from trimethylamine (TMA), a microbial metabolite of dietary precursors including L-carnitine. Dietary polyphenols have been proposed to reduce TMAO production, but mechanistic evidence and pharmacokinetic studies investigating the effect of polyphenols on TMAO production are limited.

Objectives: The research described here aimed to determine whether a polyphenol-rich pomegranate extract can reduce microbial TMA production from L-carnitine and lower circulating TMAO concentrations, and to elucidate several possible underlying mechanisms involved.

Approaches: *In vitro* colon models were used to test the effects of a pomegranate extract on microbial L-carnitine metabolism. A two-phase, double-blind, randomised, placebo-controlled, crossover intervention study in 16 healthy adults investigated the effect of a pomegranate extract on plasma TMAO levels. Mechanistic experiments involved colony forming unit (CFU) counting, quantitative polymerase chain reaction (qPCR) assays, and a metagenomic analysis.

Results: The extract and its main polyphenol, punicalagin, substantially suppressed TMA production. Mechanistic experiments suggested that the effects were not due to general or selective antibacterial activity. The intervention study showed that a single 1.6 g dose of the pomegranate extract did not significantly reduce plasma TMAO production from an oral L-carnitine challenge in the overall study population. However, in male and premenopausal female participants, the extract modestly reduced TMAO production.

Conclusion: Together, these findings provide evidence that a pomegranate extract and punicalagin inhibit microbial TMA formation and may attenuate TMAO responses in specific population subgroups. These results support the broader concept that polyphenol-rich foods could represent a dietary strategy for reducing TMAO exposure and potentially CVD risk. Further work is required to clarify underlying mechanisms and determine the effects among different population groups through larger, longer-term intervention studies.

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Abbreviations

ADME	Absorption, distribution, metabolism, and excretion
B-ME	β-mercaptoethanol
CKD	Chronic kidney disease
C_{max}	Maximum concentration
CV	Coefficient of variation
CVD	Cardiovascular disease
DAD	Diode array detection
DMB	3,3-dimethyl-1-butanol
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
EDTA	Ethylenediamin tetra-acetic acid
eGFR	Estimated glomerular filtration rate
ESI	Electrospray ionisation
FC	Folin & Ciocalteu's phenol
FFQ	Food frequency questionnaire
GAE	Gallic acid equivalent
GFP	Green fluorescent protein
HCl	Hydrochloric acid
HFBA	Heptafluorobutyric acid
HHDP	Hexahydroxydiphenoyl
HPLC	High performance liquid chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IQR	Interquartile Range
LBA	Luria-Bertani agar
LC-MS/MS	Liquid chromatography-tandem mass-spectrometry
LDL	Low-density lipoproteins
MACE	Major adverse cardiovascular event
MCC	Microcrystalline cellulose

MCS	Multiple cloning site
MeOH	Methanol
MRM	Multiple reaction monitoring
m/z	Mass-to-charge ratio
NaOH	Sodium hydroxide
OCRT	Oral carnitine response test
PBS	Phosphate-buffered saline
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
ppm	Parts per million
PVP	Polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
Q-TOF-MS	Quadrupole time-of-flight mass spectrometry
RNase	Ribonuclease
rpm	Revolutions per minute
RRF	Relative response factor
RT	Retention time
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SCFA	Short-chain fatty acid
$t_{1/2}$	Half-life
TCA	Trichloroacetic acid
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
T_{max}	Time of maximum concentration
TOF-MS	Time-of-flight mass spectrometry
UM	Urolithin metabotype
UPLC	Ultra Performance Liquid Chromatography
USDA	United States Department of Agriculture
γ-BB	γ-butyrobetaine

List of Publications

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Haarhuis JE, Gamal El-Din M, Kroon PA. Data files supporting the manuscript 'Punicalagin is the Key Pomegranate Polyphenol Inhibiting Gut Microbial Trimethylamine (TMA) Production from L-Carnitine' [Data set]. In Food & Function (Version 1). *Zenodo*. 2025. doi: 10.5281/zenodo.17296135

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Haarhuis JE, Ahn-Jarvis J, Ferns C, Savva G, Quince C, James R, Hughes S, Triller S, Hayhoe A, Kroon P. Can a polyphenol-rich pomegranate extract reduce the risk of heart disease by changing the gut microbiome? Nutrition Workshop Symposium (**Kilifi, Kenya, April 2025**).

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Acknowledgements

Although it would be impossible to thank everyone who has supported me throughout this academic and personal journey, I would like to express my gratitude to several people in particular.

First and foremost, I would like to thank Dr Paul Kroon, my primary supervisor and an incredible mentor. Paul has taught me to think like a scientist, challenging me to question my data, design follow-up experiments, and ensure I truly understood the methods I was using. Thanks to him, I developed the most valuable research skills: critical thinking and problem-solving. His trust in me, and the freedom he gave me to take ownership of this project, has made me truly enjoy the PhD. On top of that, the countless conferences he encouraged me to attend made this PhD a very rewarding experience. Beyond the science, I greatly appreciated Paul's kindness and openness on a personal level. Thank you, Paul, I could not have had a better supervisor.

A sincere thank you also goes to Dr Priscilla Day-Walsh and Dr Emad Shehata for their invaluable support and friendship, which formed the foundation of this project. Priscilla first introduced me to the methodological and theoretical aspects of the project, and Emad continued to guide me after her departure. Both of them have been pillars of patience and mentorship.

To Dr Barbora Peck, Dr Olla Al-Jaibaji, Dr Mariam Gamal-El-Din, and Marianthi Firoglani-Moschi, thank you for your friendship, laughter, and emotional support during this journey. You showed me that one of the most valuable parts of a PhD is when your colleagues become your friends.

There are several people I would like to thank for making the TESSA study possible. Dr Jennifer Ahn-Jarvis was a guiding star. Her expertise and dedication were essential for the success of the ethics process and execution of the study. The Clinical Research Facility (CRF) team was vital in enabling the clinical side of the work, and I would especially like to thank Clare Ferns, Teresa Kieloch, and Judith Gowlett for coordinating the clinical aspects of the study, and Anna Ilgunas, Laura Staff, Csilla Dabasi, and Kinga Oroszlan for treating every participant with such kindness, patience, and professionalism. I am also grateful to Sarah Hughes and Silas Triller for their invaluable support during study visits, recruitment, and sample processing.

To all the participants of the TESSA study, thank you for your commitment, effort, and willingness to donate your time and samples. Without you, this study would not have been possible. I am also thankful to the participants in the QIB Colon Model Study, who donated samples for the *in vitro* colon models.

My thanks also go to Mark Philo, Dr Shikha Saha, and Dr Robert James for their most valuable technical support.

I am grateful to the Wellcome Trust and the Biotechnology and Biological Science Research Council for their financial support.

Last, but certainly not least, I would like to thank my parents, Adam and Lucy, who have never held me back in my eagerness to study abroad, even when that meant being far away from them.

Contributions

The work presented in this thesis is original research by the author. Pieces of work described in this thesis were collaborative. Significant contributions were made by other researchers and are summarised below.

Chapter 2 describes the use of the *in vitro* batch colon model and the quantification of L-carnitine and its metabolites using LC-MS/MS. The LC-MS/MS method was established by Dr Priscilla Day-Walsh, Dr Emad Shehata, and Dr Shikha Saha [1]. Part of the experimental work described in Chapter 2 was carried out by Dr Priscilla Day-Walsh and Dr Emad Shehata, in which case their contributions are indicated in the figure legend. Part of the statistical analyses were carried out in collaboration with Dr George Savva, which is indicated in the relevant figure legends.

Chapter 3 describes the characterisation of the pomegranate extract using LC-MS/MS and HLPC-DAD-MS. The characterisation by LC-MS/MS was carried out in collaboration with Mark Philo and the characterisation by HPLC-DAD-MS was in collaboration with Professor Francisco Tomás-Barberán (CEBAS-CSIC, Murcia, Spain) according to a published method [2]. The experimental colon model work described in Chapter 3 was carried with support from Dr Mariam Gamal-El-Din and Kristel-June Sartagoda (PhD rotation student). Chapter 3 also describes a flow cytometry experiment which was conducted with support from Dr Dimitra Lamprinaki.

Chapter 4 describes the development of a quantitative polymerase chain reaction (qPCR) standard and primers and the optimisation of a qPCR assay, which were carried out with support from Dr Robert James. This chapter also describes a metagenomic analysis of colon model samples, for which Dr Robert James prepared the sample libraries and Dr Sébastien Raguideau (Earlham Institute) performed the co-assembly of the data. Final analysis of the assembled data was carried out by the author.

Chapter 5 describes the development and results of the TESSA study. The author, Dr Priscilla Day-Walsh, Dr Jennifer Ahn-Jarvis, and Dr Paul Kroon conceptualised the study, including the writing and submission of the ethics protocol to HRA REC. The author, Dr Jennifer Ahn-Jarvis and Dr Paul Kroon organised the trial. The recruitment process was managed by the author and Sarah Hughes. Study talks and obtaining of informed consents were carried out by the author and Sarah Hughes. Participant study visits were managed by the author, Dr Jennifer Ahn-Jarvis, Sarah Hughes, and the Clinical Research

Facility (CRF) team. All clinical procedures were carried out by the CRF team and were coordinated by Clare Ferns. Sample (blood, urine, stool) processing on study days was carried out by the author, Dr Jennifer Ahn-Jarvis, Sarah Hughes, Silas Triller, Dr Olla Al-Jaibaji, and Emrah Acaroz. Encapsulation of the pomegranate extract and placebo material was carried out by Dr Jennifer Ahn-Jarvis. Randomisation was carried out by Dr George Savva and blinding by Dr Natalia Perez. All the sample analyses and the data collection were carried out by the author. The data collection of polyphenol metabolites in the urine was supported by Mark Philo.

Chapter 1

General Introduction

Chapter 1: General introduction

1.1 Scope of this thesis

Some evidence suggests that dietary polyphenols can inhibit gut microbial trimethylamine (TMA) production from dietary L-carnitine and choline and subsequently reduce proatherogenic trimethylamine N-oxide (TMAO) levels. However, the body of evidence is limited to only a few polyphenols and has largely focused on the inhibition of baseline TMAO levels rather than the direct inhibition of a TMAO response from a precursor such as L-carnitine. Moreover, an understanding of the mechanism via which polyphenols can reduce TMA and TMAO production is currently lacking. Hence, the research presented in this thesis focuses on the effects of a polyphenol-rich pomegranate extract, which is high in ellagitannins and ellagic acid, on microbial L-carnitine metabolism in the colon. Several possible underlying mechanisms are also investigated. Additionally, this thesis reports the effect of the pomegranate extract on the inhibition of TMAO production from L-carnitine in healthy adults.

1.2 Structure of this thesis

This thesis consists of six chapters. This chapter (**Chapter 1**) provides a general introduction to cardiovascular disease (CVD), the role of gut microbiota in CVD risk, the production of TMAO from L-carnitine, and the potential of dietary polyphenols to modulate TMAO-producing pathways. **Chapter 2** describes *in vitro* batch colon model experiments testing the effects of a polyphenol-rich pomegranate extract on the microbial metabolism of L-carnitine. In **Chapter 3** it is reported which pomegranate polyphenols are responsible for the observed effects of the pomegranate extract on microbial L-carnitine metabolism to TMA. **Chapter 4** provides mechanistic insights into the observed effects of the pomegranate extract and its polyphenols on TMA production from L-carnitine. **Chapter 5** describes how the *in vitro* findings can be translated into a healthy study population, which was investigated using a double-blind, randomised, placebo-controlled, crossover study to estimate the effect of the pomegranate extract on blood plasma TMAO after an L-carnitine dose, measured over 48 hours. Finally, **Chapter 6** summarises the main findings and discusses the impact and prospect of the research.

1.3 CVD and atherosclerosis

CVD is a leading cause of death worldwide, affecting approximately one-third of the global population [3]. In 27 European Union (EU) countries, 62 million people today live with CVD and 4,600 people die from CVD each day [3]. The European Society of Cardiology (ESC) stated that CVD is the most common cause of death in the ESC member countries (55 countries) with over 3 million deaths per year [4]. In addition to the health burden, CVD results in a substantial economic burden. CVD-associated health and social care costs across countries in the EU accounted for €155 billion in 2021; 11% of the total EU healthcare expenditure [4]. Adding costs of formal care (i.e., opportunity cost of unpaid care) and costs related to CVD morbidity and mortality losses (e.g., lost productivity and workforce participation) results in a total estimate of €282 billion in 2021 and 20% of the total EU healthcare expenditure [3].

The primary cause of CVD is atherosclerosis [5]. Atherosclerosis is the accumulation of lipids and fibrous compounds in combination with calcification within the arteries [6]. Atherosclerosis develops within the arterial wall, where the vascular endothelium plays a central role. The endothelium lines all blood vessels and forms the first barrier between circulating blood and vascular tissues. In large arteries, the vessel wall consists of three layers: the intima (endothelium with supporting connective elements), the tunica media (vascular smooth muscle cells and elastic tissue), and the tunica adventitia (dense connective tissue). The endothelium detects mechanical forces such as wall shear stress, as well as changes in metabolic factors, and converts these signals into biochemical responses that can affect vascular permeability, contraction, and inflammation [6]. Atherosclerosis starts when the endothelium becomes dysfunctional (**Figure 1.1**). First, low-density lipoproteins (LDL) accumulate in the intima, where the LDL undergoes chemical changes such as oxidation [5, 7]. These modified forms of LDL are recognised as “non-self” and therefore stimulate endothelial cells to recruit circulating monocytes into the vessel wall [6, 7]. The released monocytes become macrophages that take up the chemically changed LDL, transforming the macrophages into foam cells [6]. The build-up of foam cells, along with the activation of inflammatory signalling pathways, can form fatty streaks in the arteries (**Figure 1.1**) [6], which is one of the earliest visible signs of atherosclerosis. Eventually, fatty streak formation can lead to the development of atherosclerotic plaque [6]. During the development of plaque, the atherosclerotic

necrotic core, a cell-free and lipid-rich area, is covered by fibres [6]. This is called the fibrous cap and represents a hallmark of advanced atherosclerosis.

Atherosclerosis can lead to thrombosis when an atherosclerotic plaque becomes unstable and its fibrous cap ruptures [5]. This rupture releases the plaque contents into the circulating blood. When these pro-thrombotic substances come in contact with blood coagulation components, it results in blood clot formation (i.e., thrombus) [5, 8]. The thrombus can obstruct blood flow, causing an infarction. Gradual narrowing of arteries due to plaque growth contributes to CVD, but the major CVD risk arises from atherothrombosis triggered by the rupture of atherosclerotic plaque [5].

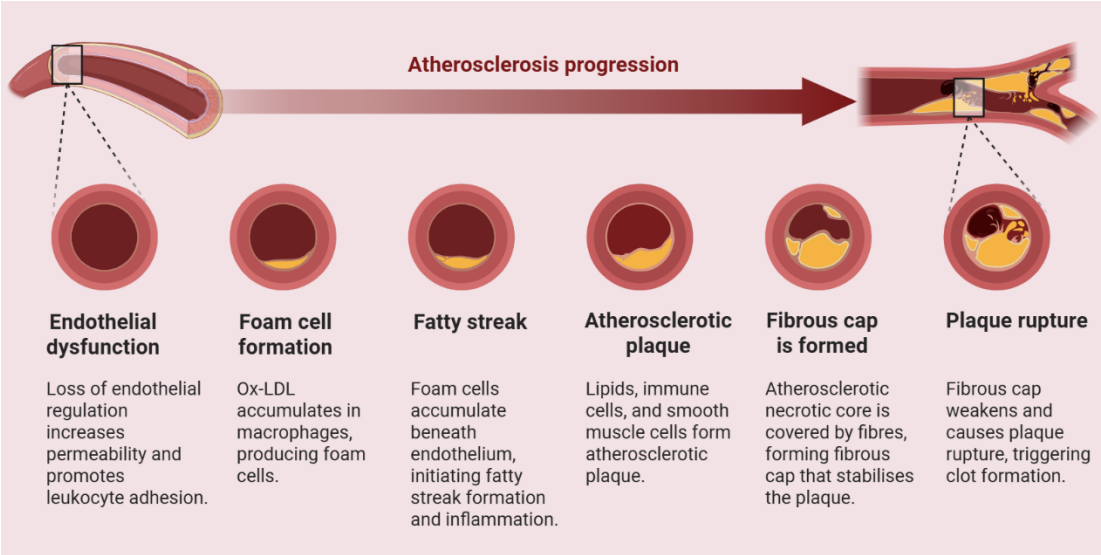

Figure 1.1. Progression of atherosclerosis from endothelial dysfunction to plaque rupture. *Ox-LDL, oxidised low-density lipoproteins. The figure was created using BioRender.com.*

1.4 Diet is a major risk factor for CVD

CVD risk factors include family history of CVD, older age, male sex, and south Asian, Black African or African Caribbean ethnicity [4, 9]. These risk factors are considered non-modifiable risk factors. However, CVD has several modifiable risk factors, including unhealthy dietary habits, alcohol consumption, use of tobacco products, and physical inactivity [4]. Since these are modifiable, they can be targeted to reduce CVD risk. Among these, dietary habits are particularly important, with large-scale analyses showing that poor diet contributes to approximately 45.4% of all cardiometabolic deaths

in the United States [10]. Globally, CVD was found to be the leading cause of diet-related deaths, resulting in an estimated 10 million CVD deaths per year due to poor diet [11]. This makes dietary modification one of the most powerful strategies for CVD prevention.

The 2019 EAT-*Lancet* Commission report described a meta-analysis of prospective studies showing that the consumption of red meat (e.g., beef, pork, lamb), particularly processed red meat, was associated with an increased CVD mortality risk [12]. This is further supported by a large meta-analysis of 13 cohort studies (encompassing 1,674,272 individuals), showing that individuals in the highest category of processed meat consumption and red meat consumption have, respectively, an 18% and 16% higher risk of CVD mortality [13]. A recent, prospective cohort study also showed that the highest category for red meat intake (≥2.0 servings/day) among 148,506 adults was associated with an 18% increased risk of CVD incidence [14]. A cohort study among 29,682 adults in the United States showed that the intake of processed meat, red meat, and poultry was significantly associated with the incidence of CVD [15]. These findings were further confirmed by a meta-analysis of 43 observational studies (comprising of 4,462,810 individuals), investigating the associations between red and processed meat intake and CVD risk, which showed that red meat and processed meat consumption were, respectively, associated with an 11% and 26% greater risk of CVD [16]. On the other hand, fish and dairy intake were associated with a reduced risk of CVD [17, 18]. Similarly, plant-based foods such as nuts, fruits, and vegetables were also found to reduce CVD risk [12]. These studies show that specific dietary patterns are associated disease risk.

1.5 The gut microbiome is a potential intermediate between the diet and CVD risk

The diet directly impacts the gut microbiome; the collection of all microbial genomes in the gastrointestinal tract comprising of about three million genes, over 100-fold more than the number of human genes [19]. An individual's gut microbiome contains roughly 10 to 100 trillion microbial cells [20], which are mostly bacteria [21], but also include viruses, fungi, parasites, and archaea [21, 22]. The diet can impact the gut microbiome by shaping its composition and function through the availability of nutrients such as fibre, fat, and protein [23]. The diet influences which microbes thrive and determines the

production of metabolites, including short-chain fatty acids (SCFAs) from fibre and secondary bile acids from fat [19]. Via microbial metabolites, the gut microbiome is involved in key biological functions of the (human) host, including metabolism and the immune system [6, 22], and also aids maintaining gut barrier integrity [19].

Growing evidence suggests that the gut microbiome influences CVD risk [20, 24, 25]. Several microbial metabolites have been linked to increased CVD risk, including phenylacetylglutamine [26], imidazole propionate [27], and TMA [28, 29]. These metabolites are produced by gut microbiota from nutrients present in our diets. For instance, phenylacetylglutamine is produced from the amino acid phenylalanine, present in protein-rich foods such as meat, fish, eggs, dairy, nuts, and seeds [30], and is detectable in human blood plasma [26]. Imidazole propionate is derived from dietary histidine, also abundant in protein-rich foods [31], and plasma levels were increased in subjects with subclinical atherosclerosis compared with controls [27]. However, the most extensively studied microbially-derived metabolite in relation to CVD is TMA and its oxidised form, TMAO [6]. TMA is produced by the gut microbiota from dietary L-carnitine, choline, phosphatidylcholine (lecithin), and betaine, which are predominantly found in animal-based foods such as meat, fish, eggs, and dairy, although betaine is mainly found in plant-based foods including spinach, beets, and grains [32].

Gut dysbiosis has also been associated with CVD [20, 33]. Dysbiosis is defined as a disruption of the gut microbial balance, often characterised by reduced diversity, loss of beneficial microbiota, or the increase in potentially harmful microbes [34]. Importantly, dysbiosis frequently coincides with elevated TMAO levels; patients with pre-eclampsia exhibited both dysbiosis and increased plasma TMAO, together with an increase in the pathogen-associated [35] phylum *Proteobacteria*, lower alpha diversity, and elevated lipopolysaccharides (LPSs), compared with healthy controls [36]. Similarly, individuals with chronic kidney disease (CKD) exhibited dysbiosis marked by reduced alpha diversity, increased *Proteobacteria*, and higher TMAO levels [37]. These findings suggest that dysbiosis may contribute simultaneously to elevated TMAO and increased CVD risk.

To summarise, diets high in red meat are strongly associated with greater CVD mortality risk [12-16]. Red meat is a major source of the TMAO precursor L-carnitine [32, 38]. Although red meat also contains small amounts of the precursors of imidazole propionate and phenylacetylglutamine (histidine and phenylalanine), TMAO remains the most extensively studied microbial metabolite in relation to CVD [6]. Hence, the research

presented in this thesis focuses on TMAO as a promising dietary target to reduce CVD risk.

1.6 The role of TMAO in CVD development

1.6.1 Epidemiological evidence

An untargeted metabolomics analysis in 2011 was the first to establish a connection between TMAO and CVD [28]. Subsequent research has demonstrated that higher circulating TMAO levels are associated with a greater risk of CVD and increased all-cause mortality. The combined data from 36 observational studies showed that high levels of TMAO were associated with a 74% greater risk of a major adverse cardiovascular event (MACE) and data from 12 studies showed an association with a 50% greater risk of CVD [39]. From observations in patients with a history of coronary artery disease, it appeared that TMAO plasma levels above 6.18 µM were associated with a 88% greater risk of MACE within three years of follow-up compared with subjects who had plasma TMAO levels below 2.43 µM, when adjusted for traditional risk factors [40].

A prospective cohort study of 6,767 U.S. adults from different ethnicities (38% white, 28% black, 22% Hispanic, and 12% Chinese) found that plasma TMAO levels were dose-dependently associated with an increased risk of atherosclerotic CVD [41]. Participants were free of CVD at baseline and over a median follow-up of 11.3 years 852 incident atherosclerotic CVD events were recorded. Time-varying Cox proportional hazards models, adjusted for demographics, lifestyle factors, medical history, lipid measures, antibiotic use, and dietary habits, showed that CVD risk increased across TMAO quintiles. Compared with the lowest quintile (1.75 µM), hazard ratios increased by 2% at 2.61 µM, 17% at 3.56 µM, 23% at 4.98 µM, and 33% at 9.20 µM (*P*-trend < 0.01) [41], indicating a dose-dependent association between TMAO levels and incident atherosclerotic CVD.

In a human intervention study, healthy participants received a daily dose of choline for two months [42]. After one month of the intervention, there was a greater than 10-fold increase in fasting plasma TMAO levels, which persisted around the same level for the remainder of the intervention. Additionally, there was an increase in adenosine diphosphate-induced platelet aggravation, which significantly correlated to the increase in plasma TMAO levels [42].

1.6.2 Mechanistic studies

The observations from epidemiological data are substantiated by mechanistic studies. Several mechanistic pathways are at play that contribute to atherosclerosis and CVD (**Figure 1.2**) [43]. First, TMAO promotes the formation of plaque by stimulating the development of macrophages into foam cells inside the arterial intima [28, 29]. This happens as TMAO reduces the levels of high-density lipoproteins (HDLs), resulting in the inhibition of the reverse cholesterol transport (RCT), which is an important process for the transport of cholesterol to the liver and its secretion as bile acids [43]. Subsequently, cholesterol starts to accumulate in the macrophages. At the same time, TMAO increases the expression of scavenger receptor cluster of differentiation 36 (CD36) and scavenger receptor-A1 (SR-A1) [44, 45], leading to an increased flow of cholesterol to the macrophages. Consequently, high TMAO levels stimulate the formation of foam cells [43].

Second, TMAO contributes to endothelial dysfunction and vascular inflammation. In mice, a choline-rich diet leading to elevated TMAO levels was associated with dyslipidaemia, hyperglycaemia, and vascular endothelial damage [46]. Clinical studies have further demonstrated that high plasma TMAO correlates with impaired endothelial function and vascular inflammation [47]. Mechanistic studies showed that TMAO promotes inflammation by activating the nuclear factor-kappa B (NF-κB) pathway, which leads to the increase of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [48]. When the NF-κB pathway gets activated in endothelial cells, there is a subsequent increased expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) [43]. The vascular inflammation together with the increased expression of adhesion molecules increases the adhesion of macrophages to vascular cells [43].

Third, TMAO contributes to plaque instability [49], which can lead to a rupture of the atherosclerotic plaque [5]. Clinical studies showed that elevated plasma TMAO levels are strongly associated with plaque instability. For instance, in patients with coronary artery disease, elevated TMAO levels were found in those with ruptured plaques compared with those without rupture [50].

Finally, TMAO has been shown to enhance platelet hyperreactivity and independently predict a higher risk of thrombosis [51]. Its levels are dose-dependently associated with

platelet activation [42], partly by promoting thrombin-induced intracellular calcium in platelets, a key trigger of platelet aggregation [51]. Elevated TMAO also increases platelet adhesion to collagen and responsiveness to agonists (e.g., thrombin or collagen), thereby accelerating thrombus formation [51].

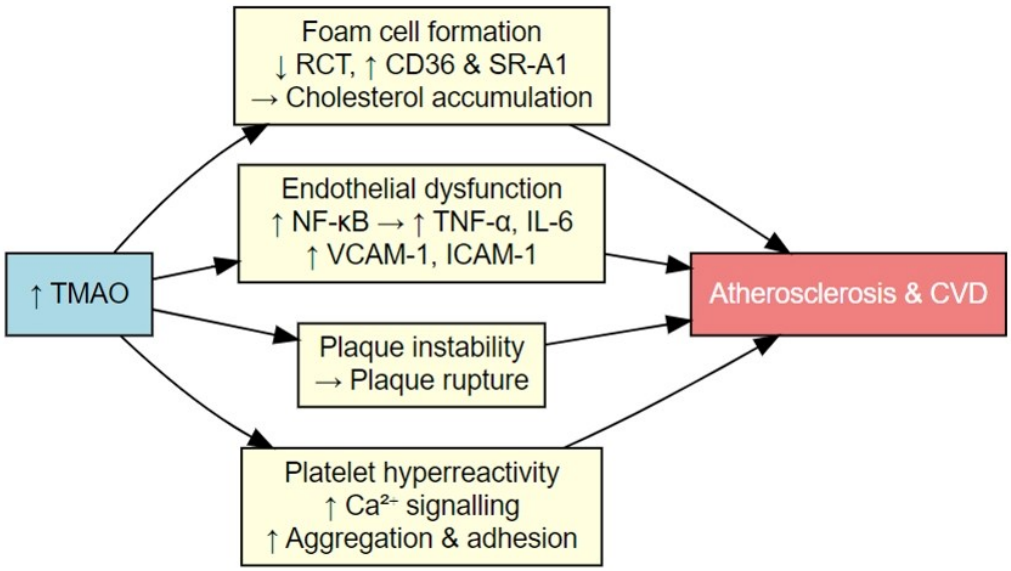

Figure 1.2. Proposed mechanistic pathways by which trimethylamine N-oxide (TMAO) promotes atherosclerosis and cardiovascular disease (CVD). *High plasma TMAO levels contribute to atherosclerosis via multiple pathways: (i) stimulation of macrophage scavenger receptors (CD36, SR-A1) and inhibition of reverse cholesterol transport (RCT), leading to foam cell formation and cholesterol accumulation; (ii) activation of NF-κB signalling pathway in endothelial cells, increasing pro-inflammatory cytokines (TNF-α, IL-6) and adhesion molecules (VCAM-1, ICAM-1), thereby promoting endothelial dysfunction and vascular inflammation; (iii) contribution to plaque instability, increasing the risk of plaque rupture; and (iv) increase of platelet calcium signalling, adhesion, and aggregation, resulting in thrombus formation. Collectively, these mechanisms accelerate the development and progression of atherosclerosis and CVD. Created in RStudio with 'DiagrammeR' [52].*

1.6.3 The controversial role of TMAO in CVD

It should also be noted that controversy exists for the role of TMAO in CVD, with some evidence showing that TMAO has a protective role in CVD. For instance, a report indicated that high TMAO levels were inversely correlated with the development of atherosclerosis in mice [53]. Several studies suggest that TMAO counteracts oxidative stress; oral L-carnitine supplementation increased plasma TMA and TMAO levels in 31 haemodialysis patients while reducing circulating markers of vascular injury and oxidative stress [54]. Similarly, TMAO administration mitigated oxidative stress in diabetic rats [55]. A proposed explanation is that TMAO may serve as an electron acceptor, thereby neutralising electron leakage from the mitochondrial transport chain and limiting the generation of reactive oxygen species [56]. Furthermore, TMAO has a well-established role in maintaining cellular homeostasis across species, where it functions to stabilise cell volume under osmotic and hydrostatic pressure stress [56].

However, the body of evidence suggesting that TMAO increases CVD risk is backed by large prospective cohort studies and is substantially more comprehensive than the evidence indicating that TMAO has a protective role in CVD. A study in patients with pulmonary embolism demonstrated a U-shaped association between TMAO levels and mortality, with the lowest risk observed around 4 µM [57]. They showed that TMAO levels above and below the optimal ~4 µM concentration were linked to increased mortality. This finding is broadly in line with findings in 4,007 patients with coronary artery disease, where the median plasma TMAO of those who developed MACE within three years of follow-up was 5 µM, compared with 3.5 µM in those who did not [40]. Furthermore, in a large, prospective, multi-ethnic cohort, individuals with plasma TMAO levels of 4.98 µM and above significantly increasing the risk of atherosclerotic cardiovascular disease compared to individuals with plasma TMAO levels of 1.75 µM, while the risk was not significantly increased with TMAO levels of 2.61 and 3.56 µM [41]. Taken together, these findings tentatively suggest that moderate levels of TMAO may be physiologically advantageous, whereas excessive concentrations could increase the risk of CVD. Thus, the goal may not be to eliminate TMAO entirely, but to maintain it within an optimal physiological range.

1.7 TMAO formation from L-carnitine

1.7.1 L-Carnitine is a major contributor to TMAO formation

The thesis focuses on TMA and TMAO production from L-carnitine. Although TMAO can be produced from other nutrients, such as (phosphatidyl)choline, a substantial amount of choline is absorbed in the small intestine (>90%) [58] and therefore a large proportion does not undergo microbial metabolism to TMA. The bioavailability of L-carnitine, on the other hand, ranges between 54-87% from the diet [59] and 14-18% from supplements [60]. The lower bioavailability from supplements is due to the absorption at higher supplement doses relying mainly on passive transport (i.e., diffusion) across enterocyte membranes, whereas L-carnitine from the diet involves both active (i.e., via specialised transport proteins in the enterocytes) and passive transport [60]. As a result, a substantial proportion of the ingested L-carnitine transits through the gastrointestinal tract and reaches the colon where it can be metabolised by microbiota to TMA. This makes L-carnitine a significant contributor to subsequent TMAO levels in omnivores [26]. To illustrate, a human study investigating the effects of a red meat-diet, white meat-diet and non-meat diet on TMAO levels, showed that plasma TMAO levels in participants who consumed the red meat-diet for one month were significantly higher, which was predominantly attributed to the high L-carnitine content of red meat [61].

1.7.2 L-Carnitine is essential for the human body

L-Carnitine has essential functions in the human body, with its primary function being the facilitation of long-chain fatty acid transport across the mitochondrial membrane for β-oxidation [38]. Additionally, L-carnitine plays a role in the maintenance of the CoA/acyl-CoA ratio in the mitochondria, the utilisation of ketone bodies, and the β-oxidation of fatty acids in peroxisomes (i.e., membrane-bound organelles in cells responsible for metabolic functions such as lipid metabolism, bile acid synthesis, and the detoxification of reactive oxygen species (ROS)) [38]. Many additional functions have been attributed to L-carnitine [62]. However, L-carnitine is not an essential nutrient as it can be produced endogenously from two essential amino acids: lysine and methionine [63]. Lysine is methylated to form N-trimethyllysine (TML), which is then converted

through several enzymatic steps into γ-butyrobetaine (γ-BB) [64]. The final step, catalysed by γ-butyrobetaine hydroxylase, produces L-carnitine [64]. L-Carnitine synthesis occurs mainly in the liver, with small amounts being synthesised in the kidneys and brain [59, 63]. Cardiac and skeletal muscle cells, however, are unable to synthesise L-carnitine despite their high demand, and therefore rely on the uptake from the systemic circulation [59]. L-Carnitine is not catabolised within human cells but is either utilised or excreted via the kidneys, with a substantial amount being reabsorbed to maintain homeostasis [65]. Unabsorbed L-carnitine in the gut is converted by gut microbiota into γ-BB and TMA, with bacterial metabolism increasing at higher L-carnitine intake as a larger proportion reaches the colon [63, 66].

1.7.3 The microbial metabolism of L-carnitine to TMA

When L-carnitine reaches the gut microbiota in the gastrointestinal tract, it is first metabolised to γ-BB and finally to TMA [67]. Microbially-produced TMA is efficiently absorbed into the blood and reaches the liver where TMA is oxidised to TMAO by enzymes called flavin-containing monooxygenases (FMOs) (**Figure 1.3**), primarily through the action of FMO3, while FMO1 plays only a minor role due to its low expression in hepatic tissue in adults [49]. Once TMAO is produced, it is circulated and eventually excreted in the urine. An additional pathway is the conversion of L-carnitine → *trans*-crotonobetaine → γ-BB → TMA and L-carnitine → *trans*-crotonobetaine → TMA [68]. However, the production of trans-crotonobetaine was slower and significantly less than the production of γ-BB.

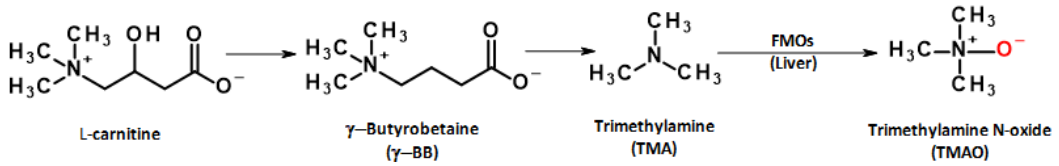

Figure 1.3. Simplified diagram of L-carnitine metabolism to trimethylamine N-oxide (TMAO). *L-Carnitine is first metabolised to its intermediate, γ-butyrobetaine (γ-BB), which is then converted to trimethylamine (TMA). In the liver, TMA is being metabolized to TMAO by flavin-dependent monooxygenases (FMOs), and TMAO has been reported to be associated with incidence of various cardiometabolic and other diseases and to cause thrombosis and vascular inflammation. Adapted from Haarhuis & Day-Walsh et al. (2025). Mol Nutr Food Res [69].*

Microbial enzymes responsible for the metabolism of L-carnitine to TMA under aerobic conditions are Riekse-type *CntA/B* enzymes carried by microbes. However, the colon is mainly anaerobic, so a more likely pathway of L-carnitine metabolism is the metabolism of L-carnitine to γ-BB by microbes carrying the *caiTABCDE* genes [70]. Common microbes containing the *caiTABCDE* genes include *Escherichia coli*, *Proteus mirabilis*, and several members of the *Citrobacter* genus. A different set of microbial genes is responsible for the second step of the metabolic pathway, converting γ-BB to TMA [70]. This set of genes belongs to the *gbuABCDEF* gene cluster (also referred to as *bbu*), which has only been defined in a small number of human gut microbes, including *Emergencia timonensis* and *Ihubacter massiliensis* [67, 71, 72]. Anaerobic metabolism of L-carnitine by microbes containing the *caiTABCDE* genes results in γ-BB production, but TMA was only produced in the presence of *E. timonensis*. Moreover, *E. timonensis* alone could not metabolise the first step, converting L-carnitine to γ-BB [70]. A later study confirmed this finding [72].

The presence of *E. timonensis* in humans who produced high levels of TMAO was confirmed by a human trial that profiled the gut microbiome of participants who underwent an L-carnitine challenge [71]. Of the high-TMAO producers, 25.5% presented *E. timonensis* in their microbiome, while none of the low TMAO producers had this microbe [71]. Moreover, this study also detected that *Ihubacter massiliensis* is likely to play a role in the metabolism of γ-BB to TMA. They identified a synergistic effect of *E. timonensis* together with *I. massiliensis* on plasma TMAO levels [71].

1.8 Treatments to reduce TMA and TMAO formation

One straightforward approach to lowering circulating TMAO would be to reduce the intake of its dietary precursors. Yet, restricting precursors such as choline and L-carnitine might not be beneficial. For instance, although choline is produced endogenously, the endogenous production is not sufficient to meet total requirements, especially during periods of high demand (e.g., pregnancy, lactation, and growth) [73]. Choline deficiency can lead to liver and muscle tissue damage and even result in non-alcoholic fatty liver disease [74]. Similarly, several population groups benefit from L-carnitine supplementation, such as neonates, patients on long-term parenteral nutrition or dialysis, and individuals with certain genetic disorders affecting L-carnitine metabolism [64, 75, 76]. For this reason, targeting the formation of TMAO rather than limiting its

precursors is preferred. This can be achieved either by blocking microbial production of TMA in the gut or by preventing the hepatic conversion of TMA to TMAO via FMOs. To date, most studies have focused on inhibiting the microbial production of TMA.

1.8.1 Pharmaceutical interventions to reduce TMA and TMAO formation

Several pharmaceutical interventions to reduce the microbial production of TMA, and subsequently TMAO, have been suggested by Dr Stanley Hazen's group, which has been instrumental in the understanding of L-carnitine and choline metabolism to TMA and the atherosclerotic risk of TMAO [28, 40, 77, 78]. Their suggested interventions include broad-spectrum antibiotics [78] and the choline analogue 3,3-dimethyl-1-butanol (DMB) [40]. Broad-spectrum antibiotics almost completely inhibited the formation of TMAO from the precursor phosphatidylcholine in 40 healthy adults who underwent an oral phosphatidylcholine challenge [78], confirming that the formation of TMAO is entirely microbiota-driven. However, in a rodent model the effectiveness of the prolonged administration of broad-spectrum antibiotics on TMAO formation wears off after 6 months [78], which is likely due to the emergence of antibiotic-resistant gut microbiota. Regarding DMB, this choline analogue inhibits TMA lyase enzymes, resulting in the inhibition of TMA formation *in vitro* using batch colon models and the reduction of plasma TMAO levels *in vivo* in mice [78, 79]. Yet, its TMAO reducing effects could not be replicated in later studies [1, 80].

Another research group suggested the use of meldonium, an anti-ischemic and anti-atherosclerotic drug with an L-carnitine-lowering activity, to reduce TMAO levels [81]. It was shown in eight healthy adults that treatment with meldonium, in combination with a TMA-rich diet, increased the urine excretion of TMAO while reducing plasma TMAO levels compared to the TMA-rich diet alone [81]. However, meldonium decreases L-carnitine absorption in the small intestine by blocking its uptake via the inhibition of OCTN2 transporters [82], which can have detrimental effects on the essential functions of L-carnitine. The same research group also investigated the use of metformin, an oral medication described to treat type 2 diabetes mellitus, to reduce TMAO levels in db/db mice with type 2 diabetes [83]. This study showed that metformin decreased plasma TMAO levels in mice fed with the TMAO-precursor choline. However, the use of metformin has a high incidence of side effects, in particular gastrointestinal

side effects, which were reported in 20-30% of patients [84]. Generally, pharmaceutical interventions can have several side effects. Therefore, it is vital to explore low-risk treatments to reduce TMA and, subsequently, TMAO.

1.8.2 The use of dietary polyphenols to reduce TMA and TMAO formation

Dietary polyphenols have emerged as promising low-risk compounds for modulating gut microbial metabolism. Since their uptake in the small intestine is relatively limited [85], significant amounts reach the colon, where they can interact directly with the gut microbiota. Beyond this, polyphenols are known to exert antibacterial [86], anti-inflammatory [87, 88], and anti-oxidant [87] effects. Therefore, dietary polyphenols might be able to directly interact with gut microbiota involved in the L-carnitine metabolism to TMA. To date, the use of dietary polyphenols and polyphenol-rich extracts to reduce TMA and TMAO formation has been tested in a few studies (**Table 1.1**).

Evidence from *in vitro* fermentation models demonstrated that several polyphenol-rich juices, including pomegranate juice, orange juice, and grapefruit juice, can suppress TMA generation from L-carnitine or choline [89]. Additionally, several individual polyphenols have been shown to inhibit the microbial formation of TMA from L-carnitine, including a hydroxycinnamic acid (ferulic acid) and two flavonoids (cyanidin-3-glucoside, hesperidin) [89]. The TMA production from choline was inhibited by several other polyphenols: hydroxycinnamic acids (*p*-coumaric acid, caffeic acid, chlorogenic acid), flavonoids (catechin, epicatechin), benzoic acid derivatives (vanillic acid, gallic acid), phenylpropionic acids (3-(3,4-dihydroxyphenyl)propionic acid, 3-(3,4-dihydroxyphenyl) acetic acid), and phenylacetic acid (**Table 1.1**) [80, 90], of which chlorogenic and gallic acid were shown to reduce TMA production in a dose-dependent manner [90].

Chlorogenic acid was also found to reduce serum TMAO levels by respectively 19.6 and 44.4% in mice which received a daily chlorogenic acid dose of 200 or 400 mg per kg body weight for 12 weeks, compared to the control group [91]. A study in mice found that the daily supplementation with resveratrol (a stilbene) for 30 days also significantly reduced both plasma TMA and TMAO levels [92]. Another study in mice showed that the daily supplementation of 1% polymethoxyflavones (flavonoids) to drinking water for six weeks substantially reduced plasma TMAO [92]. Other studies showed a reduction in circulating

TMAO after the daily administration of a honeysuckle berry extract, Oolong tea, a black raspberry extract, a hickory nut extract, and blueberries in rodents for 6-12 weeks (**Table 1.1**) [93-97]. The study showing a reduction in plasma TMAO in mice after the daily consumption of blueberries for 12 weeks also reported that strawberries, on the other hand, were not effective in reducing plasma TMAO [96]. Although both blueberries and strawberries are rich in anthocyanins, blueberries but not strawberries contain large amounts of hydroxycinnamic acids, in particular chlorogenic acid [96]. This observation is consistent with other studies showing that chlorogenic acid reduced microbial TMA production *in vitro* [90] and serum TMAO in mice [91].

Some of the observations in animal studies were replicated in human studies. In two intervention studies, a crossover study and a two-arm parallel study, participants consumed a grape extract high in stilbenes, such as resveratrol, and flavonoids, such as flavan-3-ols [98, 99]. The daily consumption of 600 mg grape extract (32 mg total polyphenols) reduced serum TMAO levels by 63.6% after four weeks [98] and the daily consumption of 300 mg grape extract (16 mg total polyphenols) reduced serum TMAO levels by 78.6% after eight weeks [99]. Similarly, the daily consumption of 125 g (fermented) apple puree, providing a total of 47-62 mg polyphenols, resulted in reduced plasma TMAO levels by 63.1% after eight weeks [100]. Apples are also a rich source of chlorogenic acid, as well as flavonoids (flavan-3-ols, anthocyanins, flavonols) and dihydrochalcones [100]. Another study reported that the daily consumption of 30 g black raspberry extract, providing more than 2.2 g of polyphenols, significantly reduced plasma TMAO levels after four weeks [101]. However, a study investigating the effects of flavonoid-rich cocoa and green tea (180-900 mg total polyphenols) did not show a significant reduction in plasma TMAO after five days (**Table 1.1**) [102].

Collectively, these results suggest that polyphenols can modulate TMAO metabolism *in vivo*, although most evidence currently comes from animal studies, often with relatively small sample sizes. For human studies, there is a large variability in study design, participant health status, polyphenol dose, and type of polyphenols. This complicates the interpretation of these studies. Furthermore, while most of the studies in rodents included a TMAO-precursor such as L-carnitine or choline, none of the human studies tested the effect of the polyphenol-rich intervention in combination with a TMAO-precursor. Investigating the TMAO response to a precursor such as L-carnitine is crucial, as it assess the acute effects of polyphenols on microbially-dependent TMA production and subsequent TMAO formation.

Table 1.1. Summary of* in vitro*, animal, and human intervention studies that tested the effects of polyphenols and polyphenol-rich foods on TMA and TMAO production. *3HOBA, 3-hydroxybenzoic acid; 3HOPAA, 3-(3-hydroxyphenyl)acetic acid; 3HOPPA, 3-(3-hydroxyphenyl)propionic acid; 4HOPAA, 3-(4-hydroxyphenyl)acetic acid; 4HOPPA, 3-(4-hydroxyphenyl)propionic acid; Caf, caffeic acid; Cat, catechin; CGA, chlorogenic acid; Cit, citric acid; Cya, cyanidin-3-glucoside; DMB, 3,3-dimethyl-1-butanol; DiHOBA, 3,4-dihydroxybenzoic acid; DiHOPAA, 3-(3,4-dihydroxyphenyl)acetic acid; DiHOPPA, 3-(3,4-dihydroxyphenyl)propionic acid; EC, epicatechin; Fer, ferulic acid; Gal, gallic acid; Hes, hesperidin; Hip, hippuric acid; HVan, homovanillic acid; Nar, narirutin; PAA, phenylacetic acid; pCou, p-coumaric acid; PhVal, phenylvaleric acid; PMF, polymethoxyflavone; PPA, 3-phenylpropionic acid; Res, resveratrol; Van, vanillic acid.*

Study reference	Model/study population	Intervention	Results
***In vitro* studies**			
Bresciani *et al.* (2018) [89]	*In vitro* fermentation model, with 45% faecal inoculum and 100 µM L-carnitine or choline.	Coffee, black tea, red wine, pomegranate juice, orange juice, extra virgin olive oil, grapefruit juice, pink grapefruit juice, Cya, Hes, Fer, Nar, Cit, limonene, limonin (5%).	↓ TMA production after 24 h for orange juice, grapefruit juice, pomegranate juice from choline and L-carnitine. ↓ TMA production after 24 h for Fer, Cya, Hes, and Cit from L-carnitine.
Iglesias-Carres *et al.* (2021) [90]	*In vitro* fermentation model, with 20% faecal inoculum and 100 µM choline.	CGA or Gal (2, 5, 10 mM). DMB as positive control (10 mM).	↓ TMA production dose-dependently (5-10 mM) at 12 h for CGA and Gal.
Iglesias-Carres *et al.* (2022) [80]	*In vitro* fermentation model, with 20% faecal inoculum and 100 µM choline.	pCou, Caf, Fer, Cat, EC, Hip, PhVal, Van, HVan, DiHOPPA, 4HOPPA, 3HOPPA, PPA, DiHOPAA, 4HOPAA, 3HOPAA, PAA, DiHOBA, or 3HOBA (2 mM). DMB as positive control (10 mM).	↓ TMA production at 12 h for pCou, Caf, Cat, EC, Van, DiHOPPA, DiHOPAA, and PAA.
McAmis *et al.* (2025) [103]	*In vitro* fermentation model, with 2% faecal inoculum and 100 µM choline.	CGA (0.05-0.3 µM after fermentation), whole blueberries (~133 g), and sugar (~10 g, mixture of glucose, fructose, sucrose)	↓ TMA production throughout 28 hours with whole blueberries and sugar.

***In vivo* (animal) studies**			
Chen *et al.* (2016) [92]	Female C57BL/6J ApoE$^{-/-}$ mice (n = 10).	Chow diet with Res (0.4%) and choline (400 mg/kg body weight) for 30 days.	↓ TMA and TMAO plasma levels ($P < 0.01$).
Liu *et al.* (2018) [93]	Male Sprague-Dawley rats (n = 18).	Standard diet including 2% cholesterol, 0.5% cholic acid, and 8% lard with or without (control) honeysuckle berry extract (75, 150 or 300 mg/kg body weight/day) for 12 weeks.	↓ Serum TMAO ($P < 0.05$) by 46.1% in honeysuckle berry extract group (300 mg/kg body weight) compared with the control group at 12 weeks.
Chen *et al.* (2019) [94]	Female C57BL/6 (B6) mice (n = 30).	Five groups followed a diet for six weeks: (i) standard diet; (ii) 1.3% L-carnitine water; (iii) 1.3% L-carnitine water with 1% Oolong tea; (iv) 1.3% L-carnitine water with 1% PMFs; (v) 1.3% L-carnitine water with antibiotic cocktail.	↓ Plasma TMAO ($P < 0.05$) for Oolong tea, PMFs, and antibiotic group compared with the control (1.3% L-carnitine water) group.
Lim *et al.* (2020) [95]	Female Sprague-Dawley rats (n = 40).	Four groups followed a diet for 8 weeks: standard diet, high-fat diet, high-fat + 1.5% choline diet, high fat + choline + 0.6% black raspberry extract diet.	↓ Serum TMAO ($P < 0.05$) for the high-fat + choline + black raspberry diet compared with the high-fat + choline diet.
Zhang *et al.* (2021) [91]	Male Kunming mice (n = 32).	3% L-carnitine water for 12 weeks with or without (control) CGA (200 or 400 mg/kg body weight/day).	↓ Serum TMAO by 19.6% (200 mg/kg group, $P < 0.05$) and 44.4% (400 mg/kg group, $P < 0.01$) compared with control group after 12 weeks.
Babu *et al.* (2024) [96]	Male C57BL/6J mice (n = 8).	Standard diet containing 3.8% (w/w) freeze-dried blueberries or 2.4% (w/w) freeze-dried strawberries, versus standard diet control group, for 12 weeks.	↓ Plasma TMAO by 48% in the blueberry group compared with control ($P < 0.001$).
Jiang *et al.* (2024) [97]	Female C57BL/6 J mice (n = 18).	Six groups followed a diet for 12 weeks: (i) standard diet; (ii) high-fat diet with choline (HFD-c); (iii) HFD-c and pravastatin (100 mg/kg, positive control); (iv) HFD-c and low dose hickory nut polyphenol extract	↓ Serum TMAO level in HFD-c + high dose hickory nut extract group compared with HFD-c group ($P < 0.05$), with a dose-response trend on serum TMAO reduction as hickory nut extract

		(400 mg/kg); (v) HFD-c and moderate dose hickory nut extract (800 mg/kg); (vi) HFD-c and high dose hickory nut extract (1,200 mg/kg).	dose increased.
***In vivo* (human) studies**			
Suh *et al.* (2013) [101]	Two-arm parallel study (n = 38 healthy men).	30 g (2,221 mg total polyphenols) of freeze-dried Korean black raspberry sample versus placebo, daily for 4 weeks.	↓ Plasma TMAO ($P < 0.05$) between baseline and week 4 in black raspberry group.
Angiletta *et al.* (2018) [102]	Crossover study (n = 20 men and women with obesity and at risk for insulin resistance).	Five flavonoid treatments: Cocoa control (30 mg flavonoids), low-flavonoid (180 mg) cocoa, medium-flavonoid (400 mg) cocoa, high-flavonoid (900 mg) cocoa, green tea beverage (300 mg flavonoids), daily for 5 days.	↔ No change in plasma TMAO across treatments.
Annunziata *et al.* (2019) [98]	Crossover study (n = 20 healthy men and women).	Grape extract (600 mg, 32 mg total polyphenols) or placebo control daily for 4 weeks.	↓ Serum TMAO in the treatment group compared to placebo (63.6% vs. 0.54%, $P < 0.0001$) after 4 weeks.
Annunziata *et al.* (2019) [99]	Two-arm parallel study (n = 65 men and women with obesity/overweight).	Grape extract (300 mg, 16 mg total polyphenols) or grape extract (300 mg) + pectin (300 mg), daily for 8 weeks.	↓ Serum TMAO between baseline and 8 weeks by 78.6% ($P = 0.006$) in grape extract group and by 76.8% ($P = 0.001$) in grape extract + pectin group.
Tenore *et al.* (2019) [100]	Three-arm parallel study (n = 90 men and women with high CVD risk).	Apple puree (125 g, 47 mg total polyphenols) versus *Lactobacillus* fermented apple puree (125 g, 62 mg total polyphenols) versus *Lactobacillus* (3.0×10^8 CFU), daily for 8 weeks.	↓ Plasma TMAO between baseline and week 8 by 63.1% ($P = 0.004$) in *Lactobacillus* fermented apple puree (125 g) group, 42.3% ($P = 0.003$) in the apple puree group, and 25.8% ($P = 0.003$) in the *Lactobacillus* group.

1.8.3 Polyphenols and ellagitannins: dietary sources, metabolism, and urolithin metabotypes

Polyphenols are a diverse group of plant secondary metabolites found in fruits, vegetables, nuts, seeds, teas, coffee, and red wine [104]. Several groups of polyphenols have been associated with cardiovascular benefits, including flavonoids (e.g., flavan-3-ols and anthocyanins), stilbenes (e.g., resveratrol), and phenolic acids (e.g., chlorogenic acid) [104]. Ellagitannins, a class of hydrolysable tannins, represent an underexplored but promising group of polyphenols for targeting TMAO reduction. Ellagitannins have high molecular weights and complex structures [105], which limits their absorption in the small intestine, resulting in the majority reaching the colon intact. In the colon, gut microbiota first convert punicalagin to punicalin and subsequently to ellagic acid [106, 107]. Ellagic acid is then converted to various urolithins by the gut microbiota (**Figure 1.4**), predominantly by *Gordonibacter* species [88, 107-111]. Urolithins have been associated with microbiota-modulating properties, acting as prebiotics for certain microbial species while inhibiting others [106].

Three different urolithin metabotypes have been defined: metabotype A only produces urolithin A, metabotype B produces urolithin A, isourolithin A and/or urolithin B, and metabotype 0 does not produce urolithins [112]. The most common metabotype is metabotype A, with 25-80% of human volunteers belonging to this category, followed by metabotype B (10-50%), and metabotype 0 (10-25%) [111, 112].

Unlike many other dietary polyphenols, which undergo partial absorption in the small intestine and reach the system circulation, ellagitannins are poorly absorbed due to their high molecular weight and structural features that largely conform to Lipinski's rule of 5 for poor absorption [113]. The rule of 5 predicts that compounds are least absorbed if they contain over 5 hydrogen bonds and over 10 hydrogen donors, their molecular weight exceeds 500 g/mol, and they have a partition coefficient (CLogP) greater than 5 [113]. Ellagitannins are mostly compliant with the rule of 5 for poor absorption except for their CLogP. Similarly, ellagic acid, the main hydrolysis product of ellagitannins, shows limited intestinal uptake, with only low plasma concentrations detected within two hours of consuming pomegranate juice or black raspberry extract [109, 114, 115]. This contrasts with other polyphenol classes, such as flavonoids, which can achieve measurable systemic levels after intestinal absorption [116, 117]. The poor bioavailability of ellagitannins and ellagic acid increases the likelihood that they reach the colon, where

they may directly interact with TMA-producing gut microbiota, making them of particular interest for strategies targeting TMAO modulation.

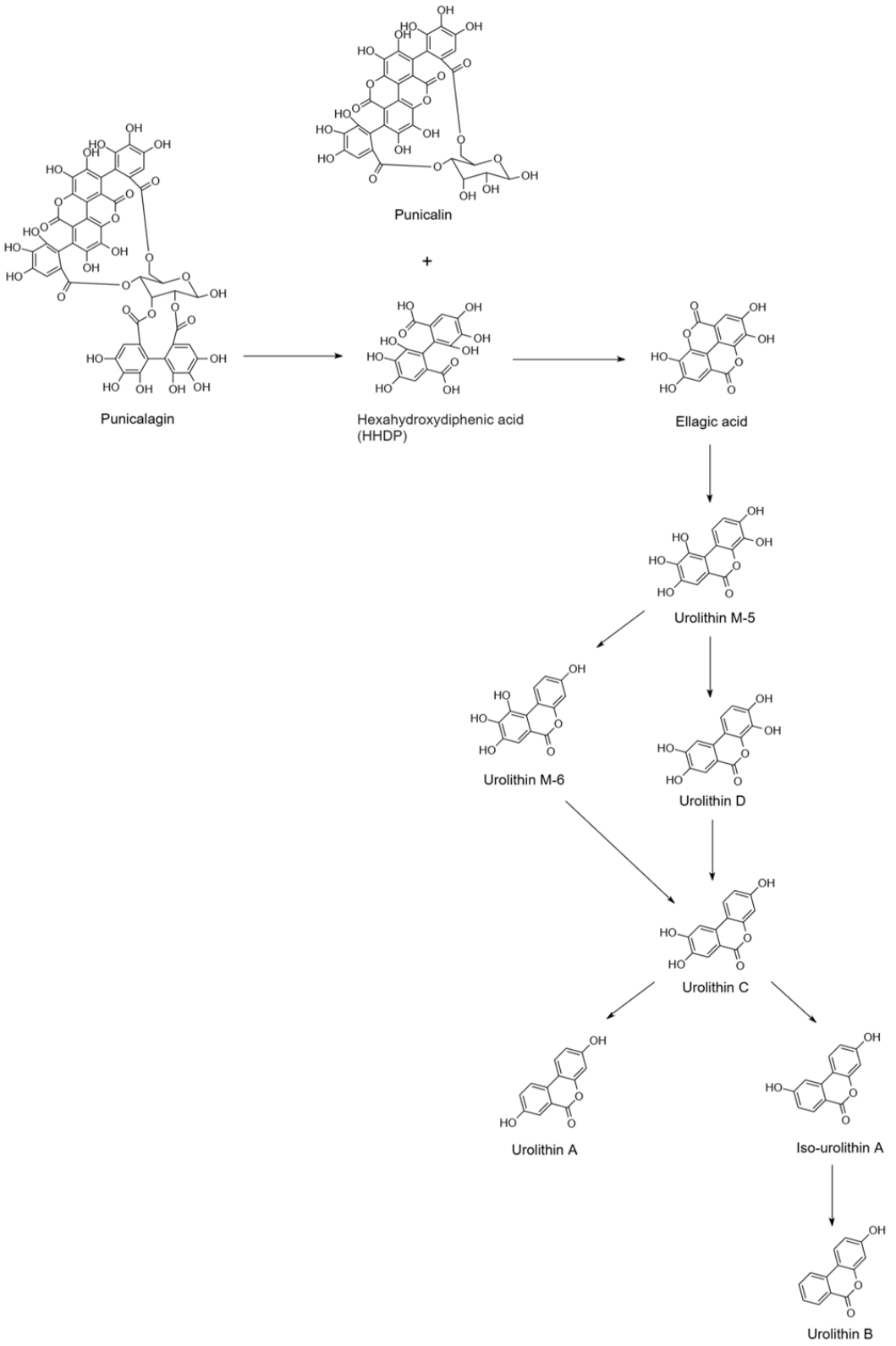

Figure 1.4. Schematic overview of the breakdown of punicalagin to urolithins. *In the human gut, punicalagin is first converted to punicalin and subsequently to ellagic acid. Within the colon, ellagic acid is converted to various urolithins by the gut microbiota.*

1.8.4 Pomegranate polyphenols as potential TMAO-reducing treatment

A well-known source of ellagitannins and ellagic acid is pomegranate [106]. Pomegranate has been reported to inhibit clostridia and increase bifidobacteria [120]. Many species belonging to the clostridia genus contain proteins of the betaine-choline-carnitine-transporter (BCCT) family. BCCT proteins are bacterial transport proteins, which contain the L-carnitine/γ-butyrobetaine antiporter, *caiT*, and other Cai proteins [121, 122]. This suggests that ellagitannin- and ellagic acid-rich pomegranate might reduce TMA production, and subsequently TMAO, by selectively inhibiting microbiota containing BCCT proteins.

Furthermore, pomegranate juice and extract were found to reduce systolic blood pressure, LDL, and pro-inflammatory cytokines in rodents [123-126]. The main ellagitannin in pomegranate, punicalagin, also protects against oxidative damage, inflammation, and apoptosis in rats [127]. These effects might be (partly) attributed to modulation of the gut microbiota, as pomegranate polyphenols were found to simultaneously reduce chronic low-grade inflammation, enhance tight junction protein expression (maintaining gut barrier integrity), and modulate the gut microbiota *in vivo* [128]. Beyond potential TMAO reduction, pomegranate polyphenol metabolites, urolithins, may contribute to cardiovascular benefits. For instance, urolithin A promotes angiogenesis [129, 130], while urolithin B-glucuronide has been shown to counteract TMAO-induced impairment of cardiomyocyte contractility and calcium handling in rats [131]. This suggests that, beyond reducing TMA formation, pomegranate polyphenol metabolites may also mitigate the harmful effects of circulating TMAO.

Only one animal study and one human study to date have investigated the TMAO-reducing effects of a black raspberry extract [95, 101], which is high in ellagic acid [118] and potentially contains ellagitannins considering that red raspberries are ellagitannin-rich sources [119]. Furthermore, after screening several polyphenols [132] and polyphenol-rich sources, preliminary data from Dr Paul Kroon and colleagues showed that a pomegranate extract was effective in reducing TMA production *in vitro*. Taken together, ellagitannin- and ellagic acid-rich sources, such as pomegranates, provide a potential dietary intervention strategy to reduce the risk of CVD, potentially through the reduction of TMAO levels.

1.9 Research aims, hypotheses and objectives

There is growing evidence that dietary polyphenols can inhibit the microbial conversion of L-carnitine and choline into TMA, and subsequently its oxidised form, TMAO. Among these precursors, L-carnitine is a major contributor to TMAO production because of its low absorption in the small intestine and extensive metabolism by gut microbiota to TMA. However, no studies have examined the direct effects of polyphenols or polyphenol-rich foods on TMAO production following the intake of an L-carnitine dose. Furthermore, the potential role of ellagitannins, polyphenols that reach the colon and directly interact with the gut microbiota, remains unexplored. The body of evidence is therefore limited, and the mechanisms by which polyphenols may suppress TMA production are poorly understood. This thesis addresses these knowledge gaps by investigating the effects of an ellagitannin-rich pomegranate extract on microbial L-carnitine metabolism to TMA and subsequent TMAO formation.

The overall aim of this research was to determine whether an ellagitannin-rich pomegranate extract can reduce microbial TMA production from L-carnitine and lower circulating TMAO concentrations, and to elucidate several of the possible underlying microbial mechanisms involved.

The hypothesis was that pomegranate polyphenols, specifically ellagitannins, inhibit microbial TMA production from L-carnitine and thereby reduce plasma TMAO. It was further hypothesised that these effects are mediated through selective suppression of bacteria involved in L-carnitine metabolism. To test the hypothesis, the following objectives were addressed:

Objective 1: Evaluate the inhibitory potential of pomegranate extract on microbial L-carnitine metabolism *in vitro*.

This included assessment of its dose-response effects on L-carnitine conversion to γ-BB, TMA and related intermediates using batch colon models, and estimating interindividual variability in microbial responses. This objective is addressed in **Chapter 2**.

Objective 2: Identify and characterise the polyphenols responsible for the inhibitory effects of pomegranate extract.

This included the characterisation of the polyphenolic composition of the extract, measuring the microbial metabolism of its main polyphenols in batch colon models,

developing a small-scale colon model, and testing individual pomegranate polyphenols for their effects on L-carnitine metabolism. This objective is addressed in **Chapter 3**.

Objective 3: Investigate the possible microbial mechanisms underlying the effects of pomegranate extract and its main polyphenol, punicalagin.

This involved quantifying total viable bacteria, assessing the effect on *cai* gene copy number and expression, and determining the impact on microbiota composition and diversity using metagenomic sequencing. This objective is addressed in **Chapter 4**.

Objective 4: Translate *in vitro* findings to humans by investigating the effects of pomegranate extract on TMAO pharmacokinetics in healthy adults.

This was achieved in the TESSA study, a double-blind, placebo-controlled, crossover trial, which examined plasma TMAO responses to an oral carnitine challenge with a pomegranate extract versus a placebo. The study also investigated the pharmacokinetics of L-carnitine, γ-BB, and pomegranate metabolites, and correlations between the plasma TMAO response with diet, kidney function, and urolithin metabotypes. This objective is addressed in **Chapter 5**.

Chapter 2

The inhibitory effect of a pomegranate extract on the microbial metabolism of L-carnitine to trimethylamine (TMA)

Chapter 2: The inhibitory effect of a pomegranate extract on the microbial metabolism of L-carnitine to trimethylamine (TMA)

2.1 Abstract

Background: High plasma levels of TMAO can have proatherogenic effects. L-Carnitine is a major dietary precursor of TMAO, which is metabolised by the gut microbiota to γ-BB and finally to TMA. In the liver, TMA is converted to TMAO. Several interventions have been suggested to reduce the microbial production of TMA, including broad-spectrum antibiotics. However, the effectiveness of broad-spectrum antibiotics wears off after 6 months, and they may contribute to antimicrobial resistance.

Aim and approach: The overall aim was to investigate the effects of a polyphenol-rich pomegranate extract on the microbial metabolism of L-carnitine to TMA, and to understand the dose-response relationship and interindividual variability underlying this process. *In vitro* batch colon models, mimicking the distal colon (anaerobic, pH 6.6-7.1, 37 °C), were inoculated with L-carnitine or γ-BB, different doses of a pomegranate extract (0, 5.7, 11.4, or 22.8 mg/mL), and 1% human faecal inoculum. Samples were collected over 24 or 48 hours and methylamines were quantified using liquid chromatography-tandem mass-spectrometry (LC-MS/MS) with isotopically labelled internal standards. Linear mixed models and logistic growth and decay models were used for statistical analyses.

Results: The polyphenol-rich pomegranate extract reduced the production of γ-BB and TMA from L-carnitine in a dose-dependent manner. Increasing concentrations of the extract progressively delayed the inflection points for both L-carnitine utilisation and γ-BB formation. Additionally, it was shown that a pomegranate extract inhibited not only the metabolism of L-carnitine to γ-BB, but also independently inhibited the metabolism of γ-BB to TMA and L-carnitine to crotonobetaine.

Conclusion: This study showed that a polyphenol-rich pomegranate extract dose-dependently inhibited the microbial metabolism of L-carnitine and γ-BB to TMA *in vitro*. However, large inter- and intra-individual variation exists in the metabolism of γ-BB to TMA.

2.2 Introduction

TMAO has been linked to various cardiometabolic diseases and all-cause mortality risk [39]. A major dietary precursor of TMAO is L-carnitine. Gut microbiota metabolise L-carnitine to γ-BB and then to TMA. Finally, TMA is converted to TMAO in the liver (**Chapter 1, Figure 1.3**). Additional pathways have been reported. One involves the microbial metabolism of L-carnitine to crotonobetaine before it is metabolised to γ-BB [68]. Another involves the metabolism of L-carnitine to crotonobetaine which is then directly converted to TMA [68].

The most effective reported interventions to reduce microbial γ-BB and TMA production from L-carnitine are broad-spectrum antibiotics [78, 133]. Long term use of broad-spectrum antibiotics, however, can contribute to antimicrobial resistance and the TMA-reducing effects have been shown to wear off after 6 months [78]. Therefore, effective, low-risk strategies to reduce microbial TMA production are urgently needed to help lower the possible health risks associated with high circulating TMAO levels.

Reducing TMA production using a dietary intervention offers a promising approach due to the relatively low risk and low cost compared to (novel) pharmaceutical interventions. Furthermore, dietary interventions will have minimal regulatory hurdles compared to pharmaceutical interventions. An important condition for these compounds to be effective is that they must reach the distal colon, where microbial TMA production from L-carnitine occurs, and in sufficient concentrations.

Dietary components that are poorly absorbed from the small intestine include dietary polyphenols [85]. Indeed, polyphenols meet the definition of prebiotics put forward by the International Scientific Association for Probiotics and Prebiotics (ISAPP); 'a substrate that is selectively utilized by host microorganisms conferring a health benefit' [134]. Therefore, most of the ingested polyphenols will reach the colon where they could directly interact with the gut microbiota. Additionally, polyphenols have known antibacterial [86], anti-inflammatory [87, 88], and anti-oxidative [87] effects. This provides a potential for dietary polyphenols as a low-risk TMA-reducing strategy.

Few reports provide evidence that dietary polyphenols and polyphenol-rich extracts can reduce TMA production and TMAO levels (**Chapter 1**, **Table 1.1**). One study using an *in vitro* fermentation model found that blonde orange juice, grapefruit juice, and pomegranate juice significantly reduced TMA production from L-carnitine [89]. A

limitation of this study is the lack of pH control, which is required to study the metabolism of choline and L-carnitine [1]. The authors of the study noted that the simple sugar content exerted the greatest effect on TMA production, which could be the result of a reduced pH. Another *in vitro* study found that chlorogenic acid and gallic acid reduced TMA formation from choline [90]. Reports in rodents show that resveratrol [92], chlorogenic acid [91], and two polyphenol-rich extracts – a suckle berry extract (*Lonicera caerulea*) [93] and a blueberry extract [96] – reduced circulating TMAO levels. Furthermore, several human intervention studies showed that the daily supplementation of polyphenol-rich foods for four to eight weeks reduced circulating TMAO levels [98-101] (**Chapter 1, Table 1.1**).

A group of polyphenols that are particularly poorly absorbed in the small intestine include ellagitannins and ellagic acid [109], and anthocyanins [135, 136]. When they reach the colon, polyphenols can directly interact with the gut microbiota, hence they may affect microbial TMA production. Pomegranates are an excellent dietary source of ellagic acid and ellagitannins. As far as the author is aware, to date only one study has investigated the effect of a pomegranate product (juice) on TMA production using an *in vitro* batch colon model, but the model was not pH controlled [89]. Unpublished data from Dr Paul Kroon and colleagues have shown that a low pH can inhibit the metabolism of L-carnitine and choline. Therefore, the aim of this research was to study the effect of a polyphenol-rich pomegranate extract on the microbial metabolism of L-carnitine to TMA using an *in vitro* batch colon model that is also pH-controlled.

2.3 Objectives

The overall aim of the research described in this chapter was to investigate the potential of a polyphenol-rich pomegranate extract to inhibit the microbial metabolism of L-carnitine to TMA in an *in vitro* batch colon model, and to understand the dose-response relationship and interindividual variability underlying this process.

To achieve this aim, the following objectives were established:

1. To evaluate the effect of the pomegranate extract on the microbial conversion of L-carnitine to γ-BB and TMA.

2. To determine whether the extract directly affects the second step of L-carnitine metabolism (the conversion of γ-BB to TMA) in the absence of L-carnitine.
3. To investigate inter- and intra-individual variability in TMA production across different stool donors.
4. To explore the effect of the extract on the microbial production of crotonobetaine, a reported intermediate on the metabolic pathway of L-carnitine to γ-BB/TMA.

2.4 Materials and methods

2.4.1 Materials

The pomegranate extract (Dermogranate®) was purchased from Medinutrex (Catania, Italy) in June 2023. Solvents were at least of high-performance liquid chromatography (HPLC) grade. Water was 18 MΩ·cm Milli-Q. Trimethylamine hydrochloride (CAS 593-81-7), γ-BB (commercially known as 3-(Carboxypropyl)trimethylammonium chloride, CAS 6249-56-5), trichloroacetic acid (TCA), glacial acetic acid, and heptafluorobutyric acid (HFBA) were obtained from Merck. L-Carnitine (CAS 541-15-1), ammonium acetate and all solvents were obtained from Fisher Scientific Limited. Crotonobetaine hydrochloride was obtained from LGC Standards (CAS 6538-82-5). Isotopically labelled standards were obtained from Cambridge Isotope Laboratories (L-carnitine-(trimethyl-d9), CAS 126827-79-0), LGC Standards (trimethylamine-d9 hydrochloride, CAS 18856-86-5), Santa Cruz Biotechnology (γ-Butyrobetaine-d9, CAS 479677-53-7), and Merck (Dimethyl-d6-amine hydrochloride, CAS 53170-19-7).

2.4.2 *In vitro* batch colon model experimental design

Faecal samples were collected from subjects who consented to participate in the QIB Colon Model Study (NCT02653001, ClinicalTrials.gov). The study was approved by the Human Research Governance Committee at Quadram Institute Bioscience (QIB) and the London-Westminster Research Ethics Committee (15/LO/2169). Donors included adult

men and women, who are based within 10 miles of the Norwich Research Park and have a normal bowel habit, with an average stool type between 3 and 5 on the Bristol Stool Chart [137]. Exclusion criteria were (i) diagnosis with gastrointestinal diseases such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), or coeliac disease, (ii) gastrointestinal complications within the 72 hours prior to donation such as vomiting or diarrhoea, (iii) use of antibiotics or probiotics within the four weeks prior to donation, (iv) ongoing pregnancy or lactating, and (v) recently undergone anaesthetics. On the day of inoculation of the *in vitro* batch colon models, the donors delivered a fresh faecal sample. In a class II microbiological safety cabinet (MSC), 10% w/v faecal slurries were prepared by making a 1:10 dilution in phosphate-buffered saline (PBS). The solution was homogenised using a Stomacher® 400 EVO for 2 × 30 seconds at 230 revolutions per minute (rpm). To maintain donor anonymity, the QIB Colon Model Study manager assigned each donor a unique subject code, which was the only identifier disclosed to us for this research. Similarly, no subject codes are used in this thesis.

In vitro batch colon models were inoculated with different concentrations of the pomegranate extract (0, 5.7, 11.4, and 22.8 mg/mL), 2 mM L-carnitine and a final concentration of 1% faecal inoculum. *In vitro* batch colon models mimic the fermentation conditions in the human colon and the basic conditions were previously reported by Parmanand *et al.* (2019) [138]. Briefly, a batch culture medium was prepared containing peptone water, yeast extract, NaCl, K_2HPO_4, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}6H_2O$, $NaHCO_3$, cysteine·HCl, bile salts, Tween80, hemin, and vitamin K_1. After autoclaving, a concentrated stock of sterile filtered D-glucose was added to the media, reaching a final concentration of 1%. Glass vessels of 300 mL volume were sealed and autoclaved. The vessels were connected to a circulating water bath (Grant Instruments Ltd, Shepreth, United Kingdom), such that the temperature of the vessels was kept at 37 °C. The pH of vessels was maintained between 6.6-7.1 using a FerMac 260 pH Controller (Electrolab Biotech Limited, Gloucestershire, United Kingdom), which was connected to sodium hydroxide (NaOH, 0.5 M) and hydrochloric acid (HCl, 0.5 M) pumps to maintain the pH within the pre-set range. Furthermore, anaerobic conditions were secured through the continuous supply with oxygen-free nitrogen.

Prior to the faecal inoculations, 100 mL of autoclaved batch culture medium was added to each vessel and the oxygen was removed from the vessels overnight, using a continuous oxygen-free nitrogen flow. The vessels were then inoculated with 11 mL fresh faecal inoculum (1% w/v final concentration of faecal matter), together with the

pomegranate extract and the L-carnitine or γ-BB. Samples for microbial metabolite analyses were taken at different time points over 24 or 48 hours after inoculation. Samples were stored at -80 °C directly after collection until LC-MS/MS analysis.

2.4.3 Dosage information

The selected doses of pomegranate extract (5.7, 11.4, and 22.8 mg/mL) correspond to polyphenol quantities of 0.68, 1.37, and 2.74 mg/mL respectively for the increasing doses of pomegranate extract, based on a polyphenol content of 12% w/w estimated in **Chapter 3** (Section **3.5.1**). Considering that the average volume of the human colon, including the ascending, transverse, and descending sections, is approximately 561 mL [139], these doses would equate to polyphenol amounts ranging from around 381.5 to 1,535 mg in the colon. This range is consistent with polyphenol intake levels reported in previous human studies on pomegranate juice or extract supplementation, which typically fall between 435 and 2,660 mg of gallic acid equivalents (GAE) [140-143]. The highest dose used in this chapter can be achieved by 350 mL pomegranate juice, containing approximately 1,540-1,790 mg of polyphenols [142, 144], with the medium and low dose being achieved by a 175 mL and 87.5 mL pomegranate juice, respectively.

2.4.4 Preparation of colon model samples for LC-MS/MS analysis

LC-MS/MS was used to quantify γ-BB, L-carnitine, TMA, and crotonobetaine concentrations in the samples collected at each time point. A mixture containing 25 µM of each of the isotopically labelled internal standards (L-carnitine-d9, γ-BB-d9, TMA-d9) was prepared in 0.2 M acetic acid. An internal crotonobetaine standard was not obtained as only a single, explorative experiment to test for crotonobetaine was conducted. Instead, crotonobetaine was compared against an isotopically labelled dimethylamine (DMA) standard (DMA-d6).

Colon model samples were centrifuged at 14,000 rpm for 10 minutes. In a 96-well plate (4titude, Wotton, United Kingdom), 5 µL of the sample was added to 25 µL TCA and 70 µL of the mixture of internal standards. On a separate 96-well plate, external standards (L-carnitine, γ-BB, TMA, crotonobetaine) were prepared via serial dilutions in a matrix

containing 1% faecal inoculum and colon model media. The 96-well plates were then centrifuged for 2 minutes at 14,000 rpm. Next, from each well 5 µL of supernatant was transferred to a Chromacol vial (Thermo Scientific) containing 95 µL distilled water. All vials were capped and stored at -20 °C until analysis.

2.4.5 Metabolite quantification using LC-MS/MS

A detailed description of the metabolite quantification using LC-MS is described elsewhere [1]. In brief, targeted analysis was performed using a 1290 Infinity II LC System, coupled to a 6490 Triple Quad LC-MS (Agilent Technologies). The metabolites were separated through an Acquity UPLC BEH C8 1.7µm column (Waters, Massachusetts, United States), using a mobile phase consisting of 10 mM acetate and 0.05% HFBA in Mili-Q water (solution A) and 10 mM acetate and 0.05% HFBA (dissolved in 50 mL Mili-Q water) in methanol (solution B). The gradient for solvent B started with 2% and increased by 10% within 1.54 minutes, with 0.2 mL/minute flow rate, followed by a wash for 4 minutes and equilibration for 2.5 minutes. The run per sample lasted approximately 8 minutes.

Additionally, crotonobetaine concentrations were quantified using the same LC-MS system. The metabolites were separated through a Luna Omega 1.6µm Polar C18 100A 100 x 2.1 mm column (Phenomenex UK Ltd), using a mobile phase consisting of Mili-Q water with 0.1% formic acid (solution A) and acetonitrile with 0.1% formic acid (solution B). The injection volume was 5 µL.

For all LC-MS analyses, the wash buffer consisted of 50% methanol, 25% mili-Q water, and 25% acetonitrile. The column temperature was maintained at 35 °C. The data were extracted and analysed using MassHunter Workstation Quantitative Analysis for GC-MS and LC-MS, version 10.0 (Agilent Technologies). The peak area of the sample was divided by the peak area of the internal standard to obtain peak area ratios. Absolute concentrations were calculated from the peak area ratios using the standard curves obtained from the non-labelled standards (TMA, γ-BB, L-carnitine, crotonobetaine) serial dilutions.

2.4.6 Method precision

To assess method precision, replicate colon model experiments were conducted using the same faecal donor sample. Three independent vessels (biological replicates) were run under identical conditions for both the control and pomegranate extract treatments. At each time point, five technical replicates were collected per vessel, resulting in a total of 15 measurements per treatment per time point. This setup allowed evaluation of variability between biological replicates, and technical variation in quantitative analyses of samples for trimethylamines, providing insight into the reproducibility and reliability of the *in vitro* batch colon model under controlled conditions.

Overall, technical variance was low (**Table 2.1**), except for metabolites that were present at very low concentrations, which is as expected as small absolute values increase sensitivity to variability. Hence, the data from the technical replicate samples (n = 5) taken through analysis show that variation in the quantitative analysis of methylamines is low and certainly acceptable.

There were numerous examples where biological variance was substantial. Biological variance at baseline (Time 0) was low for L-carnitine but higher for γ-BB and TMA, which were at low concentrations (**Table 2.1, Figure 2.1**). At later time points, once fermentation had occurred and L-carnitine concentrations declined, biological variance was higher. For γ-BB and TMA, biological variance was somewhat higher, but at higher concentrations of analytes it was typically 10% or less. Biological variances >10% were usually associated with low absolute concentrations of analytes.

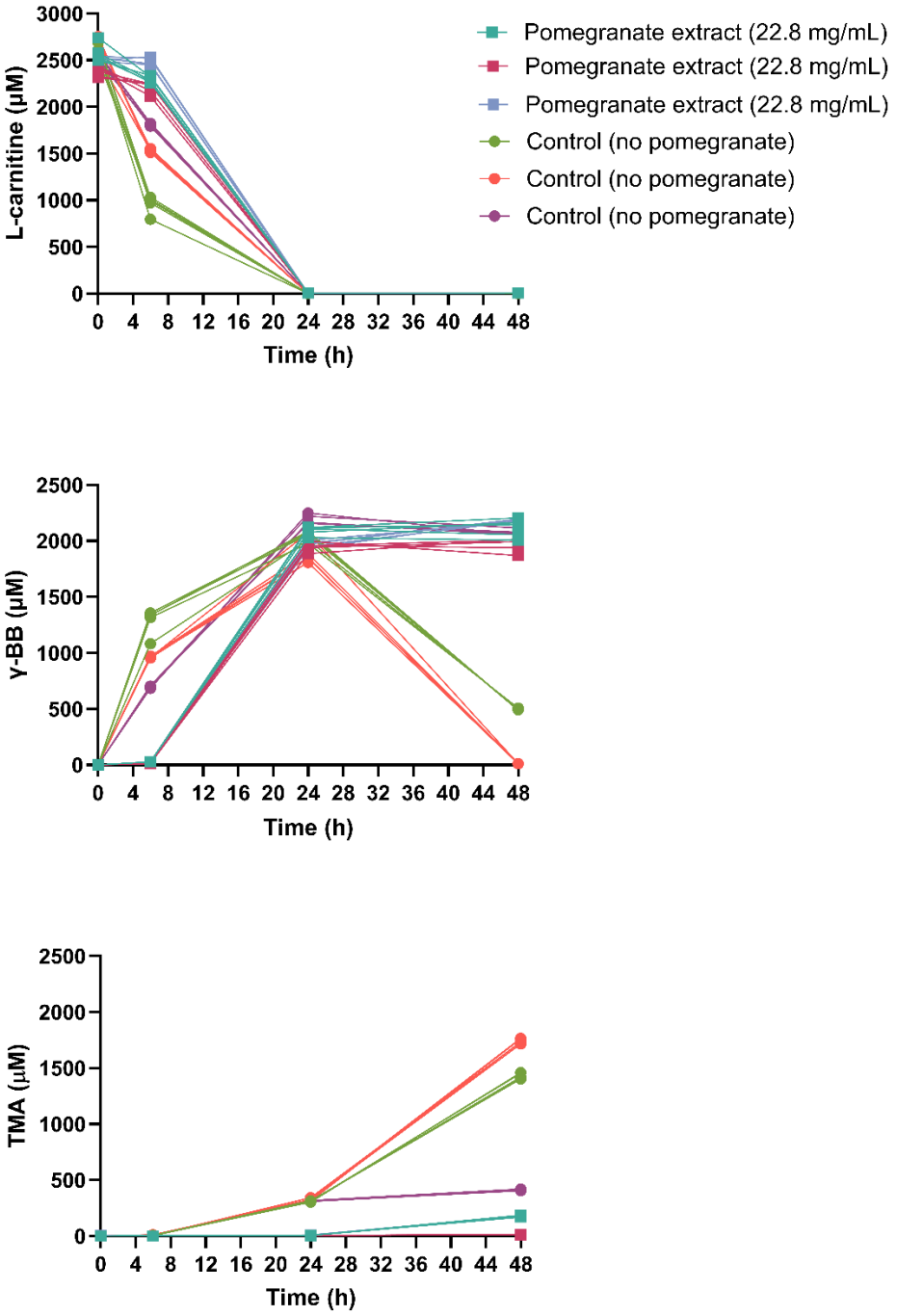

Figure 2.1. Reproducibility experiment of L-carnitine, γ-butyrobetaine (γ-BB), and trimethylamine (TMA) concentrations in in vitro batch colon models from a single faecal donation treated with a pomegranate extract. *Trajectories of (**A**) L-carnitine, (**B**) γ-BB, and (**C**) TMA concentrations are shown for three replicate vessels with each five replicate samples at every time point, comparing colon model vessels treated with the pomegranate extract (22.8 mg/mL) to control vessels (no pomegranate). In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and a pomegranate extract (0 or 22.8 mg/mL). Samples were collected at multiple time points over 48 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

Table 2.1. Variation (var.) in L-carnitine, γ-butyrobetaine (γ-BB), and trimethylamine (TMA) concentrations between vessels and treatments (pomegranate or control) in in vitro batch colon models from a single faecal donation treated with a pomegranate extract. *Vessel and treatment means and standard deviations (SD) are shown for L-carnitine, γ-BB, and TMA concentrations, obtained from three replicate vessels with five replicate samples per vessel per time point, comparing colon model vessels treated with the pomegranate extract (22.8 mg/mL) to control vessels (no pomegranate). In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and a pomegranate extract (0 or 22.8 mg/mL). Samples were collected at multiple time points over 48 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

			L-Carnitine				y-butyrobetaine				TMA			
Time	**Treatment**	**Vessel**	**Vessel mean ± SD**	**Vessel var. (%)**	**Treat-ment mean ± SD**	**Treat-ment var. (%)**	**Vessel mean ± SD**	**Vessel var. (%)**	**Treat-ment mean ± SD**	**Treat-ment var. (%)**	**Vessel mean ± SD**	**Vessel var. (%)**	**Treat-ment mean ± SD**	**Treat-ment var. (%)**
0	Control	1	2622 ± 74	2.8	2616 ± 76	2.9	0.9 ± 0.1	11	0.9 ± 0.1	9.3	0.3 ± 0.5	157	0.4 ± 0.2	57
	Control	2	2689 ± 92	3.5			1.0 ± 0.1	9.7			0.2 ± 0.4	224		
	Control	3	2538 ± 34	1.3			0.8 ± 0.1	6.7			0.6 ± 0.5	86		
	Pomegranate	1	2580 ± 95	3.7	2491 ± 105	4	1.0 ± 0.1	13	0.9 ± 0.1	10	0.5 ± 0.8	162	1.2 ± 1.0	85
	Pomegranate	2	2375 ± 53	2.2			0.8 ± 0.1	14			0.7 ± 0.9	132		
	Pomegranate	3	2518 ± 22	0.9			0.9 ± 0.1	12			2.3 ± 1.2	55		
6	Control	1	960 ± 94	9.8	1434 ± 434	30	1286 ± 115	8.9	984 ± 294	30	5.7 ± 1.6	28	7.2 ± 1.5	20
	Control	2	1530 ± 17	1.1			964 ± 7.0	0.7			8.7 ± 1.9	22		
	Control	3	1812 ± 13	0.7			670 ± 9.2	1.3			7.1 ± 1.4	19		
	Pomegranate	1	2294 ± 33	1.4	2328 ± 144	6.2	28 ± 0.7	2.5	22 ± 8.1	37	1.6 ± 2.2	132	1.5 ± 0.5	31
	Pomegranate	2	2204 ± 59	2.7			13 ± 0.5	4.2			1.8 ± 2.1	114		
	Pomegranate	3	2486 ± 38	1.5			26 ± 0.6	2.5			0.9 ± 1.0	108		
24	Control	1	0.2 ± 0.0	19	0.3 ± 0.2	49	2041 ± 45	2.2	2041 ± 148	7.3	310 ± 4.7	1.5	317 ± 8.6	2.7
	Control	2	0.5 ± 0.3	53			1894 ± 104	5.5			327 ± 10	3.1		
	Control	3	0.3 ± 0.3	72			2190 ± 42	1.9			314 ± 4.0	1.3		

	Pomegranate	1	5.8 ± 0.6	10	6.1 ± 1.7	27	2091 ± 38	1.8	2002 ± 76	3.9	6.1 ± 1.0	17	6.0 ± 0.6	10
	Pomegranate	2	7.8 ± 0.9	11			1948 ± 40	2.1			5.4 ± 0.4	6.6		
	Pomegranate	3	4.5 ± 0.4	9.6			1967 ± 29	1.5			6.6 ± 1.5	23		
48	Control	1	0.0 ± 0.1	1484	0.0 ± 0.1	1877	497 ± 7.8	1.6	861 ± 1079	125	1428 ± 27	1.9	1194 ± 696	58
	Control	2	-0.1 ± 0.1	-132			11 ± 0.4	3.4			1742 ± 21	1.2		
	Control	3	0.1 ± 0.2	290			2074 ± 25	1.2			410 ± 5.6	1.4		
	Pomegranate	1	7.1 ± 0.7	10	7.3 ± 0.9	13	2115 ± 82	3.9	2089 ± 113	5.4	178 ± 7.3	4.1	124 ± 96	77
	Pomegranate	2	8.3 ± 0.4	4.6			1965 ± 61	3.1			13 ± 2.0	15		
	Pomegranate	3	6.4 ± 1.3	20			2185 ± 9.7	0.4			180 ± 3.0	1.7		

2.4.7 Statistical analyses

Trajectories of L-carnitine and metabolite concentrations over time are presented as the mean ± SD across donors. Outcomes were considered statistically significant if $P < 0.05$.

The effects of the pomegranate extract on concentrations of L-carnitine and its metabolites were estimated using linear mixed models, with treatment (i.e., 0, 5.7, 11.4, and 22.8 mg/mL pomegranate extract) as fixed effects and random intercepts of donors. Independent models were fitted for each outcome at each time point. Due to limited data within donors, more complex models, such as those with random slopes or non-linear dose-response curves, could not be reliably estimated. Therefore, p-values based on random intercept should be interpreted with caution. Linear dose-response effects were obtained using equally spaced numerical codes to represent the increasing doses.

Additionally, logistic growth curves were fitted to model the L-carnitine and its metabolite concentrations over time. The inflection point and growth rate were also estimated and compared across conditions using linear mixed models. However, in many cases, the data were insufficient to produce reliable curve fits. The γ-BB production from L-carnitine at the highest pomegranate dose for donors 1 and 3 was delayed to the extent that the upper asymptote could not be estimated, requiring a manually set upper bound. Adjusting this bound did not affect the analytical results.

All graphs were generated using GraphPad Prism version 10.4.2, and R version 4.4.1 (R Core Team, 2024) in RStudio (Posit Software, PBC, Boston, MA). Statistical analyses were performed using RStudio with 'lme4' [145], 'lmerTest' [146], and 'broom.mixed' [147] packages for mixed modelling and 'minpack.LM' [148] for non-linear modelling.

2.5 Results

2.5.1 The microbial metabolism of L-carnitine to TMA

When *in vitro* batch colon models were inoculated with L-carnitine, the concentration of L-carnitine started to decline after 4 hours and was metabolised completely between 12-20 hours after inoculation (**Figure 2.2**). Simultaneously, γ-BB concentrations were first

detected after 4 hours and reached a peak between 12-20 hours after inoculation. TMA was slowly produced over time, starting to appear after 10 hours with an increasing trend and reflecting the decline in γ-BB concentrations.

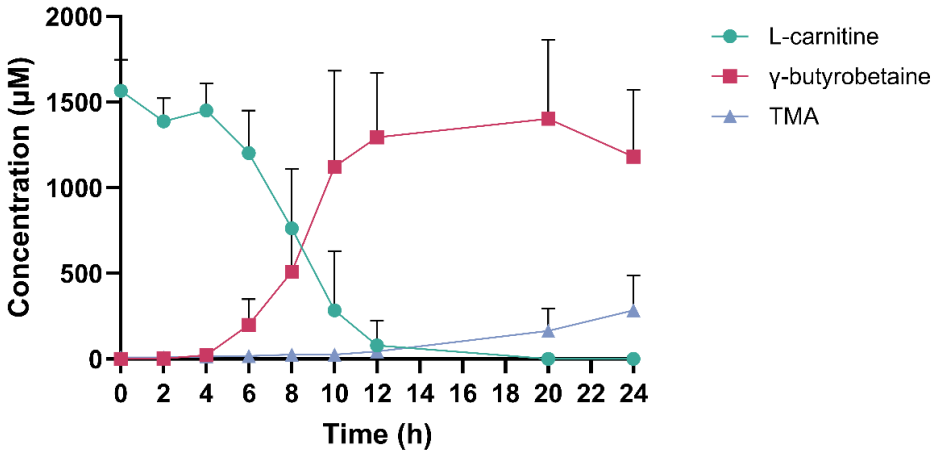

Figure 2.2. L-Carnitine metabolism to γ-butyrobetaine (γ-BB) and trimethylamine (TMA) in in vitro batch colon models. *Average concentrations of L-carnitine, γ-BB and TMA are shown across 8 donors over 24 hours. Data are combined from several experiments conducted by the author, Dr Priscilla Day-Walsh and Dr Emad Shehata, and are presented as mean ± SD. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine. Samples were collected at multiple time points over 24 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

2.5.2 The pomegranate extract inhibits the metabolism of L-carnitine

The pomegranate extract at 5.7 mg/mL slowed down the conversion of L-carnitine to γ-BB, delaying the complete utilisation of L-carnitine by 4-8 hours compared with the control condition (**Figure 2.3A**). Delays in the production of γ-BB were also observed, which are consistent with the reduced metabolism of L-carnitine (**Figure 2.3B**). Significant differences in L-carnitine and γ-BB concentrations were detected at 6, 8, 10, and 12 hours (**Figure 2.3A-B**). TMA concentrations were significantly different between the treatments only at 6 hours (**Figure 2.3C**). TMA concentrations at 24 hours were relatively low compared to L-carnitine and γ-BB concentrations, suggesting that there may be further increases in TMA concentrations after 24 hours.

However, inconsistent patterns were observed across individual trajectories (**Appendix 1, Supplementary Figure S1**).

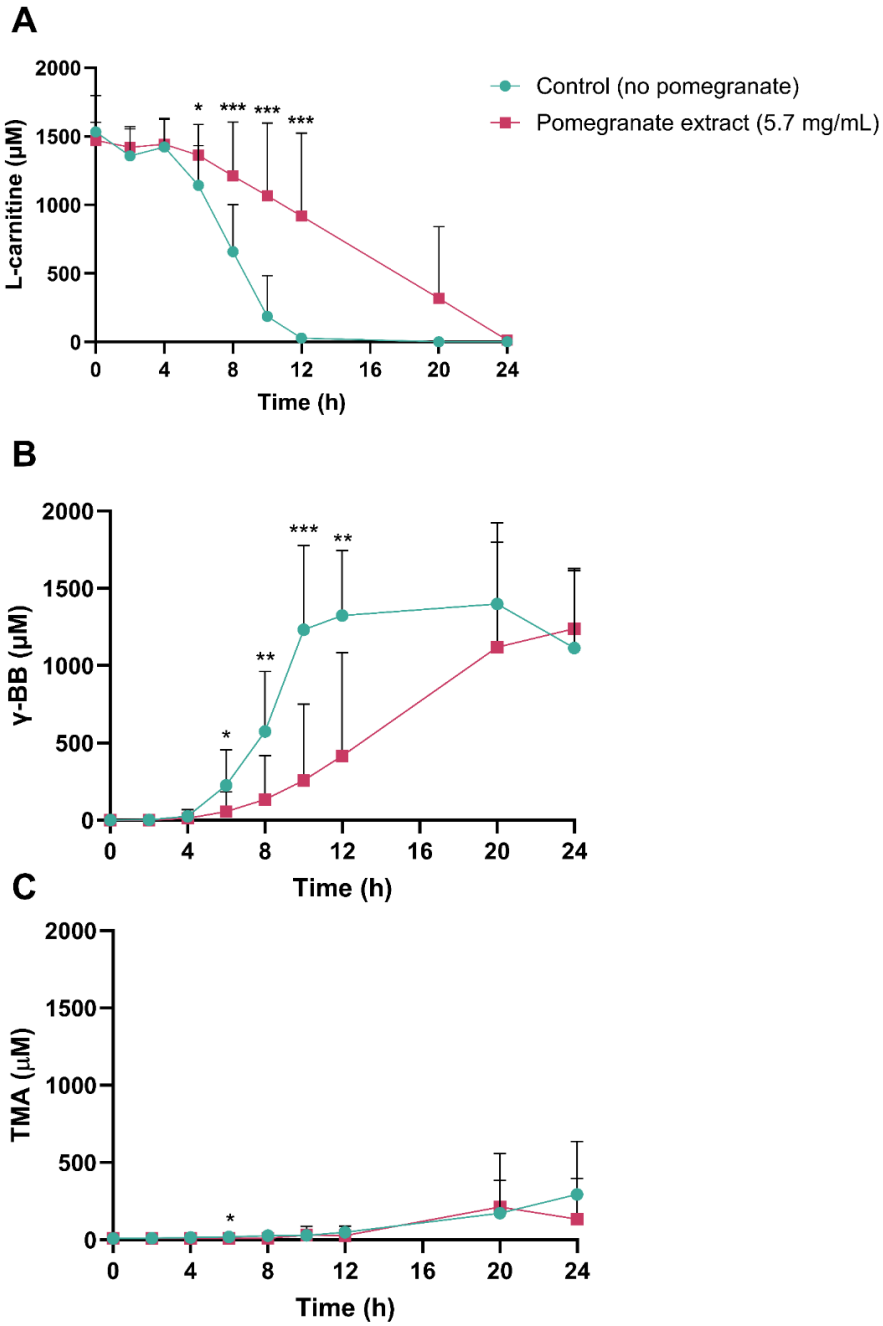

Figure 2.3. L-Carnitine, γ-butyrobetaine (γ-BB), and trimethylamine (TMA) concentrations in in vitro batch colon models treated with a pomegranate extract. *Average concentrations of (**A**) L-carnitine, (**B**) γ-BB, and (**C**) TMA are shown across 7 donors (with two replicates per donor), comparing colon model vessels treated with pomegranate extract (5.7 mg/mL) to control vessels at each time point over 24 hours. Data are combined from several experiments conducted by the author, Dr Priscilla Day-Walsh and Dr Emad Shehata, and are presented as mean ± SD. Statistical analysis was performed using linear mixed models, with treatment (control or pomegranate extract) as fixed effects and random intercepts of donors (* P < 0.05, ** P < 0.01, *** P < 0.001). In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and a pomegranate extract (0 or 5.7 mg/mL). Samples were collected at multiple time points over 24 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

2.5.3 The pomegranate extract inhibits L-carnitine metabolism dose-dependently

These data show that the pomegranate extract at 5.7 mg/mL can significantly delay the metabolism of L-carnitine to γ-BB, but the data could not capture the full trajectory of increasing TMA concentrations. Therefore, a second set of experiments was conducted over 48 hours to capture the full TMA trajectory, and with a range of pomegranate extract doses (0, 5.7, 11.4, and 22.8 mg/mL) to explore potential dose-response effects.

When a range of pomegranate extract doses was used, a strong dose-dependent delay on the metabolism of L-carnitine to γ-BB was observed (**Figure 2.4A-B**), with statistically significant average dose-response effects in all curves at different time points. This appeared to be largely consistent across donors (**Appendix 1, Supplementary Figure S2**).

TMA concentrations remained relatively low over 48 hours and the dose-dependent effect of the pomegranate extract on TMA production was less apparent (**Figure 2.4C**), likely due to large variations in TMA production between donors (**Appendix 1, Supplementary Figure S2**). In fact, at 48 hours the lowest pomegranate extract dose resulted in the greatest TMA reduction, in particular for donors 2 and 3 (**Table 2.2**). At 48 hours, there was no significant dose-dependent effect of pomegranate extract on TMA concentrations. A linear mixed-effects model, including donor as a random effect, showed that increasing pomegranate dose was not associated with a significant change in TMA levels at 48 hours after inoculation (β = -8.83, SE = 7.85, P = 0.285).

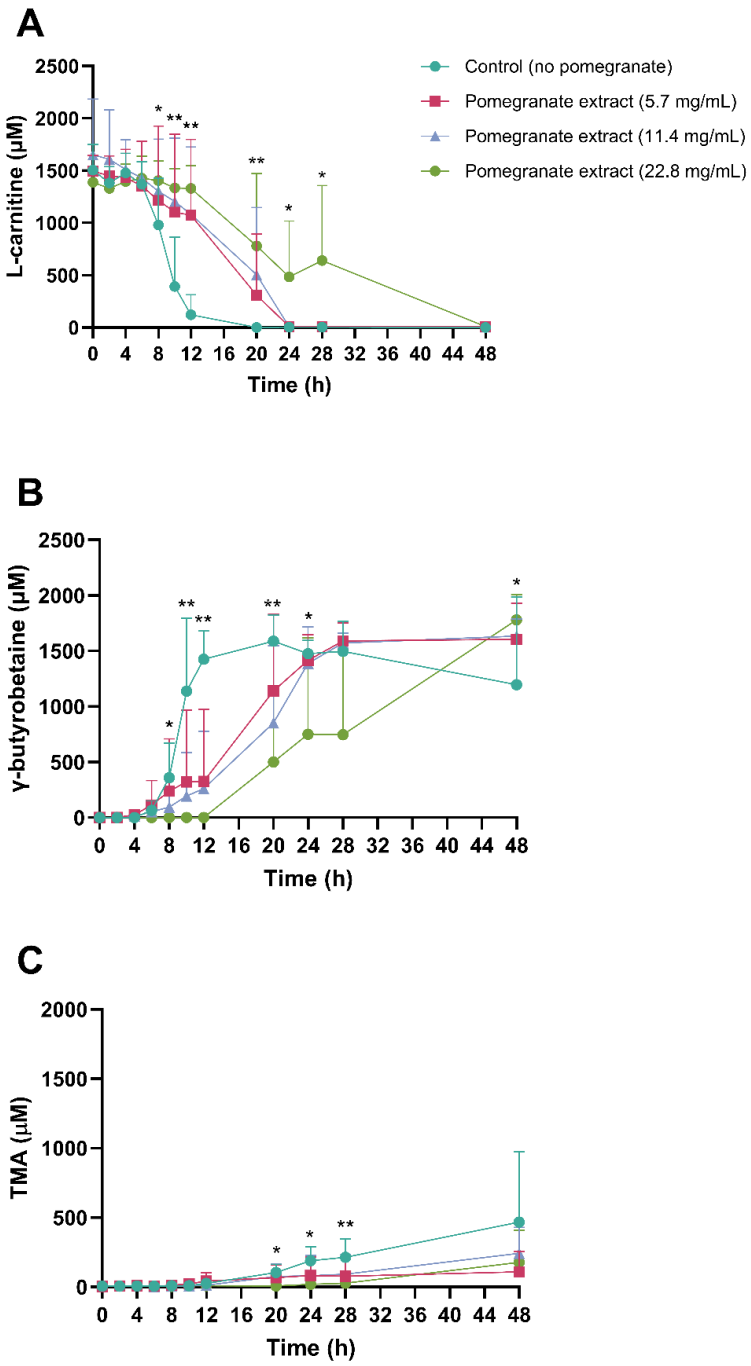

Figure 2.4. L-Carnitine, γ-butyrobetaine (γ-BB), and trimethylamine (TMA) concentrations in in vitro batch colon models treated with a pomegranate extract at increasing doses. *Average concentrations of (**A**) L-carnitine, (**B**) γ-BB, and (**C**) TMA are shown across 4 donors, comparing colon model vessels treated with the pomegranate extract at different doses (5.7, 11.4, 22.8 mg/mL) to a control vessel at each time point over 48 hours. Data are presented as mean ± SD. Statistical analysis was performed using linear mixed models, with treatment (control or pomegranate extract dose) as fixed effects and random intercepts of donors (* P < 0.05, ** P < 0.01). In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and a pomegranate extract. Samples were collected at multiple time points over 48 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

Table 2.2. Total trimethylamine (TMA) production (µM) per donor and pomegranate extract dose at 48 hours after inoculation. *In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and a pomegranate extract (0, 5.7, 11.4, 22.8 mg/mL). Samples were collected at 48 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

Donor	Control (0 mg/mL)	Low dose (5.7 mg/mL)	Medium dose (11.4 mg/mL)	High dose (22.8 mg/mL)
Donor 1	0.0	0.0	0.0	0.0
Donor 2	204	65	251	103
Donor 3	500	49	456	516
Donor 4	1164	325	262	90
Average[1]	**467**	**110**	**242**	**177**

[1]A linear mixed-effects model including dose as a numeric variable and donor as a random effect found no significant dose-response trend for TMA at 48 hours (β = -8.83, SE = 7.85, P = 0.285).

2.5.4 Logistic growth and decay modelling of the dose-dependent effect of the pomegranate extract on L-carnitine metabolism

As a secondary analysis, a non-linear regression model was fitted to estimate the conversion of L-carnitine to γ-BB and TMA using logistic growth and decay models. However, in some cases the data were not sufficient to reliably estimate a curve, which limited the estimations that could be made. **Figure 2.5** shows these curves for the metabolism of L-carnitine to γ-BB and TMA. Comparing the curve parameters (**Appendix 1, Supplementary Figure S3**) across treatments provided similar results as the primary analysis in which each outcome at each time point was individually compared.

The curve parameters show significant dose-dependent effects of the pomegranate extract on the decline in L-carnitine and the increase in γ-BB concentrations, with increasing doses of the pomegranate extract leading to a delay in the inflection point of the curve and a slower rate of growth for γ-BB and of decay for L-carnitine (**Appendix 1, Supplementary Figure S3**). The highest dose (22.8 mg/mL) resulted in a delay of 16 hours in the L-carnitine inflection point (95%CI = 8.4-24; $P = 0.0001$) and 19 hours in the γ-BB inflection point (95% CI: 11 to 27; $P < 0.01$), compared to the control condition (0 mg/mL) (**Appendix 1, Supplementary Table S1**). The effects of the pomegranate extract

on the curve parameters of TMA growth are less apparent as the production of TMA over the course of the experiment (48 hours) did not plateau for most donors.

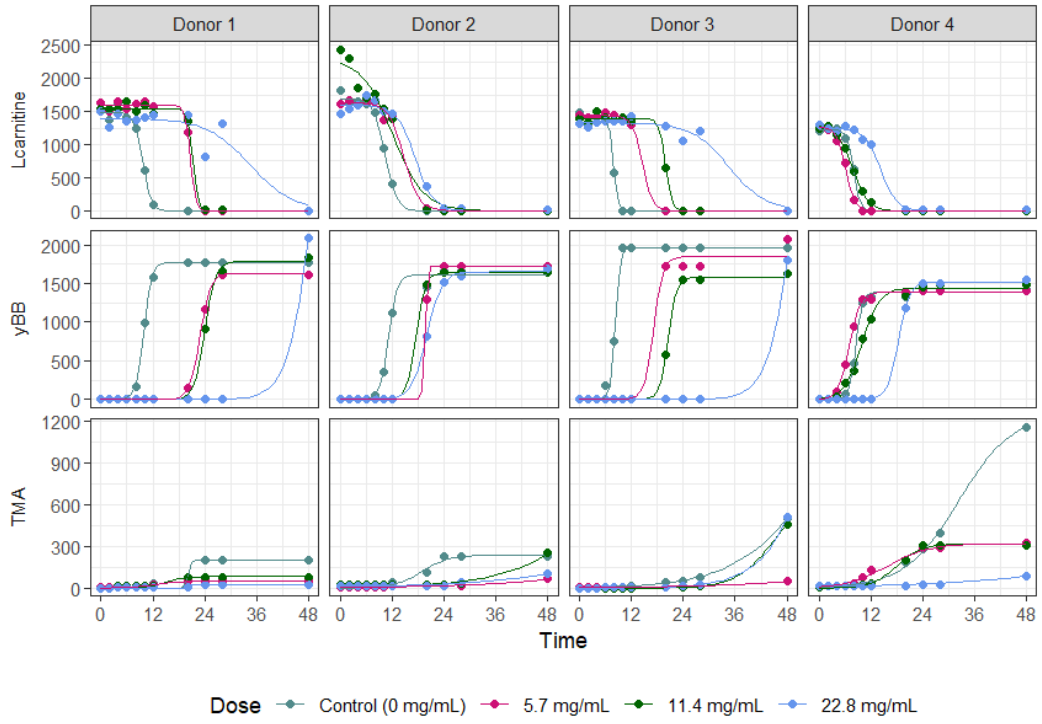

Figure 2.5. Logistic growth and decay modelling of L-carnitine, γ-butyrobetaine (γ-BB), and trimethylamine (TMA) concentrations over 48 hours in in vitro batch colon models treated with a pomegranate extract at increasing doses, stratified by donor. *Models incorporate a logistic growth and decay function to estimate the dose-dependent effect of the pomegranate extract (0, 5.7, 11.4, and 22.8 mg/mL) on L-carnitine metabolism to γ-BB and TMA. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and a pomegranate extract (0, 5.7, 11.4, or 22.8 mg/mL). Samples were collected at multiple time points over 48 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards. Experimental data were fitted to nonlinear models, with curve parameters detailed in* ***Appendix 1, Supplementary Figure S3****. This table has been generated with support from Dr George Savva.*

2.5.5 γ-BB is converted to TMA in the absence of L-carnitine

It was demonstrated that the metabolism of γ-BB to TMA can take place in the absence of L-carnitine (**Figure 2.6**), under the condition that the donated faecal sample is also able to fully convert L-carnitine to TMA (i.e., the faecal sample contains TMA-producing microbes) (**Figure 2.6A-B**), as not every donation contains the microbial environment to

conduct the second step of the metabolic pathway (**Figure 2.6C-D**). The observed decline in γ-BB concentrations corresponds to the increase in TMA (**Figure 2.6B**), where TMA was only produced when the γ-BB concentration declined. However, the faecal inoculum of one donor was able to convert γ-BB to TMA on one occasion (**Figure 2.6A**), but not on another occasion (**Figure 2.6C**). The inconsistency demonstrates that intra-individual variation in TMA production exists within donors, with TMA producing microbes being present on one occasion but not on the other occasion.

When L-carnitine and γ-BB were both present, the concentration of γ-BB accumulated (**Figure 2.6A, C**). In the presence of TMA-producing microbes, the concentration of γ-BB declined after 10 hours (**Figure 2.6A**), and TMA started to appear (**Figure 2.6B**). In the absence of TMA-producing microbes, the concentration of γ-BB continued to accumulate (**Figure 2.6C**) as no TMA was being produced (**Figure 2.6D**).

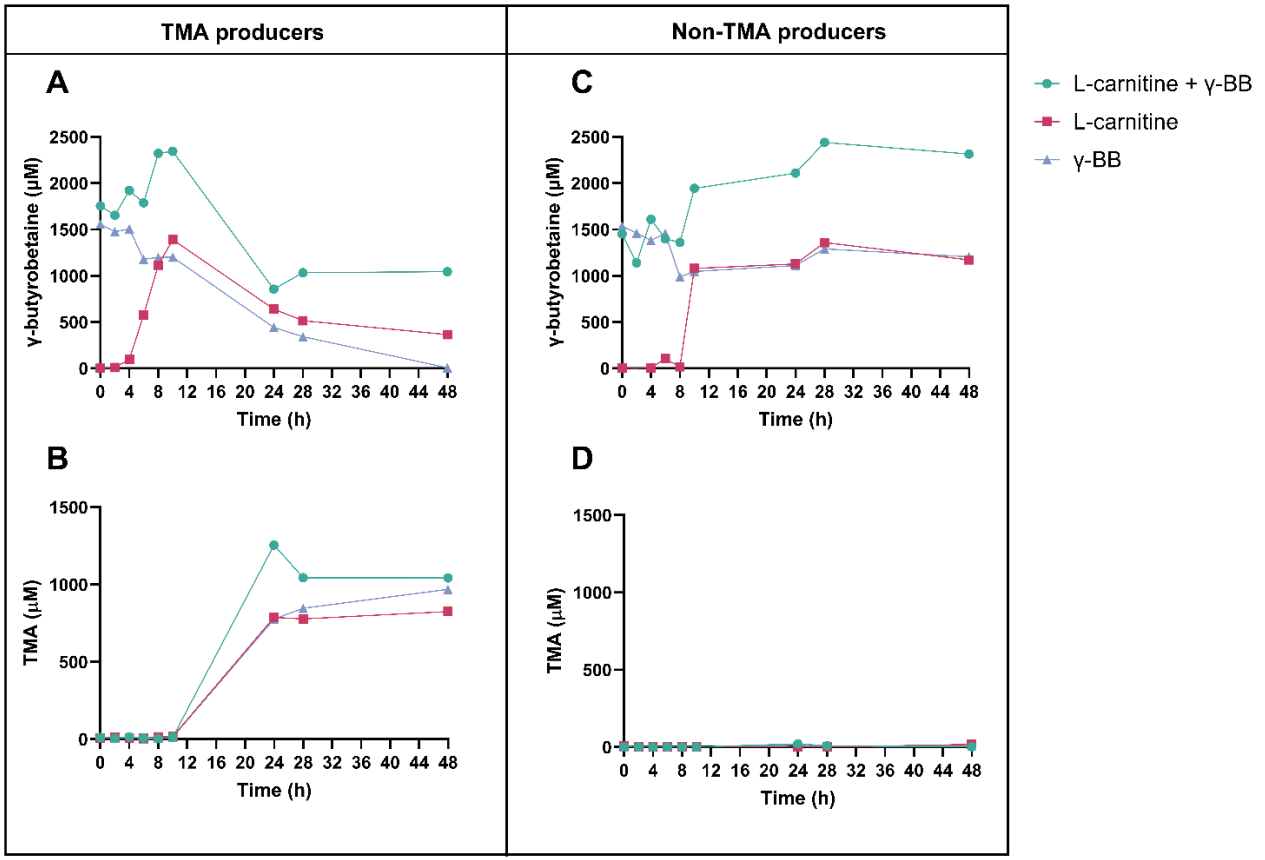

Figure 2.6. γ-butyrobetaine (γ-BB) concentrations in the in vitro batch colon models inoculated at baseline with L-carnitine, γ-BB, or both. *Average concentrations of (**A**) γ-BB and (**B**) TMA across two TMA-producing donors, and (**C**) γ-BB and (**D**) TMA across two non-TMA producers, comparing colon model vessels inoculated with L-carnitine and/or γ-BB over 48 hours. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine and/or 2mM γ-BB. Samples were collected at multiple time points over 48 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

2.5.6 The pomegranate extract inhibits the conversion of γ-BB to TMA

The effect of the pomegranate extract on the conversion of γ-BB to TMA was investigated using *in vitro* batch colon models inoculated with faecal donations from the TMA-producing donors shown in **Figure 2.6A-B**.

The inoculation of γ-BB with the pomegranate extract resulted in the complete inhibition of TMA production (**Figure 2.7**). This illustrates that the pomegranate extract not only inhibits the conversion of L-carnitine to γ-BB (and subsequently TMA) but also directly inhibits the conversion of γ-BB to TMA.

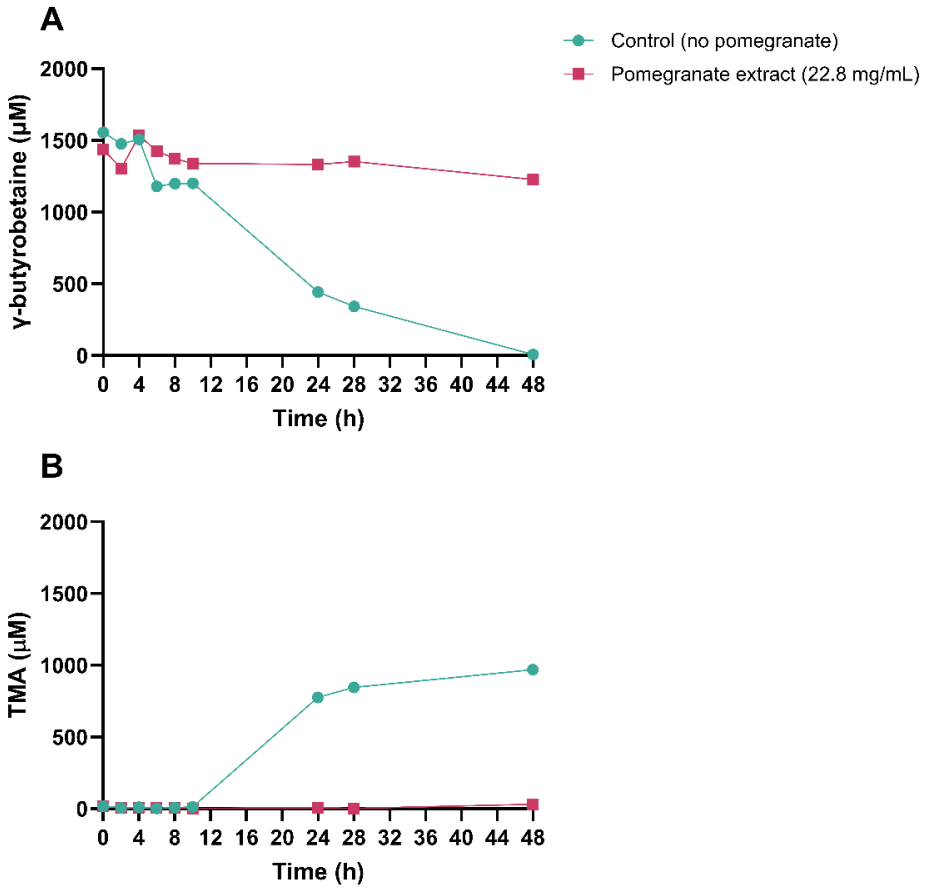

Figure 2.7. The effect of the pomegranate extract (22.8 mg/mL) on the metabolism of γ-butyrobetaine (γ-BB) to trimethylamine (TMA). *Average concentrations of (**A**) γ-BB and (**B**) TMA across two TMA-producing donors over 48 hours. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM γ-BB, and a pomegranate extract (0 or 22.8 mg/mL). Samples were collected at multiple time points over 48 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

2.5.7 Inter- and intra-individual variation in TMA production across donors

TMA production from L-carnitine is highly variable between donors (**Figure 2.8**). When *in vitro* batch colon models were inoculated with 2 mM L-carnitine, the production of TMA at 48 hours after inoculation varied between 0 to 2,214 µM. This is likely attributed to the individual differences in gut microbiota composition and ability to produce TMA from L-carnitine. Specifically, differences may exist due to the absence or presence of microbiota that contain genes responsible for the metabolism of γ-BB to TMA, such as those containing the *gbu* operon, which has only been defined in a small number of human gut microbes [70, 71].

In addition to the interindividual variation in TMA production between donors, intra-individual variation was also demonstrated within the donors that were tested on multiple occasions using a different faecal donation (**Figure 2.8**).

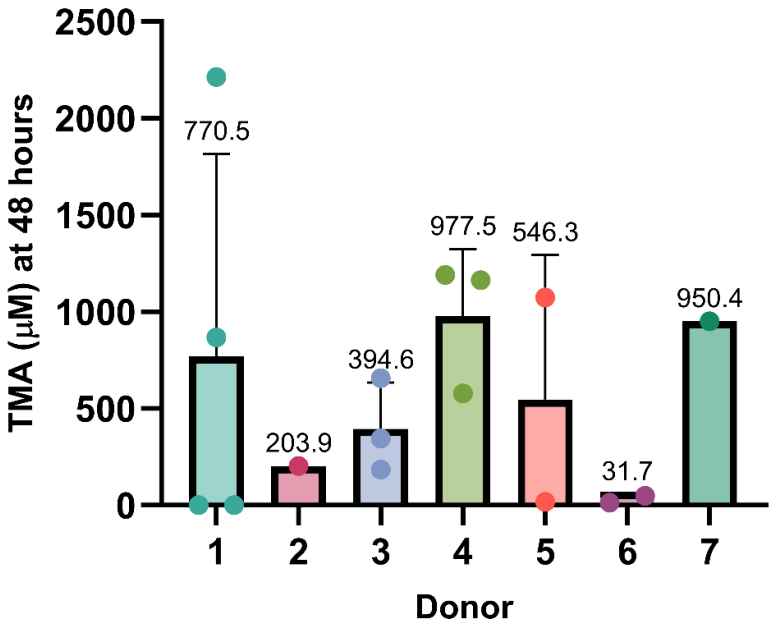

Figure 2.8. The production of trimethylamine (TMA) across 7 donors at 48 hours after inoculation with 2 mM L-carnitine in in vitro batch colon models under control (no pomegranate) conditions. *Data are combined from several experiments conducted by the author, Dr Priscilla Day-Walsh and Dr Emad Shehata, and are presented as mean ± SD where there were replicates. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine. Samples were collected at 48 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

2.5.8 The pomegranate extract inhibits the production of crotonobetaine

Crotonobetaine concentrations were only produced in small amounts (**Figure 2.9**), with a peak of 5.3 µM (± 3.5) detected at 6 hours after inoculation with L-carnitine. The pomegranate extract significantly inhibited the production of crotonobetaine.

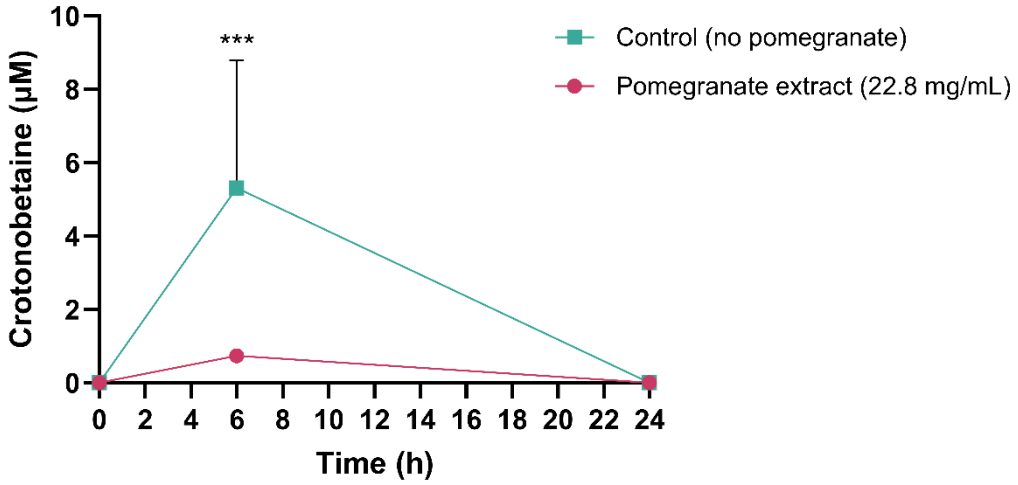

Figure 2.9. Crotonobetaine concentrations in in vitro batch colon models from a single faecal donation treated with a pomegranate extract and 2 mM L-carnitine. *Trajectory of crotonobetaine concentrations among three replicates of a single donor are shown, comparing colon model vessels treated with the pomegranate extract (22.8 mg/mL) to control vessels (no pomegranate) over 24 hours. Data are presented as mean ± SD. Statistical analysis was performed using a Student's t-test at each time point to compare treatments (control or pomegranate extract) (*** P < 0.001). In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and a pomegranate extract (0 or 22.8 mg/mL). Samples were collected at 0, 6, and 24 hours and stored at -80 °C until LC-MS/MS quantification.*

2.6 Discussion

2.6.1 Main findings

This chapter investigated the potential of a polyphenol-rich pomegranate extract to inhibit the microbial metabolism of L-carnitine to TMA using an *in vitro* batch colon model. Some reports prior to this thesis suggested that polyphenols and polyphenol-rich extracts may inhibit the formation of TMA [89-93, 95, 96, 98, 101], but the body of

evidence is limited and the effects of many polyphenols, including pomegranate polyphenols, had not been studied in pH-controlled colon models. Here it was shown that a polyphenol-rich pomegranate extract reduced the production of γ-BB and TMA from L-carnitine in a dose-dependent manner. The inhibition was largely consistent across donors and the application of logistic growth and decay models validated the dose-dependent effects of the pomegranate extract on L-carnitine metabolism. As the concentration of the extract increased, the inflection points for both L-carnitine utilisation and γ-BB production were progressively delayed. Notably, at the highest extract dose, the time required to reach 50% of maximum change (IC_{50}) was significantly extended: L-Carnitine depletion was delayed by 16 hours, and γ-BB accumulation was delayed by 19 hours compared to the untreated control. Additionally, it was demonstrated that a pomegranate extract inhibited not only the metabolism of L-carnitine to γ-BB, but also independently inhibited the metabolism of γ-BB to TMA and L-carnitine to crotonobetaine.

Several studies have shown that consuming dietary polyphenols or polyphenol-rich foods can lead to reductions in both TMA production and circulating TMAO levels. These studies vary in approach, where some focus on individual polyphenols [92, 94], others use polyphenol-rich extracts [93-95, 98, 99], and a number investigate whole foods that are naturally high in polyphenols [89, 100, 102]. Each of these approaches has its advantages and limitations. For instance, using whole foods maintains the integrity of the food matrix, which can offer more realistic insights into dietary effects. However, this also presents challenges: (i) creating a matched control that differs only in polyphenol content, and (ii) the influence of macronutrients on the gut microbiome and conditions in the colon (e.g., pH, production of gases). A study by Bresciani *et al.* (2018) illustrated the limitations of the use of whole foods. This study compared the effects of various fruit juices, including pomegranate juice, on TMA production from L-carnitine [89]. They found that sugar content, likely due to its impact on pH, played a major role in the observed TMA-reducing effects. To minimise confounding factors, the study described in this chapter used a pomegranate extract rather than whole juice and closely monitored pH in the *in vitro* batch colon model. As the pH remained stable throughout the experiment, the reduction in TMA levels cannot be explained by acidification caused by the extract.

There are several possible ways in which the pomegranate extract could exert the effects observed in this chapter. Firstly, it is possible that the polyphenols in the extract interfere

with the metabolism of L-carnitine by influencing the composition or activity of the gut microbiota. Polyphenols are known to have both antimicrobial and growth stimulating effects [149]. This gave rise to the term 'duplibiotics' to describe the simultaneously anti- and prebiotic effects observed for polyphenols [150]. The impact of polyphenols on the gut microbiota depends on both the specific compound and the microorganism in question. The extract may selectively suppress certain gut microbial populations responsible for converting L-carnitine to γ-BB and TMA. Pomegranate-derived polyphenols have demonstrated inhibitory effects on several gut pathogens including *Escherichia coli* [151], which carries the *cai* operon needed for L-carnitine metabolism [152]. Conversely, pomegranate polyphenols have also been shown to support the growth of beneficial microbes such as *Bifidobacterium* and *Lactobacillus* species, as well as *Akkermansia muciniphila* [106, 153]. These growth-promoting effects are likely due to microbial metabolism of polyphenols, releasing bioavailable compounds such as glucose and gallic or ellagic acid [149, 154]. Alternatively, the pomegranate extract may reduce total viable bacterial counts, resulting in the observed reduction in L-carnitine metabolism.

Secondly, the extract might influence the expression of microbial genes involved in L-carnitine and γ-BB metabolism. This regulation could occur at the transcriptional or translational level. Polyphenols and their microbial metabolites can interact with transcription factors or enzymes that regulate gene activity [155, 156]. Possibly, the antioxidant properties of polyphenols might also affect the redox status (i.e., the balance between free radicals and antioxidants) in microbial cells. The maintenance of redox status plays a central role in regulating microbial metabolism [157]. Alterations in redox status in microbial cells can modulate gene expression via structurally responsive (i.e., structural changes in response to a signal, enabling a functional shift) redox-sensor transcription factors [158]. The presence of polyphenols might have shifted the expression or functionality of enzymes involved in the L-carnitine metabolic pathway, due to alterations in the redox status within microbial cells.

Finally, pomegranate polyphenols may directly disrupt the activity of microbial proteins that are involved in the metabolism of L-carnitine and γ-BB, including membrane antiporters/transporters or intracellular enzymes. Some polyphenols are capable of binding to the outer regions of membrane proteins, such as porins, thereby altering membrane permeability and potentially restricting substrate access to microbial enzymes [159, 160]. By changing how easily L-carnitine or γ-BB can enter the microbial

cell or can interact with intracellular enzymes, polyphenols may reduce the efficiency of TMA production.

To the best of the author's knowledge, the pomegranate extract is the most effective dietary intervention reported to date to reduce the production of γ-BB and TMA from L-carnitine by human faecal microbiota. However, in this chapter the findings were demonstrated only in an *in vitro* model of the colon. In a study by Iglesias-Carres *et al.* (2021), which also utilised an *in vitro* colon model, it was shown that chlorogenic acid and gallic acid could reduce the microbial production of TMA, but from choline [90]. In the research presented in this chapter, however, it remains unclear which specific constituent(s) of the pomegranate extract are responsible for the inhibitory effect. The observed effects may be due to the polyphenols present in the pomegranate extract, such as punicalagin, punicalin, and ellagic acid, or due to the subsequent microbial metabolites: urolithins. In the human gut, punicalagin is first converted to punicalin and subsequently to ellagic acid [107]. Within the colon, ellagic acid is converted to various urolithins by the gut microbiota (**Figure 1.4**) [107, 109, 110]. Urolithins have been associated with microbiota-modulating properties, acting as prebiotics for certain microbial species while inhibiting others [106]. Alternatively, the observed effects may be driven by the soluble fibre gum Arabic, which was used to spray dry the pomegranate extract. Dietary fibres can be metabolised by gut microbiota and influence microbial growth and metabolic activity [161]. Research has shown that dietary fibres can inhibit the microbial conversion of several nutrients into TMA [162]. In mice, high soluble fibre diets resulted in a 40.6 and 62.6% reduction of TMA and TMAO, respectively [163]. A beneficial shift was observed towards increased numbers of beneficial gut microbiota in the mice after the administration of dietary fibre, which was particularly obvious after the administration of soluble dietary fibre. Therefore, it is important to investigate the effects of the individual pomegranate extract's constituents on L-carnitine metabolism to TMA.

As far as the author is aware, this is the first report of a dietary intervention to directly inhibit the second step of L-carnitine to TMA metabolism (γ-BB to TMA). In a study by Koeth *et al.* (2019) the direct inhibition of the second metabolic step using oral antibiotics was investigated in healthy participants [67]. They first tested the effect of an oral dose of d9-γ-BB on the production of d9-TMAO, which resulted in a significant increase of d9-TMAO. However, following a one-week treatment with a broad-spectrum oral antibiotic, the subsequent administration of d9-γ-BB did not lead to a detectable

production of d9-TMAO. In this chapter, similar effects of the pomegranate extract in an *in vitro* model of the colon were observed. In fact, the effects of the pomegranate extract on the second step of metabolism tended to be greater than the effect on the first metabolic step (L-carnitine to γ-BB), although the number of replicates and donors used in the experiments investigating the second metabolic step was limited. Additionally, our finding that the conversion of γ-BB can take place in the absence of L-carnitine is in line with the results reported by Koeth *et al.* (2019).

This chapter showed that small amounts of crotonobetaine were produced from L-carnitine. Previously, it was reported that the production of *trans*-crotonobetaine was slower and significantly less than the production of γ-BB [133], which is in line with our findings. The small concentrations of crotonobetaine produced also confirm that the production of γ-BB from L-carnitine represents the major pathway of microbial L-carnitine metabolism. However, crotonobetaine concentrations at time points between 6 and 24 hours after inoculation were not measured. Therefore, the peak of crotonobetaine production from L-carnitine may have been missed in the analysis. The production of crotonobetaine at 6 hours after inoculation was significantly inhibited by the pomegranate extract, which could be a downstream effect of the inhibition of L-carnitine utilisation.

2.6.2 Limitations

A limitation of this research is that the *in vitro* batch colon models represent fermentation in the distal colon, but do not reflect the metabolism occurring in other parts of the gastrointestinal tract. *In vivo*, components of the pomegranate extract might undergo partial metabolism in the small intestine prior to reaching the colon, potentially resulting in a compositional change of the compounds available for microbial metabolism in the colon. Previous research has indicated that ellagitannins, including punicalagin found in pomegranate, are partially converted to ellagic acid prior to entering the colon [164]. Therefore, the colon model experiments may not accurately reflect the effect of the pomegranate extract *in vivo*.

Another limitation is that there is considerable variation in L-carnitine metabolism, particularly in the second metabolic step, between individual donors, which limited the ability to detect statistically significant effects of the treatments. Furthermore, intra-

individual variation within the donors was observed. A comprehensive pathway screening using the Unified Human Gastrointestinal Genome catalog, carried out by Vital and Heinrich-Sanchez (2023), showed that the abundance of the *gbu* genes (also referred to as *bbu*) in healthy people is less than 0.1% of the total gut microbiome [165]. The *gbu* operon is responsible for the conversion of γ-BB to TMA. Given the low relative abundance of the *gbu* genes in the overall gut microbiome, it is possible that the same faecal inoculum could produce TMA in some instances but not others, despite identical conditions in the *in vitro* batch colon models. Notably, the inter- and intra-individual variation was reduced in colon models treated with the pomegranate extract. This illustrates that the inhibitory effect of the pomegranate extract is consistent between and within individuals.

Although clear dose-response effects were observed for the utilisation of L-carnitine and the appearance of γ-BB, the dose-response effect was less obvious for the appearance of TMA, especially at 48 hours after inoculation. At 48 hours, TMA concentrations were most inhibited by the lowest pomegranate dose. Hormetic responses, i.e. a biphasic dose-response where lower doses have stronger effects than higher doses, are not uncommon in microbiology [166]. Potentially, given that polyphenols can have both antimicrobial and growth stimulating effects [149], higher doses of the extract might stimulate the overall microbial growth, including the growth of microbiota containing the *gbu* operon, while the low dose did not have this capacity.

It should be noted that although the colon models were inoculated with 2 mM L-carnitine, baseline concentrations were measured at 1.5-1.7 mM. Several technical factors may account for this discrepancy. First, there is a brief delay (~30 minutes) between inoculation and sample collection due to practical constraints. Moreover, at the neutral pH of the colon model, L-carnitine may bind to glass and plastic surfaces due to its zwitterionic nature. This could result in a partial loss of L-carnitine as it might adhere to the colon model vessel or the sampling equipment (e.g., stripettes) upon collection. It is unlikely that the observation is due to a matrix-effect, because all samples are matrix matched to the baseline colon model composition. Analytical errors in the LC-MS/MS detection are also unlikely, since internal d9-L-carnitine standards were used for quantification.

2.6.3 Prospective research

The findings presented in this chapter indicate that a pomegranate extract might be able to selectively influence gut microbial pathways involved in the production of TMA, a precursor of the proatherogenic compound TMAO. Therefore, the extract could offer a dietary strategy to reduce the risk of CVD. This research suggested the need for further exploration of the effects of individual pomegranate constituents, such as polyphenols (punicalagin, punicalin, and ellagic acid), urolithins, and gum Arabic, on TMA production. Therefore, in **Chapter 3** of this thesis, the pomegranate extract is characterised and its main constituents tested for their effect on L-carnitine metabolism to TMA. Moreover, the research presented in this chapter and the limitations reported here highlight the importance of studies in human participants to validate the *in vitro* results, which has been reported in **Chapter 5** of this thesis.

2.7 Conclusion

This study has shown that a polyphenol-rich pomegranate extract significantly inhibited the microbial metabolism of L-carnitine and γ-BB to TMA *in vitro* and that this effect is dose-dependent. However, large inter- and intra-individual differences in TMA production were observed between and within donors.

Chapter 3

Assessing the effects of individual pomegranate polyphenols on microbial L-carnitine metabolism to trimethylamine (TMA)

Chapter 3: Assessing the effects of individual pomegranate polyphenols on microbial L-carnitine metabolism to trimethylamine (TMA)

3.1 Abstract

Background: In the previous chapter it was shown that a pomegranate extract dose-dependently inhibited the microbial metabolism of L-carnitine and γ-BB to TMA. However, it is not known whether the observed effect is due to a single polyphenol, a combination of polyphenols, or other (non-phenolic) compounds in the extract. Several other polyphenols have been reported for their TMA-reducing effects, but pomegranate polyphenols have not previously been studied for their effect on TMA production.

Aim and approach: The overall aim was to estimate the quantities of the polyphenols in the pomegranate extract and to test the effect on anaerobic L-carnitine metabolism of the individual pomegranate polyphenols, their metabolites (urolithins), and the spray-drying agent gum Arabic which has been used to prepare the extract. This was done in a small-scale, high-throughput *in vitro* colon model. The colon model was inoculated with L-carnitine, individual constituents of the extract (2 mg/mL), and 1% human faecal inoculum. Samples were collected over 48 hours, while continuously measuring pH, and methylamines were quantified using LC-MS/MS with isotopically labelled internal standards. LC-MS/MS was also used for the quantification of the main polyphenols in the pomegranate extract.

Results: The main polyphenols in the pomegranate extract were punicalin (5.2% w/w), punicalagin (5.1% w/w), and ellagic acid (1.1% w/w). Punicalagin, but not other constituents, significantly inhibited the conversion of L-carnitine to γ-BB ($P < 0.001$) and completely inhibited TMA production compared to the control ($P < 0.01$). Furthermore, several treatments substantially reduced the pH in the high-throughput colon model. For the pomegranate extract, which resulted in a pH drop below the optimal range (6.4-7.0), a complete inhibition of L-carnitine metabolism was observed, suggesting that acidification may also inhibit microbial L-carnitine metabolism.

Conclusion: This study is the first to show that punicalagin can completely inhibit the production of TMA from L-carnitine *in vitro*.

3.2 Introduction

In the previous chapter it was shown that a polyphenol-rich pomegranate extract dose-dependently inhibited the microbial metabolism of L-carnitine and γ-BB to TMA in an *in vitro* batch model of the human colon, which has also been published by Haarhuis & Day-Walsh *et al.* (2025) [69]. It is unknown, however, what constituents of the extract exhibited these effects.

The manufacturer (Medinutrex, Catania, Italy) stated that the pomegranate extract was derived from residues of industrial processing of whole pomegranates, such that it contains peels, membranes, and arils. The extract is high in polyphenols (>20% w/w according to the manufacturer), which could be (partially) responsible for the observed inhibitory effect of the extract on L-carnitine metabolism to γ-BB and TMA. Several other polyphenols have already been reported for their TMA-reducing effects *in vitro*, including chlorogenic acid and gallic acid [90], caffeic acid, catechin, and epicatechin [80], and there are also reports of polyphenols that exert TMAO-reducing effects in rodents, such as resveratrol [92] and chlorogenic acid [91]. However, as far as the author is aware, pomegranate polyphenols and urolithins have never been studied in isolation for their effect on TMA and TMAO production. Furthermore, most of these studies used choline as the TMA precursor, which involves a metabolic pathway that is distinct from the metabolic pathway of L-carnitine conversion to TMA. Hence, the effect of polyphenols on TMA production from L-carnitine remains understudied.

The production of the extract also included a spray drying step with gum Arabic [167, 168]. Therefore, the observed effect might be due to the gum Arabic, a soluble fibre which is unlikely to be absorbed in the small intestine. The use of dietary fibre to inhibit TMA production has been reported previously [162]. For instance, a diet high in soluble fibres successfully reduced TMA and TMAO production in mice by 40.6 and 62.6%, respectively [163]. Therefore, it is important to include gum Arabic as one of the potential bioactives that may have contributed to the observed reduction in L-carnitine metabolism when colon models were treated with the pomegranate extract.

The microbial metabolism of pomegranate polyphenols, including ellagitannins and ellagic acid, results in the production of urolithins (**Figure 1.4**). These are the main final pomegranate polyphenol metabolites found in plasma, urine, and faeces, and occur as urolithin A, urolithin B, and isourolithin A in their final forms [107, 169]. Urolithins have

been suggested to be responsible for the health benefits of pomegranates and other ellagitannin-rich foods [170]. Therefore, it is important to test whether the observed effect of the pomegranate extract on TMA production is attributed to urolithins.

To be able to test the individual pomegranate extract constituents, a small-scale, high-throughput model was developed, such that their effects on TMA production could be measured in a manner that is both rapid and inexpensive. Such a method has previously been described by Iglesias-Carres *et al.* (2021) who screened several phytochemicals for their effects on TMA production from choline [90]. However, this model did not include continuous pH monitoring. Furthermore, while the colon model medium described in **Chapter 2** includes glucose, the model described by Iglesias-Carres *et al.* does not. Therefore, further optimisation of the high-throughput colon model was necessary.

This chapter focuses on the identification and quantification of the main polyphenols in the pomegranate extract, such that these can be tested in isolation using a high-throughput, pH controlled, *in vitro* colon model.

3.3 Objectives

The overall aim was to establish which component(s) present in the pomegranate extract, and/or their major colonic metabolites, are responsible for the observed effect of the whole extract on L-carnitine metabolism.

To achieve this aim, the following objectives were established:

1. To characterise the polyphenol profile of the pomegranate extract, including the identification and quantification of the main polyphenols.
2. To assess the microbial metabolism of the polyphenols in the pomegranate extract over 48 hours in the *in vitro* model of the colon described in **Chapter 2**.
3. To optimise a small-scale, high-throughput *in vitro* colon model which is suitable to test the effects of individual (pomegranate) polyphenols on microbial metabolism.
4. To evaluate the effects of the main constituents of the pomegranate extract on the microbial conversion of L-carnitine to γ-BB and TMA over 48 hours.

3.4 Materials and methods

3.4.1 Materials

The pomegranate extract (Dermogranate®) was purchased from Medinutrex (Catania, Italy). Solvents were at least of high-performance liquid chromatography (HPLC) grade. Water was 18 MΩ·cm Milli-Q. Trimethylamine hydrochloride (CAS 593-81-7), γ-BB (commercially known as 3-(Carboxypropyl)trimethylammonium chloride, CAS 6249-56-5), urolithin A (CAS 1143-70-0), urolithin B (CAS 1139-83-9), chlorogenic acid (CAS 327-97-9), gallic acid (CAS 149-91-7), ellagic acid (CAS 476-66-4), Folin & Ciocalteu's phenol (FC) reagent, TCA, dimethyl sulfoxide (DMSO), glacial acetic acid, and HFBA were obtained from Merck. L-Carnitine (CAS 541-15-1), ammonium acetate, piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), and all solvents were obtained from Fisher Scientific Limited. Isotopically labelled standards were obtained from Cambridge Isotope Laboratories (L-carnitine-(trimethyl-d9), CAS 126827-79-0), LGC Standards (trimethylamine-d9 hydrochloride, CAS 18856-86-5), and Santa Cruz Biotechnology (γ-Butyrobetaine-d9, CAS 479677-53-7). Punicalagin was obtained from BOC Science (CAS 65995-63-3) and punicalin from Apollo Scientific (CAS 65995-64-4). Gum Arabic (CAS 9000-01-5) was provided by Willy Benecke (Hamburg, Germany).

3.4.2 Characterisation of the pomegranate extract using LC-TOF and LC-MS/MS

50 mg of pomegranate extract was dissolved in 1 mL 70% v/v aqueous methanol, shaken for one hour, centrifuged at maximum speed for 10 minutes, and filtered through a 0.22 µm filter. To identify which polyphenols are present in the extract, the extract was analysed using the Waters SYNAPT G2-Si instrument, which combines Ultra Performance Liquid Chromatography (UPLC) and Time-of-flight mass spectrometry (TOF-MS). The mobile phase consisted of distilled water with 1% formic acid (solution A) and acetonitrile with 1% formic acid (solution B). The metabolites were separated using an Acquity Premiere HSS T3 C18 1.8 µm, 100 x 2.1 column (Waters, Wilmslow, UK) at 35 °C. The flow rate was 0.4 mL/min, running from 97% of solution A (and 3% of solution B) to 5% of solution A (and 95% of solution B) within 13 minutes, and back to 97% solution A within 2 minutes. In total, the run per sample lasted 15 minutes. The analysis was

performed separately in negative and positive electrospray ionisation (ESI) modes. Analysis of spectra was made using Waters MassLynx software. The phenolic compounds were then identified based on their mass and retention time (RT) as reported in the literature and using the METLIN database [171].

To quantify the most abundant polyphenols in the pomegranate extract (punicalagin, punicalin, ellagic acid, and gallic acid), serial dilutions for each polyphenol standard were prepared in 50% v/v aqueous methanol, each starting at the following concentrations: punicalin at 100 µg/mL, punicalagin and ellagic acid at 50 µg/mL, and gallic acid at 5 µg/mL. Prior to dilution, punicalagin and punicalin were initially dissolved in pure methanol, while ellagic acid was first dissolved in DMSO and ultrasonicated for 45 minutes, followed by dilution in 50% v/v aqueous methanol. For the extract itself, 50 mg of the pomegranate extract was dissolved in 500 µL of DMSO and subsequently diluted 20-fold with 50% aqueous methanol, yielding a final concentration of 500 µg/mL. The pomegranate extract samples were prepared in triplicate and filtered through 0.22 µm PVDF filters before analysis.

Finally, the concentrations of the main polyphenols present in the extract were estimated using the Waters TQ Absolute (Wilmslow, UK), which combines UPLC and triple quadrupole MS. The mobile phase consisted of 0.1% formic acid in distilled water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). 2 µL of each sample was run through a Luna Omega Polar C18 100A column (100 x 2.1 mm; particle size 1.6 µm) at 35 °C and a flow rate of 0.4 mL/min. The gradient was 3% solvent B for 5 minutes, after which solvent B was increased to 20% for 4 minutes, and then further increased to 50% for 2 minutes, lastly solvent B was increased to 95% for 1 minute before it was re-equilibrated to 3% for 2 minutes. In total, each sample ran for 14 minutes. Polyphenols were identified against the matching standard using the RT and m/z ($[M-H]^-$). Peak assignment and quantification were conducted using SYNAPT G2-Si software.

3.4.3 Validation of pomegranate extract polyphenol profile by a third party

The polyphenol content of the pomegranate extract was also estimated by an independent lab in Spain (CEBAS-CSIC in Murcia, Spain, led by Professor Francisco Tomás-Barberán) using HPLC-DAD-MS according to a published method [2]. Briefly, 75 mg of the extract was dissolved in 5 mL of methanol/DMSO/H_2O (40:40:20, v/v/v)

containing 0.1% HCl. The sample was vortexed and filtered through a 0.22 µm PVDF filter prior to analysis using HPLC-DAD-MS. An Agilent 1100 HPLC system, equipped with a photodiode array detector and an ion-trap mass spectrometer detector (Agilent Technologies, Waldbronn, Germany), was used for the analysis. The mobile phase consisted of distilled water with 1% formic acid (solvent A) and acetonitrile (solvent B). 10 µL of sample was run through a Pursuit XRs C18 column (250 mm × 4 mm, 5 µm) (Agilent Technologies, Waldbronn, Germany) at room temperature and a flow rate of 1 mL/min. The gradient was 5-30% solvent B for 20 minutes, after which solvent B increased to 30-55% for 10 minutes, and then further increased to 55-90% for 8 minutes and stayed at 90% for 2 more minutes, before returning again to the initial conditions.

3.4.4 Determination of total polyphenol content in the pomegranate extract using a Folin-Ciocalteu assay

Triplicate samples of the pomegranate extract were prepared at a concentration of 6 mg extract per 1 mL of pre-heated 70% methanol and left at 60 °C for 30 minutes. The solution was then centrifuged at 3,000 rpm for 10 minutes. Additionally, a gallic acid standard curve was prepared in 70% methanol, ranging from 0 to 2,000 µg. 10 µL of each of the samples and standards was added to 50 µL Folin-Ciocalteu reagent, 100 µL 20% sodium carbonate, and 800 µL distilled water. The samples were shaken and then left in the dark at room temperature for 90 minutes. Finally, 200 µL of the samples were transferred into a 96-well plate and measured on a UV-Vis plate reader (Molecular Devices, LLC VersaMax, California, United States) at a wavelength of 760 nm.

3.4.5 Quantification of pomegranate polyphenols in the *in vitro* batch colon model

In vitro batch colon models were set up as described in **Chapter 2** (Section **2.4.2**) and inoculated with the pomegranate extract at 22.8 mg/mL, 2 mM L-carnitine and 1% faecal inoculum from a healthy donor (n = 2). Samples were collected over 48 hours and were stored at -80 °C until the LC-MS/MS analysis.

To prepare the batch colon model samples for LC-MS/MS, they were briefly vortexed and then spun down at 4,000 rpm for 10 minutes. 5 µL sample was added to 25 µL TCA and

left on ice for 5 minutes before 70 µL 0.2 M acetic acid was added. Samples were mixed and 10 µL sample was transferred to a chromacol vial with 90 µL distilled water.

Standards were prepared, containing urolithin A, urolithin B, punicalin, punicalagin, and ellagic acid. To achieve this, a stock was first prepared for each compound in LC-MS grade methanol at a concentration of 1 mg/mL, except for ellagic acid which was prepared in DMSO at 0.5 mg/mL and ultrasonicated for 30 minutes. Then, standards were serially diluted in 0.2 M acetic acid. Lastly, 10 µL of each dilution was transferred to a chromacol vial with 90 µL distilled water.

The Waters TQ Absolute (Wilmslow, UK) was used to quantify the most abundant polyphenols in the pomegranate extract (punicalagin, punicalin, ellagic acid, and gallic acid) as described in section **3.4.2**.

3.4.6 Optimisation of a high-throughput *in vitro* colon model to test effects of pomegranate extract constituents on L-carnitine metabolism

A small scale *in vitro* colon model was developed based on a high-throughput fermentation model reported by Iglesias-Carres *et al.* (2021) [90]. The fermentations were performed on a 1.2 mL 96-well plate (LGC Genomics, Cat. No KBS-7001-130) in an anaerobic cabinet which was set at 37 °C. Each well was inoculated with 2 mM L-carnitine and a final concentration of 1% faecal slurry from individual donors (QIB Colon Model Study, Section **2.4.2**). The medium contained peptone water, yeast extract, NaCl, K_2HPO_4, KH_2PO_4, $MgSO_4$, $CaCl_2$, $NaHCO_3$, L-cysteine, bile salts, hemin, Tween80, and vitamin K_1. Additionally, a PIPES buffer (50 mM) was added to the media. The pH was brought to 7.0 using 10 M NaOH at which the PIPES is within its buffering range and highly soluble [172]. After autoclaving, a concentrated stock of sterile filtered D-glucose was added to the media, reaching a final concentration of 0, 0.1 or 1.0%. Finally, the pH was measured again and adjusted accordingly with filtered 0.5 M HCl or 0.5 M NaOH to reach a pH of 7.1 and left in an anaerobic cabinet overnight to reduce the oxygen.

Prior to faecal inoculation, 792 µL of autoclaved, pH stable medium was added to each well. The wells were then inoculated with 90 µL fresh faecal inoculum (1% final concentration of faecal matter), together with 2 mM L-carnitine and the treatment (at 2 mg/mL, dissolved in PBS): punicalagin, punicalin, ellagic acid, gallic acid, gum Arabic, urolithin A, or urolithin B. Additionally, an L-carnitine control was prepared, containing

the faecal inoculate and L-carnitine but without any of the pomegranate constituents. A faecal control was also included, containing the faecal inoculate but no L-carnitine nor pomegranate constituents. Samples for microbial metabolite analyses were taken at over 48 hours after inoculation and stored at -80 °C directly after collection until LC-MS/MS analysis.

The pH of the high-throughput *in vitro* colon model was continuously monitored. At 3-9 time points over 48 hours, 75 µL from the wells was transferred into 0.6 mL microtubes and pH was measured using a pH electrode InLab Micro Pro-ISM (Mettler Toledo, Leicester, UK). To avoid removing excessive volumes from individual wells, pH measurements at each time point were taken from alternating replicate well for each donor.

3.4.7 Dosage information

The selected doses of pomegranate extract constituents (2 mg/mL) were based on the manufacturer's Certificate of Analysis (CoA) (**Appendix 3**) stating that the punicalagin and ellagic acid (derivatives) in the extract are at least 7 and 10%, respectively. This corresponds to polyphenol quantities between 1.6-2.3 mg/mL for the high pomegranate dose (22.8 mg/mL) reported in **Chapter 2**. When assuming an estimated colonic volume of 561 mL [139], this corresponds to approximately 1,122 mg polyphenols reaching the colon. A 500 mL pomegranate juice typically provides 2,200-2,560 mg of polyphenols [140-143], meaning that the doses reported in this chapter can be realistically achieved through a 220-255 mL pomegranate juice.

3.4.8 Methylamine quantification using LC-MS/MS

The sample preparation and methylamine (L-carnitine, γ-BB, TMA) quantifications by LC-MS/MS were performed as described in **Chapter 2** (Section **2.4.5**). In brief, targeted analysis was performed with isotopically labelled standards for L-carnitine, γ-BB, and TMA. A 1290 Infinity II LC System was used, coupled to a 6490 Triple Quad LC-MS (Agilent Technologies). The metabolites were separated through an Acquity UPLC BEH C8 1.7µm column (Waters, Massachusetts, United States), using a mobile phase consisting

of 10 mM acetate and 0.05% HFBA in Mili-Q water (solution A) and 10 mM acetate and 0.05% HFBA (dissolved in 50 mL Mili-Q water) in methanol (solution B).

3.4.9 Statistical analyses

Trajectories of L-carnitine and metabolite concentrations, as well as pH measurements, over time are presented as the mean ± SD across donors. Outcomes were considered statistically significant if $P < 0.05$.

Linear mixed models were used to estimate the effects of (pomegranate) polyphenols, urolithins, and gum Arabic on L-carnitine and metabolite concentrations, with treatment (e.g., polyphenols) as fixed effects and random intercepts of donors. Independent models were fitted for each outcome at each time point. The TMA concentrations that appeared in the faecal control models were subtracted from the treatment models.

pH values were analysed using a linear mixed-effects model with fixed effects for treatment, time point, and their interaction, and a random intercept for donor to account for repeated measurements over time. Time was treated as a categorical variable to allow estimation of treatment effects at each specific time point. *Post hoc* pairwise comparisons between each treatment and the control group were conducted at each time point using estimated marginal means (EMMs). P-values were adjusted for multiple comparisons using the Dunnett's test to control for Type I error across comparisons with the control.

All graphs were generated using GraphPad Prism version 10.4.2, and R version 4.4.1 (R Core Team, 2024) in RStudio (Posit Software, PBC, Boston, MA). Statistical analyses were performed using RStudio with ‘lme4’ [145], ‘lmerTest’ [146], and ‘broom.mixed’ [147] packages for mixed modelling and ‘minpack.LM’ [148] for non-linear modelling.

3.5 Results

3.5.1 The phenolic composition of the pomegranate extract

The polyphenols present in the pomegranate extract were measured in an initial, qualitative screening using LC-TOF. The main polyphenols that were identified include punicalin, punicalagin, galloyl-hexahydroxydiphenoyl (HHDP)-glucose, and ellagic acid (**Table 3.1**). Two peaks were measured for punicalin at RTs of 3.75 and 4.06 minutes, and for punicalagin at 5.23 and 5.69 minutes (**Table 3.1, Appendix 2, Supplementary Figure S4**), indicating that the pomegranate extract contains both alpha and beta isomers of punicalin and punicalagin.

Table 3.1. Main polyphenols present in the pomegranate extract. *The pomegranate extract was dissolved in 70% v/v aqueous methanol and shaken for one hour before analysis. Polyphenols were identified using the Waters Synapt G2-Si Q-TOF-MS. The mobile phase consisted of distilled water with 1% formic acid (solution A) and acetonitrile with 1% formic acid (solution B). Samples were run through an Acquity Premiere HSS T3 C18 1.8 µm, 100 x 2.1 column (Waters). Suggested compounds were identified by their mass using the METLIN database. Retention time (RT) was derived from diode array detection (DAD) chromatogram, which can be found in* ***Appendix 2, Supplementary Figure S4*** *along with the Base Peak Intensity (BPI) chromatogram.*

Suggested phenolic compound	RT (min)	$[M-H]^-$
Gallic acid	2.84	169.021
Punicalin α + β	3.70, 3.97	781.055
Punicalagin α + β	5.17, 5.64	1083.06
Galloyl-HHDP-glucoside	6.36	633.074
Ellagic acid	7.56	300.999

Abbreviations: RT, retention time; HHDP, hexahydroxydiphenoyl.

After the identification of the main polyphenols in the pomegranate extract, standards were obtained to quantify gallic acid, ellagic acid, punicalin, and punicalagin. Upon quantification, several peaks were detected on the chromatogram between RT 2.18 and 7.31 minutes, with gallic acid appearing first and ellagic acid last (**Table 3.2**). Again, two peaks were detected for punicalagin (**Appendix 2, Supplementary Figure S5**), while for punicalin a split peak was measured (**Appendix 2, Supplementary Figure S6**), likely corresponding to the presence of a mixture of alpha and beta isomers.

***Table 3.2. Estimated quantities of the main polyphenols in the pomegranate extract**. The main polyphenols were quantified using LC-MS/MS on a Waters TQ Absolute triple quadrupole mass spectrometer. Triplicate samples of the pomegranate extract were dissolved in 5% dimethyl sulfoxide (DMSO) and aqueous methanol (50:50, v/v) prior to analysis. The mobile phase consisted of 0.1% formic acid in distilled water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Samples were run through a Luna Omega Polar C18 100A column. The polyphenols were identified against the matching standard using the retention time (RT) and m/z ([M-H]-).*

Compound	**RT (min)**	**$[M-H]^-$**	**Amount (µg/mg extract)**
Gallic acid	2.18	169	2.7
Punicalin α + β	3.37	781	51.5
Punicalagin α + β	4.81/5.31	1083	50.8
Ellagic acid	7.31	301	11.3

Gallic acid, ellagic acid, punicalin, and punicalagin together make up 11.63% of the total dry weight of the extract (**Figure 3.1**), of which punicalin and punicalagin make up the largest proportion, with 5.15% and 5.08% w/w, respectively. Ellagic acid made up 1.13% of the total weight of the pomegranate extract, while gallic acid only made up a small proportion. These concentrations are lower than what was indicated on the provided Certificate of Analysis (CoA) (**Appendix 3**), which reported that ellagic acid should be present in amounts greater than 10% w/w and punicalagin in amounts greater than 7% w/w. However, the concentration of ellagic acid quantified here is at almost a 10-fold lower concentration (1.13%, w/w) than reported by Medinutrex. The concentration of punicalagin was only slightly lower than reported, contributing to 5.08% w/w of the extract. A significant quantity of punicalin is present in the extract (5.15%, w/w), which has not been specified on the product label (**Appendix 3**).

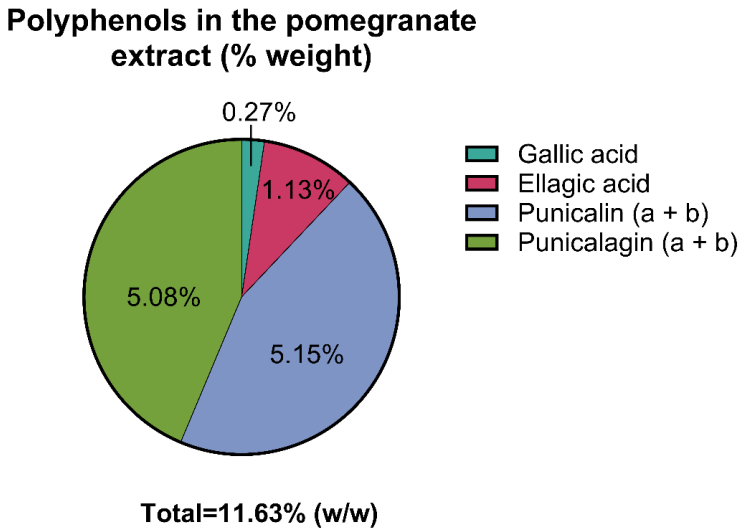

Figure 3.1. Estimates of the main polyphenols in the pomegranate extract as a percentage of the extract's dry weight*. The polyphenols were quantified using LC-MS/MS on a Waters TQ Absolute triple quadrupole mass spectrometer. Triplicate samples of the pomegranate extract were dissolved in 5% dimethyl sulfoxide (DMSO) and aqueous methanol (50:50, v/v) prior to analysis. The mobile phase consisted of 0.1% formic acid in distilled water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Samples were run through a Luna Omega Polar C18 100A column.*

3.5.2 Validation of the polyphenol estimates by a third party

Due to the discrepancy in the reported quantities by Medinutrex and the findings reported here, the results were validated by an independent laboratory at CEBAS-CSIC (Murcia, Spain). This is a world-leading group regarding the analysis of pomegranate polyphenols. CEBAS-CSIC measured multiple ellagic acid and ellagitannin derivatives in the pomegranate extract (**Table 3.3**). The polyphenols appeared on the chromatogram between RT 1.0 and 16.1 minutes, with HHDP-hexoside appearing first and the ellagic acid derivatives last. Two peaks appeared for punicalagin and for punicalin, corresponding to the alpha and beta isomers of both compounds (**Table 3.3**).

At CEBAS-CSIC other ellagic acid forms in addition to the aglycone were detected. Therefore, the quantity of ellagic acid measured by CEBAS-CSIC was slightly higher than the quantity measured by the author, at Quadram Institute Bioscience (QIB) (**Figure 3.2**). The quantity of punicalagin measured at CEBAS-CSIC was slightly higher than at QIB, but the difference was less than 10%. For punicalin, however, the estimated quantity was significantly different between the two analytical labs (**Figure 3.2**).

Table 3.3. Quantification of ellagic acid and ellagitannin (derivatives) in the pomegranate extract by an independent laboratory. *Polyphenols were extracted by dissolving the extract in methanol/dimethyl sulfoxide/water (MeOH/DMSO/H_2O, 40:40:20 v/v/v) containing 0.1% hydrochloric acid (HCl). The sample was vortexed, filtered through a 0.22 µm PVDF filter, and analysed in a HPLC-DAD-MS IT. Analysis was performed by CEBAS-CSIC (Murcia, Spain) and led by Prof Francisco Tomás-Barberán.*

Compound	RT (min)	$[M-H]^-$	Quantity (µg/mg extract)
HHDP-hexoside	1.0	481	4.61
HHDP-hexoside	1.4	481	2.32
Punicalin	1.6	781	5.49
Punicalin	1.8	781	27.12
Pedunculagin I	2.4	783	6.83
Punicalagin α + β	4.3/6.6	1083	55.32
Pedunculagin I	5.1	783	0.74
Pedunculagin I	7.5	783	2.63
Pedunculagin I	8.8	783	1.07
Pedunculagin II	9.3	785	0.80
Pedunculagin I	10.0	783	0.48
Galloyl-HHDP-hexoside	10.4	633	2.59
Ellagic acid hexoside	11.5	463	2.63
Gallagic acid dilactone	12.0	601	6.82
Galloyl-ellagic acid hexoside	14.1	615	0.11
Ellagic acid pentoside	14.3	433	0.06
Ellagic acid	14.8	301	11.55
Dimethyl ellagic acid hexoside	16.1	491	0.35

Abbreviations: RT, retention time; HHDP, hexahydroxydiphenoyl.

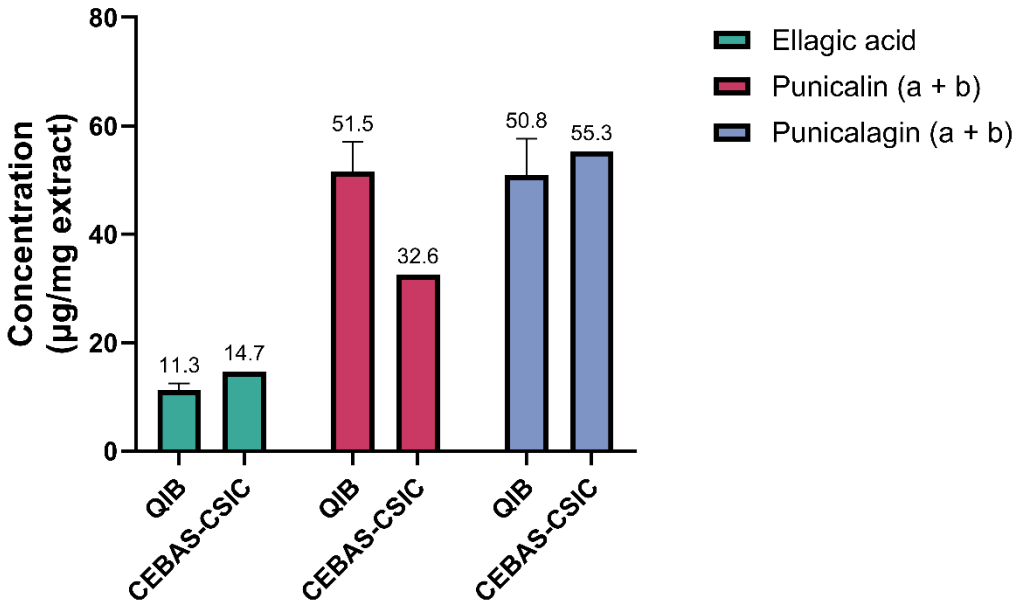

Figure 3.2. Comparison of estimated polyphenol quantities in the pomegranate extract measured by the author at Quadram Institute Bioscience (QIB) and measured by Prof Francisco Tomás-Barberán at CEBAS-CSIC. *The author used LC-MS/MS and dissolved the extract in 5% dimethyl sulfoxide (DMSO) and aqueous methanol (MeOH, 50:50, v/v). The mobile phase consisted of 0.1% formic acid in distilled water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Samples were run through a Luna Omega Polar C18 100A column. CEBAS-CSIC used HPLC-DAD-MS IT and dissolved the extract in MeOH/DMSO/H_2O (40:40:20, v/v/v) containing 0.1% hydrochloric acid (HCl). The mobile phase consisting of distilled water with 1% formic acid (solvent A) and acetonitrile (solvent B). 10 µL of sample was run through a Pursuit XRs C18 column.*

The difference in estimates obtained by the author and by CEBAS-CSIC could be explained by the difference in polyphenol extraction technique, where CEBAS-CSIC used greater volumes of DMSO. Therefore, an FC assay was performed to obtain the GAEs following two different polyphenol extraction techniques which each approximate the extraction techniques used by the author (extraction in aqueous methanol) and by CEBAS-CSIC (extraction in high volumes of DMSO and methanol). The FC assays showed that the estimated GAEs tended to be higher when the pomegranate extract is treated with a mixture of methanol and DMSO (50:50, v/v) (**Figure 3.3**), although this was not statistically significantly different from the extraction with 70% aqueous methanol ($t(4) = 1.582$, $P = 0.189$).

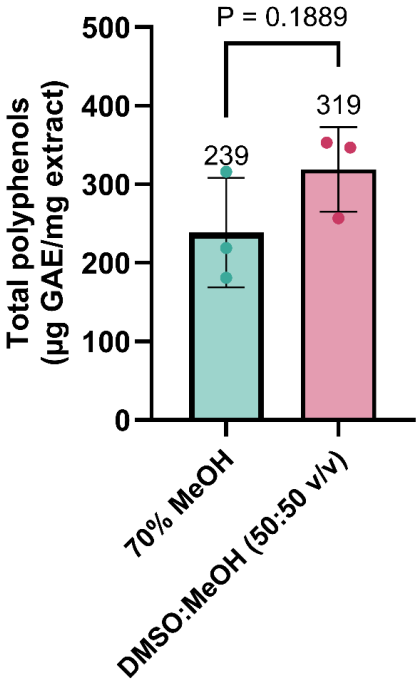

Figure 3.3. Total polyphenols, estimated as gallic acid equivalents (GAEs), in the pomegranate extract following two different extraction methods. *Data are presented as mean ± SD (n = 3). Statistical analysis was performed using an unpaired Student's t-test. The pomegranate extract was left in pre-heated 70% v/v aqueous methanol or DMSO:methanol (50:50, v/v) at 60 °C for 30 minutes. The solution was then centrifuged at 3,000 rpm for 10 minutes and filtered through at 0.45 µm PVDF filter. GAEs were estimated using a Folin-Ciocalteu (FC) assay. A standard curve was prepared in 70% methanol. Samples and standards were added to FC reagent, 20% sodium carbonate, and distilled water. The samples were shaken and then left in the dark at room temperature for 90 minutes. Finally, 200 µL of the samples was transferred into a 96-well plate and measured on a UV-Vis plate reader at a 760 nm wavelength.*

3.5.3 Metabolism of pomegranate polyphenols in the *in vitro* batch colon model

Directly after inoculation of the *in vitro* batch colon models with the pomegranate extract (22.8 mg/mL), the pomegranate polyphenols punicalagin, punicalin and ellagic acid were detected (**Figure 3.4**). At baseline, 4.85% w/w of the extract appeared as punicalagin (1,064 µg/mL), 4.93% w/w as punicalin (1,125 µg/mL), and 1.8% w/w as ellagic acid (415 µg/mL). The quantities measured in the colon models correspond with the quantities that were estimated in the pomegranate extract itself (**Figure 3.1**).

The polyphenols were rapidly metabolised during the first 10 hours (**Figure 3.4**). After 10 hours, the metabolism of the polyphenols slowed down, and their metabolism did not reach completion by 48 hours. Somewhat surprisingly, urolithins did not appear in the *in*

vitro colon models. To test if there is a matrix-effect that masks the presence of urolithins, four colon model samples were spiked with urolithins A and B at 100 parts per million (ppm) and urolithins were quantified using LC-MS/MS. Both urolithins could be accurately quantified (**Appendix 2, Supplementary Figure S7**), indicating that the absence of urolithins was not due to a matrix-effect but rather due to the absence of urolithin production in the colon models.

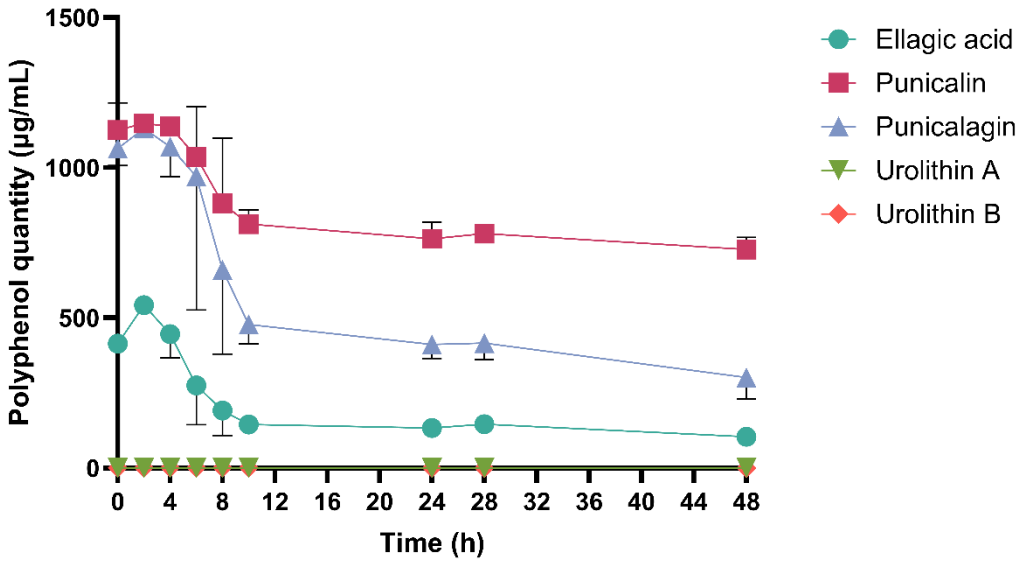

Figure 3.4. Metabolism of polyphenols from the pomegranate extract in in vitro batch colon models over 48 hours. *Data are presented as mean ± SD from two donors. In vitro colon models were inoculated with the pomegranate extract (22.8 mg/mL) and 1% faecal inoculum from a healthy donor. Samples were collected over 48 hours and stored at -80 °C until LC-MS/MS analysis.*

3.5.4 Optimisation of a high-throughput *in vitro* colon model

The aim of initial optimisation experiments of the small-scale, high-throughput *in vitro* colon model, as reported by Iglesias-Carres *et al.* (2021) [90], was to develop a model that reflects the metabolism of L-carnitine as observed in the batch colon models reported in **Chapter 2**. The target pH of the *in vitro* colon model is between 6.4-7.0. This resembles the pH of the transit from the transverse to the distal colon (6.4-6.6 for the transverse colon and 7.0 for the distal colon) [173, 174].

When concentrations of 1% glucose were used, the pH reached suboptimal levels (**Figure 3.5**). The drop in pH was accompanied by a significant reduction in bacterial viability compared to the 0% glucose control at 24 hours (P = 0.031) and 48 hours (P = 0.004),

with <1% of total cells being viable at both time points (**Appendix 2, Supplementary Figure S8**). For the 0.1% glucose treatment, the pH remained within the target range (**Figure 3.5**) and maintained bacterial viability for 24 hours, although bacterial viability slightly reduced compared to the control condition at 48 hours (22% vs. 28%, *P* = 0.037) (**Appendix 2, Supplementary Figure S8**). These data suggest that a high glucose condition (1%) can result in a large pH drop accompanied by a greatly reduced bacterial viability, while 0.1% glucose better reflects the conditions (pH and glucose concentration) of the batch colon model.

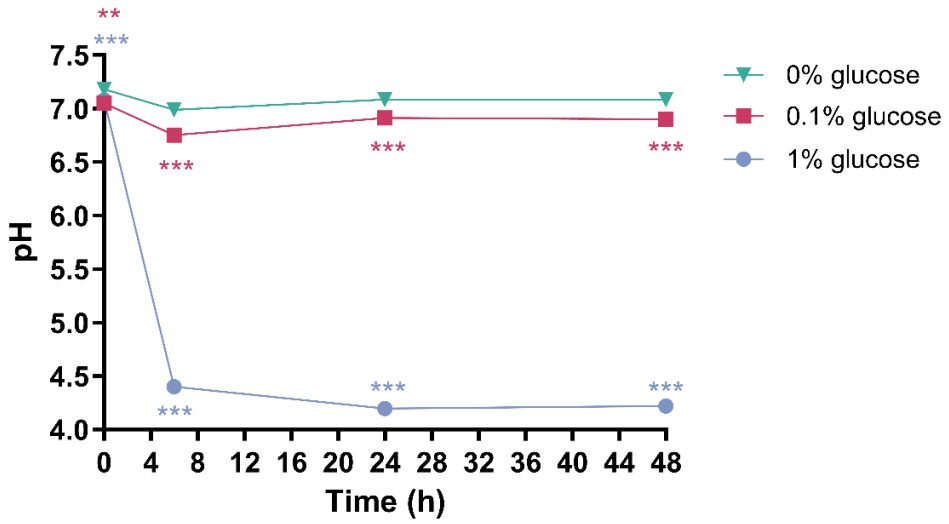

Figure 3.5. pH trajectories in the high-throughput in vitro colon model treated with 0, 0.1, and 1% glucose over 48 hours. *Data are presented as mean of three replicates from a single donor (SD too small to observe). Statistical analysis was performed using linear mixed models comparing glucose treatments to control (0% glucose), with treatment as fixed effects and random intercepts of biological replicates (** P < 0.01, *** P < 0.001). In vitro colon models were inoculated with glucose (0, 0.1, or 1%) and 1% faecal inoculum from a healthy donor. Samples were collected over 48 hours and pH was directly measured using a pH electrode InLab Micro Pro-ISM.*

Using the optimised colon model containing 0.1% glucose, the reproducibility of previously reported TMA-reducing polyphenols (chlorogenic acid and gallic acid [90]) was tested. Chlorogenic acid and gallic acid did not have a significant effect on TMA production by 48 hours (**Figure 3.6**), although chlorogenic acid significantly inhibited the metabolism of γ-BB by 48 hours (47.6, 95% CI: 5.22 to 89.98, *t*(17.45) = 2.37, *P* = 0.030). The reduction in γ-BB metabolism might be because chlorogenic acid reduced the pH to the lower end of the target range (**Figure 3.10**) which was significantly lower than the control treatment at later time points (**Table 3.4**).

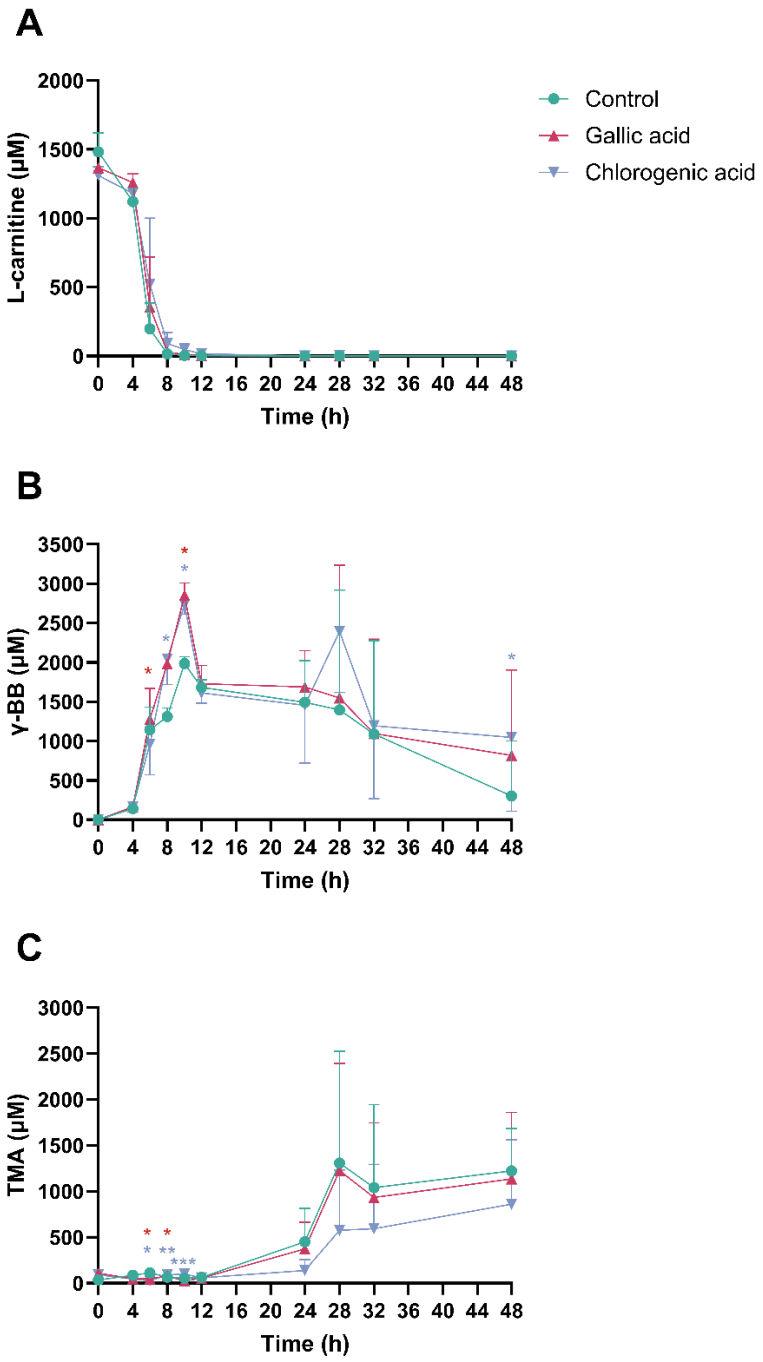

Figure 3.6. L-Carnitine, γ-butyrobetaine (γ-BB), and trimethylamine (TMA) concentrations in the high-throughput in vitro colon model treated with chlorogenic or gallic acid (2 mg/mL). *Average concentrations of (**A**) L-carnitine, (**B**) γ-BB, and (**C**) TMA are shown across two donors, comparing treatments of chlorogenic acid or gallic acid (2 mg/mL) to a control condition at each time point over 48 hours. Data are presented as mean ± SD. Statistical analysis was performed using linear mixed models, with treatment (chlorogenic acid, gallic acid, or control) as fixed effects and random intercepts of donors (* P < 0.05, ** P < 0.01, *** P < 0.001). High-throughput in vitro colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and the treatment (pomegranate extract, punicalagin, or neither). Samples were collected at multiple time points over 48 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

Next, the reproducibility of findings from the *in vitro* batch colon model reported in **Chapter 2** was tested. Using the high-throughput colon model, the pomegranate extract completely inhibited L-carnitine metabolism to γ-BB and no TMA was accumulated (**Figure 3.7**). This is likely due to a significant drop in pH to a suboptimal level of 4.9 by 48 hours, well below the target range of 6.4-7.0, within the pomegranate extract-treated conditions (**Figure 3.10, Table 3.4**). However, a drop in pH below a mean of 6.4 only occurred when the model was treated with the pomegranate extract. For treatments with isolated polyphenols the mean pH did not drop below the target range of 6.4-7.0 (**Figure 3.10**), indicating that the model is suitable for experiments using isolated polyphenols but not for experiments using the pomegranate extract.

The metabolism of L-carnitine under untreated conditions was slightly faster in the high-throughput model, with L-carnitine being metabolised between 10-12 hours (**Figure 3.7**), compared with the batch colon model described in **Chapter 2**, where L-carnitine was completely metabolised within 12-20 hours (**Figure 2.2**). This observation is likely due to the difference in glucose concentrations between the models: 1% in the batch model and 0.1% in the high-throughput model.

Individual trajectories showed that only one of two donors produced TMA (**Appendix 2, Supplementary Figure S9**). For this donor (Donor 2), punicalagin tended to have an inhibitory effect on TMA production, although a dose-dependent effect was not observed.

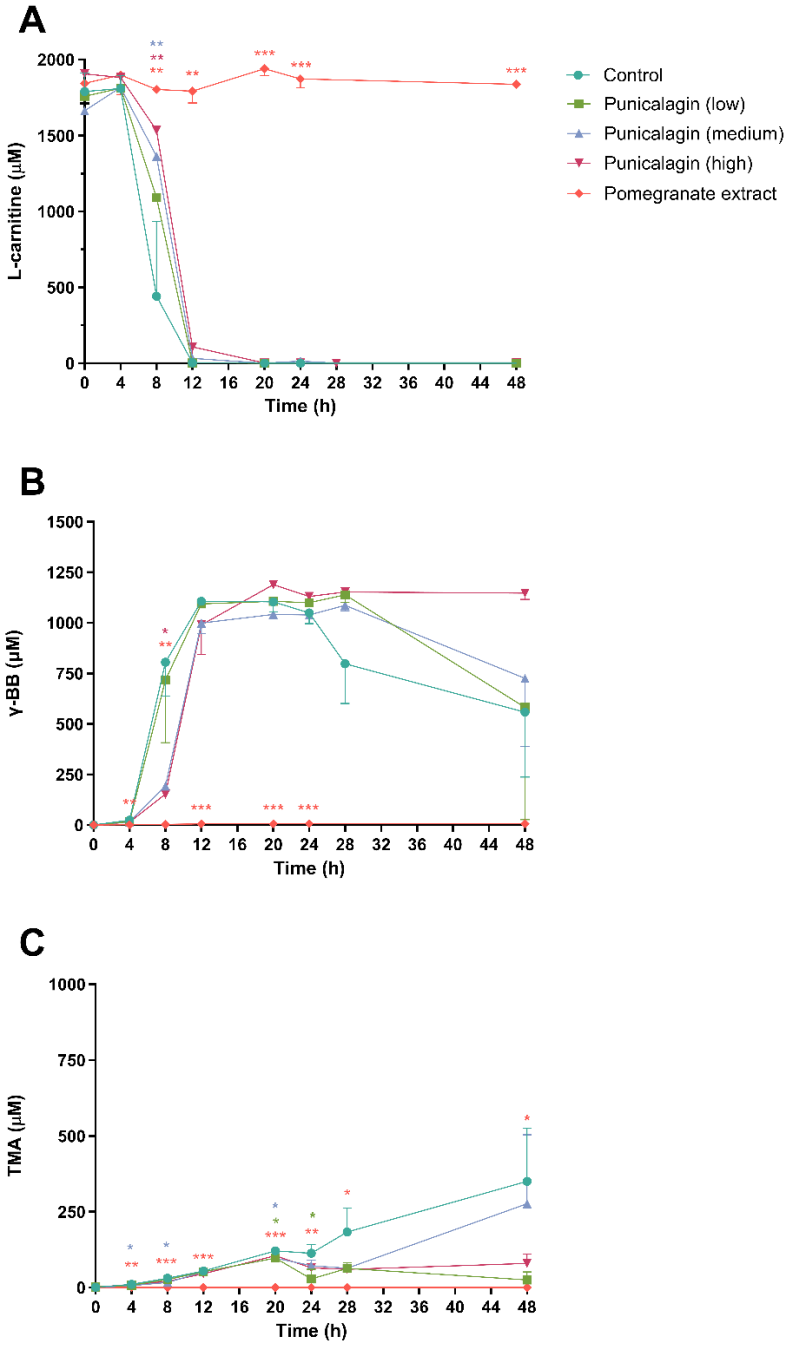

Figure 3.7.L-Carnitine, γ-butyrobetaine (γ-BB), and trimethylamine (TMA) concentrations in the high-throughput in vitro colon model treated with a pomegranate extract and punicalagin at increasing doses. *Average concentrations of (**A**) L-carnitine, (**B**) γ-BB, and (**C**) TMA are shown across two donors, comparing treatments with the pomegranate extract (22.8 mg/mL) and increasing doses of punicalagin (0.4, 0.8, 1.6 mg/mL) to a control condition at each time point over 48 hours. Data are presented as mean ± SD. Statistical analysis was performed using linear mixed models, with treatment (punicalagin, pomegranate, or control) as fixed effects and random intercepts of donors (* P < 0.05, ** P < 0.01, *** P < 0.001). High-throughput in vitro colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and the treatment (pomegranate extract, punicalagin, or neither). Samples were collected at multiple time points over 48 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

3.5.5 The effects of the pomegranate extract constituents on L-carnitine metabolism

After testing the suitability of the high-throughput colon model for experiments with isolated polyphenols, further experiments were conducted using the main constituents of the pomegranate extract (ellagic acid, punicalin, punicalagin, and gum Arabic), as well as the microbial metabolites of ellagitannins and ellagic acid, urolithin A and urolithin B.

Some differences in starting concentrations between the treatments were found, and therefore the metabolic trajectories are shown as the percentage from the starting concentration of the substrate (L-carnitine).

Optimisation experiments for the high-throughput model already showed a potential dose-response effect for punicalagin on the metabolism of L-carnitine to γ-BB and TMA (**Figure 3.7**). In line with earlier observations, a significant inhibitory effect of punicalagin on L-carnitine metabolism was observed in subsequent experiments (**Figure 3.8**). Punicalagin inhibited the conversion of L-carnitine to γ-BB, with a 43.6 and 23.6 percentage point increase in remaining L-carnitine at 8 and 10 hours, respectively, compared to the control ($P < 0.001$). Moreover, punicalagin almost completely inhibited TMA production, with 0.4% TMA production from L-carnitine at 48 hours, compared to 39.2% TMA production for the control ($P < 0.01$). These data show that punicalagin is particularly effective on the second step of the L-carnitine → γ-BB → TMA pathway. The effect of punicalagin was consistent across all individual donors (**Appendix 2, Supplementary Figure S10**).

Urolithin A and B did not have a significant effect on TMA production from L-carnitine (**Figure 3.9**). At 28 hours urolithin A showed a higher TMA production than the control condition ($P < 0.05$). This indicates that there is no inhibitory effect for urolithins on the metabolism of L-carnitine to γ-BB and TMA.

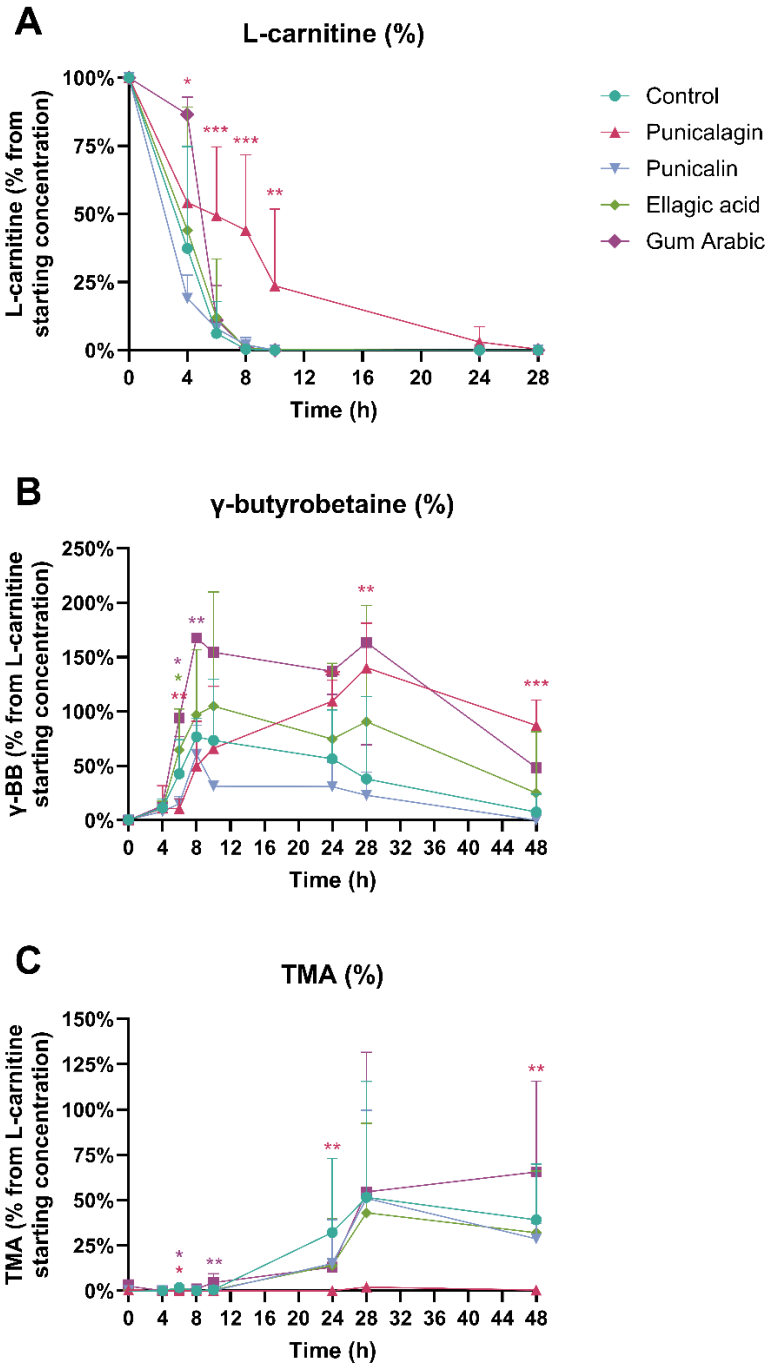

Figure 3.8. Effects of the pomegranate polyphenols and gum Arabic at 2 mg/mL on L-carnitine metabolism in vitro. *Average percentages of (**A**) L-carnitine, (**B**) γ-butyrobetaine (γ-BB), and (**C**) trimethylamine (TMA) from L-carnitine starting concentration are shown over 48 hours. Data are presented as mean ± SD from 2-5 donors with 1-4 replicates each. Statistical analysis was performed using linear mixed models, with treatment (punicalagin, punicalin, ellagic acid, gallic acid, gum Arabic, or control) as fixed effects and random intercepts of donors (* P < 0.05, ** P < 0.01, *** P < 0.001). High-throughput in vitro colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine. Samples were collected at multiple time points over 24 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

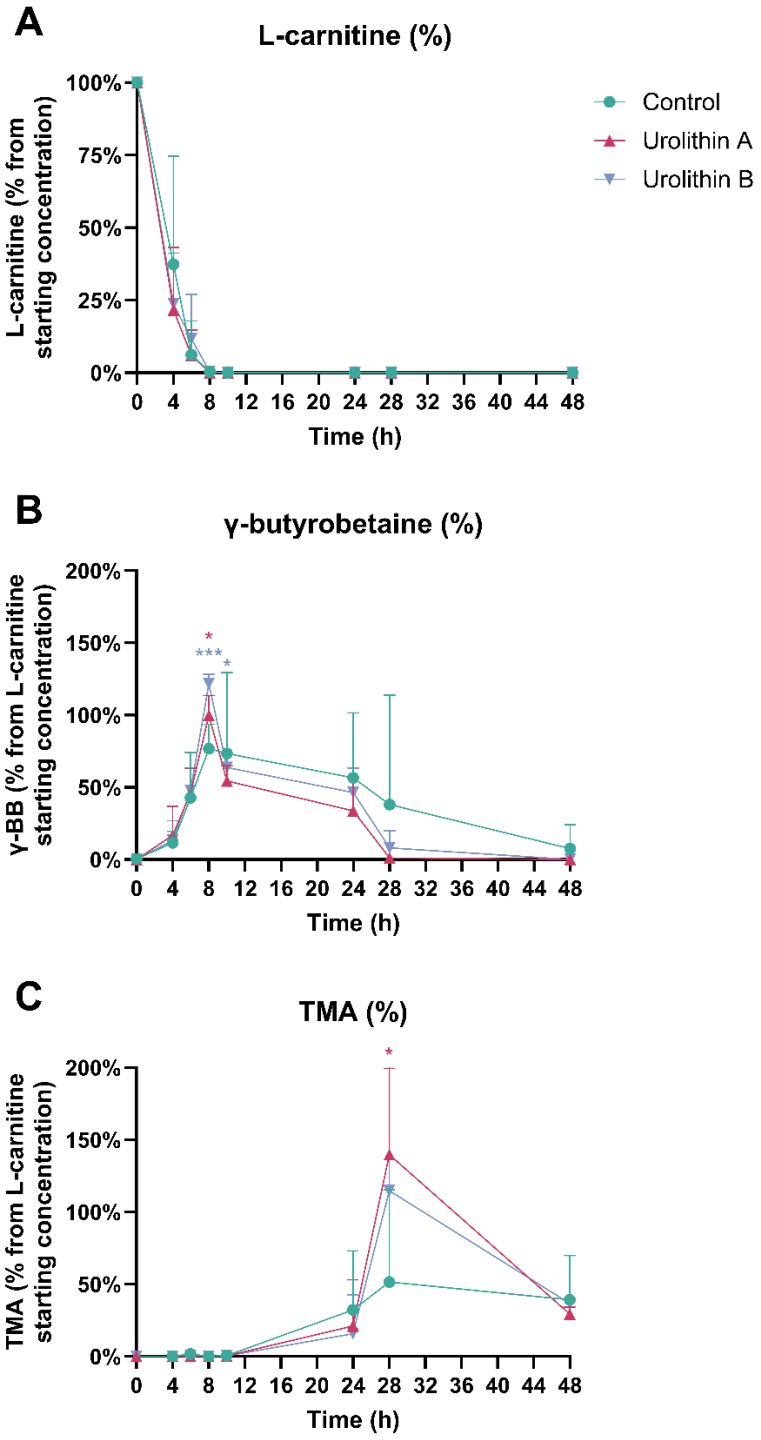

Figure 3.9. Effects of urolithins A and B at 2 mg/mL on L-carnitine metabolism in vitro*. Average percentages of (**A**) L-carnitine, (**B**) γ-butyrobetaine (γ-BB), and (**C**) trimethylamine (TMA) from L-carnitine starting concentration are shown over 48 hours. Data are presented as mean ± SD from 3-5 donors with 1-4 replicates each. Statistical analysis was performed using linear mixed models, with treatment (urolithin A, urolithin B, or control) as fixed effects and random intercepts of donors (* P < 0.05, ** P < 0.01, *** P < 0.001). High-throughput in vitro colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine. Samples were collected at multiple time points over 48 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

For most treatments there was an initial 0.2-0.3 drop in pH between baseline and at four hours, except for the urolithins, which started at a lower pH at baseline (**Figure 3.10**). After this initial drop, the pH of all treatments slightly increased and remained stable at around a mean pH of 6.7-6.8, except for gum Arabic, which continued to decrease but did not drop below a mean pH of 6.4.

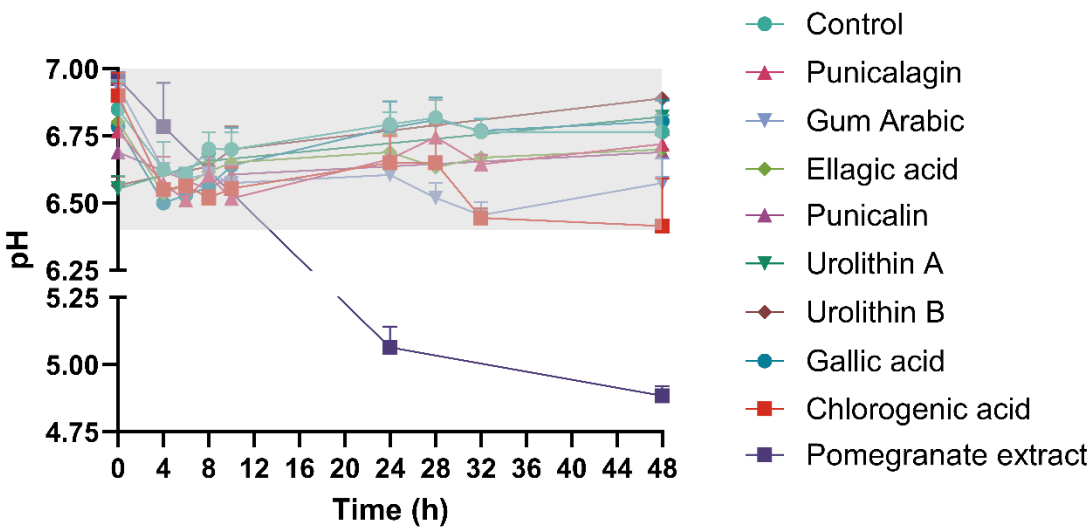

Figure 3.10. Trajectories of the pH in a high-throughput in vitro colon model treated with the pomegranate extract, the different constituents and metabolites of the extract, and the previously tested compounds, chlorogenic and gallic acid, over 48 hours. *The target pH range of 6.4-7.0 is highlighted in grey. Data are presented as mean ± SD of 2-7 donors. High-throughput in vitro colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and one of the treatments: pomegranate extract (22.8 mg/mL), punicalagin (0.4-2 mg/mL), gum Arabic (2 mg/mL), ellagic acid (2 mg/mL), punicalin (2 mg/mL), urolithin A (2 mg/mL), urolithin B (2 mg/mL), gallic acid (2 mg/mL), chlorogenic acid (2 mg/mL), or none (control). Samples were collected over 48 hours and pH was directly measured using a pH electrode InLab Micro Pro-ISM.*

Table 3.4. Estimated pH decrease relative to control at different time points over 48 hours. *Data are presented as the estimated mean pH decrease relative to the control from replicates of 2-7 donors per treatment. Statistical analysis was performed using linear mixed models comparing treatments to control at each time point, with treatment and time as fixed effects and donors as random intercepts (* P < 0.05, ** P < 0.01, *** P < 0.001). High-throughput in vitro colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and one of the treatments: pomegranate extract (22.8 mg/mL), punicalagin (0.4-2 mg/mL), other pomegranate extract constituents (2 mg/mL), or none (control). Samples were collected over 48 hours and pH was directly measured using a pH electrode InLab Micro Pro-ISM.*

Time (h)	0	4	6	8	10	24	28	32	48
Punicalagin	-0.09	- 0.04	- 0.09	-0.10 *	-0.18 **	-0.13 **	-0.07	-0.12	-0.04
Gum Arabic	0.06	- 0.001	- 0.05	-0.09	-0.16	-0.19 **	-0.28 ***	-0.31 ***	-0.17 *
Ellagic acid	-0.04	- 0.06	-0.04	-0.05	-0.05	-0.07	-0.16 *	-0.10	-0.04
Punicalin	-0.18 **	N/A	N/A	-0.15 *	-0.12	N/A	N/A	N/A	-0.07
Urolithin A	-0.31 ***	N/A	N/A	-0.04	N/A	N/A	N/A	N/A	0.06
Urolithin B	-0.30 ***	N/A	N/A	-0.06	-0.03	N/A	N/A	N/A	0.13
Gallic acid	-0.09	- 0.13	- 0.11	-0.14	-0.09	-0.01	0.01	0.01	0.06
Chlorogenic acid	0.03	- 0.08	- 0.07	-0.18 *	-0.18 *	-0.14	-0.15	-0.32 ***	-0.33 ***
Pomegranate extract	0.09	0.16 *	N/A	N/A	N/A	-1.73 ***	N/A	N/A	-1.86 ***

3.6 Discussion

3.6.1 Main findings

The aim of the research presented in this chapter was to establish which component(s) present in the pomegranate extract, and/or their primary microbial metabolites, are responsible for the observed inhibition of L-carnitine metabolism by the pomegranate extract. The main finding reported in this chapter was that, of all the phenolic and non-phenolic components of the pomegranate extract that were tested, only punicalagin inhibited TMA production from L-carnitine.

TMA production was completely inhibited by punicalagin, indicating that punicalagin is the main bioactive in the pomegranate extract responsible for the inhibitory effects of the extract on L-carnitine metabolism reported in **Chapter 2** and published by Haarhuis & Day-Walsh *et al.* (2025) [69]. Punicalagin has gained increasing scientific interest for its many beneficial properties, including beneficial effects on the gut microbiome, such as promoting interactions between gut microbiota and reversing microbial dysbiosis [175]. However, the beneficial effects of punicalagin have been partially attributed to its breakdown products, including ellagic acid and urolithins [169, 176]. In this chapter it was shown that punicalin, ellagic acid, and urolithins did not significantly inhibit L-carnitine metabolism and TMA production, suggesting that the effects observed here are solely attributable to punicalagin.

An important question that remains is how punicalagin inhibits the conversion of L-carnitine to γ-BB and γ-BB to TMA. A number of possible mechanisms has been discussed in **Chapter 2**: a) reduction of the total viable bacterial cells, b) reduction of the abundance of microbes harbouring genes responsible for L-carnitine metabolism, c) inhibition of the transcription of these genes, and d) inhibition of the proteins that carry out the conversion of L-carnitine to γ-BB and γ-BB to TMA (e.g., enzymes and transporters). An additional possibility for ellagitannins (e.g., punicalagin and punicalin) is that they may be able to bind nitrogenous molecules, such as choline, L-carnitine, γ-BB, and TMA [177]. For example, tannic acid has been shown to form non-covalent complexes with choline [178]. Punicalagin has many hydroxyl groups and aromatic rings (**Figure 1.4**), which can donate hydrogen bonds to polar sites and form cation-π interactions [177, 179]. L-Carnitine is a zwitterion, meaning it has both a positive and negative charge, and is highly polar. The multiple polar sites of L-carnitine can form hydrogen bonds with punicalagin's hydroxyl groups. Additionally, the positive charge of L-carnitine on the trimethylammonium group can form cation-π interactions with punicalagin's aromatic rings. Since the molarity of punicalagin (1.8 mM) in the colon model was similar to the molarity of L-carnitine (2 mM), punicalagin might have inhibited L-carnitine metabolism due to non-covalent binding with L-carnitine and γ-BB, making these substrates less available to microbial enzymes and preventing TMA production.

In contrast to punicalagin, punicalin is smaller (782 Da, compared to punicalagin which is 1084 Da) and has less potential to form hydrogen bonds, as punicalin has approximately 12 hydrogen donors and 21 acceptors, while punicalagin has approximately 17 hydrogen donors and 30 acceptors (counting all hydroxyl and carbonyl groups). Additionally,

punicalagin has a greater number of aromatic rings than punicalin and is thus more likely to form cation-π interactions. Therefore, larger ellagitannins with more hydroxyl groups and aromatic rings (punicalagin > punicalin) have a greater potential to bind L-carnitine, and may explain why the inhibitory effect on L-carnitine metabolism was observed for punicalagin but not punicalin.

In the research reported in this chapter, the addition of chlorogenic or gallic acid at 2 mg/mL did not reduce TMA production from L-carnitine. However, a report by Iglesias-Carres *et al.* (2021) described that the addition of gallic acid or chlorogenic acid at concentrations greater than 5 mM (approximately 0.9 and 1.8 mg/mL, respectively) significantly reduced TMA production from choline using a similar high-throughput colon model [90]. This demonstrates that, although the end product of both substrates (choline and L-carnitine) is TMA, these are metabolised to TMA through distinct metabolic pathways that each require specific treatments to inhibit TMA production. This is also consistent with findings reported by Haarhuis & Day-Walsh *et al.* (2025), which showed that the pomegranate extract more effectively inhibited TMA production from L-carnitine than from choline [69].

Data presented here suggest that changes in pH can dramatically affect L-carnitine metabolism. It was observed that the use of the pomegranate extract and chlorogenic acid led to the most significant decrease in pH, regardless the addition of 50 mM PIPES buffer to increase the buffering capacity. Unpublished data from Dr Paul Kroon and colleagues showed that a lower pH can result in the inhibition of L-carnitine and choline metabolism to TMA, suggesting that pH plays a role in the observed inhibitory effects. This would explain the total inhibition of L-carnitine metabolism to γ-BB and TMA when the high-throughput colon model was treated with the pomegranate extract, in combination with the significant drop in pH well below the target pH range (6.4-7.0). In alignment with these findings, a high-glucose condition (1%) substantially reduced the pH, well below the target range of 6.4-7.0, and resulted in a reduction of microbial viability to less than 1% of total bacterial counts at 48 hours of fermentation. A recent study comparing whole blueberries, sugar, and different doses of chlorogenic acid using the high-throughput colon model established by Iglesias-Carres *et al.* [90] showed that whole blueberries and sugar completely inhibited TMA production, but chlorogenic acid did not [103]. Importantly, the authors did not monitor pH nor increase the buffering capacity of the colon model, making it likely that the observed TMA inhibition was caused by the high sugar content, leading to acidification and subsequent reduction in

microbial viability. pH can significantly alter the gut microbiome composition, with a lower pH resulting in a decrease in microbial community structure and abundance of specific microbial taxa [180]. Microbial metabolism, growth, and enzyme activity are determined by several factors including pH [181]. Microbial growth is hampered when the environmental pH deviates from the optimal growth pH. For instance, it has been shown that a one unit decrease of pH can lower the metabolic activity of microbes by up to 50% [181]. Therefore, a pH decline within the high-throughput colon model below the target range may lead to compromised microbial viability, consequently suppressing L-carnitine metabolism.

In the *in vitro* batch colon models no urolithins were observed during the metabolism of the pomegranate extract. Moreover, the metabolism of punicalagin, punicalin, and ellagic acid slowed down after 10 hours. The absence of urolithins was unexpected considering that ellagic acid is broken down into urolithins, which are the final metabolites in the fermentation process of ellagitannins [170]. A matrix effect was ruled out as an additional experiment showed that spiked-in urolithins at 100 ppm could be accurately detected. In a previous report of fermentation experiments incubating ellagic acid with faecal donations from six different donors, urolithin A was detected after five hours in two donors and reached peak levels at 48 hours [169]. For a third and fourth donor, urolithin A was detected at very low levels from 48 and 72 hours, while two other donors only produced trace amounts of urolithin A [169]. In a later study by the same research group, ellagic acid was fermented with the faecal donations of two known urolithin producers [182]. For one donor, urolithin A was detected after 14 hours, while for the other donor isourolithin A was detected after 5 hours. These reports demonstrate the large variability in urolithin production between donors. It is therefore possible that the two donors reported in this chapter to investigate the metabolism of pomegranate polyphenols in the *in vitro* batch colon model do not produce urolithins (urolithin metabotype 0), or 48 hours may not have been sufficient for urolithin production. Three different urolithin metabotypes have been defined: metabotype A only produces urolithin A, metabotype B produces urolithin A, isourolithin A and/or urolithin B, and metabotype 0 does not produce urolithins [112]. The most common metabotype is metabotype A, with 25-80% of human volunteers belonging to this category, followed by metabotype B (10-50%), and metabotype 0 (10-25%) [111, 112].

The observation that ellagic acid was metabolised but no urolithins were produced, points to the absence of specific dehydroxylating bacteria, such as *Gordonibacter,*

needed to cleave the lactone ring into urolithins. Studies show that metabotype 0 individuals only produce the lactone intermediate 6H-Dibenzo[b,d]pyran-6-one, also called urolithin M-5 (**Figure 1.4**), from ellagic acid [183]. Alternatively, ellagic acid may have been metabolised into other metabolites that are currently not well characterised in the literature. For instance, it is possible that the ellagic acid has been converted into nasutins [176]. Ellagic acid can undergo microbial dihydroxylation without being subjected to lactonase-decarboxylase bacterial enzymes, such that the opening of the lactone ring necessary to produce urolithins is prevented [176]. However, to date, nasutins have not been detected in human samples [184], but only in animals [185, 186]. Nevertheless, the presence of nasutins in human samples should not be ruled out [111] and should be a focus point for future *in vitro* studies.

A difference was observed between the estimated quantity of punicalin by the author and the quantity estimated by Prof Tomás-Barberán's laboratory. The discrepancy could be due to different polyphenol extraction techniques. Prof Tomás-Barberán dissolved the extract in higher volumes of DMSO. The Folin-Ciocalteu assay reported here showed that the estimated GAEs tended to be greater when the extract was left in a mixture of DMSO and methanol at a high volume (50:50, v/v). Since DMSO is excellent in dissolving punicalin, it is possible that the higher volume of DMSO used by Prof Tomás-Barberán led to a higher estimate of quantified punicalin. Furthermore, Prof Tomás-Barberán has slightly acidified the extract by adding 0.1% HCl, which stabilises ellagitannins such as punicalagin and punicalin [187]. The accurate identification and quantification of ellagitannins is not straightforward due to the strong tendency of ellagitannins to form dimeric and oligomeric derivatives [2]. This can limit the solubility of ellagitannins such as punicalin and therefore hamper accurate and precise quantification.

3.6.2 Limitations

A limitation of the work presented here is that the pomegranate extract could not be tested in the high-throughput *in vitro* colon model due to the large drop in pH. Therefore, the effects of the individual pomegranate constituents on microbial L-carnitine metabolism could not be directly compared with the pomegranate extract (as a positive control). Nevertheless, punicalagin was able to nearly completely inhibit the

TMA production, suggesting that the effect of punicalagin is more substantial than the effect observed for the pomegranate extract itself.

The drop in pH within the high-throughput colon model is likely caused by constituents other than the polyphenols present in the extract. Gum Arabic was used in the spray-drying process of the extract, which is a soluble fibre metabolised by gut microbiota into organic acids, such as SCFAs that in turn correlate with a lower colonic pH [188]. However, the use of gum Arabic alone resulted in a slight pH drop but did not reflect the large pH drop observed when the pomegranate extract was introduced into the high-throughput model. The large drop in pH might be better explained by the presence of carbohydrates, which make up a large proportion (90-95%) of the extract's macronutrient content (**Appendix 3**). Studies using *in vitro* fermentation models showed that the use of carbohydrates leads to a production of SCFAs and a reduction in pH [189]. Therefore, carbohydrate-rich products might not be suitable for testing of their effects on L-carnitine metabolism using the high-throughput colon model. This work also highlights the importance of monitoring the pH throughout the experiment when using colon models that are not pH controlled.

When testing the effects of individual pomegranate constituents on L-carnitine metabolism, differences in starting concentrations of L-carnitine between the treatments were observed. Furthermore, some of the estimated γ-BB and TMA concentrations exceeded 100% of the starting concentration of L-carnitine. The observed discrepancies may have been caused by a matrix-effect. Matrix effects are common in chromatography and refer to the effect of components in the sample matrix on the ionisation of the analyte (e.g., γ-BB, TMA), which subsequently affects the estimated quantity [190]. Here, the standard curve was prepared in a matrix consisting of the colon model medium and 1% faecal slurry, reflecting the matrix at baseline of the experiment, without the presence of any of the treatments. Hence, the standard curve matrix cannot accurately reflect the matrix of samples at later time points during the experiment, nor the matrix after the addition of any of the treatments. This can result in a matrix containing microbial metabolites such as SCFAs and phenolic catabolites. These metabolites can distort LC-MS/MS quantification due to ion suppression or enhancement. Nevertheless, the issue of differences in L-carnitine starting concentrations was largely bypassed by showing the metabolic trajectories as the percentage from the starting concentration of the substrate (L-carnitine), allowing for metabolic trajectories to be compared between each other. Essentially, the matrix is constantly changing over time and will be different

in each colon model vessel. Therefore, to matrix-match the standards a separate standard curve would need to be prepared for each time point and each colon model vessel, which is not feasible.

3.6.3 Prospective research

The findings presented in this chapter show that punicalagin can almost completely inhibit TMA production from L-carnitine. Since punicalagin may be able to donate hydrogen bonds to polar sites and form cation-π interactions with L-carnitine and γ-BB, it might make these substrates less accessible to microbial enzymes and prevent TMA production. Computational biology models (e.g., molecular docking) could be used to simulate interactions between punicalagin and L-carnitine/γ-BB to further understand the underlying mechanism behind this observation. As emphasised in the discussion of **Chapter 2** of this thesis, it is important to also investigate the ability of the pomegranate extract and/or punicalagin to influence the composition or activity of the gut microbiota, the expression of microbial genes involved in L-carnitine metabolism, and the activity of microbial proteins involved in L-carnitine metabolism (e.g., membrane antiporters/transporters or intracellular enzymes). Several of these potential mechanisms will be explored in **Chapter 4** of this thesis.

3.7 Conclusion

Here it was shown that punicalagin, the second most prominent polyphenol (5.1% w/w) in the pomegranate extract, completely inhibited the production of TMA from L-carnitine in a high-throughput colon model. However, none of the other components in the pomegranate extract inhibited L-carnitine metabolism.

Chapter 4

Mechanistic insights into the inhibition of trimethylamine (TMA) formation from L-carnitine by the pomegranate extract

Chapter 4: Mechanistic insights into the inhibition of trimethylamine (TMA) formation from L-carnitine by the pomegranate extract

4.1 Abstract

Background: The previous chapters of this thesis showed that the pomegranate extract and its main polyphenol, punicalagin, reduced the microbial production of γ-BB and TMA from L-carnitine. The aim of the research presented in this chapter was to investigate the potential mechanisms underlying the observed effects.

Aim and approach: This chapter focuses on the effect of the pomegranate extract on total viable bacterial counts, *cai* gene abundance and expression, and the identification of species containing the *cai* operon in *in vitro* colon models. Total viable bacterial counts were assessed by colony forming unit (CFU) counting. The abundance and expression of *cai* genes in colon models were determined by quantitative real-time polymerase chain reaction (qPCR) and reverse transcription (RT)-qPCR using a vector-based *cai* PCR standard. The effect of the pomegranate extract on microbial diversity and relative abundance in colon models was explored using shotgun metagenomic sequencing.

Results: The pomegranate extract increased the total viable bacterial counts in the *in vitro* colon models, reaching 2.68×10^{9} CFUs at 24 hours (versus 5.89×10^{7} CFUs in controls; 46-fold increase). It was observed that the pomegranate extract substantially inhibited the DNA extraction efficiency and downstream PCR assay, but this was eliminated by extracting DNA with the PowerFecal Pro Kit (Qiagen). Using the optimised method, it was found that the pomegranate extract and punicalagin increased *cai* gene abundance, with the pomegranate-treated colon models containing over 41,500 times more copies than the control models at 24 hours (7.9×10^{8} vs 1.9×10^{4} copies). Furthermore, in an explorative analysis, the extract increased the abundance of the *cai*-carrying *Escherichia coli*, but the overall diversity was decreased.

Conclusion: The pomegranate extract increased bacterial viability and the abundance of *cai* genes, demonstrating that the extract did not act as a general antibacterial nor selectively inhibited *cai*-carrying bacteria. Finally, seven key recommendations for future research investigating the underlying mechanisms are proposed.

4.2 Introduction

The previous chapters of this thesis showed that the pomegranate extract reduced the production of γ-BB and TMA from L-carnitine. Several mechanisms underlying the observed effects were proposed:

a) Reduction of total viable bacterial counts.
b) Specific inhibition of gut microbes carrying the *cai* and *gbu* (*bbu*) operons, such as *Escherichia coli* and *Emergencia timonensis*, needed for the complete anaerobic conversion of L-carnitine to TMA.
c) Inhibition of translation and/or transcription of the microbial genes involved in the conversion of L-carnitine to γ-BB and γ-BB to TMA.
d) Inhibition of the proteins that carry out the conversion of L-carnitine to γ-BB and γ-BB to TMA, including enzymes and transporters.
e) Reduction of L-carnitine and γ-BB access to microbial enzymes by punicalagin donating hydrogen bonds to polar sites and forming cation-π interactions with L-carnitine and γ-BB.

Understanding the underlying mechanisms enables the development of targeted treatments to reduce microbial TMA production and subsequent TMAO formation. In this chapter, the proposed mechanisms a-c are addressed.

Firstly, the effect of the pomegranate extract on total viable bacterial counts needed to be established. Polyphenols are well-known for their antibiotic and prebiotic effects [149] and have therefore been termed 'duplibiotics' [150]. The pomegranate polyphenols may have reduced the total viable bacterial counts in the colon models, resulting in the observed reduction in L-carnitine metabolism. However, whether the effect of polyphenols is anti- or prebiotic depends on the type of polyphenol and the type of microbe. For instance, pomegranate polyphenols have been reported to promote the growth of probiotic species such as *Bifidobacterium breve, B. infantis*, other *Bifidobacterium* species, *Lactobacillus* sp., and *Akkermancia muciniphila* [106, 153], likely due to the enzymatic release of glucose and free gallic and ellagic acid from the polyphenols [149, 154]. On the other hand, pomegranate polyphenols have strong antimicrobial effects on gut microbial species such as *Escherichia coli*, *Proteus* species, and *Salmonella* species [151, 153]. Some of these are known carriers of the *cai* operon (*caiTABCDE*), which catalyses the first step of anaerobic L-carnitine metabolism, the

conversion of L-carnitine to γ-BB [59, 67]. Therefore, it is also possible that the pomegranate polyphenols selectively inhibited *cai*-carrying bacteria.

The operon responsible for the second step of metabolism, the conversion of γ-BB to TMA, has recently been uncovered by two research groups independently, which named the operon *bbu* and *gbu* [72, 191]. The *gbu* (*bbu*) operon (*gbuABCDEF*) is only known to be present in a few species, including *Emergencia timonensis* and *Ihubacter massiliensis* [67, 71, 72]. Although it is important to investigate the effect of the pomegranate extract and punicalagin on the abundance and expression of the *gbu* operon, the research reported in this chapter focuses solely on the *cai* operon.

Secondly, the pomegranate extract and punicalagin may influence the transcription or translation of *cai* and *gbu* (or *bbu*) genes, involved in the metabolism of L-carnitine to γ-BB and γ-BB to TMA, respectively [59, 72, 191]. Interactions between polyphenols and transcription factors or enzymes that regulate gene activity have been previously reported [155, 156]. Hence, pomegranate polyphenols might affect the expression of enzymes that are involved in the metabolism of L-carnitine to γ-BB and TMA.

The research described in this chapter focuses on the effect of the pomegranate extract on total viable bacterial counts, the abundance and expression of the *cai* genes, and the identification of species containing the *cai* operon in *in vitro* colon models.

4.3 Objectives

The main aim was to explore some of the possible underlying mechanisms behind the reduced microbial production of γ-BB and TMA from L-carnitine by the pomegranate extract and its main polyphenol, punicalagin.

To achieve this aim, the following objectives were established:

1. To determine the effect of the pomegranate extract on the total number of viable bacterial cells in the *in vitro* batch colon model.
2. To assess the effect of a pomegranate extract and punicalagin on the number of bacteria involved in L-carnitine metabolism by quantifying bacterial *cai* gene copies using a suitable DNA extraction technique and a qPCR assay.
3. To assess the effect of a pomegranate extract on the expression of *cai* genes using an RT-qPCR assay.

4. To explore the effect of a pomegranate extract on the microbial diversity and relative abundance within *in vitro* batch colon models using shotgun metagenomic sequencing.

4.4 Materials and methods

4.4.1 Materials

All solvents were obtained from Fisher Scientific Limited. Water was 18 MΩ·cm Milli-Q. Punicalagin was obtained from BOC Science (CAS 65995-63-3). The pomegranate extract (Dermogranate®) was purchased from Medinutrex (Catania, Italy). β-mercaptoethanol (B-ME) and polyvinylpyrrolidone-10 (PVP-10) were obtained from Merck.

4.4.2 *In vitro* colon models

In vitro batch colon models were conducted as described in **Chapter 2** (Section **2.4.2**). Briefly, the batch colon models were inoculated with 2 mM L-carnitine and 1% faecal inoculum from a healthy donor, with or without the pomegranate extract (22.8 mg/mL).

In vitro high-throughput colon models were conducted as described in **Chapter 3** (Section **3.4.6**). Briefly, the high-throughput models were inoculated with 2 mM mM L-carnitine and 1% faecal inoculum from a healthy donor, with or without punicalagin (2 mg/mL).

Samples were collected over 24 or 48 hours and processed for microbial plating and DNA and RNA extractions for PCR assays and shotgun metagenomic sequencing.

4.4.3 Microbial plating to count CFUs

Plates were prepared using autoclaved colon model media with 1% agar and left in an anaerobic cabinet overnight to make them anaerobic. Colon model samples were taken at 0, 6, and 24 hours and immediately diluted in the anaerobic cabinet, with serial

dilutions ranging from 10^{-1} to 10^{-7}. From each of the serial dilutions, 5 µL was transferred onto the plate. Each series of dilutions from each vessel was plated in triplicate. After 24 hours the CFUs were counted.

4.4.4 Design of *caiT* PCR primers and probe

To estimate the abundance and expression of the *cai* genes, a real-time PCR probe and primers were specifically designed to target *caiT*, which is part of the *cai* operon and encodes for the L-carnitine/γ-butyrobetaine antiporter responsible for the transport of L-carnitine into the cytoplasm and the export of γ-BB out of the cytoplasm (**Figure 4.1**) [59].

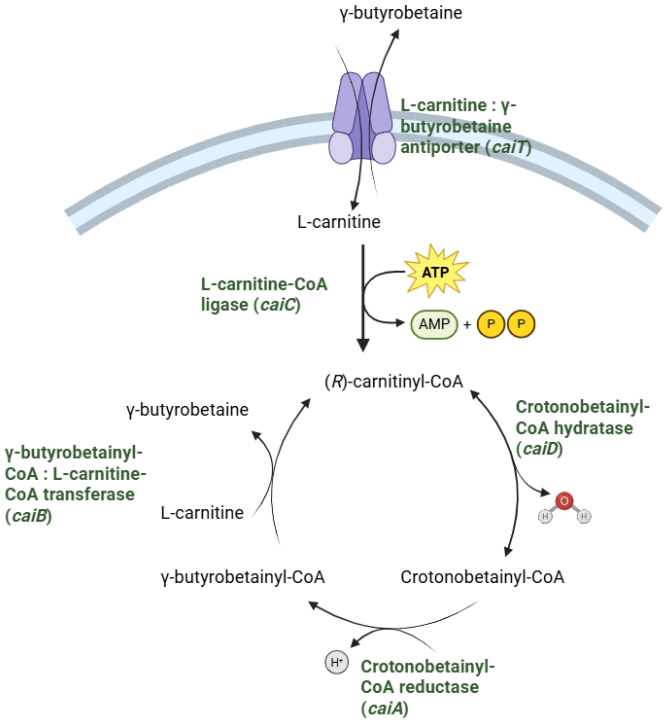

Figure 4.1. Anaerobic L-carnitine degradation pathway in gut bacteria. *Information to illustrate the pathway was derived from the BioCyc Database (EcoCyc/Escherichia coli K-12 substr. MG1655 reference genome)* [192]*, and the figure was created using BioRender.com.*

Gene sequences of *caiT* were retrieved from the NCBI Primer-BLAST tool (https://blast.ncbi.nlm.nih.gov/) [193]. To design suitable degenerate primers for a TaqMan qPCR, the sequences were initially analysed using Primer3Plus

(https://www.primer3plus.com/) [194] to identify potential primer regions. Multiple sequence alignment (MSA) was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) [195] to determine conserved regions within the *caiT* gene sequences and assess phylogenetic relationships. Based on the MSA results, degenerate primers were designed using the rprimer Shiny application (https://sofpn.shinyapps.io/rprimer/) to account for sequence variability across bacterial species. The final degenerate primer set consisted of *caiT*-F (5'-CCTGATGCTSGGYTGGGT-3') and *caiT*-R (5'-ACCCACCAYGCCCAGTAG-3'), along with a TaqMan probe (5'-FAM-TCAGCGGYGCCAGCTTCATCATGAA-BHQ1-3'), producing an expected amplicon size of 174 bp. A BLAST® search against the core nucleotide database was performed to confirm the specificity of the primers. All oligonucleotides were synthesized by Integrated DNA Technologies (IDT, IA, USA).

The degenerate primers were tested by PCR amplification followed by agarose gel electrophoresis using *caiT*-positive template DNA from *E. coli*, which is known to contain the *caiT* gene [152], *caiT*-negative DNA, and a no-template control (ribonuclease (RNase)-free water). Reactions were prepared according to the New England Biolabs (NEB, MA, United States) Q5® High-Fidelity DNA Polymerase (M0491) protocol. Primers were hydrated in RNase-free water to obtain 10 µM stock solutions. Each 25 µL PCR reaction contained 1 µL DNA template, 2.5 µL of each primer (10 µM), Q5 Reaction Buffer, 10 mM dNTPs, Q5 High-Fidelity DNA Polymerase, and RNase-free water. Amplification was performed according to the NEB-recommended thermocycling conditions on a Techne Flexigene Thermal Cycler.

PCR products were loaded on an agarose gel prepared in 1× TBE (Tris/Borate/EDTA) buffer with 1.5 µL SYBR Safe DNA Gel Stain (Invitrogen, MA, United States). 5 µL of each PCR product was mixed with 1 µL Purple Gel Loading Dye (NEB) and applied to the gel alongside a Quick-Load Purple 100 bp Plus DNA Ladder. Electrophoresis was conducted at 110 V for 35 min, and gels were imaged on a Syngene U:Genius3 system.

In the *caiT*-positive *E. coli* template, a band was observed at ~174 bp (**Figure 4.2**), matching the expected amplicon size. In the *caiT*-negative template, off-target amplification was detected. However, the use of a TaqMan probe is expected to negate the off-target amplification, because any off-target amplification should not have the probe binding site.

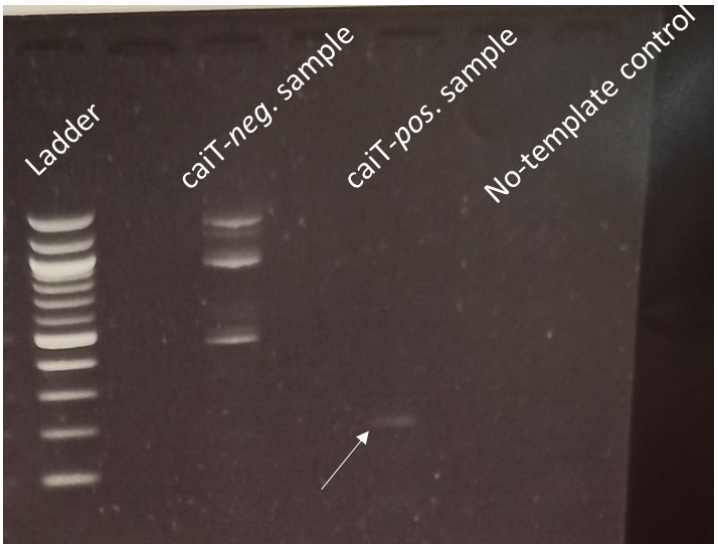

Figure 4.2. Agarose gel electrophoresis of PCR products generated with degenerate caiT primers (2.5 µL of each primer from 10 µM stocks) using caiT-positive and caiT-negative DNA templates, and a no-template control. *An agarose gel was prepared in 1× TBE (Tris/Borate/EDTA) buffer with 1.5 µL SYBR Safe DNA Gel Stain. Amplification was performed using Q5® High-Fidelity DNA Polymerase (M0491) protocol and thermocycling on a Techne Flexigene Thermal Cycler.*

4.4.5 DNA extractions from *in vitro* colon model samples

Samples from the *in vitro* colon models were collected at several time points after inoculation. For initial experiments, DNA extractions were carried out using the FastDNA™ SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's instructions. Briefly, 1 mL colon model samples were spun down for 10 minutes at maximum speed and the supernatant decanted. The pelleted samples were then processed through bead-beating lysis using a FastPrep-24™ bead beating instrument, followed by protein precipitation and silica-based DNA purification. DNA was eluted in 100 µL of sterile water.

Since polyphenols are known PCR inhibitors and can inhibit the PCR or degrade DNA [196], pomegranate polyphenols might interfere with DNA extractions and PCR amplification. PVP and B-ME are commonly used for the removal of polyphenols [196, 197]. B-ME functions as a reducing agent, while PVP can bind polyphenols through hydrogen bonds [197]. Therefore, further experiments were carried out to determine the effectiveness of PVP and B-ME at different concentrations on DNA yield from control colon models and colon models treated with the pomegranate extract (22.8 mg/mL). PVP-10 and B-ME were added at 1-2% (w/v) and 0.2-1% (v/v), respectively, during DNA

extractions using the FastDNA™ SPIN Kit for Soil. Colon model samples was spun down at maximum speed for 10 minutes, the supernatant decanted, and the pellet resuspended in 950 µL sodium phosphate buffer, after which 300 µL was transferred into three different tubes (i.e., 300 µL in each tube). Then, 678 µL sodium phosphate buffer, 122 µL MT buffer, and 0, 1, or 2% PVP-10 (w/v), or 0, 0.2, or 1% B-ME (v/v) was added to each tube. The samples were further processed according to the manufacturer's instructions.

To confirm whether or not the pomegranate extract interfered with the DNA yield, an experiment was carried out by adding the pomegranate extract at 22.8 mg/mL to control colon model samples directly prior to DNA extractions using the FastDNA™ SPIN Kit for Soil. Control colon model samples were spun down at maximum speed for 10 minutes, the supernatant decanted, and the pellet resuspended in 725 µL sodium phosphate buffer, after which each sample was split into three different aliquots of 225 µL. One of the aliquots was treated with the pomegranate extract (22.8 mg/mL) and incubated for 5 minutes. All aliquots were then vortexed and spun down for 10 minutes at 17,000 rpm, after which the supernatants were decanted, and the pellets resuspended in 978 µL sodium phosphate buffer with 0 or 1% PVP-10 (w/v). The samples were further processed according to the manufacturer's instructions.

For further optimisation, alternative DNA extraction techniques were tested, including the RNeasy Pro Kit (Qiagen) and a published phenol-chloroform extraction method [196]. For the method using the RNeasy PowerFecal Pro Kit, manufacturer's instructions were followed, but the deoxyribonuclease (DNase) treatment was omitted to retain the DNA. Briefly, samples were subjected to mechanical and chemical lysis using the provided bead solution and lysis buffer, followed by inhibitor removal and nucleic acid binding to the silica-based spin column. After washing steps to remove contaminants, total nucleic acids were eluted in 100 µL RNase-free water.

For the phenol-chloroform extractions, the protocol reported by Rezadoost *et al.* (2016) was followed [196]. Briefly, colon model samples were centrifuged at maximum speed for 10 minutes, after which the supernatant was decanted. The samples were resuspended in 400 µL of a buffer containing Tris-HCl (pH 7.5, 200 mM), NaCl (1.4 M), 0.5% (v/v) Triton X-100, 3% (w/v) CTAB, and 0.1% (w/v) PVP-10 and left in a heat block at 60 °C for 30 minutes. Samples were treated with 400 µL phenol chloroform (24:1, v/v), shaken for 2 minutes and centrifuged at 10,000 rpm for 15 minutes. 300 µL supernatant was transferred into a new tube with 150 µL buffer containing Tris-HCl (50 mM),

guanidine HCl (2 M), 0.2% (v/v) B-ME and 0.2 mg/mL Proteinase K, and left in a heat block at 40 °C for 15 minutes. 225 µL 4 M NaCl was added, and the samples were shaken and left in the fridge overnight. 1,350 µL cold isopropanol was added, left at room temperature for 2 minutes, and centrifuged at 8,000 rpm for 15 minutes. The pellet was washed with 75% (v/v) ethanol, dried, and dissolved in 100 µL Tris-EDTA buffer. DNA yields were confirmed using Qubit™ dsDNA High Sensitivity Assay Kit.

4.4.6 RNA extractions from *in vitro* colon model samples

From the same *in vitro* batch fermentations, samples for RNA extractions were collected at five different time points, 0, 4, 8, 10 and 24 hours after inoculation. 1.5 mL was taken from the fermentation models and transferred to a 2 mL collection tube. The collection tube was spun down and supernatant removed, before adding 200 µL of RNALater to preserve the RNA. Upon treatment with RNALater, the tubes were transferred to 4 °C overnight and then transferred to -80 °C until further analysis. RNA extractions were then performed using the RNeasy PowerFecal Pro Kit (Qiagen). Manufacturer's instructions were followed. Briefly, samples were subjected to mechanical and chemical lysis using the provided bead solution and lysis buffer, followed by inhibitor removal and nucleic acid binding to the silica-based spin column. After washing steps to remove contaminants, total nucleic acids were eluted in 100 µL RNase-free water. RNA yields were confirmed using the Qubit™ RNA HS High Sensitivity Assay Kit.

4.4.7 Construction of a vector-based *caiT* PCR standard

To quantify *caiT* gene copies in colon models, a vector-based PCR standard was developed using the pGEM®-T Easy Vector System (Promega, Southampton, UK).

The *caiT* gene was amplified using Q5® High-Fidelity DNA Polymerase (NEB, M0491) following the protocol described in Section **4.4.4**. The resulting PCR product was purified and used as an insert for vector construction. Ligation reactions were performed according to the manufacturer's protocol. Briefly, reaction mixtures containing 5 µL of 2X Rapid Ligation Buffer (Promega), 50 ng pGEM®-T Easy Vector, 1 µL T4 DNA Ligase,

purified *caiT*-positive PCR product, and RNase-free water (to a final volume of 10 µL) were incubated overnight at 4°C to obtain transformants.

Ligation products were transformed into competent *E. coli* DH5α cells and plated onto Luria-Bertani agar (LBA) containing ampicillin (100 µg/mL), 5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside (X-GAL, 40 µg/mL), and and isopropyl-β-D-1-thiogalactopyranoside (IPTG, 0.1 mM) for blue-white screening. Plates were incubated overnight at 37°C. The pGEM®-T Easy Vector contains a *lacZ* gene within the multiple cloning site (MCS) that is disrupted upon successful insertion of the *caiT* gene. Successful transformants appear as white colonies due to the loss β-galactosidase expression, while unsuccessful ligations result in blue colonies expressing β-galactosidase. White colonies were selected and the presence of the *caiT* insert was confirmed by PCR amplification using the same conditions described in Section **4.4.4**, followed by agarose gel electrophoresis. Clones showing the strongest amplification were selected for further analysis.

Plasmid DNA was extracted from selected clones using the QIAprep Spin Miniprep Kit (Qiagen, Manchester, UK) and quantified using a high-sensitivity Qubit™ dsDNA assay. The copy number of *caiT* genes per vector was calculated based on the total DNA (ng/µL) and the molecular weight of the vector-insert construct (3,015 bp pGEM®-T Easy vector + 174 bp *caiT* insert = 3,189 bp). A stock of 2×10^7 copies/µL was stored at -20 °C.

4.4.8 qPCR and RT-qPCR assays to measure *caiT* expression and abundance

DNA and RNA extracts were used for the qPCR and RT-qPCR reactions, respectively, with a Luna® Universal Probe (New England Bioscience) and the designed *caiT* primers and TaqMan probe (Section **4.4.4**). A standard curve was prepared using the vector-based *caiT* PCR standard, as described in the previous section (Section **4.4.7**). Serial dilutions of the stock were prepared to generate a standard curve from 1×10^7 to 1×10^1 copies per well. A negative control containing just RNase-free water was included.

The PCR reactions were carried out in a MicroAmp Fast Optical 96-well reaction plate (Applied Biosystems), while kept on ice. Each reaction was performed in triplicate. The plate was covered using an optical cover and analysed in the OneStep Plus PCR System (Applied Biosystems). The samples underwent 40 cycles, with rapid heating to 95 °C to denature the DNA, followed by cooling to 60 °C. The samples were inspected using

StepOne Software v2.3 and then analysed and visualised using the Standard Curve (SC) application module within the Applied Biosystems® qPCR Analysis Modules on the Thermo Fisher Cloud (Thermo Fisher Scientific).

4.4.9 Determining PCR inhibition by pomegranate polyphenols

To test the inhibitory effect of the pomegranate extract on the qPCR assay, a plasmid control was spiked in each assay reaction. The plasmid was obtained from an *E. coli* JM109 containing a unique *M. bovis* primer + green fluorescent protein (GFP) probe. The *E. coli* was grown on LBA plates supplemented with ampicillin, IPTG, and ChromoSelect Selective Agar. After the colonies were grown, selected colonies were further grown in a Super Optimal broth with Catabolite repression (SOC) medium, and the vector was extracted. Presence of the GFP insert was confirmed by PCR amplification using Q5® High-Fidelity DNA Polymerase (NEB, M0491) with GFP primers, following the protocol described in Section **4.4.4**. The mass of the vector was derived from the DNA yield measured using a Qubit™ dsDNA High Sensitivity Assay Kit. Finally, the copy number of vector per µL was derived from the vector mass.

DNA extracts obtained through three different methods (MP Biomedicals, Qiagen, and phenol-chloroform), as described in Section **4.4.6**, were diluted in RNase-free water at concentrations of 1:10 and 1:100. For each DNA extract an undiluted (raw) sample was included as well. Then, 10 µL of $2x10^5$ copies/µL of the GFP vector was spiked in into 90 µL of each of the samples and dilutions. A qPCR plate was prepared containing 15 µL Luna® Universal Probe qPCR Master Mix (New England Biolabs) with 5 µL ($1x10^5$ copies/well final concentration of the vector) of the samples and dilutions. Each reaction was performed in triplicate and analysed in the OneStep Plus PCR System as described in Section **4.4.8**.

4.4.10 Gut microbiome composition and diversity analysis

The KEGG (Kyoto Encyclopedia of Genes and Genomes) database (https://www.genome.jp/kegg/) [198, 199] was searched for the *caiT* gene (K05245), encoding for the L-carnitine/gamma-butyrobetaine antiporter, on 13 August 2025.

Presence of species in the gut microbiome was confirmed using the GMrepo database [200].

Samples from two *in vitro* batch colon models were collected for shotgun metagenomic sequencing. Batch colon models were carried out as described in **Chapter 2** of this thesis (Section **2.4.2**). The batch colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine, with or without a pomegranate extract (22.8 mg/mL). Samples were collected at 0, 4, 6, 8 and 12 hours and DNA was extracted using the FastDNA™ SPIN Kit for Soil.

Library preparation for shotgun metagenomic sequencing was performed by Dr Robert James. The pooled library was sequenced by the Technical Genomics group at the Earlham Institute (Norwich, UK) using the Low Input, Transposase Enabled (LITE) protocol at a sequencing depth of 10 Gb per sample on the Illumina NovaSeq X platform. Paired-end reads were obtained and co-assembly was performed by Dr Sébastien Raguideau. Briefly, the reads were assembled into contigs and metagenome-assembled genomes (MAGs) were derived using binning algorithms, which group contigs based on their sequence composition and coverage patterns across samples. Based on the MAGs, the author obtained coverage depth values by dividing the total coverage of each sample to obtain relative abundance. Furthermore, Shannon index and inverse Simpson index were obtained from the relative abundance data using the 'vegan' package in R [201].

4.4.11 Statistical Analyses

All graphs were generated using GraphPad Prism version 10.4.2, and R version 4.4.1 (R Core Team, 2024) in RStudio (Posit Software, PBC, Boston, MA). Statistical analyses were performed in GraphPad Prism. One-Way ANOVA with Dunnett's post-hoc test was used for comparing multiple treatment groups to a single control group. Two-Way ANOVA with Tukey's post-hoc test was used when analysing multiple independent variables. Values of $P < 0.05$ were considered significant.

4.5 Results

4.5.1 The effect of a pomegranate extract on microbial viability

Total viable bacterial counts were estimated by counting CFUs (**Figure 4.3**). Microbial growth was greatest during the first 6 hours, increasing viable microbial numbers in the control and pomegranate condition to 2.94×10^{8} and 7.34×10^{8}, respectively. Between 6 and 24 hours, CFU counts decreased in the control condition, but increased in the pomegranate condition (**Figure 4.3**). At 24 hours, the pomegranate-treated colon models had 2.68×10^{9} CFUs and the control colon models 5.89×10^{7} CFUs. These data demonstrate that the pomegranate extract increased, rather than decreased, the overall microbial viability over 24 hours in the colon models. The next step was to investigate whether the extract may selectively inhibit the microbes responsible for L-carnitine metabolism.

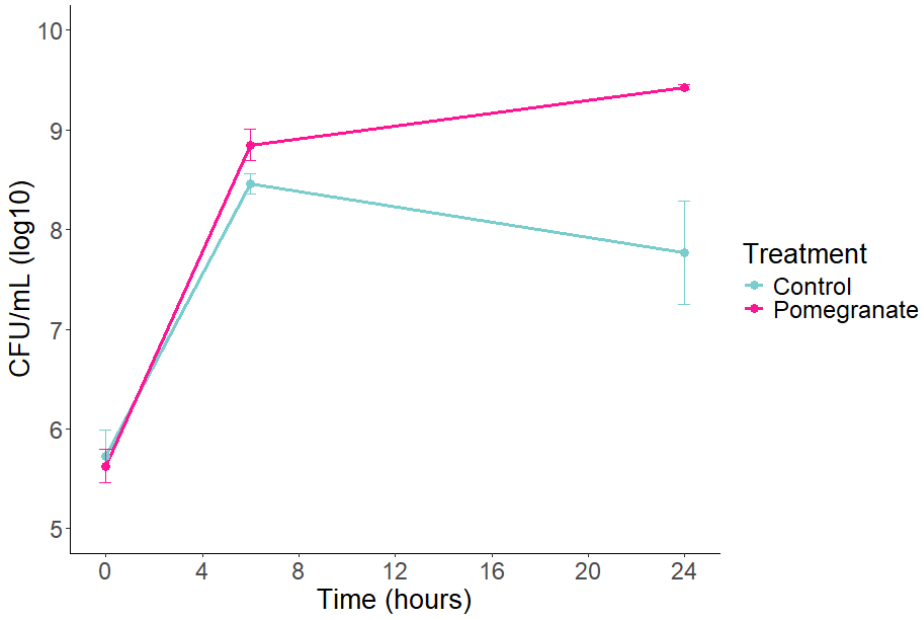

Figure 4.3. Bacterial growth in in vitro batch colon models measured over 24 hours in log_{10} converted colony forming units (CFUs) per mL colon model sample. *Data are presented as mean ± SD of three biological replicates from one donor. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine, with or without a pomegranate extract (22.8 mg/mL). Samples were collected at 0, 6, and 24 hours. Serial dilutions (10^{-1} to 10^{-7}) were prepared in an anaerobic cabinet, and 5 µL of each dilution was plated in triplicate onto anaerobic agar plates. CFUs were counted after 24 hours of incubation.*

4.5.2 Initial experiment testing the effect of the pomegranate extract on *caiT* abundance

The DNA yields from *in vitro* colon models that were inoculated with different doses of the pomegranate extract decreased dose-dependently (**Figure 4.4**). Polyphenols can bind to DNA and hamper DNA extractions due to co-precipitation with the nucleic acids [197], which might explain the observation that inoculation with the pomegranate extract dose-dependently reduced DNA yield.

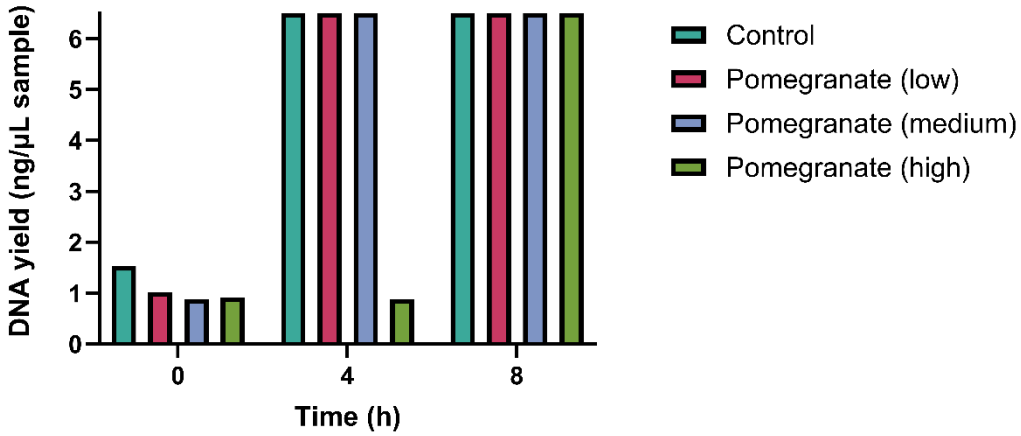

Figure 4.4. The effect of increasing pomegranate extract doses on DNA yield (ng/µL sample) from colon model samples at 0, 4, and 8 hours of fermentation. *In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and different doses of a pomegranate extract (0, 5.7, 11.4, or 22.8 mg/mL). One sample per pomegranate extract dose was collected at each time point. Samples were directly stored at -80 °C, until DNA extractions were carried out using the FastDNA™ SPIN Kit for Soil (MP Biomedicals). DNA yield was estimated using a Qubit™ dsDNA High Sensitivity Assay Kit, with a detection limit of 100 ng/µL (6.5 ng/µL sample).*

The initial qPCR analysis of the extracted DNA showed that the abundance of the *caiT* gene was dose-dependently decreased in the pomegranate-treated colon models at 4 hours of fermentation compared to controls (**Figure 4.5**). However, this pattern reversed at 8 hours of fermentation, where the *caiT* abundance increased dose-dependently with each pomegranate dose, with the highest dose (22.8 mg/mL) exceeding the *caiT* abundance measured in controls (**Figure 4.5**). These data show that the high dose pomegranate extract increased the total abundance of the *caiT* gene, even though the extract inhibited the L-carnitine metabolism to γ-BB and TMA, as shown in **Chapter 2** of this thesis. The observed increase in *caiT* abundance could be due to an increase in the

number of bacteria carrying the gene or an increase in gene copy number per bacterium (e.g., via plasmid amplification).

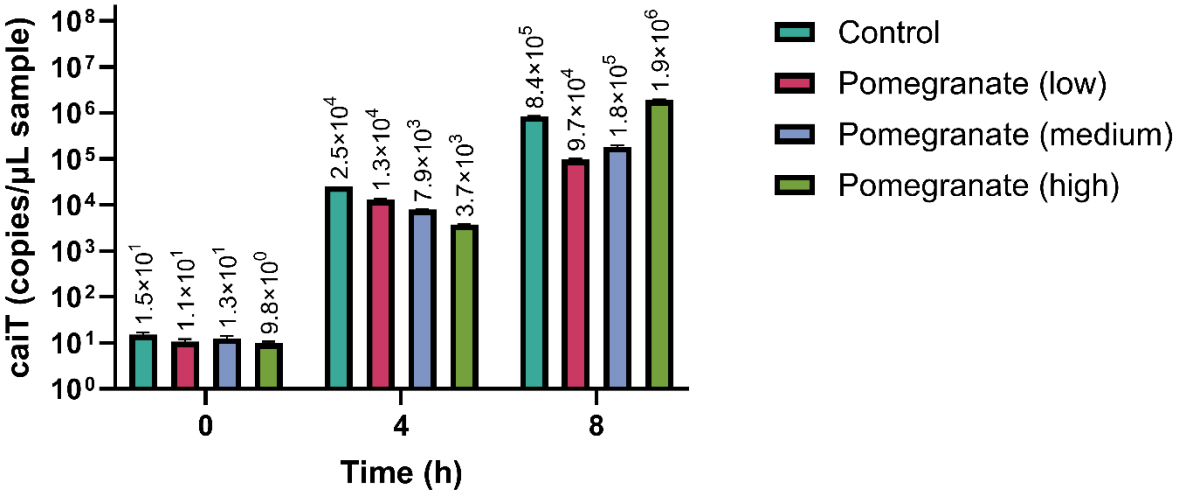

Figure 4.5. Abundance of the caiT gene (copies/µL sample), which encodes for the L-carnitine/γ-butyrobetaine antiporter, in colon models at different time points. *Data are presented as mean ± SD of three technical replicates from one donor. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and different doses of a pomegranate extract (0, 5.7, 11.4, or 22.8 mg/mL). Samples were collected at different time points and stored at -80 °C until DNA was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals). qPCR reactions were carried out using a Luna® Universal Probe (New England Bioscience) with a caiT TaqMan probe and primers in a OneStep Plus PCR System (Applied Biosystems). The samples underwent 40 cycles, with rapid heating to 95 °C to denature the DNA, followed by cooling to 60 °C.*

However, differences in DNA extraction efficiency between pomegranate-treated and control colon models can obscure the comparison of the abundance of the L-carnitine metabolic genes between colon models. Therefore, optimisation of the extraction efficiency was necessary before drawing conclusions from the initial PCR analyses.

4.5.3 The optimisation of DNA extraction techniques to enhance DNA yield from *in vitro* colon models treated with a pomegranate extract

An explorative experiment was performed with the addition of different B-ME and PVP-10 concentrations to the DNA extractions carried out using FastDNA™ SPIN Kit for Soil (MP Biomedicals). B-ME is a reducing agent, while PVP-10 removes polyphenolic

contamination by binding it through hydrogen bonds [197]. Neither of the B-ME concentrations (0.2 or 1%) could improve the DNA yield from pomegranate-treated colon model samples, while B-ME slightly improved the DNA yield from control colon model samples (**Figure 4.6B**). The addition of 1% PVP-10 tended to increase the DNA yield from pomegranate-treated colon model samples, approaching the DNA quantity found in control colon models (**Figure 4.6A**). Since this was an explorative experiment (n = 1), the addition of 1% PVP-10 to enhance the DNA extractions was further investigated.

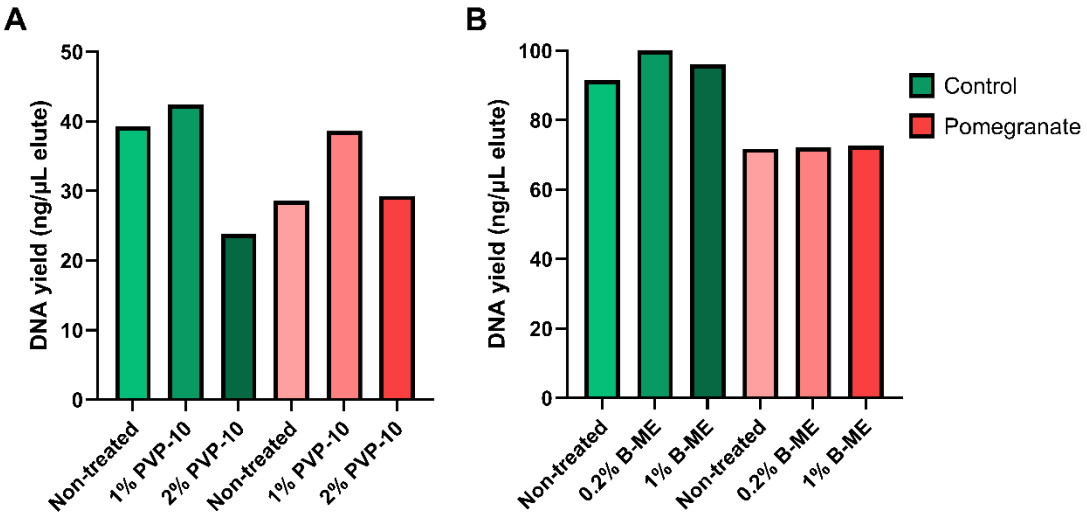

Figure 4.6. The effect of polyvinylpyrrolidone-10 (PVP-10) and β-mercaptoethanol (B-ME) on DNA yield (ng/µL elute) from colon model samples at 12 hours of fermentation. *DNA was extracted from colon model samples using the FastDNA™ SPIN Kit for Soil (MP Biomedicals) with the addition of different concentrations of (**A**) PVP-10 and (**B**) B-ME at 12 hours of fermentation. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine, without (control) or with a pomegranate extract (22.8 mg/mL). One sample per condition (control or pomegranate) was collected at a single time point (12 hours) and stored at -80 °C until DNA was extracted. Prior to DNA extractions, samples were split in three. DNA yield was measured using Qubit™ dsDNA High Sensitivity Assay Kit, with a detection limit of 100 ng/µL. DNA extractions using B-ME and PVP-10 were each performed on a different day, with different colon model samples.*

The effect of 1% PVP-10 was further tested on control colon model samples that were split in three: One part was treated with the pomegranate extract (22.8 mg/mL) before the DNA extractions, one part with both the pomegranate extract (22.8 mg/mL) and 1% PVP-10, and another part was used as a control. The addition of the pomegranate extract reduced the DNA yield relative to the control at all time points (**Figure 4.7**), with approximately a 9.1, 28.3, and 14.9% reduction at 2, 4, and 10 hours after inoculation,

respectively. However, 1% PVP-10 did not improve the DNA yield and further suppressed the DNA yield relative to the sample with the pomegranate extract at 10 hours of fermentation (**Figure 4.7**). These data confirm that the pomegranate extract hampers the DNA extraction efficiency and that this could not be improved with the addition of 1% PVP-10.

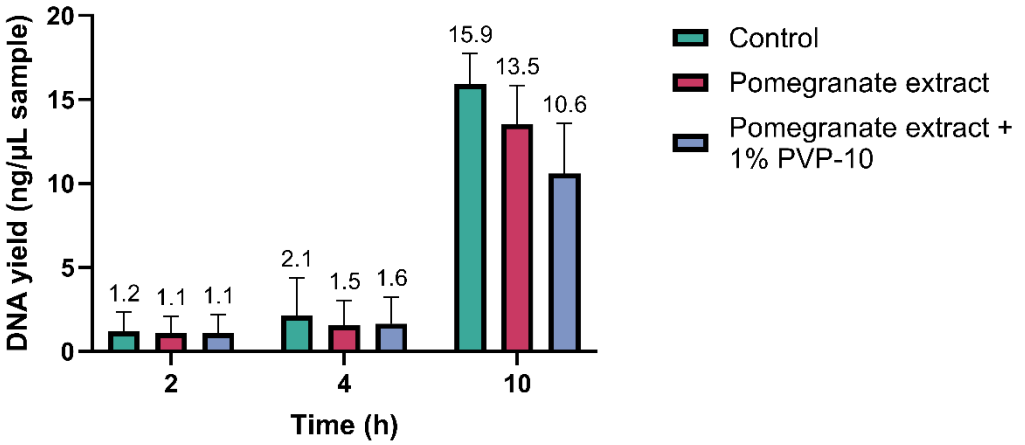

Figure 4.7. DNA yield (ng/µL sample) after the addition of a pomegranate extract (22.8 mg/mL) with or without 1% polyvinylpyrrolidone-10 (PVP-10) to a control colon model sample. *Data are shown as the mean ± SD of two biological replicates. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine. Samples were collected at 2, 4, and 10 hours and stored at -80 °C until DNA was extracted. Prior to DNA extractions, samples were split in three: one control, one with added pomegranate extract, and one with added pomegranate extract and PVP-10. DNA was extracted from the samples using the FastDNA™ SPIN Kit for Soil (MP Biomedicals) and DNA yield was measured using Qubit™ dsDNA High Sensitivity Assay Kit.*

Several other DNA extraction methods were tested for their efficiency to extract DNA from *in vitro* colon models treated with the pomegranate extract. The greatest DNA yield was obtained using the Qiagen PowerFecal Pro Kit, with a mean (± SD) total DNA yield of 549 ng (± 14), while the phenol-chloroform method resulted in a DNA yield of just 67 ng (± 17) (**Figure 4.8**). The greatest variance was observed for the DNA extracted using the MP Biomedicals FastDNA™ SPIN Kit for Soil, with a mean DNA yield of 413 ng (± 119) (**Figure 4.8**). The MP Biomedicals kit and the Qiagen kit yielded significantly higher DNA concentrations than the phenol-chloroform extraction method (P = 0.002 and P = 0.0004, respectively). Based on these data, the Qiagen kit would yield the highest concentration of DNA with the least variance.

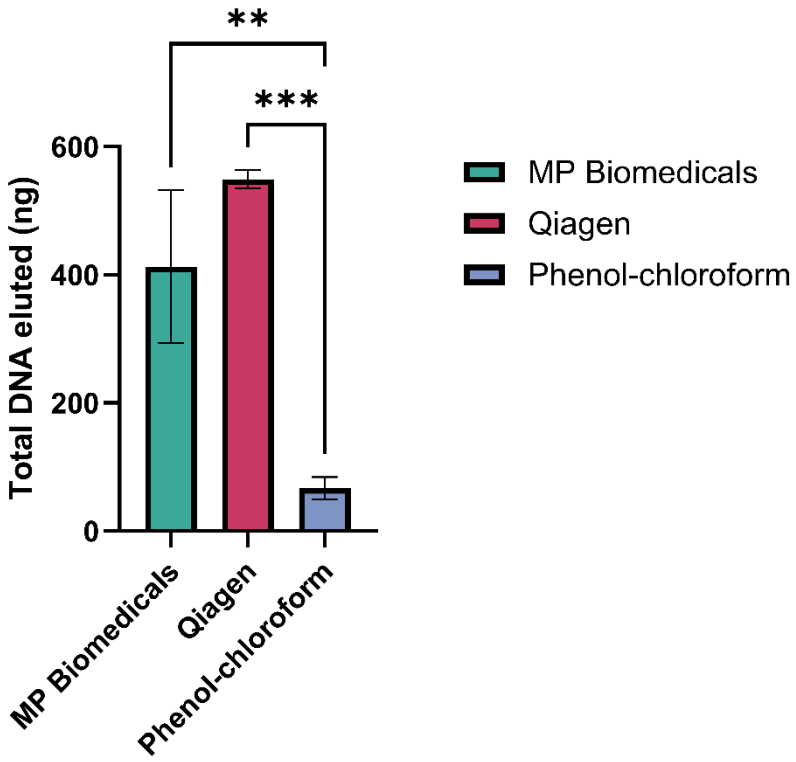

Figure 4.8. Total DNA yield (ng) from colon model samples (approx. 300 µL) using three different DNA extraction techniques. *Data are presented as mean ± SD of three technical replicates from one colon model. Statistical analysis was performed using One-Way ANOVA (** P < 0.01, *** P < 0.001). In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and a pomegranate extract (22.8 mg/mL). Samples were collected at a single time point (4 hours) and stored at -80 °C until DNA was extracted. DNA was extracted from the samples using the MP Biomedicals FastDNA™ SPIN Kit for Soil, the Qiagen Powerfecal Pro kit, and a phenol-chloroform extraction. DNA yield was measured using Qubit™ dsDNA High Sensitivity Assay Kit.*

4.5.4 Inhibition of PCR amplification by pomegranate polyphenols

DNA extracts from all three extraction techniques (MP Biomedicals kit, Qiagen kit, phenol-chloroform) were subjected to qPCR analysis. To assess PCR inhibition, samples were serially diluted (1:10 and 1:100) and spiked with a GFP gene vector at 1×10^5 copies per sample. The rationale was that any PCR inhibitors present in the undiluted (raw) sample would suppress GFP amplification, with a higher recovery expected in the diluted samples where inhibitors are reduced.

The PCR inhibition was most obvious for the DNA extracted using the MP Biomedicals kit, with inhibition decreasing progressively with each dilution (**Figure 4.9**). This pattern suggests potential carry-over from components of the pomegranate extract that

interfered with PCR amplification. In contrast, samples extracted using the Qiagen kit and phenol-chloroform method showed no substantial differences across dilutions (**Figure 4.9**), indicating minimal PCR inhibition when these extraction techniques were used. GFP vector recovery was significantly higher with both the Qiagen and phenol-chloroform extraction techniques compared to the MP Biomedicals technique across all dilutions (1:10 and 1:100; $P < 0.0001$). Combined with the DNA yield results, these findings demonstrate that the Qiagen extraction technique results in the highest DNA yield with minimal PCR inhibition.

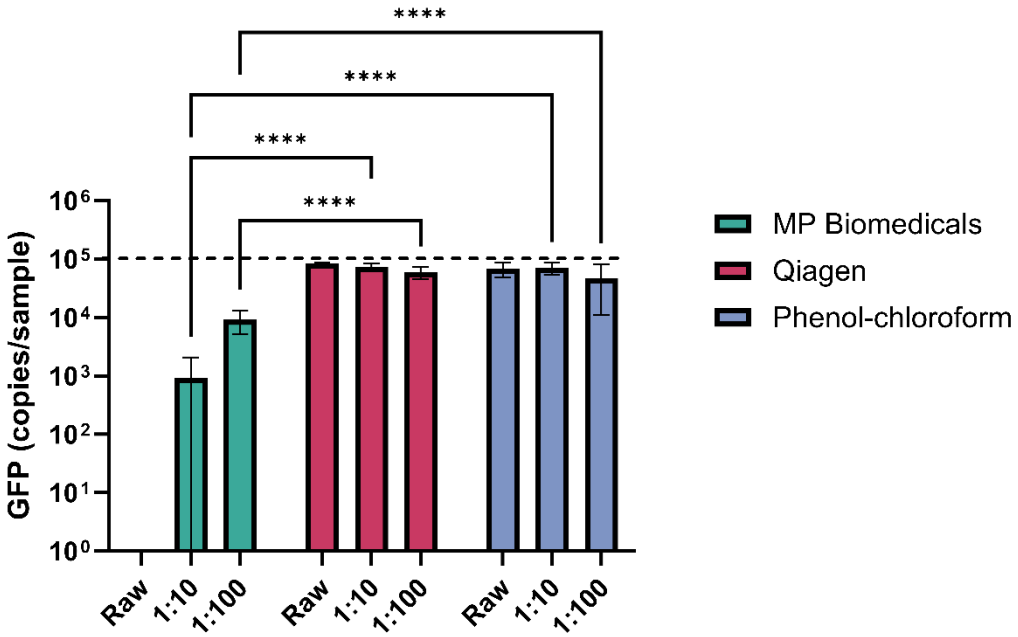

Figure 4.9. Abundance of the green fluorescent protein (GFP) gene, which is present in the vector that was spiked in at 1x10^5 copies/sample into each of the dilutions. *Data are presented as mean ± SD (n = 3). Statistical analysis was performed using Two-Way ANOVA with a post-hoc pairwise comparisons between the different extraction techniques within each dilution (**** P < 0.0001). Amplification could not be detected for the raw MP Biomedicals DNA extract. DNA was extracted from in vitro colon model samples using three different DNA extraction techniques: using the MP Biomedicals FastDNA™ SPIN Kit for Soil, the Qiagen Powerfecal Pro kit, and a phenol-chloroform extraction. qPCR reactions were carried out using a Luna® Universal Probe (New England Bioscience) with a GFP probe and primers in a OneStep Plus PCR System (Applied Biosystems). The samples underwent 40 cycles, with rapid heating to 95 °C to denature the DNA, followed by cooling to 60 °C.*

4.5.5 The effect of the pomegranate extract on the abundance of the *caiT* gene

After the optimisation of DNA extraction techniques, the Qiagen RNeasy Powerfecal Pro kit was used to extract DNA and RNA from colon model samples. For DNA extractions, the DNase treatment as part of the Qiagen RNeasy Powerfecal Pro kit was omitted to retain the DNA.

At 4 hours of fermentation, slightly more *caiT* gene copies were measured in the control colon models, while at 8, 10, and 24 hours of fermentation the *caiT* gene copies were increased in the pomegranate-treated colon models (**Figure 4.10**). At 24 hours, 7.9×10^{8} *caiT* copies were estimated in the pomegranate-treated colon models, and 1.9×10^{4} in the control models, meaning that at 24 hours the pomegranate-treated colon models had over 41,500 times more *caiT* gene copies than the control models. These data are in line with findings from the initial qPCR analysis (**Figure 4.5**), where the high pomegranate dose increased the *caiT* gene abundance.

DNA yield was lower in the pomegranate-treated colon models but reached the same level as the control models at 24 hours of fermentation (**Appendix 4, Supplementary Figure S11**).

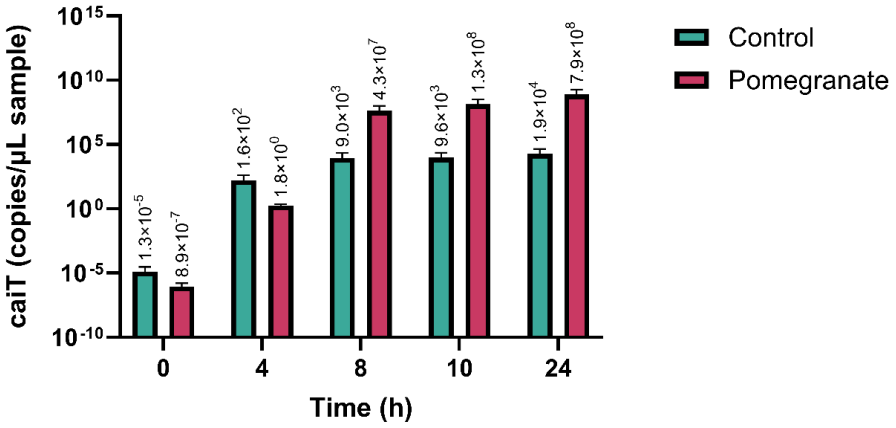

Figure 4.10. Abundance of the caiT gene (copies/µL sample) on a log_{10} scale, which encodes for the L-carnitine/γ-butyrobetaine antiporter, in colon models at different time points. *Data are presented as mean ± SD from 2 donors. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine, with or without a pomegranate extract (22.8 mg/mL). Samples were collected at different time points and stored at -80 °C until DNA was extracted using the Qiagen Powerfecal Pro kit. qPCR reactions were carried out using a Luna® Universal Probe (New England Bioscience) with a caiT TaqMan probe and primers in a OneStep Plus PCR System (Applied Biosystems). The samples underwent 40 cycles, with rapid heating to 95 °C to denature the DNA, followed by cooling to 60 °C.*

4.5.6 The effect of the pomegranate extract on *caiT* expression

After it was established that the pomegranate-treated colon models increased the copy number of the *caiT* gene, the expression of *caiT* was investigated. It is possible that, even though a high number of *caiT* copies was present, the transcription is lower in the pomegranate-treated condition, which could explain the inhibited L-carnitine metabolism.

Similar to the initial experiment testing the effect of the pomegranate extract on DNA yields, the RNA yields from *in vitro* colon models that were inoculated with different doses of the pomegranate extract decreased dose-dependently (**Figure 4.11**).

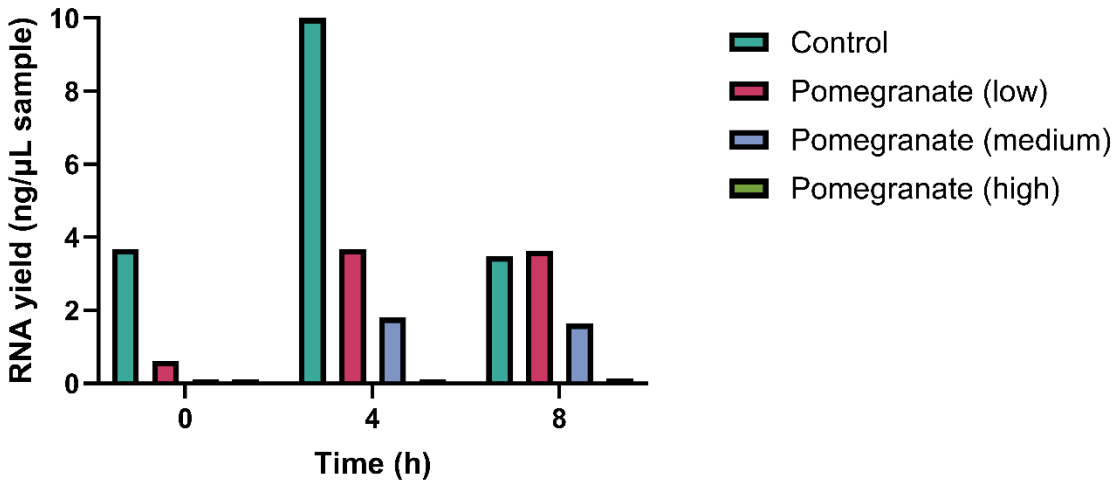

Figure 4.11. The effect of increasing pomegranate extract doses on RNA yield (ng/µL sample) from colon model samples at 0, 4, and 8 hours of fermentation. In vitro *batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and different doses of a pomegranate extract (0, 5.7, 11.4, or 22.8 mg/mL). One sample per pomegranate extract dose was collected at each time point. Samples were first resuspended in RNAlater and left at 4 °C for 24 hours before being stored at -80 °C until RNA extractions were carried out using the RNeasy PowerFecal Pro Kit (Qiagen). RNA yield was estimated using a Qubit™ RNA HS High Sensitivity Assay Kit with a detection limit of 100 ng/µL (10 ng/µL sample).*

The initial RT-qPCR analysis corroborated the *caiT* gene abundance patterns observed at the DNA level, showing dose-dependent suppression at 4 hours in pomegranate-treated models (**Figure 4.12**). At 8 hours, the highest pomegranate concentration again produced the greatest *caiT* transcript abundance. Notably, transcript levels remained substantially

lower than gene copy numbers across all conditions, with transcript-to-DNA ratios ranging from 0.03-0.3%, meaning that *caiT* transcription remains minimal relative to its abundance. The most pronounced effect was observed for the low pomegranate dose at 8 hours, which increased the transcript-to-DNA ratio 10-fold compared to the control (0.3% vs. 0.03%).

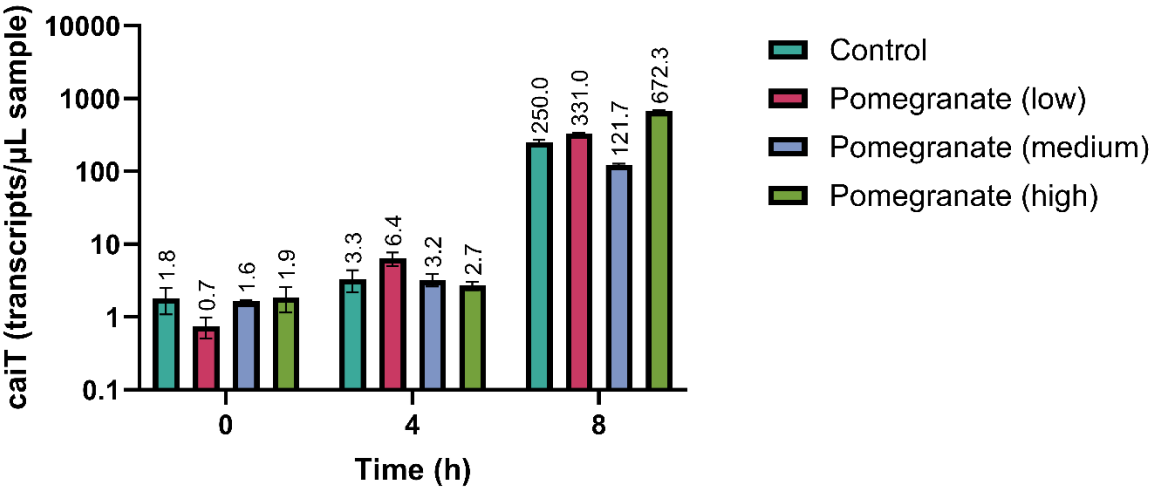

Figure 4.12. Expression of the caiT gene (transcripts/µL sample), which encodes for the L-carnitine/γ-butyrobetaine antiporter, in colon models at different time points. *Data are presented as mean ± SD of three technical replicates from one donor.* In vitro *batch colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and different doses of a pomegranate extract (0, 5.7, 11.4, or 22.8 mg/mL). Samples were collected at different time points, resuspended in RNAlater and left at 4 °C for 24 hours before being stored at -80 °C until extractions were carried out using the RNeasy PowerFecal Pro Kit (Qiagen). RT-qPCR reactions were carried out using a Luna® Universal Probe (New England Bioscience) with a caiT TaqMan probe and primers in a OneStep Plus PCR System (Applied Biosystems). The samples underwent 40 cycles, with rapid heating to 95 °C to denature the DNA, followed by cooling to 60 °C.*

In a second experiment, testing up to 24 hours post-inoculation and only testing the high pomegranate extract dose (22.8 mg/mL), the relative estimated number of *caiT* transcripts remained low for the control colon models and was higher for the pomegranate-treated colon models at 8 hours of fermentation (**Figure 4.13**). It should be noted, however, that the SD for the transcript number of the pomegranate-treated colon model at 8 hours is very large (± 588.7 transcripts/µL sample) and with only 2 biological replicates it cannot be ruled out that this was an outlier. Moreover, the *caiT* transcript

numbers reported in **Figure 4.13** are low compared to the transcript numbers observed in the initial experiment (**Figure 4.12**).

Furthermore, a large difference in RNA yield was observed between the control and the pomegranate-treated colon models (**Appendix 4, Supplementary Figure S12**), suggesting that the RNA extraction technique needs to be further optimised.

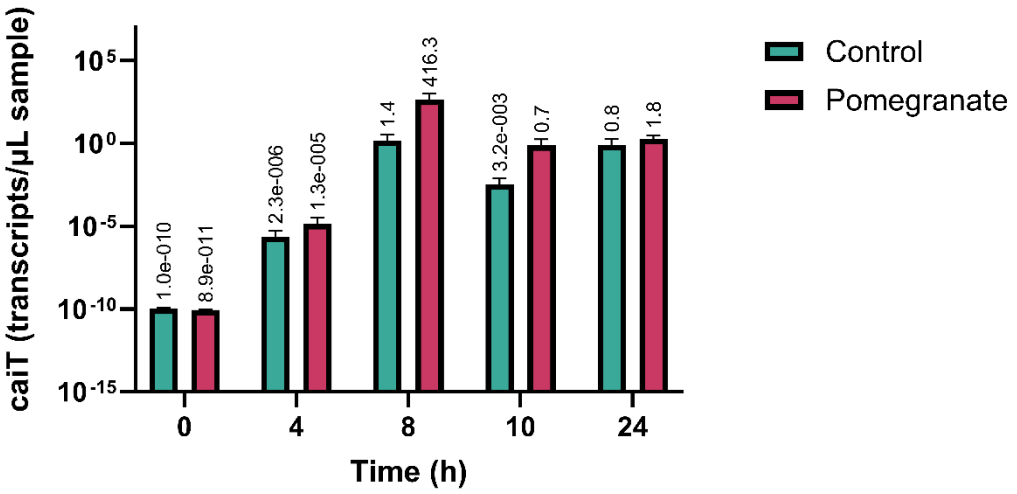

Figure 4.13. Expression of the caiT gene (transcripts/µL sample) on a log_{10} scale, which encodes for the L-carnitine/γ-butyrobetaine antiporter, in colon models at different time points. *Data are presented as mean ± SD from 2 donors.* In vitro *batch colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine, with or without a pomegranate extract (22.8 mg/mL). Samples were collected at different time points, resuspended in RNAlater and left at 4 °C for 24 hours before being stored at -80 °C until extractions were carried out using the RNeasy PowerFecal Pro Kit (Qiagen). RT-qPCR reactions were carried out using a Luna® Universal Probe (New England Bioscience) with a caiT TaqMan probe and primers in a OneStep Plus PCR System (Applied Biosystems). The samples underwent 40 cycles, with rapid heating to 95 °C to denature the DNA, followed by cooling to 60 °C.*

4.5.7 The effect of punicalagin on the abundance of the *caiT* gene

Since it was found that the pomegranate polyphenol punicalagin is responsible for the observed effect of the pomegranate extract on L-carnitine metabolism, DNA was also extracted from high-throughput colon models that were treated with punicalagin (2 mg/mL). Although it was observed in previous experiments that the pomegranate extract inhibited the DNA yield, in the case of punicalagin the DNA yield was higher compared with the control (**Figure 4.14**). Notably, the DNA yield reported here, from the

high-throughput colon model, was lower than the DNA yield from the batch colon model (**Figure 4.4, Figure 4.7**). Since the media of the high-throughput colon models contain a lower glucose concentration than the batch colon models (0.1% versus 1%), this might have affected the bacterial growth and therefore the DNA yield.

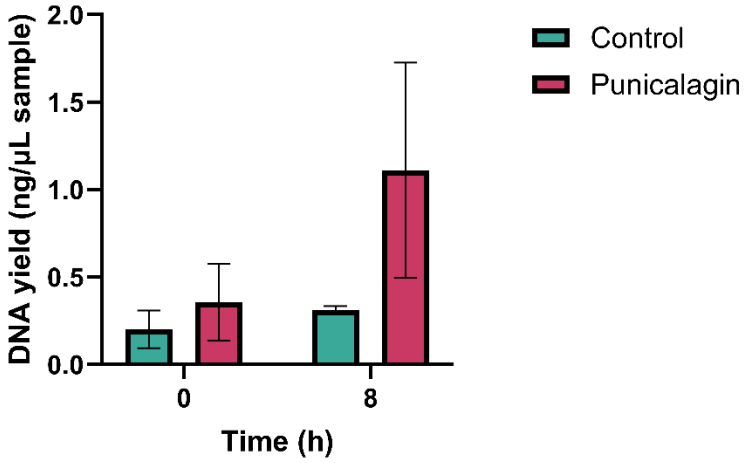

Figure 4.14. The DNA yield (ng/µL sample) from high-throughput colon model samples at 0 and 8 hours of fermentation. *Data are presented as mean ± SD from 3 donors. In vitro high-throughput colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine, with or without punicalagin (2 mg/mL). Samples were directly stored at -80 °C until DNA was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals). DNA yield was estimated using a Qubit™ dsDNA High Sensitivity Assay Kit.*

Initial *caiT* gene abundance was comparable between punicalagin-treated and control colon models (**Figure 4.15**). Following 8 hours of fermentation, *caiT* abundance in punicalagin-treated models increased substantially to 9.6×10^4 copies/µL, while the abundance was significantly lower for the control models, with 4.8×10^4 copies/µL (P = 0.0003, **Figure 4.15**Error! Reference source not found.). It should be noted, however, that these copy numbers were slightly lower than those observed in the batch colon models treated with the pomegranate extract (**Figure 4.5, Figure 4.10**). Possibly, the lower copy number is due to the lower DNA yield from the high-throughput colon models (**Figure 4.14**) compared to the batch colon models (**Figure 4.4, Figure 4.7**).

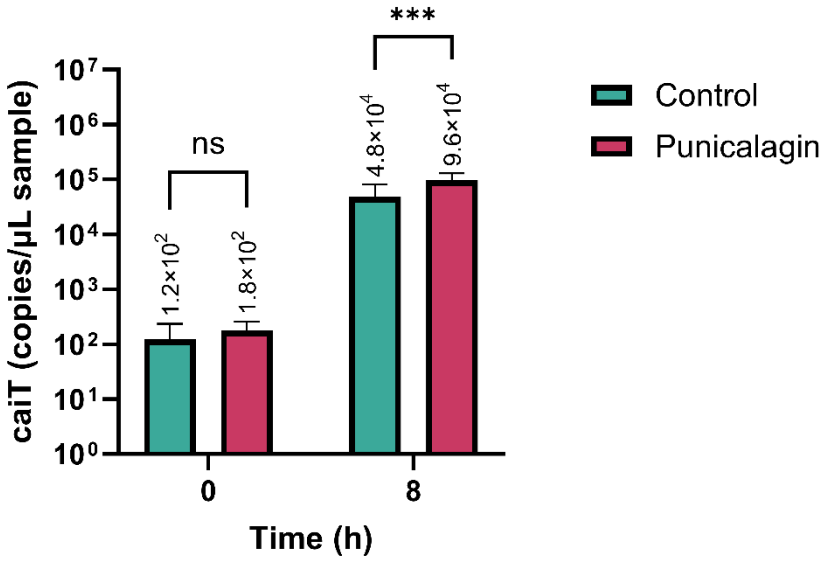

Figure 4.15. Abundance of the caiT gene (copies/µL sample), which encodes for the L-carnitine/γ-butyrobetaine antiporter, in high-throughput colon models at 0 and 8 hours of fermentation. *Data are presented as mean ± SD from 3 donors. Statistical analysis was performed using Two-Way ANOVA with post-hoc pairwise comparisons between each condition (control versus punicalagin) at each time point (*** P < 0.001). In vitro high-throughput colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine, with or without punicalagin (2 mg/mL). Samples were directly stored at -80 °C until DNA was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals). qPCR reactions were carried out using a Luna® Universal Probe (New England Bioscience) with a caiT TaqMan probe and primers in a OneStep Plus PCR System (Applied Biosystems). The samples underwent 40 cycles, with rapid heating to 95 °C to denature the DNA, followed by cooling to 60 °C.*

4.5.8 Microbial diversity and composition of *caiT*-containing bacteria in colon models

The *caiT* gene has been reported in 244 bacterial strains (KEGG database, accessed 13 August 2025). A large proportion of the reported *caiT*-containing bacteria belong to *Escherichia* and *Salmonella* and are predominantly *E. coli* and *S. enterica* strains (**Table 4.1**). In **Table 4.1**, strain identifiers were only included when the KEGG database did not specify the species. Additionally, several *Citrobacter*, *Proteus,* and *Shigella* species have been reported to contain the *caiT* gene. Several of the species are well-known gut microbiota reported in gut microbiome database GMrepo [200], including, but not limited to, *E. coli, E. fergusonii, S. enterica, S. bongori S. flexneri, S. sonnei*, *C. koseri, C. freundii, P. mirabilis, P. vulgaris, and P. penneri.*

Table 4.1. Bacterial genera and species containing* caiT *gene. *The KEGG (Kyoto Encyclopedia of Genes and Genomes) database (https://www.genome.jp/kegg/) was searched for the* caiT *gene (K05245), encoding for the L-carnitine/gamma-butyrobetaine antiporter, on 13 August 2025. For entries where KEGG database did not specify the species, the strain or isolate identifier provided in the database is shown (e.g., Escherichia sp. E4742).*

Genus	Species
Escherichia	*E. coli, E. fergusonii, E. marmotae, E. sp. E4742, E. ruysiae, E. sp. F1*
Salmonella	*S. enterica, S. bongori, S. sp. SSDFZ69*
Shigella	*S. flexneri, S. sonnei, S. dysenteriae, S. sp. PAMC 28760, S. sp. PIB*
Citrobacter	*C. rodentium, C. koseri, C. freundii, C. sedlakii, C. amalonaticus, C. sp. FDAARGOS_156, C. farmer, C. sp. CFNIH10, C. sp. CRE-46, C. pasteurii, C. telavivensis, C. tructae, C. arsenatis, C. sp. XT1-2-2, C. sp. TSA-1, C. enshiensis, C. sp. LUTT5, C. sp. TBCP-5362, C. sp. Y3*
Proteus	*P. mirabilis, P. vulgaris, P. hauseri, P. sp. CD3, P. columbae, P. terrae, P. penneri, P. appendicitidis, P. rettgeri*
Enterobacter	*E. lignolyticus*
Shewanella	*S. loihica, S. sediminis, S. pealeana, S. halifaxensis, S. piezotolerans, S. algae, S. livingstonensis. S. carassii, S. chilikensis, S. aegiceratis, S. spartinae, S. alkalitolerans, S. rhizosphaerae, S. sp. MTB7*
Desulfobaculum	*D. bizertense*
Desulforapulum	*D. autotrophicum*
Desulfitobacterium	*D. hafniense*
Desulfosporosinus	*D. orientis, D. meridiei*
Desulforamulus	*D. reducens*
Edwardsiella	*E. ictaluri, E. tarda, E. anguillarum, E. piscicida, E. hoshinae, E. sp. EA181011, E. sp. LADL05-105*
Hafnia	*H. alvei, H. paralvei*
Sporomusa	*S. termitida, S. sphaeroides, S. ovata, S. silvacetica*
Erysipelothrix	*E. inopinata, E. sp. HDW6C*
Shimwellia	*S. blattae*
Atlantibacter	*A. subterraneus*
Yokenella	*Y. regensburgei*
Phytobacter	*P. ursingii, P. diazotrophicus, P. sp. MRY16-398*
Pseudocitrobacter	*P. corydidari*
Trabulsiella	*T. odontotermitis*
Enterobacteriaceae	*E. bacterium*
Providencia	*P. heimbachae, P. huaxiensis, P. hangzhouensis, P. zhijiangensis, P. xihuensis, P. lanzhouensis*

Obesumbacterium	*O. proteus*
Limnobaculum	*L. parvum, L. zhutongyuii*
Leminorella	*L. richardii, L. grimontii*
Campylobacter	*C. pinnipediorum, C. rectus*
Malaciobacter	*M. halophilus, M. marinus*
Candidatus	*C. Formimonas warabiya*
Acididesulfobacillus	*A. acetoxydans*
Eubacterium	*E. callanderi, E. maltosivorans, E. limosum*
Blautia	*B. parvula*

To test the effect of the pomegranate extract on the presence and relative abundance of microbial genera and species, an explorative metagenomic analysis was performed on several samples from one control and one pomegranate-treated colon model.

Of the genera, *Escherichia* was most prevalent at later time points in the pomegranate-treated batch model (**Figure 4.16**). Within the *Escherichia* genus, only *E. coli* was observed. This large relative abundance of *E. coli* in the colon model at later time points can explain the higher abundance of the *caiT* gene that was observed. Nonetheless, these data should be interpreted with caution, as the samples were only derived from a single control and a single pomegranate-treated colon model.

These data demonstrate that the pomegranate extract increased, rather than inhibited, the relative abundance of *Escherichia coli*, a gut microbe containing the *cai* operon. This observation suggests that selective inhibition of microbes harbouring the *cai* operon is not the underlying mechanism responsible for the inhibitory effect of the pomegranate extract on L-carnitine metabolism.

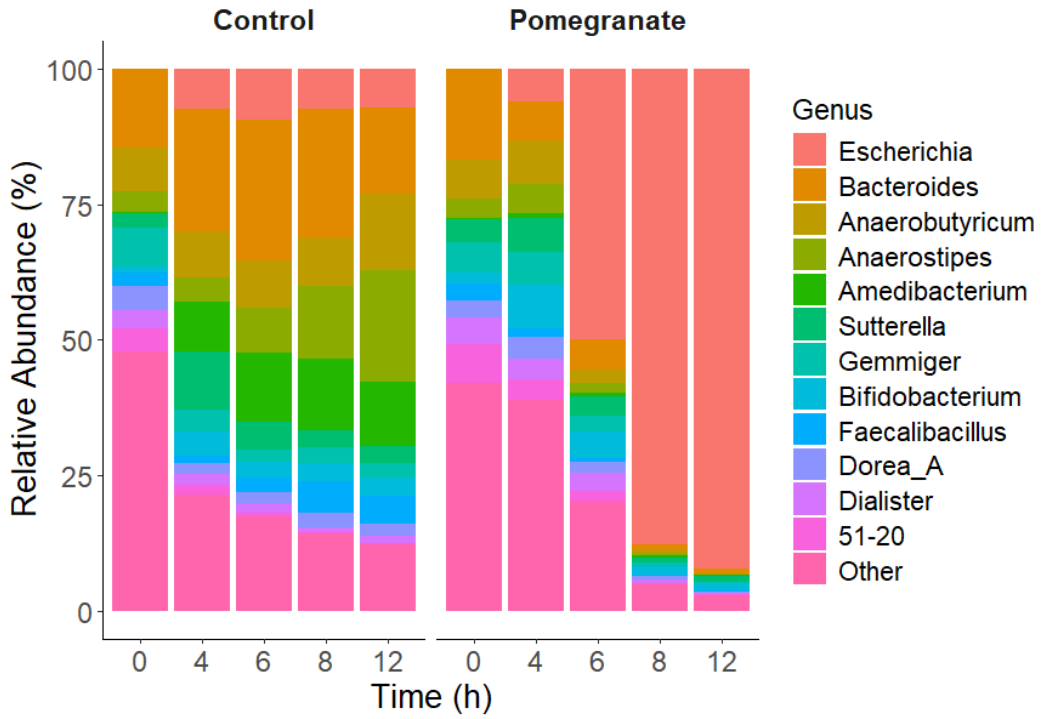

Figure 4.16. Relative abundance (%) of bacterial species in colon model samples treated with a pomegranate extract versus a control at different time points (n = 1). *In vitro colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine, with or without a pomegranate extract (22.8 mg/mL). Samples were collected at 0, 4, 6, 8 and 12 hours and DNA was extracted using the FastDNA™ SPIN Kit for Soil. Samples were sequenced by Technical Genomics group at the Earlham Institute (Norwich, UK) and metagenome-assembled genomes (MAGs) and coverage depth were derived from the reads. Coverage depth was normalised into % relative abundance for each sample. Species with a mean relative abundance < 2% were grouped in "Other". Numeric names represent unclassified or provisional taxa. Dr Robert James prepared the samples for sequencing and Dr Sébastien Raguideau performed the co-assembly of the data.*

Additionally, the variety of microbes in the colon models was estimated using the R package 'vegan' [201]. Two measures of diversity were used: the Shannon index and the Inverse Simpson index. The Shannon index considers how many different species are present and how evenly they are distributed, with higher values indicating more diverse microbial communities. The Inverse Simpson index shows how many species are dominant in the community, with higher values indicating that the community is made up of many species that are relatively similar in abundance.

Within the pomegranate-treated colon model, the Shannon and the Inverse Simpson index initially increased between 0-4 hours of fermentation, but then rapidly decreased between 4-8 hours compared with the control model (**Figure 4.17**). The reduced

diversity at later time points in the pomegranate-treated colon model could be explained by the very large relative abundance of *E. coli* (**Figure 4.16**).

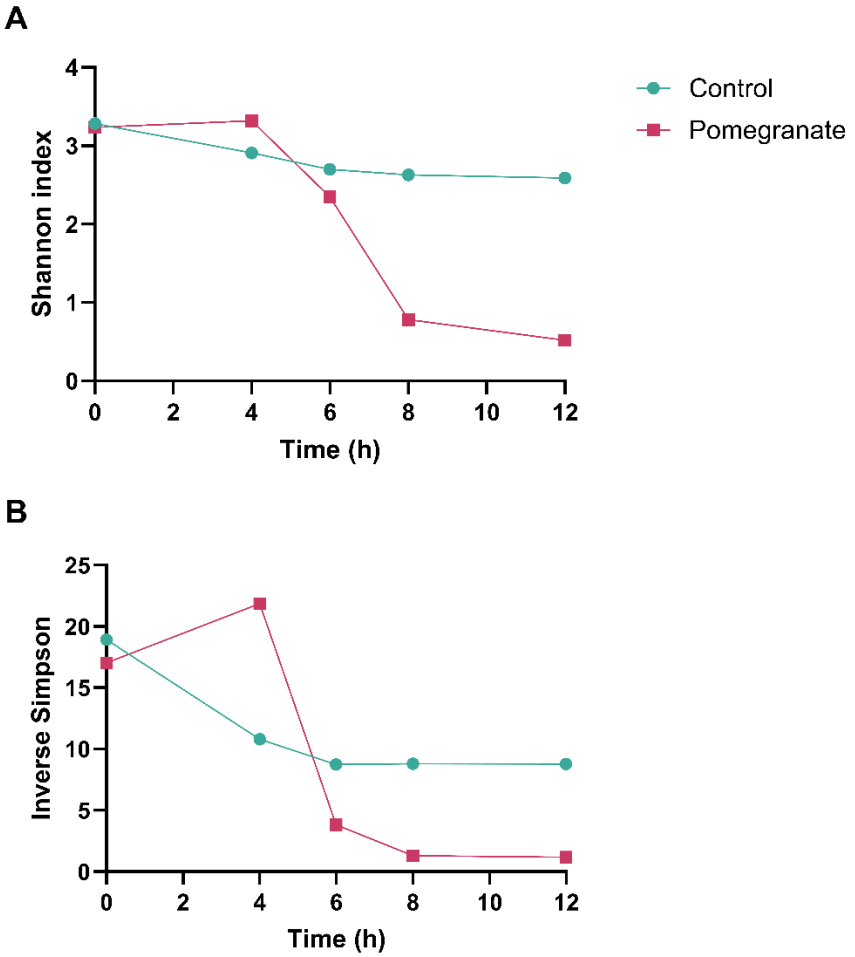

Figure 4.17. (A) Shannon diversity and (B) inverse Simpson indices. *In vitro colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine, with or without a pomegranate extract (22.8 mg/mL). Samples were collected at 0, 4, 6, 8 and 12 hours and DNA was extracted using the FastDNA™ SPIN Kit for Soil. Samples were sequenced by Technical Genomics group at the Earlham Institute (Norwich, UK)and metagenome-assembled genomes (MAGs) and coverage depth were derived from the reads. Shannon index and inverse Simpson index were obtained from the relative abundance data using the 'vegan' package in R. Dr Robert James prepared the samples for sequencing and Dr Sébastien Raguideau performed the co-assembly of the data.*

4.6 Discussion

The aim of the research presented in this chapter was to investigate several possible underlying mechanisms responsible for the inhibitory effects of the pomegranate extract and punicalagin on L-carnitine metabolism to γ-BB and TMA. The main findings reported in this chapter was that the pomegranate extract resulted in an overall increase in total viable bacteria and an increase in the abundance of *caiT* genes, involved in the anaerobic metabolism of L-carnitine to γ-BB, demonstrating that the extract is not acting as a general antibacterial, nor selectively inhibiting *caiT*-carrying bacteria.

This chapter furthermore showed that the pomegranate extract increased the abundance of *Escherichia coli*, of which many strains have been reported to contain the *caiT* gene [198, 199]. It is possible that the increase in *E. coli* resulted in the observed increase in *caiT* gene copies within pomegranate-treated colon models, although it is important to note that the increase in *E. coli* was observed in an explorative metagenomic analysis involving just one pomegranate-treated colon model and one control model. These observations contradict previous reports suggesting that pomegranate polyphenols have antimicrobial effects on microbes such as *Escherichia coli*, *Proteus* sp., and *Salmonella* species [151, 153], of which many contain the *caiT* gene [198, 199]. Based on the findings reported here, selective inhibition of microbes harbouring the *cai* operon is likely not the underlying mechanism responsible for the inhibitory effect of the pomegranate extract and punicalagin on L-carnitine metabolism, which is seen from the increase in *caiT* gene copies, as well as the increase in *E. coli* in the pomegranate-treated colon models.

This chapter also assessed the expression of *caiT* genes, but the *caiT* transcript numbers remained very low relative to the DNA copy numbers in the control and pomegranate-treated colon models. Although it is possible that transcript numbers are lower than DNA copy numbers since bacteria can have multiple copies of a gene (due to the presence of plasmids, multiple chromosomes, or gene duplications [202, 203]), the *caiT* transcript numbers were much lower than expected with transcript-to-DNA ratios ranging from 0.03-0.3% in the initial experiment and even more reduced ratios after the optimisation of the DNA extractions.

Possibly, the number of *caiT* transcripts (RNA) was lower than the *caiT* copy number (DNA) because the RNA may have been degraded during the sample collection, RNA

extractions, or RT-qPCR preparation. After all, RNA degrades much faster than DNA, because RNases are ubiquitous in nature and ribose is more susceptible to hydrolysis than deoxyribose [204, 205]. Since the RT-qPCR has an additional step, the reverse transcription, it could result in the loss of RNA. A limitation of the RT-qPCR analysis was the use of the vector-based *caiT* PCR standard without converting the DNA to RNA or complementary DNA (cDNA). Some researchers suggest that the quantification of transcripts should not be conducted using a standard curve from double-stranded DNA (dsDNA) at all [206]. Since the vector-based *caiT* PCR standard did not undergo reverse transcription, the standard does not account for reverse transcription efficiency. Instead, to increase accuracy, the standard curve should be constructed from a cDNA template. For instance, an RNA standard can be prepared by linearising the *caiT* plasmid (i.e., create a linear DNA strand). To do so, a restriction enzyme should be selected that is located on the plasmid downstream of the *caiT* insert and before the MCS promotors, T7 or SP6. From the linear DNA template, RNA can be transcribed with T7 or SP6 polymerase (depending on where the DNA was cut). Then, the RNA should be purified using a silica spin column kit (e.g., Zymo Research RNA Clean & Concentrator kit), which also includes DNase to remove DNA template that could contaminate the RNA. The RNA can be quantified using Qubit™ RNA HS High Sensitivity Assay Kit to obtain the copy number. The RNA can be used directly on the PCR plate, or first be subjected to reverse transcription to create cDNA. The benefit of using RNA as a standard is that it will better capture the reverse transcription efficiency, while the benefit of cDNA as a standard is that it is less prone to degradation than RNA and can therefore be reused in other experiments. Furthermore, the cDNA standard can be used across different experiments which allows for better normalisation of the data. In summary, first converting the vector-based *caiT* standard to an RNA or cDNA standard will capture the reverse transcription efficiency and result in a better estimation of *caiT* transcript numbers.

Moreover, to account for differences introduced during RNA/DNA extractions, sample preparation for PCR, and reverse transcription, it might be useful to include 2-3 reference (housekeeping) genes in the PCR assays to normalise *caiT* RNA transcript or DNA copy numbers to the reference genes. In future experiments, the most stable reference genes in these conditions can be selected using a tool like geNorm [207, 208]. To use geNorm, a set of candidate reference genes should first be tested across colon model samples. Candidate reference genes can include: the 16S rRNA gene, the most common and a highly conserved gene present in all bacteria which is often used as a

reference gene [209, 210]; *recA* and *recF*, genes involved in DNA repair that have also been used as reference genes in other fermentation studies [210, 211] and are supported for their use as reference genes [212]; *rpoB*, a transcription gene and commonly used reference gene [210, 213]; *proC*, a gene involved in proline biosynthesis which has been found to be relatively stable in microbial studies [214, 215]; *gyrA* and *gyrB*, ubiquitous bacterial genes encoding for subunits A and B of DNA gyrase and used as reference genes in several microbiology studies [210, 213, 215, 216]. Based on the Cq values of the candidate reference genes, geNorm can be used to rank the genes from most to least stable across conditions and time points. The 2-3 most stable reference genes should then be selected for inclusion in future PCR experiments. However, it should be taken into account that a substantial difference in total viable bacteria was observed between the control and pomegranate-treated colon models, which may then obscure the PCR results upon normalisation to reference genes.

In this chapter a large difference in RNA yield was observed between the control and the pomegranate-treated colon models. Therefore, in addition to the use of reference genes, the RNA extraction technique should be optimised to minimise inhibition by the pomegranate extract. First, additional steps should be taken when storing colon model samples from which RNA will be extracted. It is important to use tubes specifically designed for RNA storage that are airtight, watertight and RNase free [205]. It is also recommended to use water with 0.1% diethyl pyrocarbonate (DEPC) for all solutions and reagents used in the RNA extractions and RT-qPCR preparation [205]. DEPC deactivates ribonucleases, as such preventing RNA degradation. Second, a secondary RNA cleanup step could be included directly after the RNA extractions using a silica spin column kit (e.g., Zymo Research RNA Clean & Concentrator kit).

The difference in RNA yield between the pomegranate-treated and control colon models might also affect the downstream RT-qPCR assay. Therefore, it should be established to what extent the pomegranate extract inhibited the RT-qPCR assay. This can be achieved by doing a dilution experiment with a spiked-in gene similar to the experiment described in Section **4.4.9**. Briefly, RNA extracts should be obtained from pomegranate-treated colon models and serially diluted (e.g., raw, 1:10, 1:100). Then, a standardised copy number of a GFP-containing plasmid (or equivalent) can be spiked into each of the dilutions. This allows to test for the interference of pomegranate extract constituents with the RT-qPCR analysis, with greater dilutions resulting in reduced extract

interference and improved recovery of the expected plasmid copy number, thereby providing a quantitative measure of assay inhibition.

In this chapter, explorative metagenomic analyses showed that *E. coli*, which is a known carrier of the *cai* operon, was increased in pomegranate-treated models. However, only samples from one control model and one pomegranate-treated model from a single donor were included. To get a better understanding of microbial shifts within pomegranate-treated versus control colon models, and to confirm whether the pomegranate-induced increase of *E. coli* is a pattern or a single occurrence, it is important to repeat this experiment with samples from at least three donors.

Two other underlying mechanisms that were proposed previously in this thesis (Sections **2.6**, **3.6.1**, and **4.2**) have not been addressed by the research presented in this chapter: 1) the pomegranate extract/punicalagin may inhibit proteins that carry out the conversion of L-carnitine to γ-BB and γ-BB to TMA (e.g., enzymes and transporters), and 2) punicalagin may make L-carnitine and γ-BB less accessible to microbial enzymes by donating hydrogen bonds to polar sites and forming cation-π interactions with L-carnitine and γ-BB. Based on the findings reported here, that the extract is not reducing total bacterial numbers nor selectively inhibiting *caiT*-carrying bacteria, it is certainly possible that one of those two mechanisms explains the observed inhibitory effect of the pomegranate extract and punicalagin on L-carnitine metabolism to γ-BB and TMA.

For instance, punicalagin may disrupt TMA production by restricting access of L-carnitine or γ-BB to microbial enzymes. There are reports of polyphenols binding to the outer regions of membrane proteins (e.g., porins), leading to reduced membrane permeability and restricted access of substrates to enzymes [159, 160]. To test this in a future experiment, enzyme assays should be performed using bacterial cells containing the *cai* and *gbu* operons. In a previous report by Koeth *et al.* (2019), it was shown that the combination of *P. penneri* and *E. timonensis* could successfully convert L-carnitine to TMA, while neither of the strains could complete the metabolic pathway alone [67]. When a *P. penneri* culture was supplemented with L-carnitine, the substrate was completely converted to γ-BB within four hours, but no TMA was being produced. L-Carnitine supplementation in a culture only containing *E. timonensis* did not result in the production of γ-BB nor TMA, while γ-BB supplementation did result in TMA production [67]. The report demonstrated that *P. penneri* and *E. timonensis* are useful strains to study the L-carnitine → γ-BB → TMA pathway in isolation. First, the effect of the

pomegranate extract and/or punicalagin on L-carnitine metabolism should be tested in a (co-)culture of *P. penneri* and *E. timonensis.* Second, upon confirmation that the extract/punicalagin inhibit L-carnitine metabolism in the cultures, an enzyme assay should be performed to test the effect of the pomegranate extract and punicalagin on enzymes involved in L-carnitine metabolism to γ-BB and TMA. For the enzyme assays, the bacterial genes would first need to be activated by incubation with L-carnitine (for *P. penneri*) and γ-BB (for *E. timonensis*), and the cells would need to be lysed. Then, the lysate could be incubated with labelled L-carnitine or γ-BB (e.g., L-carnitine-d9 or γ-butyrobetaine-d9) such that the enzymatic conversion to γ-butyrobetaine-d9 or TMA-d9 can be measured using LC-MS/MS as described earlier in this thesis.

Furthermore, it is important to investigate the effect of the pomegranate extract and punicalagin on *gbu*-carrying bacteria. In **Chapter 2** of this thesis, it was demonstrated that a pomegranate extract inhibited the metabolism of γ-BB to TMA, independently of the metabolism of L-carnitine to γ-BB. This suggests that the pomegranate extract might also affect abundance or expression of *gbu* genes, or interfere with the proteins involved in the conversion of γ-BB to TMA. In future experiments, a vector-based *gbu* PCR standard should be developed which can then be used in qPCR and RT-qPCR experiments to quantify the abundance and expression of the *gbu* operon in colon models.

This chapter provided an overview of the next steps to be taken in further understanding the underlying mechanisms responsible for the inhibitory effects of the pomegranate extract and punicalagin on L-carnitine metabolism to γ-BB and TMA. The 7 key recommendations for future research investigating the underlying mechanisms are as follows:

1. Convert the vector-based *caiT* standard to an RNA or cDNA standard to capture the reverse transcription efficiency and improve the accuracy of *caiT* transcript quantification.
2. Include 2-3 stable reference genes, selected through geNorm from a set of candidate reference genes, in the qPCR and RT-qPCR analyses.
3. Optimise the RNA extraction technique to minimise inhibition by the pomegranate extract (e.g., improved storage conditions and adding a secondary RNA cleanup step).

4. Establish to what extent the pomegranate extract inhibited the RT-qPCR assay through serially diluting RNA samples and spiking in a standardised copy number of a gene prior to the RT-qPCR.
5. Repeat the shotgun metagenomic sequencing analysis with pomegranate-treated colon model samples from three or more donors to confirm whether the increased numbers of *E. coli* in pomegranate-treated colon models is a pattern or was a single occurrence.
6. Conduct enzyme assays using a (co-)culture of *P. penneri* and *E. timonensis*, containing the *cai* and *gbu* operons, to test the effect of the pomegranate extract and punicalagin on enzymes involved in L-carnitine metabolism to γ-BB and TMA.
7. Investigate the effect of the pomegranate extract and punicalagin on the abundance of *gbu*-carrying bacteria and the expression of the *gbu* operon through qPCR and RT-qPCR analyses with a vector-based *gbu* PCR standard.

4.7 Conclusion(s)

The pomegranate extract increased total bacterial viability and the abundance of *cai* genes, demonstrating that the extract did not act as a general antibacterial, nor selectively inhibited the growth of *cai*-carrying bacteria. To further understand the underlying mechanisms and enable the development of targeted treatments reducing microbial TMA production, and subsequent TMAO formation, more research is warranted. For future research investigating the underlying mechanisms, 7 key recommendations were proposed.

Chapter 5

The translation from bench to clinic: the effect of a pomegranate extract on plasma trimethylamine N-oxide (TMAO) levels in healthy adults

Chapter 5: The translation from bench to clinic: the effect of a pomegranate extract on plasma trimethylamine N-oxide (TMAO) levels in healthy adults

5.1 Abstract

Background: The previous chapters demonstrated that a punicalagin-rich pomegranate extract inhibits the production of TMA from L-carnitine. Some previous human studies have investigated the effects of polyphenol-rich extracts on fasting TMAO levels in healthy adults but did not investigate the effect on TMAO production from L-carnitine, nor the effect of pomegranate polyphenols.

Aim and approach: The aim of the TESSA study was to investigate the effect of a pomegranate extract on the TMAO response from an oral carnitine response test (OCRT). This dietary intervention study in healthy, omnivorous adults was two-phased. In Phase I, habitual diet was assessed using a food frequency questionnaire (FFQ), serum creatinine and estimated glomerular filtration rate (eGFR) were measured, and participants' TMAO response was measured after an OCRT of 1.5 g L-carnitine. High TMAO producers (increase ≥ 5 μM) were invited to Phase II: an 18-day double-blind, randomised, placebo-controlled, crossover study consisting of two 48-hour pharmacokinetic interventions separated by a 10-day washout period. One intervention involved an OCRT with a simultaneous administration of the pomegranate extract (1.6 g) and the other an OCRT with a placebo. Blood, stool and urine were collected during Phase II.

Results: The pomegranate extract did not reduce the TMAO area under the curve (AUC) in the full study population (n = 16). However, in all male and premenopausal female participants (n = 13) the extract reduced TMAO AUC by 15% (95% CI: 1.01 to 1.31, P = 0.036) compared to the placebo. Furthermore, a strong correlation was observed between higher serum creatinine levels, indicating impaired kidney function, and a reduced TMAO AUC by the pomegranate intervention compared with the placebo (r = 0.712, P = 0.002).

Conclusion: The pomegranate extract did not reduce plasma TMAO levels in the overall TESSA study population, although modest effects were observed in male and premenopausal participants.

5.2 Introduction

In vitro work using colon models demonstrated that a polyphenol-rich pomegranate extract and the pomegranate polyphenol punicalagin can inhibit the production of TMA from L-carnitine [69], which has been presented in Chapters 2 and 3 of this thesis.

In contrast to *in vitro* colon models which solely investigate microbial metabolism, human physiology is complex and involves absorption, distribution, metabolism, and excretion (ADME), which affects the bioavailability of dietary compounds [217]. In the case of punicalagin, research has shown that it is poorly bioavailable in the small intestine due to its large molecular weight (1084 Da) and high number of hydrogen bond donors and acceptors [106], enabling the compound to reach the colon where it can interact with the gut microbiota. Therefore, it can be hypothesised that punicalagin has the potential to inhibit microbial TMA production *in vivo*.

The bioavailability of L-carnitine ranges between 54-87% from the diet [59], compared to only 14-18% from supplements [60]. The lower bioavailability from supplements occurs because absorption at higher supplement doses relies mainly on passive transport across enterocyte membranes, whereas L-carnitine from the diet involves both active and passive transport [60]. The remaining L-carnitine transits to the colon where it is metabolised by gut microbiota to TMA. As seen in the *in vitro* colon models, the concentrations of TMA being produced are highly variable between donors, likely due to differences in gut microbiome composition and dietary habits. For instance, omnivores have a more than 20-fold greater TMAO response from L-carnitine than vegetarians [67]. A human study involving an OCRT reported high interindividual variation in plasma TMAO concentrations, especially between vegetarians and omnivores [71]. It is likely that omnivores are exposed to higher levels of L-carnitine (from meat) and therefore have a gut microbiome that is more efficient in TMA production, increasing plasma TMAO levels. For instance, studies have shown that diets higher in red meat were associated with higher levels of TMAO [218] and that L-carnitine supplementation could enhance the capacity of the gut microbiota to produce TMA [219]. Therefore, it can be hypothesised that individuals who derive more L-carnitine from the diet will produce more TMA and, subsequently, TMAO, which also makes omnivores a more relevant study population to investigate the effects of treatments to reduce TMAO production.

Some human studies investigated the effects of polyphenol-rich extracts on TMAO levels in healthy adults (**Chapter 1**, **Table 1.1**). For instance, a two-arm parallel study in 38 men showed that the daily consumption of 30 g of Korean black raspberry for four weeks significantly reduced fasting plasma TMAO levels compared to baseline [101]. Like pomegranates, black raspberries contain large amounts of ellagic acid [118], as well as anthocyanins [114]. It is also likely that black raspberries contain ellagitannins, since (red) raspberries have been reported to contain substantial amounts of ellagitannins [119]. Two other studies investigated the TMAO-reducing effects of a grape pomace extract rich in flavonoids (flavan-3-ols, flavonols, proantocyanidins), stilbenes (resveratrol), and phenolic acids (hydroxybenzoic acid, hydroxycinnamic acid). One of those studies was a crossover study in 20 healthy adults, which demonstrated that the daily consumption of 600 g grape extract for four weeks reduced serum TMAO levels compared to a placebo intervention [98]. The other study, a two-arm parallel study in 65 adults with obesity, showed that the daily consumption of 300 g grape extract for eight weeks significantly reduced serum TMAO levels compared to baseline levels [99]. A three-arm parallel study in 90 adults showed that the daily consumption of 125 g apple puree, a rich source of flavonoids (flavan-3-ols, anthocyanins, flavonols), chlorogenic acid, and dihydrochalcones, reduced fasting plasma TMAO levels by eight weeks compared to baseline [100]. However, a crossover study in 20 men and women with obesity who consumed a flavonol-rich cocoa or green tea beverage twice daily for five days did not show a significant reduction in fasting plasma TMAO levels [102].

Although these studies examined how polyphenol-rich extracts affected circulating TMAO levels, they did not investigate whether these extracts could reduce the TMAO response when co-administered with precursors such as L-carnitine or choline. Investigating the TMAO response to a precursor such as L-carnitine is crucial, as it allows the direct assessment of polyphenols on microbial TMA production, while also capturing the pharmacokinetics of TMAO formation. Moreover, only one previous study investigated the effect of a source potentially containing ellagitannins [101]. Since the *in vitro* work presented in this thesis demonstrated that punicalagin, an ellagitannin, could almost completely block TMA production, ellagitannin-rich sources are compelling treatments to further investigate *in vivo* for their effects on TMAO production.

Given the evidence from the *in vitro* work and the available literature, which suggests that a punicalagin-rich pomegranate extract might inhibit TMAO production, the TESSA (TMAO-reducing Effects of a pomegranate Supplement Simultaneously Administered

with L-carnitine) study was established to investigate the effect of a pomegranate extract on plasma TMAO levels in healthy adults.

5.3 Objectives

The TESSA study investigated how the *in vitro* findings are translated to TMAO pharmacokinetics in healthy adults. The primary aim of this double-blind, randomised, placebo-controlled, crossover study was to estimate the effect of the pomegranate extract on blood plasma TMAO over 48 hours after an OCRT. Secondarily, the TESSA study aimed to determine the absorption and excretion of the L-carnitine and pomegranate polyphenols. The study also investigated whether the L-carnitine intake from the habitual diet correlated with the TMAO response.

The following objectives are addressed in this chapter:

1. To estimate the TMAO response following an OCRT in healthy, omnivorous adults in order to identify high-TMAO producers.
2. To compare the effect of an OCRT with a single dose of pomegranate extract (1.6 g) versus an OCRT with a placebo on plasma TMAO pharmacokinetics, including area under the curve (AUC), maximum concentration (C_{max}), half-life ($t_{1/2}$), and time to reach maximum concentration (T_{max}), in high-TMAO producers.
3. To explore the pharmacokinetic parameters (AUC, C_{max}, $t_{1/2}$, T_{max}) of L-carnitine and γ-BB concentrations in blood plasma after an OCRT with a single dose of pomegranate extract (1.6 g) or a placebo.
4. To estimate the concentrations of L-carnitine, γ-BB and TMA excreted in urine and faecal samples after an OCRT with a single dose of pomegranate extract (1.6 g) or a placebo.
5. To assess the correlation between plasma TMAO AUC and urolithin metabotypes.
6. To explore the correlation between plasma TMAO AUC and creatinine levels as a marker of kidney function (*post hoc* analysis).
7. To assess the correlation between the TMAO increase from baseline to 24 hours after an OCRT and the habitual intake of L-carnitine over 30 days prior to Phase I measured by an FFQ.

5.4 Materials and methods

5.4.1 Materials

L-Carnitine capsules (Carnipure®) were obtained from VitaminExpress LCC. The pomegranate extract (Dermogranate®) was purchased from Medinutrex (Catania, Italy). Hydroxypropyl methylcellulose (HPMC) DRcaps® size 0 capsules (Capsugel, now part of Lonza, Slough, UK) were purchased from on Amazon UK (www.amazon.co.uk). Gum Arabic (CAS 9000-01-5) was provided by Willy Benecke (Hamburg, Germany). Brown food dye (rainbow dust edible powder colour) was obtained from the Cake Decorating Co. (www.thecakedecoratingcompany.co.uk) and pharmaceutical grade microcrystalline cellulose (MCC) powder (REDWELLS HEALTH LTD, London, UK) from Amazon UK. DNA/RNA Shield was purchased from Zymo Research. All solvents were at least of high-performance liquid chromatography (HPLC) grade and obtained from Fisher Scientific Limited. Water was 18 MΩ·cm Milli-Q. Trimethylamine hydrochloride (CAS 593-81-7), TMAO (CAS 1184-78-7), γ-BB (commercially known as 3-(Carboxypropyl)trimethylammonium chloride, CAS 6249-56-5), urolithin A (CAS 1143-70-0), urolithin B (CAS 1139-83-9), ellagic acid (CAS 476-66-4), TCA, DMSO, glacial acetic acid, sulfatase (CAS 9016-17-5), β-glucuronidase (CAS 9001-45-0), and HFBA were obtained from Merck. L-Carnitine (CAS 541-15-1), ammonium acetate, AnaeroGen™ sachets, and RNAlater™ Stabilization Solution were obtained from Fisher Scientific Limited. Isotopically labelled standards were obtained from Cambridge Isotope Laboratories (L-carnitine-(trimethyl-d9), CAS 126827-79-0), LGC Standards (trimethylamine-d9 hydrochloride, CAS 18856-86-5; trimethylamine-d9 N-oxide, CAS 1161070-49-0), and Santa Cruz Biotechnology (γ-Butyrobetaine-d9, CAS 479677-53-7). Punicalagin was obtained from BOC Science (CAS 65995-63-3) and punicalin from Apollo Scientific (CAS 65995-64-4).

5.4.2 Study design

This two-phased intervention trial received approval by the QIB Human Research Governance Committee (QIB01/2024) and the South East Scotland Research Ethics Committee prior to enrolment (24/SS/0047). The study is registered at Clinicaltrials.gov with ID NCT06518343. The study protocol can be found in **Appendix 5**.

Phase I comprised of two visits within a 24-hour period during which participants consumed a single dose of 1.5 g L-carnitine – an OCRT. Directly prior to and 24 hours after the OCRT, fasting blood was collected. Participants also completed an FFQ. Nurses completed a medical questionnaire, measured height, weight and blood pressure. Fasted blood plasma samples were analysed for TMAO concentrations in-house, while full blood count, blood urea, creatinine, and eGFR were tested by the Norfolk and Norwich University Hospital (NNUH) Pathology Department.

Participants who were deemed high-TMAO producers (>5 μM and >50% increase between baseline and 24-hour follow-up) and met the clinical eligibility criteria (normal kidney function and blood pressure) were invited for the Intervention Phase (Phase II). Phase II was an 18-day, double-blind, randomised, controlled crossover study consisting of two interventions: once an OCRT with a pomegranate extract (1.6 g), and once an OCRT with a placebo. At the start of Phase II, participants were randomly allocated to one of the two intervention sequences (pomegranate then placebo, or placebo then pomegranate) (**Figure 5.1**).

Phase II comprised of six study visits over the course of 18 ± 1 days. Each intervention consisted of a 48-hour run-in period, followed by a 48-hour pharmacokinetic study. The interventions were separated by a 10-day washout period. Blood, urine, and faecal samples were collected at specified time points throughout the 48-hour pharmacokinetic study. For the duration of the pharmacokinetic studies, participants consumed standardised meals provided at prescribed times.

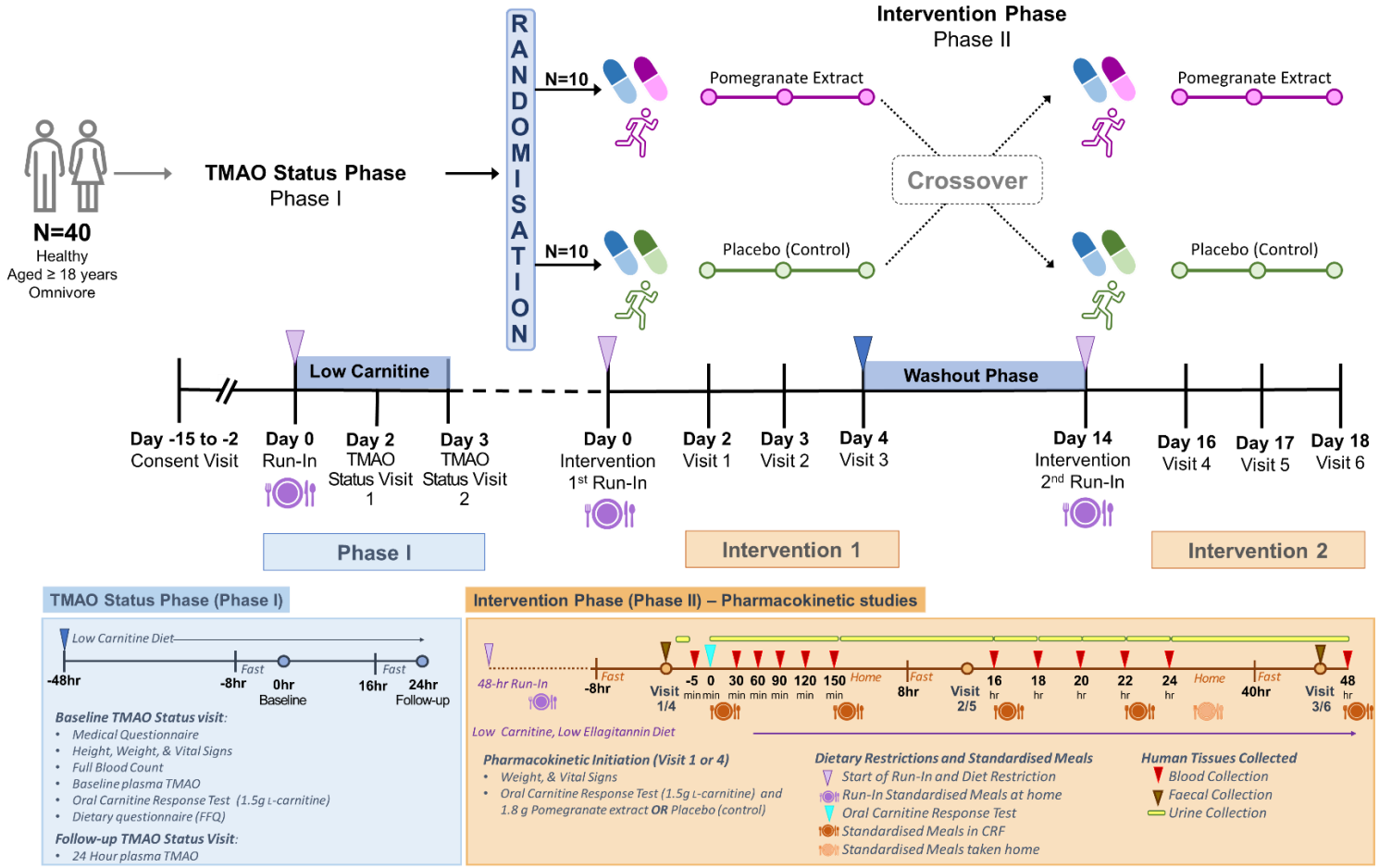

Figure 5.1. Schematic study design of the TESSA study. *The TESSA study is a two-phased study, including an 18-day, double-blind, randomised, controlled crossover study consisting of two 48-hour run-in periods and two 48-hour pharmacokinetic studies.*

5.4.3 Participants

34 healthy adults were recruited into Phase I and 20 continued to Phase II. Recruitment took place from August 2024 to March 2025. Participants were recruited through local news and social media and were included if they were healthy adults who followed an omnivorous diet (≥4 portions of meat weekly), have a BMI between 18.5-30 kg/m^2, and live within 40 miles of the Norwich Research Park. Omnivores were recruited because they were found to produce greater levels of TMAO from L-carnitine than vegetarians [67], and are therefore more likely to show an effect and to potentially benefit from a treatment to reduce TMAO levels.

Exclusion criteria were: having an allergy or intolerance for any of the ingredients in the standardised meals; having smoked in the 3 months prior to enrolment; having bowel movements less than 5 times a week; consuming more than 14 units of alcohol per week; taking medication that hinders blood clotting; abnormally high or low blood pressure (≤90/60 or ≥160/100 mmHg); abnormal kidney function (creatinine ≤59 or ≥104 µM for men and ≤45 or ≥84 µM for women, blood urea ≤2.5 or ≥ 7.8 mM); having a medical condition that might affect the primary outcome measure (e.g., diabetes, non-alcoholic fatty liver disease); taking dietary supplements that might affect the primary outcome measure (e.g., fish oil, L-carnitine); being pregnant or breastfeeding; currently undergoing active treatment for cancer or heart disease. Prior to their participation all participants gave informed consent.

5.4.4 Outcomes

The primary outcome was the effect of a single dose of pomegranate extract (1.6 g) compared with a placebo on the plasma TMAO AUC over 48 hours following an OCRT.

Secondary outcomes included:

1. Identification of high- and low-TMAO producers based on the plasma TMAO response to the OCRT in Phase I.
2. Pharmacokinetic parameters (AUC, C_{max}, T_{max}, $t_{½}$) of plasma TMAO, L-carnitine and γ-BB after the OCRT with the pomegranate extract or placebo.
3. Urinary and faecal excretion of L-carnitine, γ-BB, and TMA.

4. Correlations between the effect of the pomegranate extract on plasma TMAO AUC and urolithin metabotypes.
5. Correlations between plasma TMAO response to the OCRT and habitual dietary L-carnitine intake assessed via an FFQ.

As part of *post hoc* analyses, associations between the effect of the pomegranate extract on plasma TMAO AUC and the variables age, sex, and kidney function (measured by creatinine levels assessed during Phase I) were explored.

5.4.5 Sample size

In vitro results showed that the pomegranate extract reduced TMA production at 48 hours by 48.2-76.4%, depending on the dose (**Chapter 2**, **Table 2.2**). In a human intervention study, daily consumption of a polyphenol-rich grape extract for four or eight weeks reduced fasting plasma TMAO concentrations by 63.6% and 78.6%, respectively, compared with baseline [98, 99]. However, no studies to date have investigated whether polyphenol-rich extracts can reduce TMAO production following an OCRT, and therefore limited data were available to derive an expected effect size. A within-person standard deviation of 1.178 µM (log-transformed) for the maximum TMAO concentration after an OCRT of 1.5 g was derived from a report by Wu *et al.* (2020) [71]. Based on this variability, a sample size of 16 participants would provide 80% power to detect a 60% reduction in TMAO production at $P < 0.05$. To account for potential dropouts, 20 participants were randomised into Phase II.

5.4.6 Blinding, randomisation, and allocation

Participants who proceeded to Phase II were randomised to the order in which they received the two dietary interventions (OCRT + pomegranate extract or OCRT + placebo) using block randomisation, with a permuted block size of 20, to ensure balanced allocation. The random sequence was generated by Dr George Savva, and allocation concealment was maintained by Dr Natalia Perez-Moral, both independently of the study team. Allocation concealment was achieved by relabelling the intervention bottles with participant IDs and intervention order. Both pomegranate extract and placebo

capsules were identical in appearance, packaging, and labelling, ensuring blinding of participants and investigators. The trial was conducted as a double-blind, placebo-controlled, crossover study. Hence, the Chief Investigator, Principal Investigator, study scientists, and participants remained blinded to allocation until the data analysis was completed.

5.4.7 Preparation of the pomegranate extract and placebo capsules

The pomegranate extract and placebo capsules were prepared by Dr Jennifer Ahn-Jarvis in the Quadram Institute (QI) Clinical Research Facility (CRF) research kitchen using a Profiller 1100 (Torpac Inc., Fairfield, NJ, USA). All ingredients were commercially available for human consumption. Placebo capsules were prepared by combining 10% (w/w) gum Arabic and 1% (w/w) brown food dye with MCC. This percentage of gum Arabic reflects the percentage present in the pomegranate extract (**Appendix 3**). DRcaps® size 0 capsules were loaded into the Filler and Dermogranate® extract or placebo contents were poured onto the Filler, spread using a Powder Spreader, and capsules were closed.

Placebo and pomegranate capsules were acid resistant and designed for delayed release, such that the contents were released in the intestines rather than the stomach. Capsules were packed into a pill bottle (28 mL), which each contained four pomegranate capsules (400 mg each) or four placebo capsules, along with three L-carnitine capsules (500 mg) and a silica gel sachet. Prior to use in the intervention study, the capsules were tested at ALS Laboratories (UK) for microbiological safety.

5.4.8 Run-in diet and standardised meals

To minimise the production of TMAO throughout the study period from sources other than the OCRT, participants completed a run-in period of 48 hours during which they followed a low-carnitine and low-ellagitannin diet prior to their Phase I and II visits (**Figure 5.1**). Participants were provided with a list of foods to avoid (**Appendix 6**). Additionally, during each of the Phase II interventions, participants were provided with standardised meals which were low in L-carnitine, choline, and ellagitannins, with an upper limit of 200 mg/day for each of choline and L-carnitine, based on amounts

reported by the United States Department of Agriculture (USDA) Foods Database and available literature [32, 220-223].

All meals were provided by a commercial supplier (Tesco Plc) and were handled in accordance with the UK Environmental Health Guidelines. Meals were given to participants with clear instructions for storage and preparation, or they were prepared in the QI CRF unit kitchen.

5.4.9 Food frequency questionnaire (FFQ)

Participants completed an FFQ about their dietary intake over the 30 days prior to Phase I (VioScreen™, VioCare®) to assess their habitual L-carnitine intake. The FFQ has been validated in the USA, which reflects the Western diet, and has standardised software for its analysis. The FFQ provides information about the participant's habitual diet through online questions to assess their intake of each food group. A completed VioScreen™ FFQ automatically generates a report detailing macronutrients and micronutrients from the top foods consumed but does not include L-carnitine intake. Therefore, the total L-carnitine intake was derived by multiplying the reported L-carnitine content of food items (mg/g) reported in the literature [32, 220, 221] with the intake of L-carnitine-containing foods in grams that was established through the FFQ.

5.4.10 Collection and processing of plasma, urine, and faecal samples

Whole blood to obtain blood plasma and blood serum were collected into ethylenediamine tetra-acetic acid (EDTA) tubes and serum separating tubes (SST), respectively. EDTA tubes were immediately centrifuged at 3,000xg for 15 minutes to obtain blood plasma. Every collected plasma sample was divided into 5-6 aliquots of 350 µL and stored at -80 °C until batch analysed by LC-MS/MS. SST tubes were sent off to the Norfolk and Norwich University Hospital (NNUH) Pathology Department, who analysed serum samples for eGFR, creatinine, and urea.

Participants collected all their urine over 48 hours into provided, pre-weighed collection pots containing boric acid (0.5 g/L). Each urine collection was weighed and the specific

gravity measured using a One Step specific gravity dip stick to obtain the total volume. Urine was aliquoted into 5 tubes of 1 mL and stored at -80 °C until batch analysis.

Participants collected two faecal samples per intervention (pre- and post-intervention) in a secondary contained cooler box containing an anaerobic sachet (AnaeroGen™). Faecal samples were aliquoted into 10 vials, containing 200-500 mg each, and one extra aliquot containing excess faecal sample. Aliquots were stored according to different processing routes: DNA/RNA shield (1:2 dilution), RNAlater™ (1:5 dilution), snap freezing, or directly into Lysing Matrix E tubes (MP Biomedicals). All aliquots were directly stored at -80 °C until batch analysis of DNA and metabolites, except for aliquots stored in RNAlater™, which were first left at 4 °C for 24 hours.

5.4.11 LC-MS/MS analysis of L-carnitine metabolites in biological samples

Phase I blood plasma samples were analysed for TMAO concentrations within two weeks after collection using the Standard Addition Method (SAM). Plasma samples were split into 5 aliquots and known concentrations of TMAO were added to each aliquot at concentrations ranging between 0-20 µM. Then, 50 µL of 0.2 M acetic acid containing d9-TMAO was added to each aliquot, followed by 150 µL ice-cold TCA which was left at 4 °C for ten minutes to precipitate the protein. Finally, the aliquots were centrifuged at 13,000 rpm for ten minutes at 4 °C and 150 µL was transferred into Chromacol vials that contained 100 µL water to slightly dilute the sample.

Phase II biological samples were analysed in batch, once the last participant completed the study. Analysis of TMAO, L-carnitine, γ-BB, and TMA in Phase II samples was performed as described in **Chapter 2** (Section **2.4.5**). For the standard curve, a pooled plasma or pooled urine sample, derived from multiple participants' baseline samples. To account for baseline concentrations of analytes in the pooled sample, the concentrations were first established using SAM and final concentrations of the standard curve were adjusted accordingly.

Targeted analysis was performed with isotopically labelled standards for L-carnitine, γ-BB, TMA, and TMAO. A 1290 Infinity II LC System was used, coupled to a 6490 Triple Quad LC-MS (Agilent Technologies). The metabolites were separated through an Acquity UPLC BEH C8 1.7µm column (Waters, Massachusetts, United States), using a mobile

phase consisting of 10 mM acetate and 0.05% HFBA in Mili-Q water (solution A) and 10 mM acetate and 0.05% HFBA (dissolved in 50 mL Mili-Q water) in methanol (solution B).

5.4.12 LC-MS/MS analysis of urine and stool samples for pomegranate polyphenols and urolithins

Pomegranate polyphenols (punicalagin, punicalin, ellagic acid) and urolithins were quantified from urine and stool using LC-MS/MS. A standard curve was prepared in a pooled baseline urine from multiple participants, which was collected prior to the intervention, to matrix-match the samples. The standard curve ranged from 16, 8, 4, 2, 0 µg/mL for each compound. Urine samples and standards were transferred to a 96-well plate and taxifolin (1 µg/mL) was added as an internal standard. All samples and standards were vortexed and then centrifuged at maximum speed for 2 minutes prior to analysis on LC-MS/MS.

Samples were analysed using the Waters TQ Absolute (Wilmslow, UK), which combines UPLC and triple quadrupole MS. The mobile phase consisted of 0.1% formic acid in distilled water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). 1 µL of each sample was run through a Waters HSS T3 column (100 x 2.1 mm; particle size 1.8 µm) at 35°C and a flow rate of 0.4 mL/min. The gradient started at 3% solvent B for 5 minutes, after which solvent B to 20% for 4 minutes, and then further increased to 50% for 2 minutes, lastly solvent B increased to 95% for 1 minute before it re-equilibrated to 3% for 2 minutes. In total, each sample ran for 14 minutes. Polyphenols were identified against the matching standard using the RT and m/z ($[M-H]^-$). Peak assignment and quantification were conducted using SYNAPT G2-Si software.

Urolithin standards are only commercially available as aglycones. However, urolithins will mainly appear in the urine attached to glucuronides or sulfates, and not in their aglycone form. Therefore, prior to batch analysis, we performed a hydrolysis on one urine sample using glucuronidases and sulfatases to estimate the response ratio of the aglycone forms to the glucuronide and sulfate forms. For this experiment, a continuous 24-hour urine collection, obtained from an individual who had received the pomegranate intervention, was divided into ten 200 µL aliquots. For the standard curve, a baseline urine sample, that should not contain any pomegranate polyphenols and metabolites, was divided into five 200 µL aliquots. Standards were made up of increasing quantities of urolithin A and

B aglycones, up to 16 µg/mL. Then, 10 µL taxifolin (at 20 µg/mL) and 520 µL phosphate buffer (pH 6.8) were added to each aliquot. Half of the samples and all standards were treated with 80 µL glucuronidase (1000 U/mL) and 80 µL sulfatase (1000 U/mL), the other half remained untreated (non-hydrolysed). All aliquots were left at 37 °C for 2 hours to facilitate the hydrolysis. To stop the hydrolysis, 570 µL dimethylformamide (DMF) and 40 µL formic acid were added to the aliquots. The aliquots were vortexed and centrifuged at maximum speed for 2 minutes. Finally, 400 µL of the samples was filtered into Whatman Mini-UniPrep filters (Cytiva) of 0.45 µm and analysed using the described LC-MS/MS method above.

Sulfate forms were not detected, so based on the results only the ratios for glucuronides to aglycones were developed. Urolithin concentrations in the 24-hour urine collection were calculated by multiplying the quantities by the total volume of the urine collection and then converting these to µM. Non-hydrolysed samples contained an average of 2.5 µM urolithin A and 0.6 µM urolithin B (**Figure 5.2A**) while hydrolysed samples contained an average of 30.8 µM urolithin A and 15.3 µM urolithin B (**Figure 5.2B**). Based on these results the relative response factor (RRF) for each of the urolithins was calculated as follows:

$$RRF = \frac{C\ aglycone, hydrolysed - C\ aglycone, non\ hydrolysed}{C\ glucuronide, non\ hydrolysed}$$

For urolithin A glucuronide to urolithin A the mean RRF was 2.13 and for urolithin B glucuronide to urolithin B the mean RRF was 2.75.

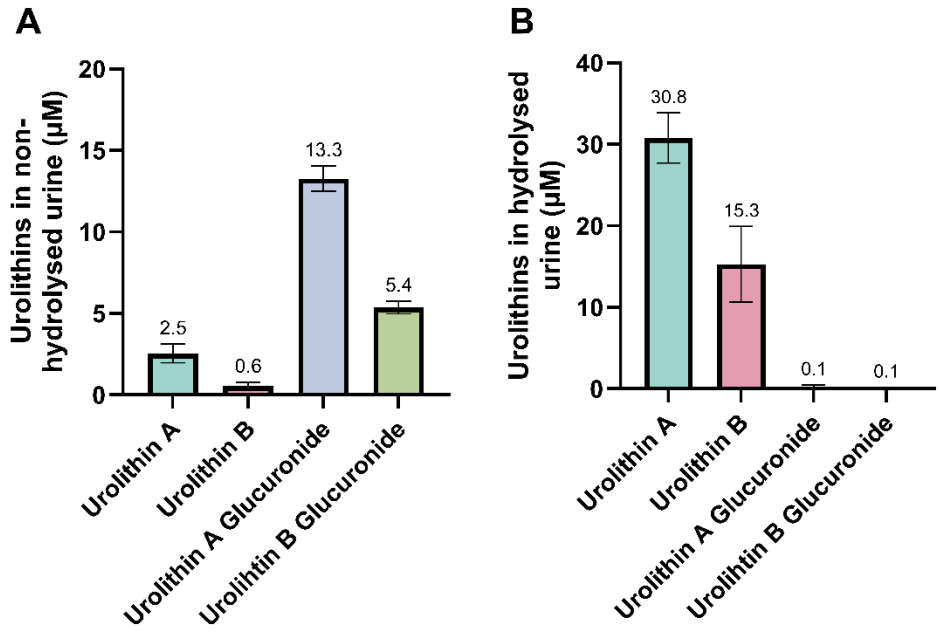

Figure 5.2. Urolithin concentrations in (A) non-hydrolysed and (B) hydrolysed urine. *A hydrolysis experiment was performed on a 24-hour urine collection after intervention with the pomegranate extract to obtain the conversion ratio for urolithin glucuronides to urolithin aglycones. Five urine aliquots were hydrolysed using glucuronidase and sulfatase (1000 U/mL) and five aliquots remained untreated (non-hydrolysed). All samples were left at 37 °C to hydrolyse and hydrolysis was blocked after 2 hours using dimethylformamide (DMF). Aliquots were analysed using LC-MS/MS and urolithins quantified using urolithin A and B standards.*

5.4.13 Moisture content measurement of faecal samples

Faecal samples were collected and the weight of the tube with the sample was recorded. The tube was then transferred to a lyophiliser and freeze-dried for 48 hours, after which the dry weight was recorded to obtain the moisture content factor. All metabolite estimates in stool samples are shown by dry faecal weight, obtained by multiplying the wet weight by the calculated moisture content factor of the faecal sample.

5.4.14 Statistical analyses

All collected data were analysed per protocol, although the data of two participants who did not complete both interventions in Phase II have been included in some analyses, in which case this is indicated. For all data analyses the observed effect was considered statistically significant if $P < 0.05$.

No participant IDs are used in this chapter. Where applicable, participants are referred to by numerical identifiers (1-16), assigned according to the rank order of the pomegranate extract's effect relative to the placebo on plasma TMAO, starting from the largest observed effect.

All graphs were generated using GraphPad Prism version 10.4.2, and R version 4.4.1 (R Core Team, 2024) in RStudio (Posit Software, PBC, Boston, MA). Statistical analyses were performed using RStudio with 'ggpubr' [224], 'FSA' [225], 'lmerTest' [146], 'emmeans' [226], and base functions from the 'stats' package.

Predefined analyses

Phase I plasma TMAO concentrations after the OCRT were evaluated by means of a paired t-test. Participants with abnormal TMAO values, identified as outliers using the interquartile range (IQR) method [227], were excluded from the t-test.

The pharmacokinetic effects of the pomegranate extract on TMAO plasma concentrations were estimated by comparing the log-transformed area under the curve (log AUC) of TMAO between the pomegranate extract and placebo interventions. For clarity, AUC values are displayed in figures on the linear scale (µM×h), while statistical interpretations are based on the log-transformed model. The AUC was calculated using the trapezium rule via the trapz() function from the 'pracma' package [228] using R version 4.4.1 (R Core Team, 2024). Where up to two data points were missing, a curve was estimated using imputed values. One participant presented abnormal baseline TMAO values in one intervention (>45 µM) for three time points which then returned to baseline levels by 1.5 hours after the OCRT. Therefore, to better capture the TMAO pharmacokinetics in response to the OCRT, the first three TMAO concentration data points were excluded from the analysis. A linear mixed model with the intervention as fixed effect and the participant as a random effect was used to estimate the difference in the TMAO AUC between the two treatments.

Other pharmacokinetic parameters, C_{max}, T_{max}, and $t_{1/2}$, were calculated from plasma TMAO and L-carnitine concentrations at each time point using R package 'pracma' [228]. C_{max} and T_{max} were obtained, respectively, as the highest observed TMAO concentration and the corresponding time point, per participant and intervention. The TMAO $t_{1/2}$ was

estimated by fitting a linear regression to log-transformed concentrations over the final three time points of each curve, corresponding to the terminal elimination phase. This approach follows standard log-linear pharmacokinetic modelling methods.

Post hoc analyses

For each participant, the coefficient of variation (CV) of baseline plasma TMAO levels was calculated across three time points (two time points for those with incomplete data) as a *post hoc* analysis. These time points were the baseline measurements taken during Phase I, Phase II intervention 1 and Phase II intervention 2 (i.e., the measurement taken during time point 0). The CV values were used to assess intra-individual variability in baseline plasma TMAO concentrations across study phases.

Additionally, *post hoc* analyses were carried out of the observed intervention effects on plasma TMAO by age groups, sex, and kidney function. Age groups were defined by a median split of participant age. A linear mixed model was fitted with TMAO log AUC as the outcome and fixed effects for intervention (placebo versus pomegranate), age group, and their interaction (intervention × age group).

To test for an interaction with kidney function, linear regression models were performed separately for each intervention arm (placebo, pomegranate) and for the difference between arms (placebo minus pomegranate). Creatinine levels, which were measured during Phase I, were used as a marker for kidney function. The kidney function marker eGFR was not used because it had a ceiling value of 90 mL/min/1.73m^2, which most participants exceeded. Each model included creatinine levels as the primary predictor, with age and sex included as covariates. Interaction terms between sex and creatinine were tested to assess sex-specific associations.

5.5 Results

5.5.1 Study CONSORT diagram and demographics

Of the 144 individuals who expressed interest and were assessed for eligibility based on inclusion and exclusion criteria, 34 started Phase I (**Figure 5.3**). Out of 32 participants who completed Phase I, 3 were not high-TMAO producers (<5 µM or <50% increase between baseline and 24-hour follow-up), meaning that 90.6% of the participants were classified as high-TMAO producers. 20 participants were randomised into Phase II. Two participants only completed one intervention before dropping out: one due to an unrelated health issue and the other due to dehydration. Two other participants dropped out at the start of Phase II, both due to overhydration. 16 participants completed the study and were included in the full analysis.

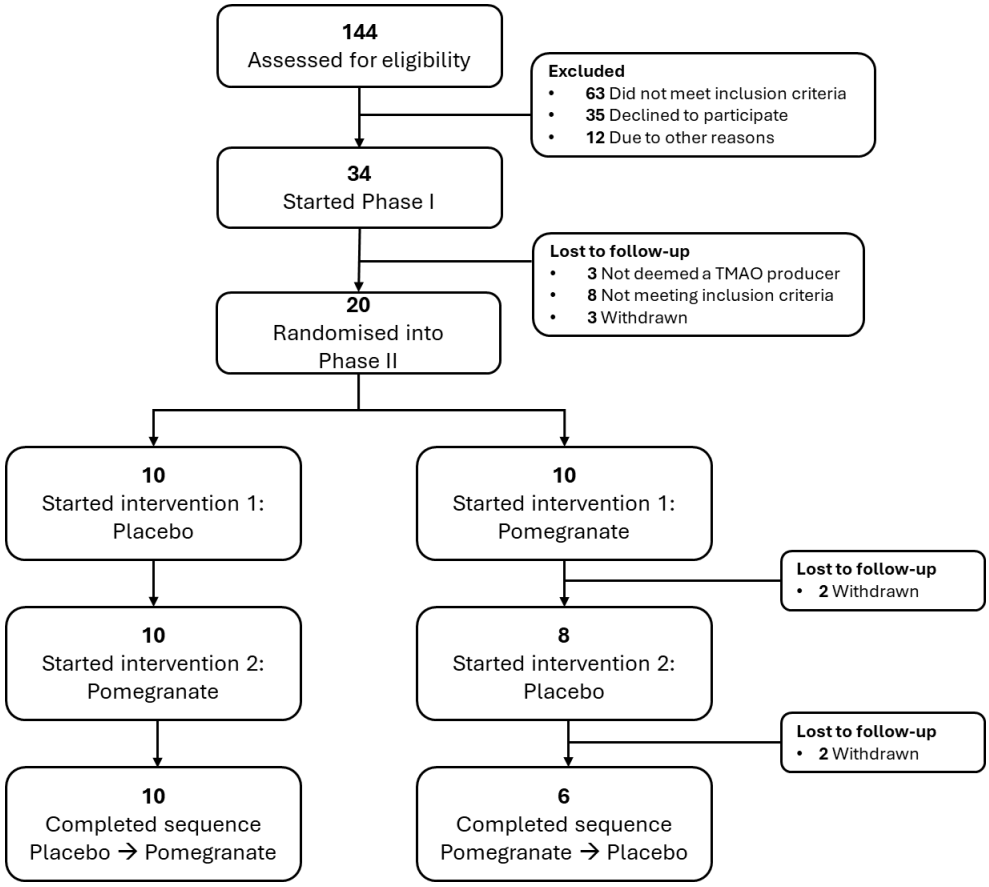

Figure 5.3. TESSA study CONSORT diagram. *This study is a two-phased dietary intervention study, of which Phase II was an 18-day, double-blind, randomised, controlled crossover study consisting of two interventions: L-carnitine (1.5 g) co-supplemented with a pomegranate extract (1.6 g), and L-carnitine (1.5) co-supplemented with a placebo.*

Respectively 56.3 and 62.5% of the participants who completed Phase I and II were male (**Table 5.1**). While the TMAO response between male and female participants who participated in Phase I tended to be similar, the increase in TMAO levels from baseline to the 24-hour follow-up was greater in female than in male participants who participated in Phase II.

Table 5.1. Demographic and anthropometric characteristics, and trimethylamine N-oxide (TMAO) levels of TESSA study participants, stratified by Phase completed and sex. *All TMAO values shown in this table were obtained during Phase I only.*

	Phase I		**Phase II**	
	Male (n = 18)	**Female (n = 14)**	**Male (n = 10)**	**Female (n = 6)**
Age (mean ± SEM)	41.2 ± 3.3	40.1 ± 4.0	39.6 ± 4.2	40.8 ± 6.0
Height (mean ± SEM)	176.4 ± 1.7	165.0 ± 1.7	177.1 ± 2.4	165.0 ± 1.9
Weight (mean ± SEM)	80.3 ± 2.5	70.1 ± 3.1	80.5 ± 3.3	67.8 ± 3.6
BMI (mean ± SEM)	25.8 ± 0.6	25.8 ± 1.0	25.7 ± 0.9	24.9 ± 1.2
TMAO at baseline, µM (mean ± SEM)	4.4 ± 0.9	4.5 ± 1.2	4.8 ± 1.7	3.3 ± 0.6
TMAO at 24-hour follow-up, µM (mean ± SEM)	41.0 ± 6.1	42.5 ± 9.0	38.7 ± 6.1	54.3 ± 9.9
TMAO response, µM (mean ± SEM)	36.6 ± 5.9	37.9 ± 8.9	33.9 ± 5.7	51.0 ± 9.4
TMAO response, % (mean ± SEM)[1]	10.1 ± 1.6	12.0 ± 2.7	9.7 ± 1.3	16.0 ± 2.0

[1] Calculated from the difference between baseline and 24-hour follow-up per participant.

5.5.2 Phase I: TMAO response following an OCRT

Prior to the OCRT, fasting blood plasma TMAO levels averaged at 4.48 µM (± 1.98 SEM), although this was skewed by two outliers exceeding the IQR outlier upper bound (>7.3 µM). Therefore, after removal of the two outliers, TMAO levels averaged at 3.48 µM (± 0.78 SEM) (**Figure 5.4**), which matches previously reported fasting plasma TMAO levels in omnivorous adults of 3.54 (± 0.96 SEM) and 4.7 µM (± 0.9 SEM) [229, 230].

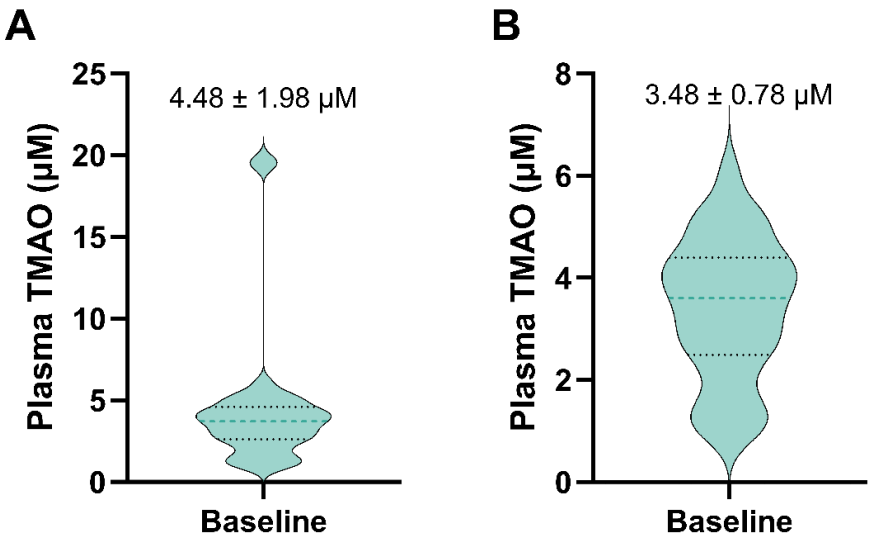

Figure 5.4. Distribution of fasting blood plasma trimethylamine N-oxide (TMAO) levels at baseline. *Data are shown as mean ± SEM (**A**) of all participants who completed Phase I (n = 32) and (**B**) after outlier exclusion based on the interquartile range (IQR) method (n = 30). Fasted blood samples were collected from TESSA participants prior to the OCRT in Phase I. Whole blood samples were centrifuged to obtain plasma and stored at -80 °C until LC-MS/MS quantification using a d9-TMAO internal standard.*

After the OCRT, 29 participants (90.6%) had an increase in plasma TMAO levels greater than 5 µM (**Figure 5.5**). Two participants exceeded the IQR upper bound for outlier detection at baseline (> 7.3 µM), possibly due to incorrectly following the dietary guidelines (**Appendix 6**), resulting in the dietary intake of foods containing high levels of L-carnitine. At 24-hour follow-up, one participant's plasma TMAO was above the IQR upper bound (>102.1 µM) (**Figure 5.5A-B**). On average, after exclusion of outliers based on the IRQ method [227], the plasma TMAO levels between baseline and 24-hour follow-up increased from 3.48 (± 0.78) to 39.16 µM (± 4.10) ($P < 0.001$) (**Figure 5.5C, Figure 5.6**).

To compare the prevalence of high-TMAO producers in the TESSA study cohort with the prevalence in other study cohorts, the TMAO cut-off reported by Wu *et al.* (2020) was applied, which was based on absolute plasma TMAO levels of 10 µM at 24 hours after a 1.5 g L-carnitine OCRT [71]. This is slightly different from the cut-off criteria used in the TESSA study, which were based on the TMAO increase between baseline and 24 hours post-OCRT (> 5 µM and >50%). The *post hoc* analysis showed that 28 participants (87.5%) exceeded 10 µM plasma TMAO at 24 hours post-OCRT (**Appendix 7, Supplementary**

Figure S16), and would therefore still be classified as high-TMAO producers based on the Wu *et al.* cut-off criteria.

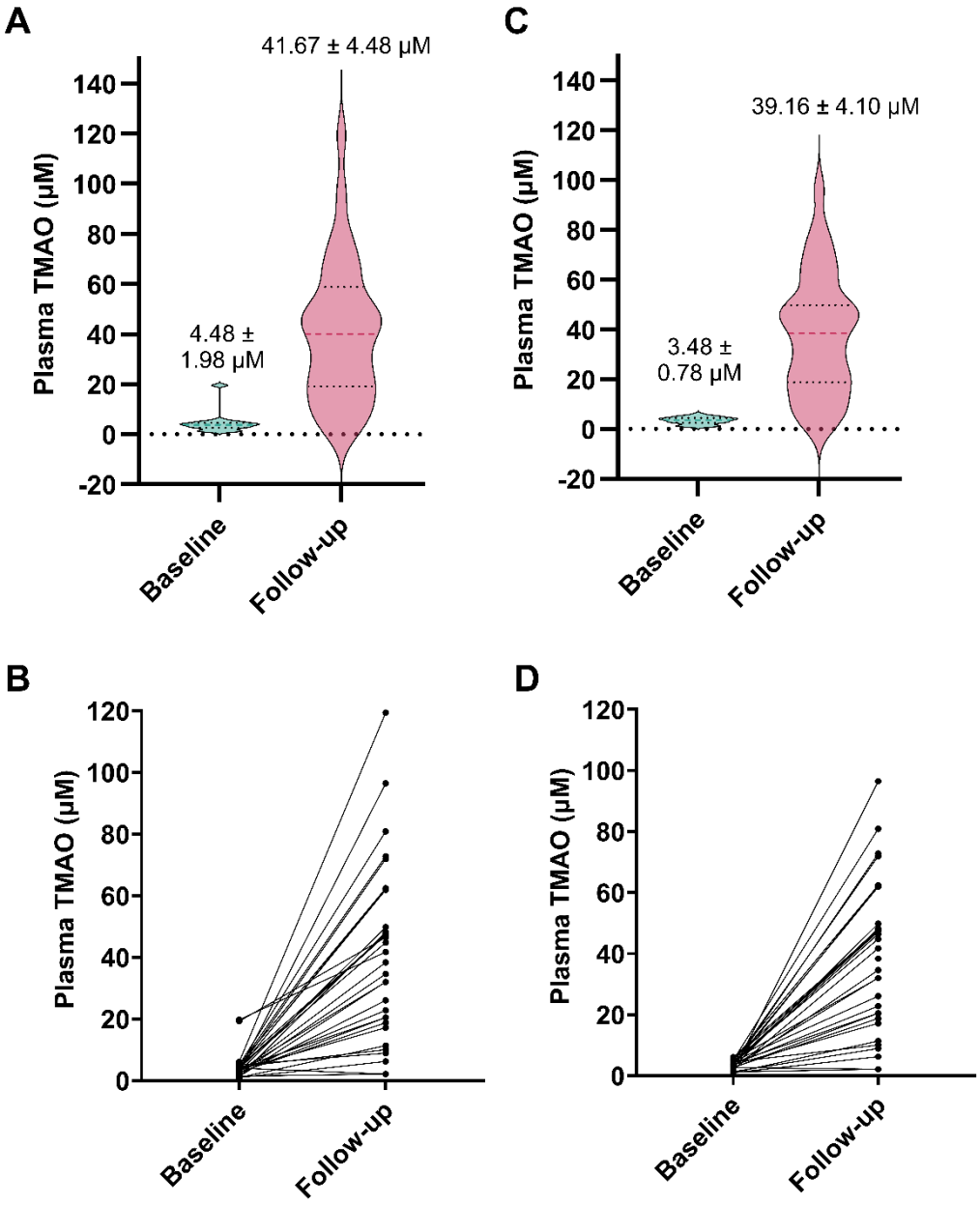

Figure 5.5. Distribution of trimethylamine N-oxide (TMAO) production after the oral carnitine response test (OCRT) in Phase I. *Distribution and individual production of TMAO (**A-B**) for all participants who completed Phase I (n = 32) and (**C-D**) after excluding statistical outliers from baseline (n = 29) and follow-up (n = 31) identified using the interquartile range (IQR) method. Values are shown as mean ± SEM. Fasted blood samples were collected from TESSA participants prior to and 24 hours after the OCRT. Whole blood samples were centrifuged to obtain plasma and stored at -80 °C until LC-MS/MS quantification using a d9-TMAO internal standard.*

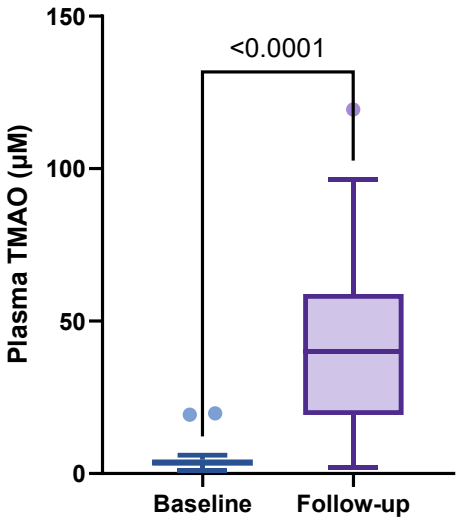

Figure 5.6. The increase in trimethylamine N-oxide (TMAO) production after the oral carnitine response test (OCRT) in Phase I. *Data are shown as mean ± 1.5×IQR of 32 participants. Statistical analysis was performed using a paired t-test. Outliers were identified using the interquartile range (IQR) method and are shown separately. Fasted blood samples were collected from TESSA participants prior to and 24 hours after the OCRT. Whole blood samples were centrifuged to obtain plasma and stored at -80 °C until LC-MS/MS quantification using a d9-TMAO internal standard.*

Additionally, for all participants who started Phase II (n = 20), the CV of baseline plasma TMAO levels was calculated across three time points as a *post hoc* analysis. Fasting baseline blood plasma TMAO was measured on three occasions: prior to the OCRT in Phase I, and prior to the OCRT of both Phase II interventions. The variation between measurements in baseline plasma TMAO was small for most participants, with over half of the participants exhibiting a CV below 50% (**Appendix 7, Supplementary Figure S14**). Four participants showed a large variation due to a single high measurement of plasma TMAO (**Appendix 7, Supplementary Figure S14B**). In comparison, data from the BERI study (Clinicaltrials.gov ID NCT03213288), which estimated the variation between six measurements of plasma TMAO when no dietary restrictions for TMA precursors were in place, showed that most participants exceeded a CV of 50% [132]. This shows that the TESSA run-in diet, starting 48 hours prior to the OCRT, maintained plasma TMAO levels within a relatively small range for the majority of the participants.

5.5.3 Demographic variables correlated with a greater TMAO response

It has been reported previously that a higher BMI and older age (tend to) correlate with a greater TMAO response [102, 231]. Therefore, Phase I data (n = 30) were explored for a correlation between the TMAO response and BMI as well as age. These data show that a higher BMI was not correlated with a greater TMAO response (**Figure 5.7**). However, a significant correlation was found between age and TMAO response (**Figure 5.8**), with older age being correlated to a greater TMAO response between baseline and 24-hour follow-up (r = 0.37, P = 0.046).

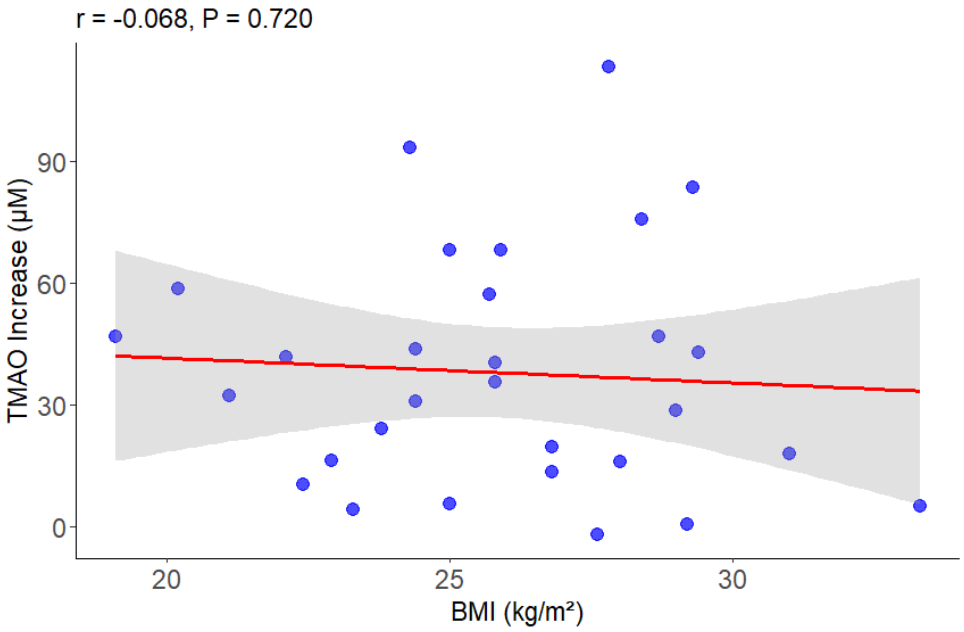

Figure 5.7. Correlation analysis between trimethylamine N-oxide (TMAO) response (µM) and body mass index (BMI). *BMI and TMAO response were assessed during Phase I. Individual data points are shown (n = 30) and a Pearson correlation was estimated. Two outliers were identified using the Interquartile Range (IQR) method and removed from the analysis. Participants underwent an oral carnitine response test (OCRT), consuming 1.5 g L-carnitine. Directly prior to the OCRT and 24 hours after the OCRT blood plasma was collected to estimate levels of TMAO.*

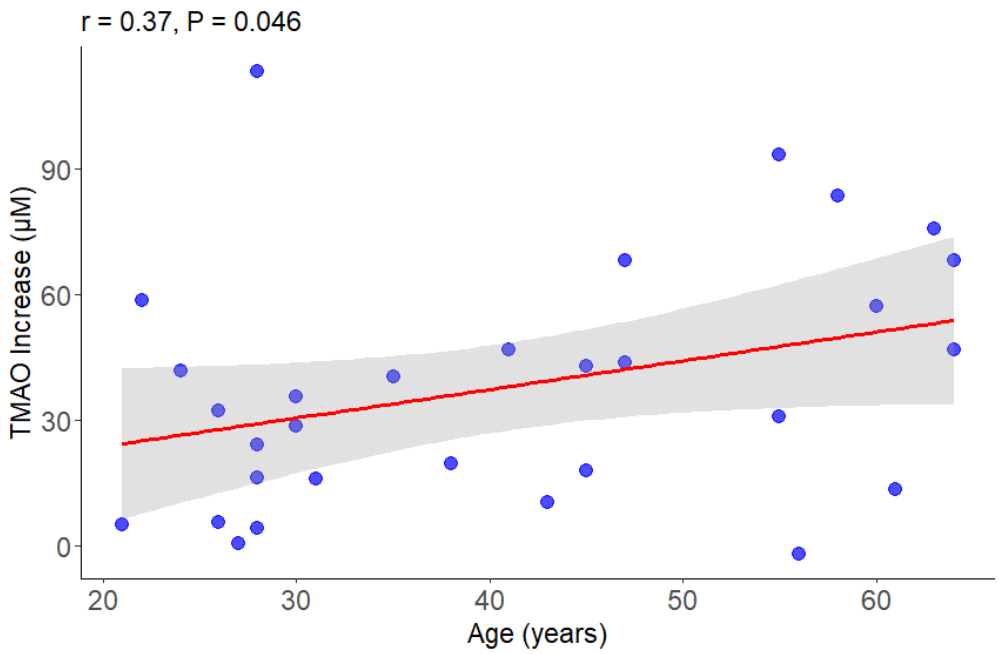

Figure 5.8. Correlation analysis between trimethylamine N-oxide (TMAO) response (µM) and age. *Age and TMAO response were assessed during Phase I. Individual data points are shown (n = 30) and a Pearson correlation was estimated. Two outliers were identified using the Interquartile Range (IQR) method and removed from the analysis. Participants underwent an oral carnitine response test (OCRT), consuming 1.5 g L-carnitine. Directly prior to the OCRT and 24 hours after the OCRT blood plasma was collected to estimate levels of TMAO.*

5.5.4 Correlation between habitual L-carnitine intake and TMAO response

Omnivores have a greater TMAO production than vegetarians and vegans, likely due to a higher dietary intake of L-carnitine among omnivores [29, 230]. The TESSA study specifically included omnivorous adults. Considering the previously reported correlation between dietary habits and TMAO production, it was hypothesised that, in this omnivorous population, greater OCRT-induced TMAO production correlates with higher habitual L-carnitine intake.

Based on the Phase I data of 30 participants, a diet containing greater amounts of L-carnitine tended to be correlated with greater TMAO increase between baseline and 24 hours (**Figure 5.9**), although this did not reach statistical significance ($r = 0.28$, $P = 0.130$).

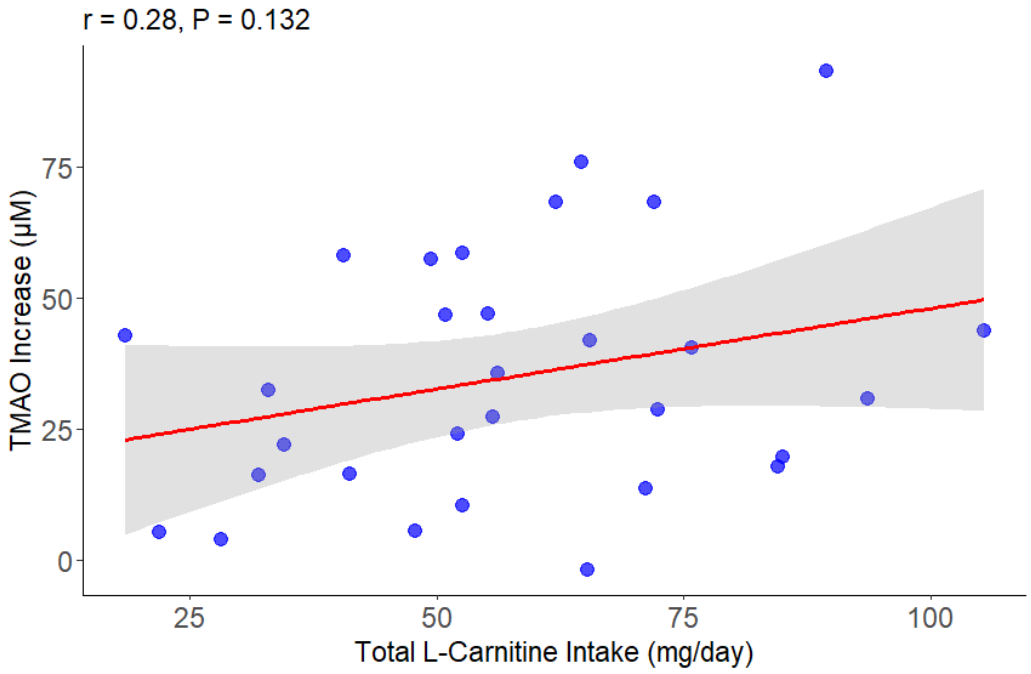

Figure 5.9. Correlation analysis between L-carnitine intake and trimethylamine N-oxide (TMAO) increase. *L-Carnitine intake was assessed during Phase I using a food frequency questionnaire (FFQ). Individual data points are shown (n = 30) and a Pearson correlation was estimated. Two outliers were identified using the Interquartile Range (IQR) method and removed from the analysis. Participants underwent an oral carnitine response test (OCRT), consuming 1.5 g L-carnitine. Directly prior to the OCRT and 24 hours after the OCRT blood plasma was collected to estimate levels of TMAO.*

5.5.5 The effect of a pomegranate extract on OCRT-induced TMAO response

10 out of 16 had a lower TMAO AUC after the pomegranate intervention compared to the placebo (**Figure 5.10**). However, linear mixed models, treating intervention (pomegranate or placebo) as a fixed effect and participant as a random intercept, showed no significant difference in TMAO log AUC between the two interventions (β = 0.0067, SE = 0.096, *P* = 0.945). Estimated TMAO AUC means were 1,041 and 1,048 μM×h for the placebo and pomegranate intervention, respectively, with a mean ratio of 0.993 (95% CI: 0.809 to 1.22). Paired TMAO AUC comparisons between the interventions are shown per participant (**Figure 5.11**). These findings suggest that a single dose of pomegranate extract did not affect plasma TMAO levels over time in this cohort.

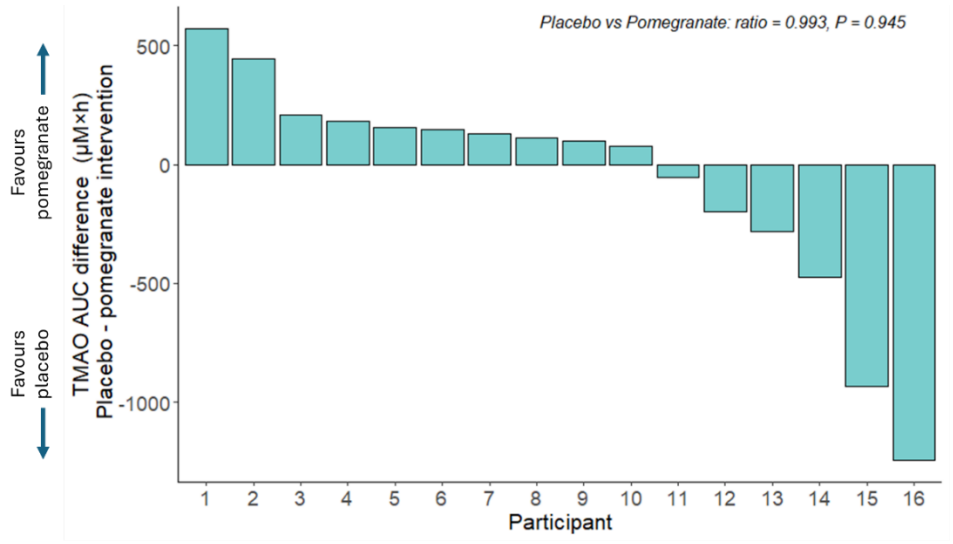

Figure 5.10. The difference between the trimethylamine N-oxide area under the curve (TMAO AUC) after the placebo versus the pomegranate extract per participant. *Participants (n = 16) underwent two oral carnitine response tests (OCRTs), consuming 1.5 g L-carnitine once with a placebo and once with a pomegranate extract. During 48 hours after the OCRT, blood plasma was collected to estimate a pharmacokinetic curve for TMAO, from which the AUC was derived. A positive value means that the TMAO AUC after the pomegranate extract supplementation was reduced compared to the placebo. For clarity, AUC values are displayed in figures on the linear scale, while statistical interpretations are based on the log-transformed model.*

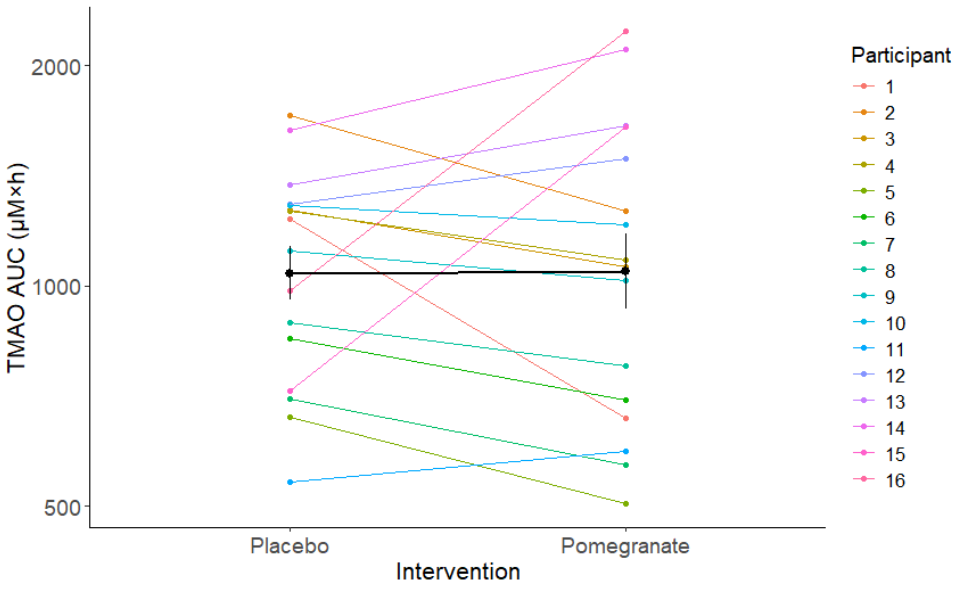

Figure 5.11. Paired comparison of trimethylamine N-oxide area under the curve (TMAO AUC) per participant. *Participants (n = 16) underwent two oral carnitine response tests (OCRTs), consuming 1.5 g L-carnitine once with a placebo and once with a pomegranate extract. During 48 hours after the OCRT blood plasma was collected to estimate a pharmacokinetic curve for TMAO, from which the AUC was derived. Paired TMAO AUC comparisons between the interventions are shown per participant, with the black line showing the mean. For clarity, AUC values are displayed in figures on the linear scale, while statistical interpretations are based on the log-transformed model.*

5.5.6 The pomegranate extract reduced the TMAO response in men premenopausal women

Since Phase I data showed a correlation between age and TMAO response, the influence of age on the effectiveness of the pomegranate intervention was further explored in Phase II. The effect of the pomegranate extract tended to be correlated with age, with younger age showing an increased effect (**Figure 5.12**), although this was not statistically significant (r = -0.422, P = 0.103).

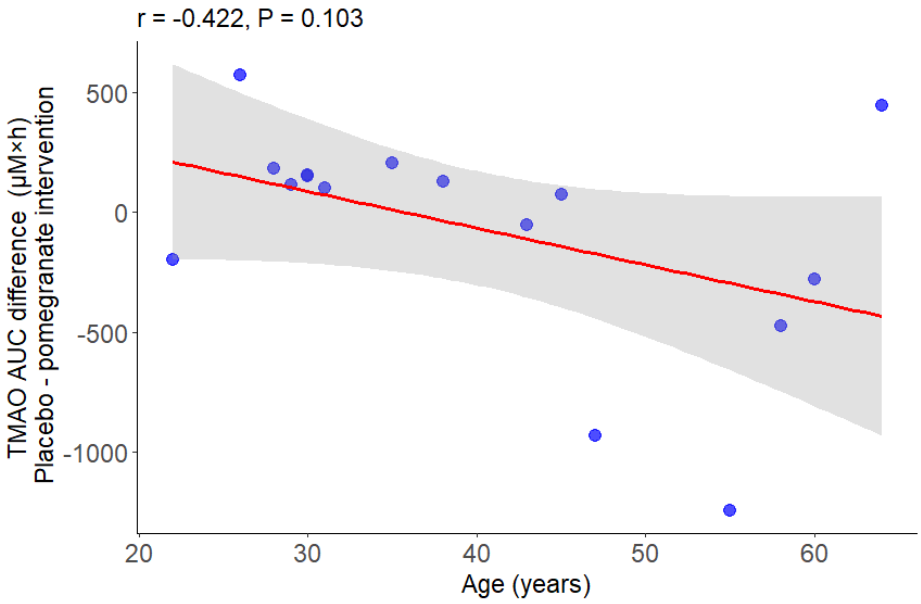

Figure 5.12. Correlation analysis between age and the difference between the trimethylamine N-oxide area under the curve (TMAO AUC) after the placebo versus the pomegranate extract. *Individual data points are shown (n = 16), and a Spearman correlation was estimated. Participants underwent two oral carnitine response tests (OCRTs), consuming 1.5 g L-carnitine once with a placebo and once with a pomegranate extract. During 48 hours after the OCRT blood plasma was collected to estimate a pharmacokinetic curve for TMAO, from which the AUC was derived.*

Next, participants were stratified by age based on a median split of participant age, creating two age groups: 22-35 years (“Younger”) and 38-64 years ("Older”). The TMAO AUC difference (µM×h) between the placebo and pomegranate intervention was compared across age groups (**Figure 5.13A**).

A linear mixed model was fitted with TMAO log AUC as the outcome, and fixed effects for intervention (placebo versus pomegranate), age group, and their interaction (intervention × age group). This revealed an interaction between intervention and age group (P = 0.037), indicating that the effect of the intervention may differ by age.

To further explore whether the intervention effect differed by both age and sex, participants within both age groups were subdivided by sex, creating four subgroups (**Figure 5.13A**). A linear model including the interaction between age group and sex revealed a significant interaction effect on TMAO log AUC difference (P = 0.004), suggesting that the effect of the intervention varied depending on the combination of age and sex. *Post hoc* comparisons showed that "older" women (n = 3) exhibited a significantly greater TMAO increase after the pomegranate intervention (mean difference = -884.8 µM×h; 95% CI: -1,219.0 to -551.0; SEM = 153) compared to all other subgroups (P < 0.01 in all comparisons involving "older" women), while "younger" men (n = 5) had the largest reduction (mean difference = +218.4 µM×h; 95% CI: -40.4 to 477.0; SEM = 119), although this was not statistically significant relative to other groups (P > 0.05 for all comparisons).

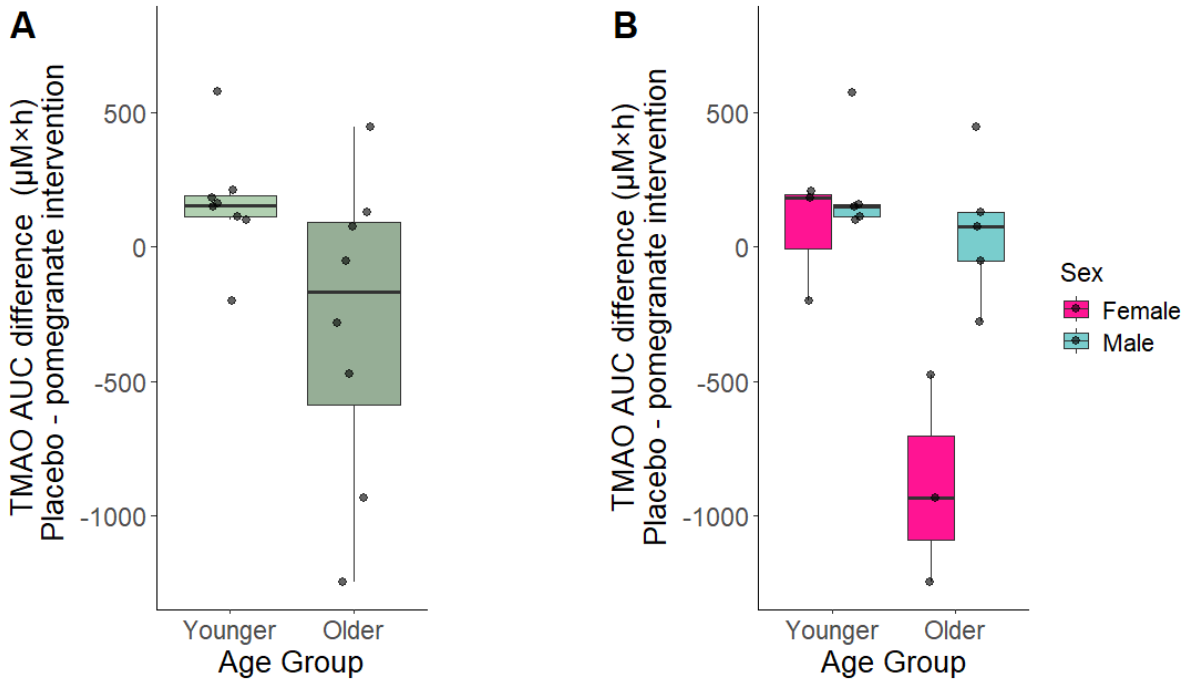

Figure 5.13. The difference between the trimethylamine N-oxide area under the curve (TMAO AUC) after the placebo versus the pomegranate extract by age group and sex. *(**A**) TMAO AUC stratified by age group, and (**B**) TMAO AUC stratified by age group and sex. Age groups were defined by a median split of participant age [median = 36.5 years (n = 8 "Younger", n = 8 "Older")]. Boxplots show the median, interquartile range, and individual data points. Participants (n = 16) underwent two oral carnitine response tests (OCRTs), consuming 1.5 g L-carnitine once with a placebo and once with a pomegranate extract. During 48 hours after the OCRT blood plasma was collected to estimate a pharmacokinetic curve for TMAO, from which the AUC was derived. Positive values indicate a greater reduction in TMAO AUC following the pomegranate intervention relative to placebo. For clarity, AUC values are displayed in figures on the linear scale, while statistical interpretations are based on the log-transformed model.*

These findings are consistent with a cross-sectional study in 51 adults with obesity, which suggested that TMAO metabolism may be altered in postmenopausal women [232]. Therefore, a *post hoc* analysis was carried out excluding "older" women (n = 3, age > 47) who may have been postmenopausal, based on reports by the WHO [233] and NHS [234] indicating that menopause typically occurs between 45-55 years of age. Among the remaining male (n = 10) and premenopausal female (n = 3) participants, the pomegranate extract significantly reduced TMAO AUC, as estimated from a linear mixed model on log-transformed AUC values, by 15% compared to the placebo (95% CI: 1.01 to 1.31, *P* = 0.036) (**Figure 5.14**). On a linear scale, this corresponds to a mean TMAO AUC of 1,039 µM×h for the placebo and 904 µM×h for the pomegranate intervention.

When analysing the data from the "older" women separately, the pomegranate extract appeared to have the opposite effect (**Appendix 7, Supplementary Figure S15**), increasing the plasma TMAO AUC for all three participants compared to the placebo.

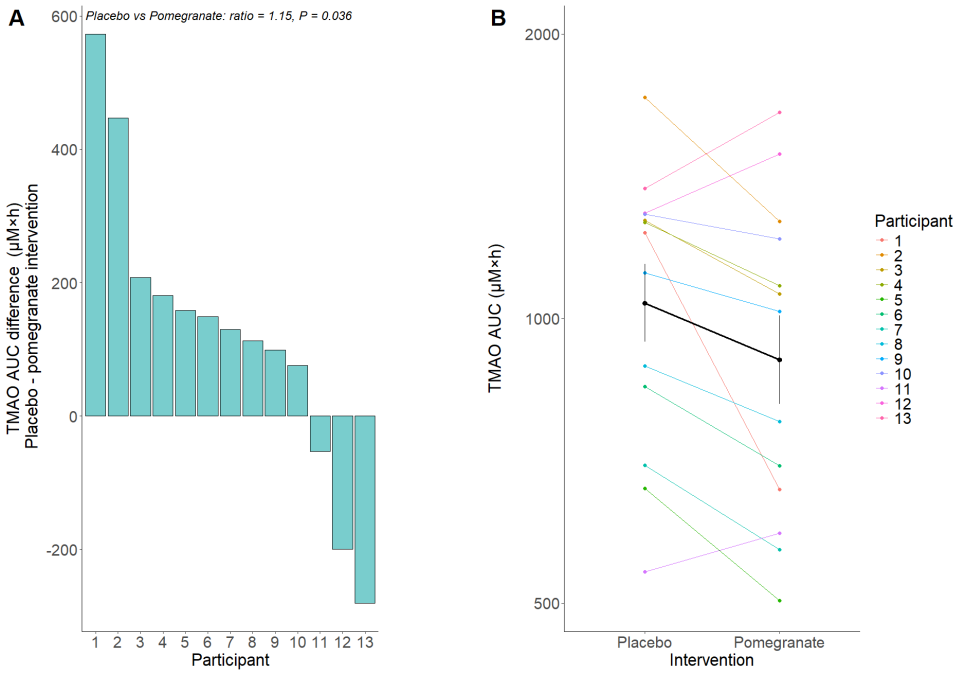

Figure 5.14. The trimethylamine N-oxide area under the curve (TMAO AUC) after the placebo versus the pomegranate extract across male and premenopausal female participants (n = 13). *(**A**) The difference between TMAO AUC after the placebo versus the pomegranate extract per participant, with positive values indicating that the TMAO AUC after the pomegranate extract was reduced compared to the placebo, and (**B**) paired comparisons of TMAO AUC per participant. Paired TMAO AUC comparisons between the interventions are shown per participant, with the black line showing the mean. Participants underwent two oral carnitine response tests (OCRTs), consuming 1.5 g L-carnitine once with a placebo and once with a pomegranate extract. During 48 hours after the OCRT, blood plasma was collected to estimate a pharmacokinetic curve for TMAO, from which the AUC was derived. For clarity, AUC values are displayed in figures on the linear scale, while statistical interpretations are based on the log-transformed model.*

5.5.7 Pharmacokinetic parameters for plasma TMAO, L-carnitine, and TMA

Pharmacokinetic parameters for plasma TMAO, L-carnitine, and TMA are presented in **Table 5.2**. Only trace amounts of γ-BB were detected in blood plasma, which corresponds to observations reported in the literature [102, 235]. Therefore, γ-BB parameters have not been included in the table.

No significant differences were observed between placebo and pomegranate conditions for TMAO C_{max}, T_{max}, or $t_{1/2}$. Prior to the TESSA study, studies investigating TMAO responses in participants after an OCRT did not measure TMAO levels at time points between 8 and 24 hours and assumed that the T_{max} was at 24 hours [29, 71]. Here, it was observed that the T_{max} was reached before 24 hours for the pomegranate extract intervention, with a mean of 21.6 hours (± 3.0) (**Table 5.2**). For the placebo intervention T_{max} was reached at a mean time of 25.8 hours (± 11.5) but varied widely between individuals, demonstrating the importance of blood collections prior to 24 hours to capture the full pharmacokinetic curve. The TMAO $t_{1/2}$ estimates were derived from the log-linear elimination phase, starting after C_{max} and were 7.8 (± 1.5) and 8.6 (± 3.4) hours for the placebo and pomegranate interventions, respectively. This indicates a relatively efficient elimination once peak TMAO levels were reached.

Pharmacokinetic parameters of L-carnitine showed a C_{max} below the expected values from a 1.5 g L-carnitine dose. As a result, the T_{max} of approximately 20 hours is well beyond the values previously reported, which range from 2 to 4.5 [236]. It is possible that the T_{max} was reached between 2.5 and 16 hours and therefore the pharmacokinetic curve did not capture the C_{max} (**Appendix 7, Supplementary Figure S13A**). This also affected an accurate determination of the $t_{1/2}$, which has been indicated in **Table 5.2** as "not determined".

TMA blood plasma concentrations were relatively low, averaging around 0.4 µM (**Appendix 7, Supplementary Figure S13B**), with a C_{max} of 0.7-0.8 µM at around 8 hours (**Table 5.2**). This is in line with previous reports that 95% of TMA is oxidised to TMAO [49], indicating that the conversion of TMA in the liver is highly efficient.

Table 5.2. Pharmacokinetic parameters of trimethylamine N-oxide (TMAO), L-carnitine, and trimethylamine (TMA) concentrations in the blood plasma. *Data are shown as mean ± SEM for all participants (n = 16) per intervention. Participants underwent two oral carnitine response tests (OCRTs), consuming 1.5 g L-carnitine once with a placebo and once with a pomegranate extract. During 48 hours after the OCRT blood plasma was collected to estimate a pharmacokinetic curve for TMAO, L-carnitine, and TMA, from which the area under the curve (AUC), maximum concentration (C_{max}), time to reach maximum concentration (T_{max}), and half-life ($t_{1/2}$) were derived. P-values were derived using a t-test (AUC) and the Wilcoxon signed-rank test (all other parameters), comparing the distribution of paired differences between the placebo and pomegranate intervention for each parameter.*

	Parameter	Placebo (mean ± SEM)	Pomegranate (mean ± SEM)	P-value
TMAO	AUC (μM×h)	1,041 ± 107	1,048 ± 108	0.945
	C_{max} (μM)	43.8 ± 3.0	48.7 ± 5.9	0.737
	T_{max} (h)	25.8 ± 2.9	21.6 ± 0.8	0.676
	$t_{1/2}$ (h)[1]	7.8 ± 0.4	8.6 ± 0.9	0.367
L-Carnitine	AUC (μM×h)	1,095 ± 114	1,161 ± 114	0.588
	C_{max} (μM)	41.3 ± 3.4	42.66 ± 2.5	0.660
	T_{max} (h)	20.4 ± 2.2	19.7 ± 0.5	0.715
	$t_{1/2}$ (h)[2]	ND	ND	ND
TMA	AUC (μM×h)	19.2 ± 2.2	17.7 ± 1.6	0.638
	C_{max} (μM)	0.8 ± 0.1	0.7 ± 0.04	0.409
	T_{max} (h)	7.8 ± 2.4	8.2 ± 3.0	1.000
	$t_{1/2}$ (h)	53.3 ± 11.1	37.8 ± 7.8	0.554

[1] Negative TMAO $t_{1/2}$ values were excluded, meaning that mean ± SEM $t_{1/2}$ values were only obtained for 14 participants.

[2] L-Carnitine $t_{1/2}$ values could not be established as the C_{max} was not captured. ND, not determined.

5.5.8 TMAO excretion in urine and faecal samples

Faecal samples for two participants were explored to study concentrations of γ-BB, L-carnitine, and TMAO. Both L-carnitine and γ-BB were detected at the µg/mg level (**Figure 5.15A-B**), while only trace amounts of TMAO were detected in two samples (**Figure 5.15C**).

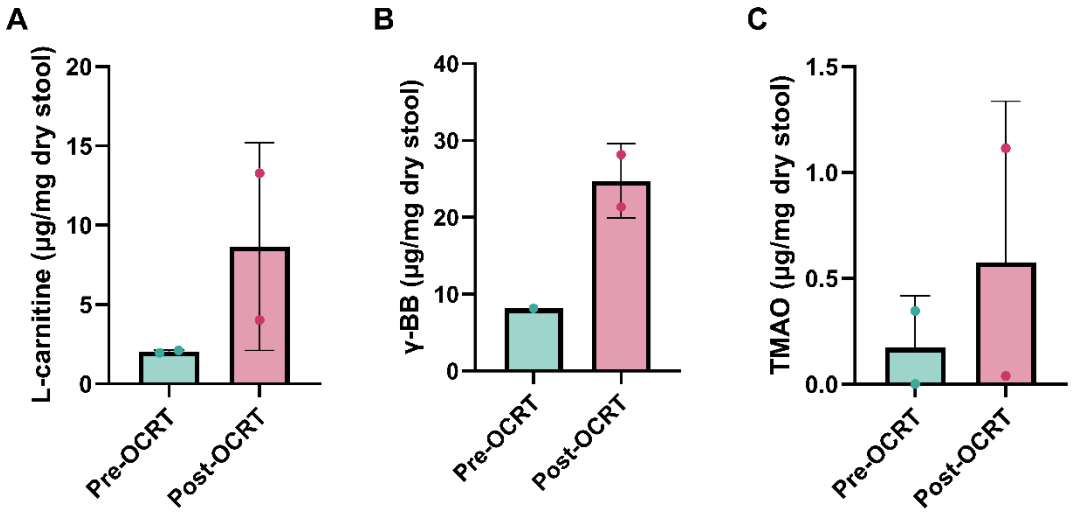

Figure 5.15. L-Carnitine, γ-butyrobetaine (γ-BB), and trimethylamine N-oxide (TMAO) concentrations in pre- and post-OCRT faecal samples. *Concentrations of (**A**) L-carnitine, (**B**) γ-BB, and (**C**) TMAO in faecal samples of one intervention for two participants in the TESSA study as an explorative analysis. Data are shown as mean ± SD. Faecal samples were diluted 1:10, vortexed for 30 minutes, centrifuged and filtered prior to LC-MS/MS analysis using isotope-labelled internal standards.*

5.5.9 Urolithin metabotypes

Varying concentrations of urolithins A and B, as aglycones and as glucuronides, were observed in urine samples. Detected amounts of urolithin glucuronides were converted to urolithins using the previously established RRF (**Figure 5.2**). The production of urolithins varies widely among participants (**Figure 5.16**). The presence of urolithins shows that the pomegranate extract reached the colon where it underwent microbial metabolism to ellagic acid metabolites.

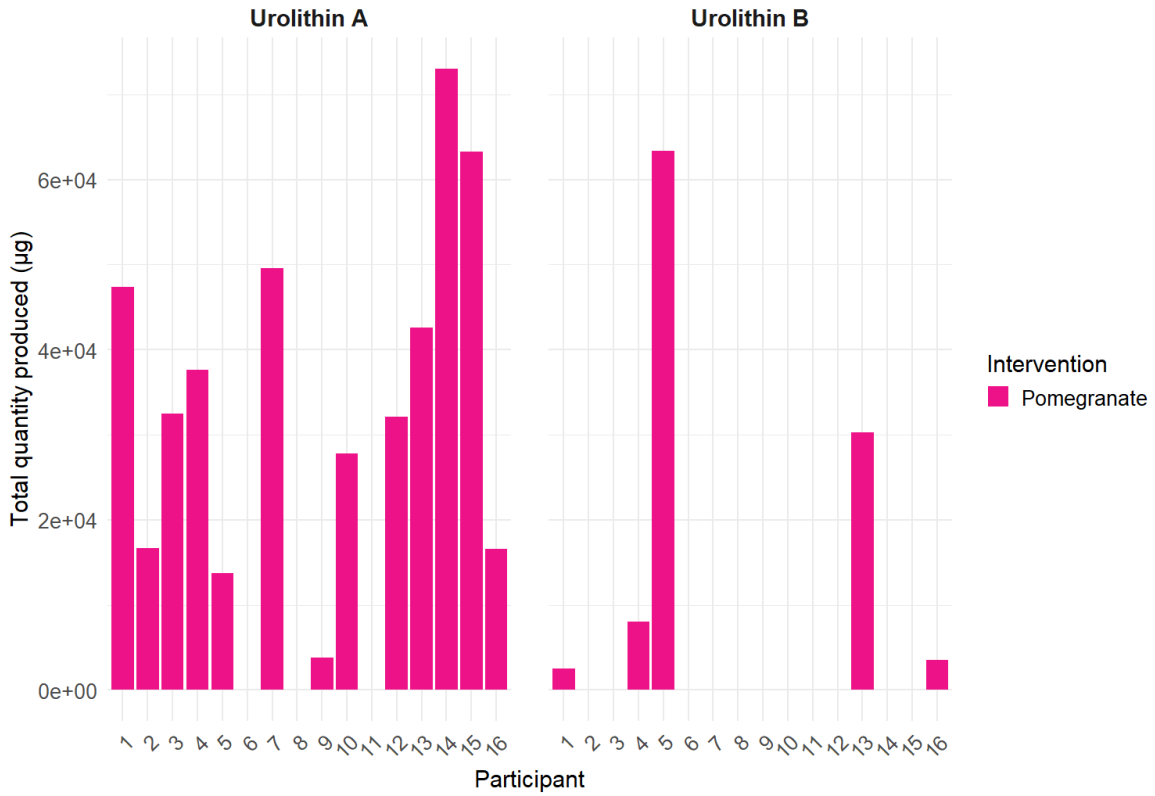

Figure 5.16. Total amount of urolithins produced per TESSA participant over 48 hours after ingesting a pomegranate extract (1.6 g). *Total urolithin quantities were estimated per participant (n = 16) by multiplying total urolithin quantities in the urine collections by the total volume of collected urine. Detected amounts of urolithin glucuronides were converted to urolithins using the previously established relative response factor (RRF) (**Figure 5.2**). Participants collected all urine over 48 hours after ingestion in of the pomegranate extract, which were aliquoted and stored at -80 °C until LC-MS/MS quantification of urolithins. Numerical participant identifiers are assigned according to the rank order of the pomegranate extract's effect relative to the placebo on TMAO AUC, from the largest to the smallest observed effect.*

Based on the urolithins measured in urine samples, urolithin metabotypes (UMs) could be established (**Figure 5.17**). Of the 16 participants completing Phase II, 3 (18.8%) did not produce any urolithins (UM-0), 5 (31.3%) produced both urolithins A and B (UM-B), and 8 (50%) produced urolithin A only (UM-A). This largely corresponds to the literature, reporting that the prevalence of UM A, B, and 0 is 25-80%, 10-50%, and 10-25%, respectively [111, 112].

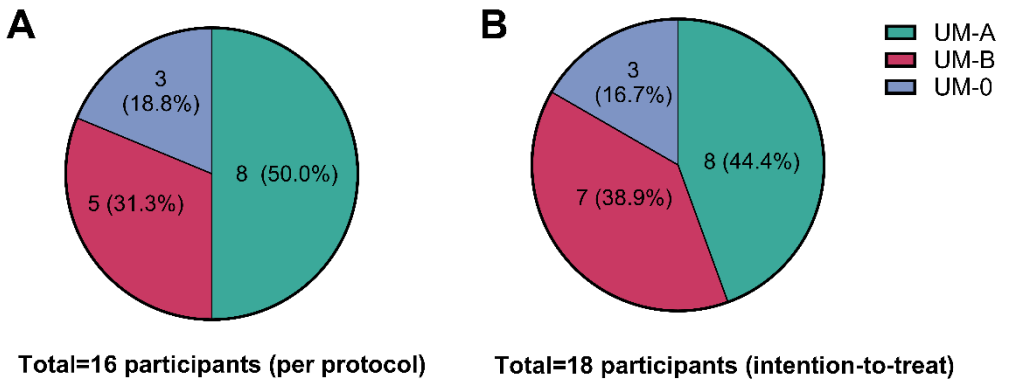

Figure 5.17. Urolithin metabotypes (Ums) among TESSA participants. *Data are derived from urolithins present in the urine after pomegranate extract supplementation in (**A**) all participants who completed Phase II (per protocol, n = 16), and (**B**) all participants who completed the pomegranate intervention (intention-to-treat, n = 18). Urolithin metabotypes are categorised into UM-A (produces only urolithin A and derivatives), UM-B (produces urolithin A and B derivatives), UM-0 (doesn't produce urolithins). A threshold of 0.3 parts per million (ppm) was used.*

To explore potential associations between interindividual variability in TMAO response to the pomegranate supplementation, plasma TMAO AUC was stratified by UM. While mean TMAO AUC values differed between UMs following the pomegranate intervention (**Figure 5.18A**), these differences were not statistically significant (**Table 5.3**). Similarly, no significant differences were observed across UMs in the change in TMAO AUC between the placebo and pomegranate interventions (**Figure 5.18B**, **Table 5.3**).

Table 5.3. Pairwise comparisons of plasma trimethylamine N-oxide area under the curve (TMAO AUC) following the pomegranate intervention, and of the difference in TMAO AUC between placebo and pomegranate interventions across urolithin metabotyptes (UMs). *Plasma TMAO responses were compared across metabotypes UM-0 (n = 3), UM-A (n = 8), and UM-B (n = 5). A Dunn's test was used for pairwise non-parametric pairwise comparisons. All p-values are adjusted using the Benjamini-Hochberg method.*

Comparison	Adjusted p-value TMAO AUC (pomegranate intervention)	Adjusted p-value TMAO AUC difference (placebo - pomegranate intervention)
UM-0 vs UM-A	0.344	1.000
UM-0 vs UM-B	0.352	0.878
UM-A vs UM-B	0.726	1.000

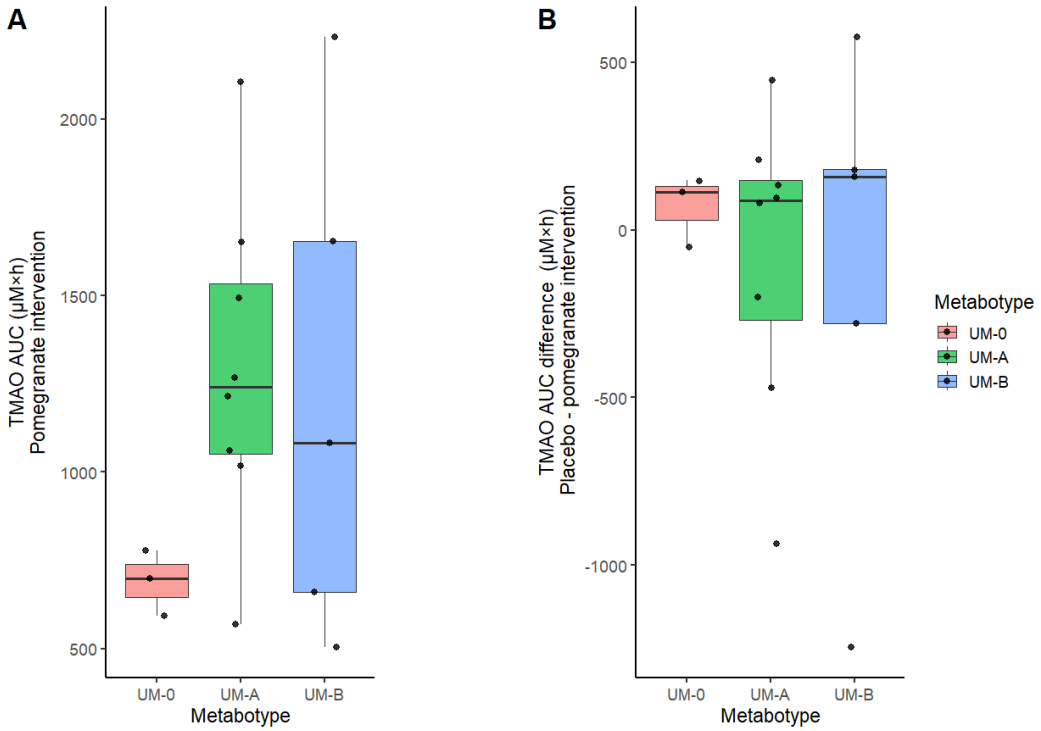

Figure 5.18. (A) Plasma trimethylamine N-oxide area under the curve (TMAO AUC) following the pomegranate intervention, and (B) the difference in TMAO AUC between placebo and pomegranate interventions, stratified by urolithin metabotypes (UMs). *Plasma TMAO responses were stratified across metabotypes UM-0 (n = 3), UM-A (n = 8), and UM-B (n = 5).*

5.5.10 Polyphenols and urolithins in a faecal sample

To explore the excretion of the pomegranate extract, a faecal sample from a TESSA participant who showed a reduced TMAO response upon treatment with the pomegranate intervention was analysed for quantities of pomegranate polyphenols and urolithins. The exploratory analysis showed that trace amounts of punicalagin, punicalin, and ellagic acid were present in the faecal sample (**Figure 5.19**). A more than 10-fold greater quantity of urolithin A was observed in the sample. This indicates that the polyphenols have undergone significant metabolism in the gastrointestinal tract. Urolithin B was not observed, corresponding to this participant's metabotype (UM-A). Urolithin A glucuronide was not detected, which was expected since glucuronide conjugates are primarily detected in urine and plasma samples [237].

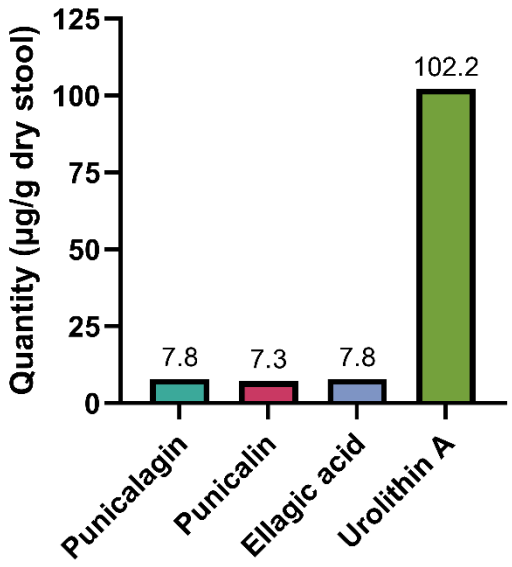

Figure 5.19. Pomegranate polyphenols and urolithins in a post-pomegranate intervention faecal sample of one participant who had a reduced trimethylamine N-oxide area under the curve (TMAO AUC) in response to the pomegranate intervention. *Explorative analysis (n = 1). A faecal sample was collected and stored at -80 °C until analysis. Prior to LC-MS/MS analysis, the faecal sample was diluted 1:10 in MeOH/DMSO/H_2O (40/40/40), vortexed for 30 minutes, centrifuged, and filtered.*

5.5.11 Correlation between kidney function and TMAO response

Since TMAO is primarily cleared from the body through renal excretion, associations between TMAO response and kidney function were explored in a *post hoc* analysis. During Phase I, data were collected from blood serum samples on eGFR and creatinine levels. Generally, a lower eGFR and higher creatinine level indicate declining kidney function. When kidney function declines, the kidneys become less efficient at filtering and excreting TMAO, potentially leading to TMAO accumulation in blood plasma. Previous studies have shown that a declined kidney function is associated with higher plasma TMAO levels [238]. Since a ceiling value of 90 mL/min/1.73m^2 was in place for eGFR, which most participants exceeded, only creatinine levels were used as a measure of kidney function.

Higher serum creatinine levels were positively associated with the TMAO AUC difference between the placebo and the pomegranate extract (β = 26.0, 95% CI: 11.4 to 40.6, *P* =

0.002); i.e., a favourable effect of the pomegranate extract on reducing TMAO levels compared to the placebo (**Figure 5.20**). This indicates that the pomegranate extract was more effective in reducing plasma TMAO AUC in participants with poorer kidney function. When adjusted for age and sex, the association between creatinine levels and the AUC difference remained significant (β = 20.8, 95% CI: 0.7 to 40.8, *P* = 0.045).

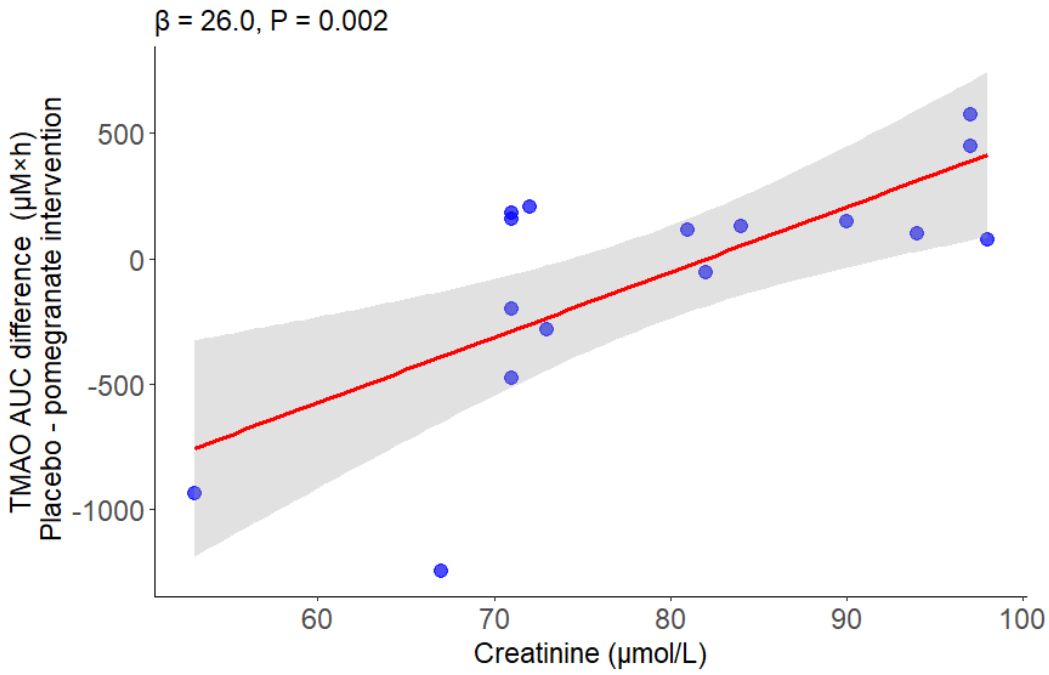

Figure 5.20. Association between serum creatinine (µmol/L) and the difference in trimethylamine N-oxide area under the curve (TMAO AUC) between placebo and pomegranate interventions. *A linear regression model showed that higher creatinine levels were associated with a reduced TMAO AUC after the pomegranate extract relative to placebo. Individual data points are shown (n = 16). Kidney function was assessed from blood serum samples collected during Phase I and TMAO AUC was assessed during Phase II. Participants underwent an oral carnitine response test (OCRT), consuming 1.5 g L-carnitine. For 48 hours after the OCRT blood plasma was collected to estimate the TMAO AUC.*

A two-sample t-test was conducted to compare serum creatinine levels between female participants in the "Older" group (n = 3) and the combined group of male and premenopausal participants (n = 13). "Older" female participants had significantly lower serum creatinine levels (63.7 ± 9.5 versus 83.2 ± 11.0 µmol/L, *t*(3.38) = -3.12, *P* = 0.045). This finding indicates that, despite being older, women in the "older" subgroup exhibited relatively better kidney function compared to the rest of the study population.

5.6 Discussion

5.6.1 Main findings

In this chapter, which describes findings of the TESSA study, the effect of a pomegranate extract on plasma TMAO levels in healthy, omnivorous adults was demonstrated. The TESSA study is the first to investigate the effect of a polyphenol-rich extract on the TMAO response from an L-carnitine challenge. Here, it was shown that the pomegranate extract did not reduce plasma TMAO levels in the overall TESSA study population (n = 16). However, the pomegranate extract reduced TMAO log AUC by 15% compared to the placebo intervention in the combined group of male and premenopausal female participants (n = 13). A significant interaction of age group and sex was observed in response to the pomegranate intervention, indicating that the intervention effect differed according to the combination of age and sex.

TESSA Phase I data showed that 90.6% of the participants were able to produce substantial amounts of TMAO from L-carnitine. This is much higher than numbers reported in a previous study; Wu *et al.* (2020) categorised 45% of participants as high-TMAO producers based on a plasma TMAO cut-off of 10 µM at 24 hours after a 1.5 g L-carnitine OCRT [71]. Although the cut-off criteria used in the TESSA study were based on the TMAO increase between baseline and 24 hours post-OCRT (> 5 µM and >50%) rather than the absolute TMAO levels 24 hours post-OCRT, a *post hoc* analysis showed that most TESSA participants (28 out of 32, 87.5%) exceeded 10 µM plasma TMAO at 24 hours post-OCRT, and would therefore still be classified as high-TMAO producers based on the Wu *et al.* cut-off criteria. The larger proportion of high producers in the TESSA study may be because the TESSA study recruited omnivores who regularly consumed meat (≥4 portions per week), whereas Wu *et al.* included both omnivores and vegetarians [71].

Studies have shown that omnivores generate more TMAO from L-carnitine than vegetarians and vegans [29, 230]. In fact, omnivores were found to have a more than 20-fold greater TMAO response from L-carnitine than vegetarians [67]. This is substantiated by a previous report that high-meat diets were associated with higher urinary TMAO levels compared to low-meat and vegetarian diets [218]. This is likely due to the gut microbiota becoming more efficient at converting L-carnitine to TMA upon the consumption of a high-meat diet. For instance, L-carnitine supplementation could

enhance the capacity of the gut microbiota to produce TMA [219]. Supporting this, the *gbuA* gene, involved in the conversion of γ-BB to TMA, was enriched in participants consuming a diet high in red meat for four weeks compared to when they consumed a non-meat diet for four weeks (n = 50), with corresponding reductions in both *gbuA* abundance and plasma TMAO upon switching to the non-meat diet [191]. These findings suggest that the abundance of the *gbu* gene cluster is shaped by habitual diet, particularly by red meat intake, which provides high levels of L-carnitine. This likely explains why the TESSA cohort, consisting of omnivores who regularly consume meat, exhibited a higher prevalence of high-TMAO producers compared with other studies. This also corresponds with the TESSA FFQ data showing that the habitual dietary L-carnitine intake tended to be correlated with the TMAO response in Phase I, although this was not statistically significant. Previously, specific gut microbial taxa have been associated simultaneously with omnivorous versus vegetarian diets and plasma TMAO concentrations [29]. During the TESSA study, faecal samples were collected to further elucidate associations between TMAO response and specific microbial taxa. This is an important avenue for future research that can be achieved with readily available TESSA study samples.

TESSA Phase I data showed that older age correlated with a greater TMAO response between baseline and 24-hour follow-up. These findings correspond to a crossover study in 20 adults, which reported that fasting plasma TMAO levels were significantly higher in participants above the median age (45.5 years) [102]. Another study showed that plasma TMAO levels were higher in older (64 ± 7 years) compared to younger (22 ± 2 years) adults [239]. Serum TMAO concentrations also increased with age in 592 healthy participants who were followed from age 11 to 26 in a longitudinal prospective cohort study [240]. TESSA Phase II data furthermore showed an interaction between age group and sex on the intervention effect on plasma TMAO levels, suggesting that the effect of the intervention varied depending on the combination of age and sex. This finding aligns with previous evidence suggesting that both age and sex influence circulating TMAO concentrations, such as a cross-sectional study in 51 adults with obesity showing that postmenopausal women (mean age 56.5 years) had higher plasma TMAO levels than premenopausal women (mean age 40.3 years) and aged-matched men [232]. However, a study specifically investigating the differences in TMAO levels across young, healthy men and women did not find any differences in plasma TMAO levels between men,

eumenorrheic women and women using contraceptives [241], suggesting that sex differences in TMAO levels may be especially apparent at older age.

The TESSA study demonstrated that the group of "older" women (n = 3, age > 47), defined based on a median split, did not exhibit a reduction in plasma TMAO AUC following the pomegranate intervention. In fact, their TMAO AUC was elevated compared to the placebo, indicating a response directionally opposite to the effect observed in male and premenopausal female participants. Although there were only three out of 16 participants in the TESSA study who were classified as "older" (possibly postmenopausal) women, and therefore no conclusions can be drawn from these observations alone, there is some evidence in the existing literature that the gut microbiome characteristics differ significantly between sexes, with women typically exhibiting greater microbial richness relative to men [242, 243]. Evidence suggests that the typical pattern of higher gut microbiome diversity observed in women compared to men during younger years may not persist into older adulthood [243], indicating that older women potentially exhibit a microbiome profile more similar to that of men. This hypothesis is supported by a study that demonstrated compositional gut microbiome differences between premenopausal women (n = 44) and men (n = 42), which were much less pronounced between postmenopausal women (n = 45) and men (n = 48) [244]. These findings may therefore be attributable to distinct microbiome profiles in older women compared with men and younger (premenopausal) women.

Nevertheless, if older age/menopause results in a microbiome profile resembling that of men, it would be expected that TESSA's male and "older" (postmenopausal) female participants showed similar treatment responses. Therefore, the observed differential TMAO response may not primarily arise from differences in microbial TMA production from L-carnitine. Alternatively, age and sex may influence hepatic TMA conversion, accounting for the observed differences between groups. After all, polyphenols have been reported to reduce the expression of FMO3, which oxidises TMA to TMAO in the liver [162], suggesting that this is a possible mechanism of action of the pomegranate extract. Experiments using male C57BL/6N mice demonstrated that aging was associated with an increased expression of FMO3 [245]. Moreover, females were shown to have a higher FMO3 expression than males [246], indicating that females have a greater ability to convert TMA to TMAO. However, if the pomegranate extract differentially affects TMAO levels across subpopulations due to varying baseline FMO3 activity, higher TMA concentrations would be expected in the blood plasma, but here it was shown that

plasma TMA concentrations averaged around 0.4 µM and were not significantly different between interventions.

The TESSA study furthermore showed a strong association between higher serum creatinine levels and an improved effect of the pomegranate extract relative to placebo on plasma TMAO AUC. Higher serum creatinine levels indicate a declining kidney function [247]. Hence, these data suggest that the pomegranate extract was more effective in reducing plasma TMAO AUC relative to the placebo in participants with poorer kidney function. However, the interpretation of serum creatinine requires caution, because creatinine levels can be influenced by various factors independent of kidney function, including muscle mass and diet (especially cooked meat) [247]. In some cases, eGFR is used as a measure for kidney function, which accounts for variables like age and sex and can therefore be a more useful measure of kidney function. However, eGFR could not be established for most participants in the TESSA study due to a ceiling of 90 mL/min/1.73m^2, which most participants (n = 12) exceeded. It has furthermore been reported that creatinine levels increase with age [248], but upon adjustment of the linear regression model for age and sex, the association between creatinine levels and the effect of the pomegranate extract on plasma TMAO AUC remained significant. This suggests that higher creatinine levels in the TESSA study participants, independently of age or sex, predicted a better response to the pomegranate extract.

In previous studies large intra-individual variation in TMAO values have been observed [132, 249]. For instance, in the BERI study (Clinicaltrials.gov ID NCT03213288), which estimated the variation between six measurements of plasma TMAO when no dietary restrictions for TMAO precursors were in place, substantial variation in plasma TMAO values was shown within participants [132], with a CV exceeding 50% for the majority of participants. In this chapter, however, the CVs of three (fasted) baseline TMAO measurements were much lower and remained below 50% for most participants. This is likely due to the dietary restrictions that were in place starting 48 hours before the baseline measurements. Several TESSA participants did exhibit a single high baseline TMAO measurement, which may suggest that they did not adhere to the dietary restrictions, or they may have consumed foods that are not known to contain L-carnitine. Unlike choline, available food composition data of L-carnitine are limited and is not reported in large food databases such as the USDA FoodData Central [250]. Therefore, L-carnitine food composition data were obtained from just three publications, and it is possible that participants unknowingly consumed foods containing L-carnitine that were

not identified in the limited available data, leading to elevated baseline TMAO levels despite dietary compliance.

5.6.2 Limitations

Although an effect of the pomegranate extract on TMAO production was observed in male and premenopausal women, the TESSA study remains a proof-of-concept study with a small study population and even smaller subgroups, having only three participants in the “older” (possibly postmenopausal) women group and three in the premenopausal women group. Since the TESSA study was not powered for stratification by age group and sex, it is important to interpret the data with caution.

A limitation of this study is the categorisation of urolithin metabotypes. Urolithin metabotypes were established in order to investigate associations with the TMAO AUC response to the pomegranate intervention. Pairwise comparisons showed that the TMAO AUC was not associated with urolithin metabotypes. However, it is possible that some participants produced isourolithin A, but not urolithin B. Since isourolithin A was not included in the analyses, certain participants might have been classified as metabotype A while they potentially belong to metabotype B (producing urolithin A and isourolithin A and/or urolithin B). The identification of isourolithin A presents several analytical challenges due to its structural similarity to urolithin A (**Figure 1.4**), resulting in close elution or co-elution in chromatographic analyses. Previous studies that successfully identified isourolithin A employed HPLC-DAD-ESI-MS with an extended gradient over a 30-minute run [170], achieving marginal separation with an RT of 15.95 minutes for isourolithin A and 16.01 minutes for urolithin A (at approximately 26.5% solvent B). In contrast, the method used in this study used a shorter, 14-minute run with a steeper gradient, detecting urolithin A at 8.1 minutes (around 38% solvent B). Therefore, under the applied chromatographic conditions, isourolithin A could not be separately detected from urolithin A.

Another limitation is that pharmacokinetic parameters for L-carnitine were difficult to establish. For instance, $t_{1/2}$ values could not be derived because plasma L-carnitine concentrations remained relatively stable over 48 hours. Since only a small proportion of L-carnitine is absorbed from supplements compared to the diet, with a reported bioavailability of just 14-18% [60], it is unlikely that all L-carnitine was absorbed in the

blood. Yet, if 18% of 1.5 g L-carnitine would be absorbed (equivalent to 270 mg L-carnitine), an increase of approximately 335 µM plasma L-carnitine would be expected, whereas the mean peak concentration observed in the TESSA study was only 42 µM. Previous ADME studies showed that the T_{max} of L-carnitine was achieved at 2-4.5 hours after administration [236]. Therefore, L-carnitine concentrations may have reached T_{max} after 2.5 hours (and before 16 hours), which has not been captured, and might have affected the determination of $t_{1/2}$. To get a better understanding of the ADME of the L-carnitine supplements, collected urine samples should be tested for the presence of L-carnitine, TMAO, and other methylamines.

5.6.2 Prospective research

The TESSA study highlights the importance of age and sex stratification in dietary intervention studies targeting TMAO reduction. The observed opposite effect in the subgroup of "older" women (n = 3) emphasises the need for future studies to explore this on a larger population scale. Postmenopausal women face elevated CVD risk compared to premenopausal women [251], making the identification of effective, low-risk interventions for TMAO reduction particularly relevant for this population.

Future studies should also investigate associations between TMAO response and specific microbial taxa. During TESSA study Phase II, participants collected faecal samples prior to and after each OCRT. These faecal samples can be tested for the abundance of L-carnitine- and γ-BB-metabolising genes (*cai* and *gbu* gene clusters), such that the abundance prior to and after the OCRT can be compared between the pomegranate versus the placebo intervention. This can confirm the findings reported in **Chapter 4**, that the pomegranate extract might promote L-carnitine-metabolising genes, while reducing TMA production, indicating a mechanistic effect on enzymes involved. Furthermore, faecal samples can be sequenced and tested for their presence of reported L-carnitine and γ-BB metabolising bacteria, such as *Escherichia coli* [152], *Emergencia timonensis* [70], and *Ihubacter massiliensis* [71]. Finally, faecal samples can elucidate associations between TMAO response and specific microbial taxa and can be compared with previously reported taxa associated with TMAO responses among omnivores and vegetarians [29].

5.7 Conclusion(s)

The TESSA study demonstrated that a pomegranate extract did not reduce plasma TMAO levels in the overall study population. However, a significant interaction of age group and sex was observed, with the pomegranate extract reducing plasma TMAO AUC by 15% in the combined group of male and premenopausal female participants.

Chapter 6

General Discussion

Chapter 6: General Discussion

6.1 Summary of the main findings

The overall aims of the research presented in this thesis were to determine the effect of an ellagitannin-rich pomegranate extract on microbial TMA production from L-carnitine, and subsequently on circulating TMAO levels, and to elucidate several of the possible underlying microbial mechanisms involved. A bench-to-clinic approach was used to achieve these aims. The main findings of the presented work can be summarised as follows:

- Pomegranate extract reduced the production of γ-BB and TMA from L-carnitine dose-dependently in an *in vitro* colon model. L-Carnitine depletion and γ-BB accumulation were delayed, and less TMA was produced.
- Pomegranate extract independently inhibited the metabolism of γ-BB to TMA and of L-carnitine to crotonobetaine, demonstrating an effect on multiple steps in the pathway (i.e., inhibiting L-carnitine → γ-BB, γ-BB → TMA, and L-carnitine → crotonobetaine).
- Punicalagin nearly completely inhibited TMA production from L-carnitine, making it the only compound in the pomegranate extract, of all the phenolic and non-phenolic compounds that were tested, that affected TMA production.
- Pomegranate extract increased the total viable bacterial count, as well as the abundance of *caiT* genes, which are involved in the anaerobic metabolism of L-carnitine to γ-BB, demonstrating that the extract is not acting as a general antibacterial, nor selectively inhibiting *caiT*-carrying bacteria.
- Pomegranate extract did not reduce plasma TMAO levels in the overall study population of a randomised, controlled crossover study (TESSA study). However, pomegranate extract reduced plasma TMAO by 15% in the combined group of men and premenopausal women.
- The effect of the pomegranate extract interacted with age and sex, indicating that the intervention effect differed according to the combination of age and sex. The effect also depended on baseline serum creatinine levels, with higher creatinine levels (generally indicating poorer kidney function) predicting a better response to the pomegranate extract, independently of age and sex.

- A large proportion of participants in the TESSA study produced substantial amounts of TMAO from L-carnitine, which was much higher than numbers reported in a previous study (87.5 versus 45%) [71].

6.2 Impact and implications

6.2.1 Novelty of this thesis

Previous *in vitro* fermentation studies have suggested that polyphenols and polyphenol-rich extracts may inhibit microbial TMA formation [89, 90, 252], but the available evidence is limited and the effects of many polyphenols, including those from pomegranates, had not been studied under pH-controlled conditions. The present thesis is the first to evaluate a punicalagin-rich pomegranate extract in colon models with continuous pH monitoring. By maintaining the pH within the physiological range, this approach allowed for the distinction between inhibitory effects due to the pomegranate extract and those due to pH-driven microbial suppression.

To be able to identify the main pomegranate bioactive that suppressed TMA production from L-carnitine, a small-scale high-throughput colon model was established. This model allowed the testing of individual pomegranate extract constituents in a rapid and cost-efficient manner. While similar methods have been described previously [90, 252], this thesis introduces critical optimisation through improved buffering capacity and additional pH monitoring, which revealed that the microbial metabolism of L-carnitine is pH-dependent. These findings highlight that low pH is a key confounder in *in vitro* fermentation studies investigating L-carnitine metabolism. After all, pH can influence microbial viability, community composition, and metabolic capacity [180, 181].

This thesis is the first report of the near-complete inhibition of TMA production by punicalagin *in vitro*. The promising *in vitro* findings led to a unique study investigating the effect of a pomegranate extract on the TMAO response to an L-carnitine challenge in healthy participants. While different studies had previously investigated the effects of polyphenol-rich interventions [98-102] and L-carnitine challenges [29, 67, 71, 230] on circulating TMAO levels, this thesis is the first to demonstrate how a polyphenol-rich intervention influences the TMAO response to an L-carnitine challenge, rather than baseline circulating TMAO levels.

6.2.2 Human variability in TMAO responses to polyphenols

The findings from the TESSA study revealed that the administration of a pomegranate extract did not reduce TMAO production in the full cohort (n = 16) but resulted in a statistically significant 15% reduction in the combined group of men and premenopausal women (n = 13). There was an interaction between age and sex on the response to the extract. The differential effect amongst different study population subgroups suggests that host characteristics, including age and sex, influence the response to polyphenols on microbial TMA production and subsequent TMAO formation. For female TESSA participants above the age of 47 (possibly postmenopausal), the pomegranate extract increased TMAO levels compared to the placebo, in contrast to men and "younger" women. Evidence from other studies showed that TMAO levels were higher in older adults and postmenopausal women compared with younger adults or premenopausal women [102, 232, 239, 240]. One plausible mechanism may be age and sex differences in microbial community diversity, which have been well established [242-244].

The TESSA study also highlighted that substantial interindividual variation exists in TMAO production from L-carnitine. Several factors appear to modulate this variation. First, habitual diet matters: omnivores in the TESSA study (≥4 portions of meat per week) showed far greater increases in plasma TMAO levels after the L-carnitine challenge than reported in studies including vegetarians or mixed diet groups [29, 67, 71, 230]. This suggests that long-term exposure to L-carnitine shapes the gut microbiome's capacity to convert L-carnitine to TMA. Second, the TESSA study showed that kidney function correlated with the intervention effect: higher serum creatinine, indicating poorer kidney function, predicted a greater reduction in TMAO AUC with the pomegranate extract intervention, independent of age and sex. This supports a view that kidney clearance capacity influences circulating TMAO levels and possibly the response to interventions. In fact, TMAO levels tend to be higher among those with poor kidney function [253, 254]. Concomitantly, poor kidney function has been associated with increased CVD risk, with CKD markedly increasing CVD risk: CVD is the leading cause of death in this population [255-257]. This raises the possibility that the pomegranate extract might be most effective among those with poor kidney function and at greater risk for CVD.

6.2.3 Are the investigated pomegranate doses achievable through the diet?

A key translational question is whether the observed effects of the pomegranate extract can realistically be achieved through the diet. The doses of pomegranate extract used for the *in vitro* work presented in this thesis (5.7, 11.4, and 22.8 mg/mL) were standardised based on a polyphenol content of at least 20% w/w stated by the manufacturer (**Appendix 3**), which is equivalent to 1.14-4.56 mg/mL of polyphenols. When assuming an estimated colonic volume of 561 mL [139], this corresponds to approximately 640-2,560 mg of polyphenols reaching the colon. A 500 mL pomegranate juice typically provides 2,200-2,560 mg of polyphenols [140-143]. Therefore, the different doses of the extract used in the *in vitro* experiments would be achievable in a 125, 250, and 500 mL of juice. The pomegranate extract dose used in the TESSA study provided approximately 320 mg of polyphenols (20% of 1.6 g pomegranate extract), which would be readily available from pomegranate juice. Thus, the intervention tested here does not require pharmacological dosing but can be achieved via the diet.

In this thesis it was shown that punicalagin, an ellagitannin, almost completely inhibited TMA production from L-carnitine at a 2 mg/mL dose, equivalent to 1,122 mg of punicalagin assuming a colonic volume of 561 mL [139]. This dose is achievable from a pomegranate extract depending on what parts of the pomegranate are used to make the extract. The pomegranate extract reported in this thesis (Dermogranate®), obtained from whole pomegranates, provided 51 mg/g (5.1%) punicalagin. Hence, 22 g extract should be consumed to achieve 1,122 mg punicalagin reaching the colon. Other studies reported a punicalagin content between 62-125 mg/g for pomegranate peel, 42-63 mg/g for pomegranate flesh, and only up to 0.7 mg/g for juice [258]. Punicalagin content varies considerably between pomegranate cultivars and juice or extract preparation methods [258, 259]. Optimisation of extraction techniques, such as high-pressure processing or solvent-free methods, could enhance punicalagin yields from pomegranates [260, 261].

The estimated 22 g Dermogranate® extract needed to achieve the punicalagin dose that was tested in **Chapter 3** (2 mg/mL) suggests that the dose used in the TESSA study (1.6 g) may have been too low for a significant effect in the overall study population. Future *in vitro* studies could investigate the minimum inhibitory concentration of punicalagin, such that potentially a higher pomegranate dose can be tested in a follow-up intervention study.

6.2.4 Punicalagin's structural advantage in TMA inhibition via multiple pathways

The findings presented in this thesis demonstrate that punicalagin, but not punicalin nor ellagic acid, suppressed TMA production, suggesting that punicalagin is the main bioactive compound responsible for TMA inhibition. In **Chapter 2** it is shown that the pomegranate extract independently inhibited the metabolism of γ-BB to TMA and of L-carnitine to crotonobetaine, demonstrating that the pomegranate extract/punicalagin influence multiple steps in the pathways (i.e., L-carnitine → γ-BB, γ-BB → TMA, and L-carnitine → crotonobetaine). Furthermore, in a study by Haarhuis & Day-Walsh et al. (2025), the pomegranate extract was also able to reduce choline metabolism to TMA [69]. These findings suggest that punicalagin affects multiple pathways of TMA production involving different gene clusters (*gbuABCDEF*, *caiTABCDE*, *cutCD*). This wide-ranging inhibitory effect is unique to punicalagin, because in **Chapter 3** of this thesis it was shown that previously reported inhibitors of the choline → TMA pathway (chlorogenic acid and gallic acid) did not inhibit the L-carnitine → γ-BB → TMA pathway.

The effective TMA inhibition by punicalagin compared to its non-effective derivatives (punicalin, ellagic acid, gallic acid, urolithins) might be attributed to its specific structural properties. As discussed in **Chapter 3**, punicalagin's larger molecular size (1,084 Da compared to punicalin's 782 Da) provides it with better capacity for potential interactions with TMA precursors. The mechanism might involve direct binding with TMA precursors through non-covalent interactions [177]. For instance, tannic acid can form non-covalent complexes with choline [178], and punicalagin's multiple hydroxyl groups and aromatic rings can donate hydrogen bonds to polar sites and form cation-π interactions [177, 179]. Since the molarity of punicalagin (1.8 mM) in the colon model was similar to the molarity of L-carnitine (2 mM), punicalagin might have inhibited L-carnitine metabolism through non-covalent binding with L-carnitine and γ-BB, making these substrates less available to microbial enzymes. In contrast to punicalagin, punicalin has less potential to form hydrogen bonds, with approximately 12 hydrogen donors and 21 acceptors, while punicalagin has approximately 17 hydrogen donors and 30 acceptors. Additionally, punicalagin has a greater number of aromatic rings than punicalin and is thus more likely to form cation-π interactions.

Therefore, larger ellagitannins with more hydroxyl groups and aromatic rings (punicalagin > punicalin) have a greater potential to bind L-carnitine, which may explain why the inhibitory effect on L-carnitine metabolism was observed for punicalagin but not

for punicalin. This structural advantage suggests that the potential of ellagitannins to inhibit TMA production may be directly related to their structural complexity and molecular weight.

6.3 Limitations

6.3.1 Methodological considerations of static *in vitro* colon models

This thesis described the use of both batch and high-throughput colon models to investigate the effects of a pomegranate extract and its constituents on microbial L-carnitine metabolism. While these models provide valuable mechanistic insights, they are static models and represent a simplified model of the human colon, and do not capture the full complexity of gastrointestinal physiology. For instance, the described colon models primarily reflect fermentation in the transverse and distal colon and do not account for metabolism in the upper gastrointestinal tract, where ellagitannins such as punicalagin might partially be hydrolysed to ellagic acid prior to reaching the colon [107]. As a result, the microbial substrates available *in vivo* may differ from those measured *in vitro*.

Furthermore, static colon models are subject to nutrient depletion and end-product accumulation over time [262], which can impact the microbial ecosystem and may explain the reduced microbial viability observed at 48 hours (**Appendix 2, Supplementary Figure S8**). This may have slowed down the rate of L-carnitine metabolism to TMA, particularly at later time points. These limitations of static colon models can be minimised using dynamic colon models, which are able to establish a relatively stable microbial ecosystem under physiologically relevant conditions [262, 263].

In addition, inter- and intra-individual variability in L-carnitine metabolism was observed across donors. The low relative abundance of the *gbu* (*bbu*) operon in the human gut microbiome (<0.1% of microbial genomes [49]) means that TMA production from γ-BB can vary even within the same inoculum under otherwise identical conditions. While the pomegranate extract appeared to reduce this variability, the inter- and intra-individual variability remains a limitation of *in vitro* colon models.

In the high-throughput colon model, the pomegranate extract itself could not be tested due to a substantial pH drop that compromised microbial metabolism (**Figure 3.10**). This effect likely arose from the high carbohydrate content (90-95%) of the extract (**Appendix 3**), which are rapidly fermented to organic acids that acidify the ecosystem [188]. Although punicalagin alone nearly completely inhibited TMA formation, suggesting a stronger effect than the extract itself, the inability to include the full extract represents a limitation in establishing comparability between experiments.

The mechanistic experiments highlighted additional methodological limitations. The RT-qPCR assays employed vector-based DNA standards that did not account for reverse transcription efficiency, potentially reducing accuracy in transcript quantification. Moreover, RNA extraction yields differed between control and pomegranate-treated models, suggesting that polyphenols may interfere with nucleic acid recovery and downstream amplification. Finally, explorative metagenomic analyses were performed on a limited number of donor samples (n = 1 per condition), impeding generalisation of findings regarding microbial shifts such as increased *E. coli* abundance.

6.3.2 Translational limitations for the pomegranate intervention

The TESSA study provided unique pharmacokinetic data on the effects of a pomegranate extract on plasma TMAO levels. However, several limitations constrain the interpretation of the data. The study population was small and not powered for subgroup analyses. The subgroups (e.g., grouped by age and sex) were underpowered, with as few as three participants per group. These findings should therefore be regarded as exploratory. Furthermore, the single-dose intervention limits the understanding about long-term effects of pomegranate supplementation on TMAO levels and CVD risk. Other studies have primarily examined the impact of a longer-term, high-dose supplementation with polyphenol-rich interventions on fasting circulating TMAO, with reported effective doses ranging from 30 g to 600 g daily for four to eight weeks [98-101]. Only one short-term intervention (60 mg flavonoids for five days) failed to demonstrate an effect [102]. In contrast, the TESSA study was distinct in its design, using an L-carnitine challenge to assess the acute pharmacokinetic effects of a pomegranate extract on TMAO production, rather than focusing solely on baseline fasting concentrations. This approach provided novel mechanistic insights but at the expense of scope: the intervention involved only a

single, relatively low dose (1.6 g) compared with the higher and longer-term interventions tested in other studies. It is therefore plausible that a more pronounced effect would be observed under conditions of prolonged supplementation at higher doses.

6.4 Future directions

This thesis identified punicalagin-rich pomegranate extract as a potential dietary strategy to reduce microbial TMA formation and thereby lower CVD risk. Future work should build on these findings through further mechanistic studies as well as intervention studies.

Further mechanistic studies are needed to advance the understanding of how punicalagin and related polyphenols interact with TMA precursors (e.g., L-carnitine, γ-BB), microbial enzymes and gene expression, and the composition of microbial communities. Approaches such as molecular docking, enzyme assays, optimised RT-qPCR assays, and further metagenomic profiling will help define whether inhibitory effects are due to direct substrate interactions, changes in microbial enzyme activity, changes in gene expression, or changes in the composition of the microbial communities. To achieve this, several methodological improvements are necessary, including the use of RNA/cDNA standards and stable reference genes for RT-qPCR assays as well as optimised RNA extraction protocols. Future mechanistic studies should also focus on the *gbu* (*bbu*) genes. After all, in this thesis it was shown that the pomegranate extract independently inhibited the metabolism of γ-BB to TMA.

The application of an optimised RT-qPCR assay and metagenomic profiling on the obtained faecal samples during the TESSA study will also advance mechanistic understandings. For instance, collected stool samples can be tested for the expression of *cai* and *gbu* genes through RT-qPCR assays or for reported species through metagenomic analyses. It has been reported that the *gbu* operon is present only in a few known bacterial species, including *Emergencia timonensis* and *Ihubacter massiliensis* [67, 71, 72]. However, a research group has recently reported that an unidentified species, belonging to the *Lachnospiraceae* family and referred to as JAGTTR01 sp018223385, contains the *gbu* operon and is responsible for a major part of the TMA production from γ-BB in the human gut [264]. Another research group confirmed that the JAGTTR01

sp018223385 contains the *gbu* gene, which was identified by a comprehensive pathway screening using the Unified Human Gastrointestinal Genome catalog (UHGG.v2.0), covering nonredundant genomes from isolates and metagenome-assembled genomes (MAGs) of the gut environment [165]. In total, they found 36 *gbu*-containing strains among seven species, including *Emergencia*, *Agathobaculum,* and *Dysosmobacter* species. Faecal samples obtained during the TESSA study can be readily tested for these species and contribute to our understanding of species capable of the conversion of γ-BB to TMA.

Moreover, for the clinical translation of the findings reported here, larger, longer-term intervention studies which are powered for stratification by age and sex are needed. Based on previous studies investigating the effects of polyphenol-rich interventions on TMAO levels, a longer-term intervention with a higher pomegranate extract dose is more likely to be effective. Alternatively, interventions using punicalagin alone may be more effective considering the near-complete TMA reduction observed *in vitro*, which resulted in greater inhibition than the pomegranate extract. Additionally, since the TESSA study showed that kidney function correlated with the intervention effect, future studies could focus on reducing TMAO levels specifically in populations with poor kidney function.

Finally, future work could move beyond pomegranates to test other ellagitannin-rich sources, including nuts (walnuts, pistachios, pistachios, cashews, pecans, and acorns), berries (raspberries, blackberries, strawberries, currants, and grapes), tropical fruits, medicinal plants, and herbal, green and black tea [105, 176]. Structurally, ellagitannins differ from punicalagin in size, the arrangement of HHDP and galloyl units, and the sugar groups to which they are conjugated [265]. These structural differences may influence their bioavailability and metabolism by the gut microbiota. The molecular size of several dimeric and oligomeric ellagitannins exceeds that of punicalagin (1,084 Da), up to 2,806 Da [105]. Some of these ellagitannins, including the dimeric sanguin H-6 and oligomeric lambertianin C, have more hydroxyl groups and aromatic rings than punicalagin, suggesting that these have similar, if not greater, structural advantages in TMA inhibition as punicalagin. To investigate this, further *in vitro* fermentation studies are warranted to screen different ellagitannin-rich sources for their effects on microbial TMA production, and any successful ellagitannin-rich sources can be tested in an intervention study to establish whether their consumption can contribute to reducing TMAO levels.

6.5 Conclusions

Overall, the work presented in this thesis provides new insights into the ability of a punicalagin-rich pomegranate extract to modulate the microbial production of TMA and its proatherogenic metabolite, TMAO. Using *in vitro* colon models and a human intervention study, this research demonstrated that the pomegranate extract and its main polyphenol, punicalagin, can inhibit L-carnitine metabolism to TMA, and that the single-dose supplementation with the pomegranate extract might moderately reduce postprandial TMAO responses in men and premenopausal women. Together, these findings provide proof-of-concept that pomegranate extract, and possibly other ellagitannin-rich foods, may represent a dietary strategy to lower TMAO exposure and reduce CVD risk.

At the same time, this thesis highlights important methodological and translational challenges. Static *in vitro* colon models cannot fully capture the complexity of host-microbiota interactions, and the small-scale intervention study was not powered to evaluate the effects across population subgroups. Addressing these challenges will require (i) further mechanistic studies and (ii) larger, longer-term intervention studies with a higher pomegranate extract dose and which are powered for stratification by age and sex.

Finally, for interventions such as a pomegranate extract to be a meaningful CVD prevention strategy, TMAO must first be validated as a reliable biomarker of CVD risk. Only then, dietary strategies targeting TMAO production can be established as a useful intervention to reduce the global burden of CVD.

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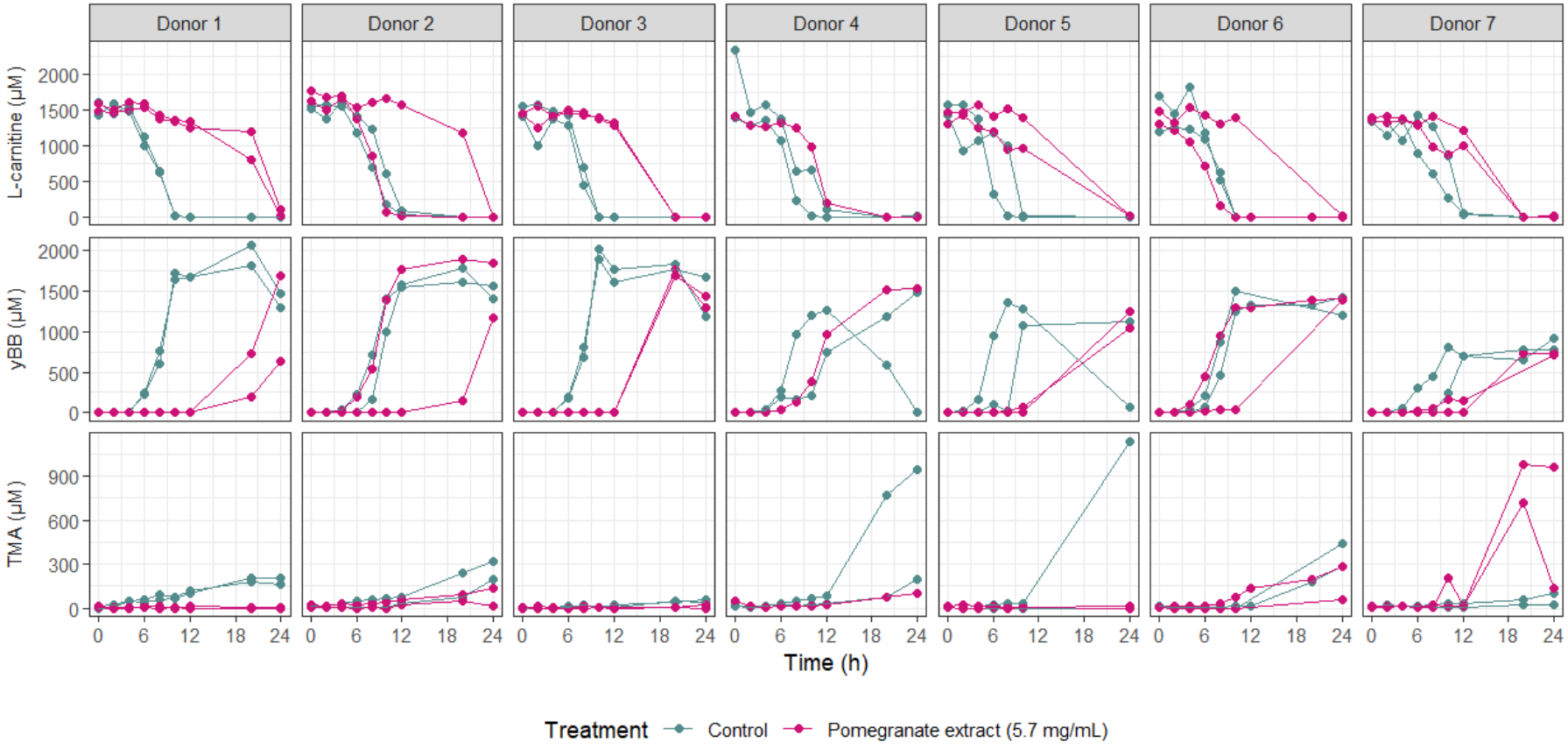
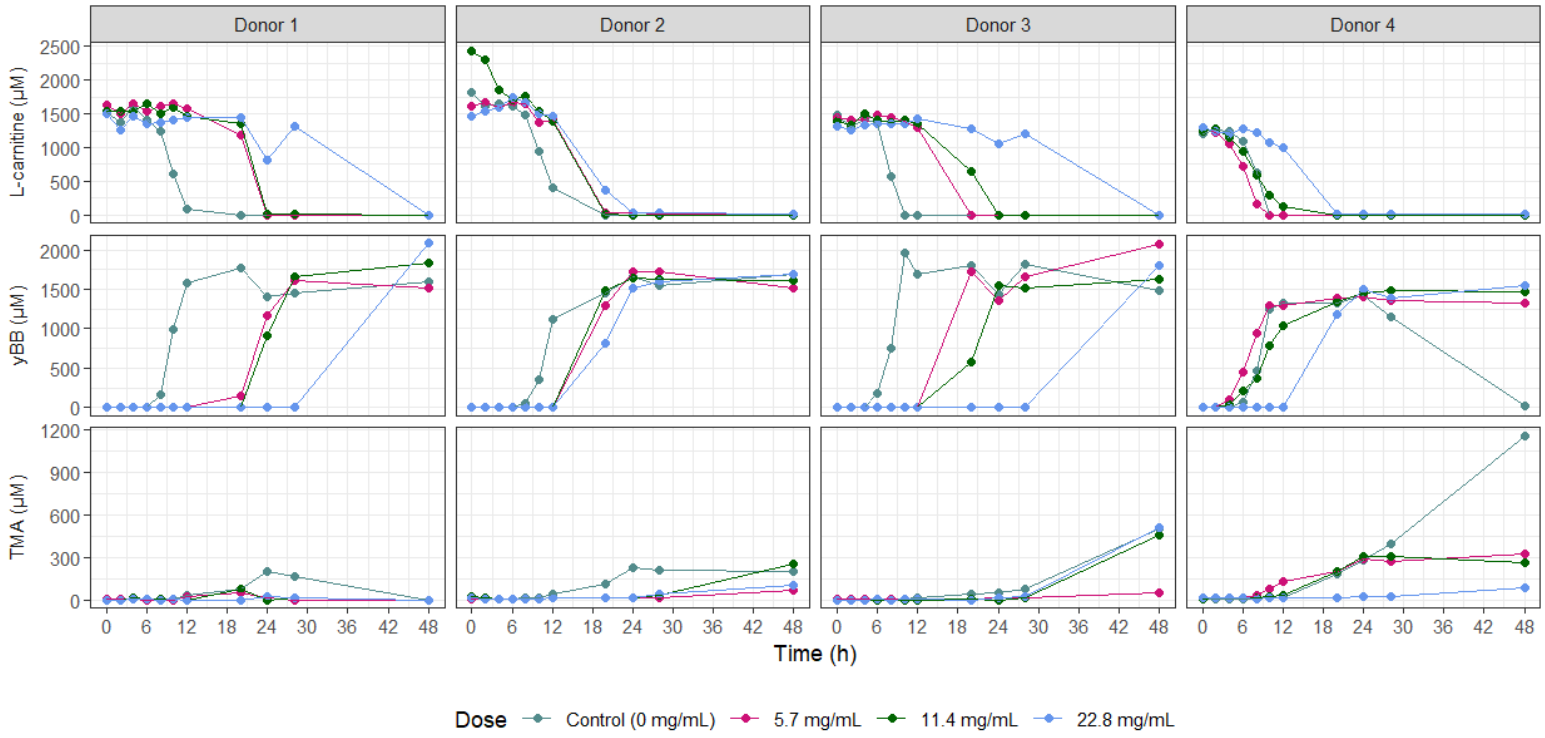
Supplementary information

Appendix 1

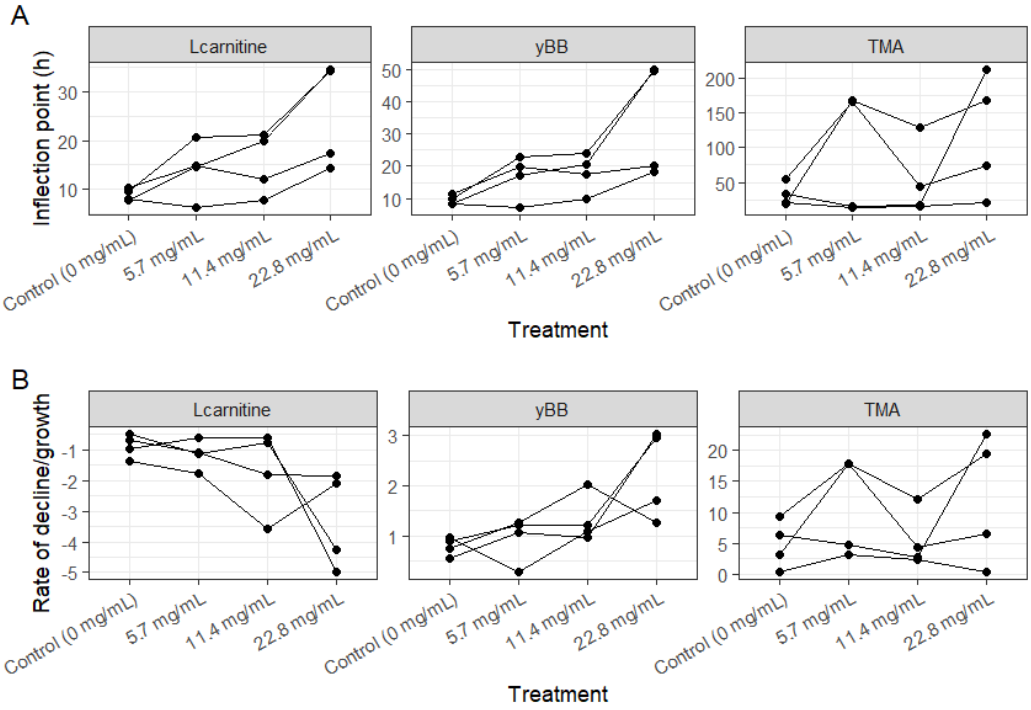
The following supplementary information are with reference to **Chapter 2**.



Supplementary Figure S1. The effects of the pomegranate extract on L-carnitine metabolism in vitro, stratified by individual donors*. Concentrations of L-carnitine, γ-butyrobetaine (γ-BB), and trimethylamine (TMA) are shown per donor over 24 hours, with two replicates per donor. Data are combined from several experiments. In vitro colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine. Samples were collected at multiple time points over 24 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*



Supplementary Figure S2. Dose-dependent effects of the pomegranate extract on L-carnitine metabolism in vitro, stratified by individual donors. *Concentrations of L-carnitine, γ-butyrobetaine (γ-BB), and trimethylamine (TMA) are shown per donor over 48 hours, with one replicate per donor. In vitro colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine. Samples were collected at multiple time points over 24 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards. This table has been generated with support from Dr George Savva.*



Supplementary Figure S3. The parameters of logistic models corresponding to the growth curves in Figure 2.4. *These curves follow the logistic function:*

$$y = \frac{A}{1 + e^{\frac{t_{50} - t}{S}}}$$

In this equation, A represents the upper asymptote of the curve, while t_{50} *denotes the time at which the curve reaches half of its maximum value. The top panels illustrate variations in* t_{50} *across different pomegranate doses within the four individual donors, highlighting the time point at which 50% of the total increase or decrease occurs. The bottom panels show how the shape parameter S, which influences the steepness of the curve, changes in response to pomegranate treatment. Larger absolute values of S indicate a more gradual rate of change. This table has been generated by Dr George Savva.*

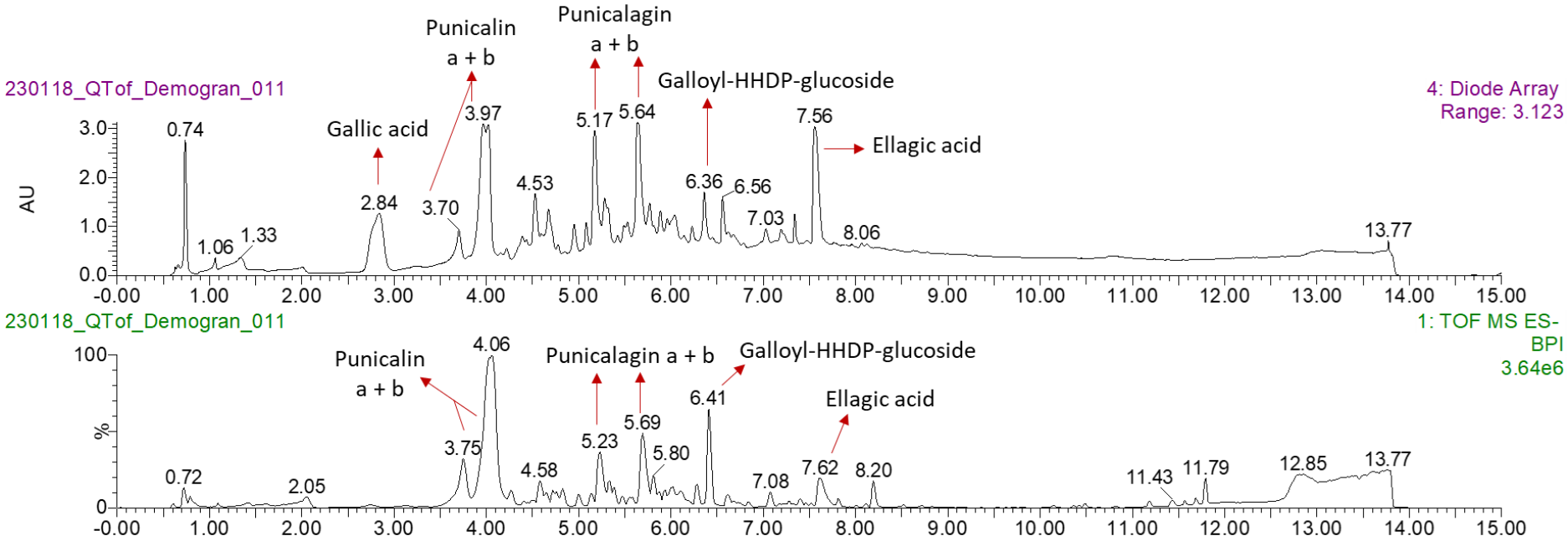
Supplementary Table S1. The effect of the different pomegranate extract doses on L-carnitine metabolism delay, γ-BB growth delay, and on L-carnitine hill slope. *Beta represents the time at which the concentration of the metabolites reached the mid-point (t_{IC50}).*

	L-carnitine disappearance time to midpoint, t_{IC50} (h)			**γ-BB accumulation time to midpoint, t_{IC50} (h)**		
Characteristic	**Beta**	**95% CI [a]**	**p-value**	**Beta**	**95% CI [a]**	**p-value**
(Intercept)	9.0	0.59, 17	0.039	9.6	1.2, 18	0.031
Treatment						
Control	-	-	-	-	-	-
Pomegranate extract (5.7 mg/mL) vs Control	5.1	-2.7, 13	0.200	6.1	-1.8, 14	0.120
Pomegranate extract (11.4 mg/mL) vs Control	6.3	-1.5, 14	0.100	8.8	0.93, 17	0.032
Pomegranate extract (22.8 mg/mL) vs Control	16	8.4, 24	0.001	19	11, 27	<0.001

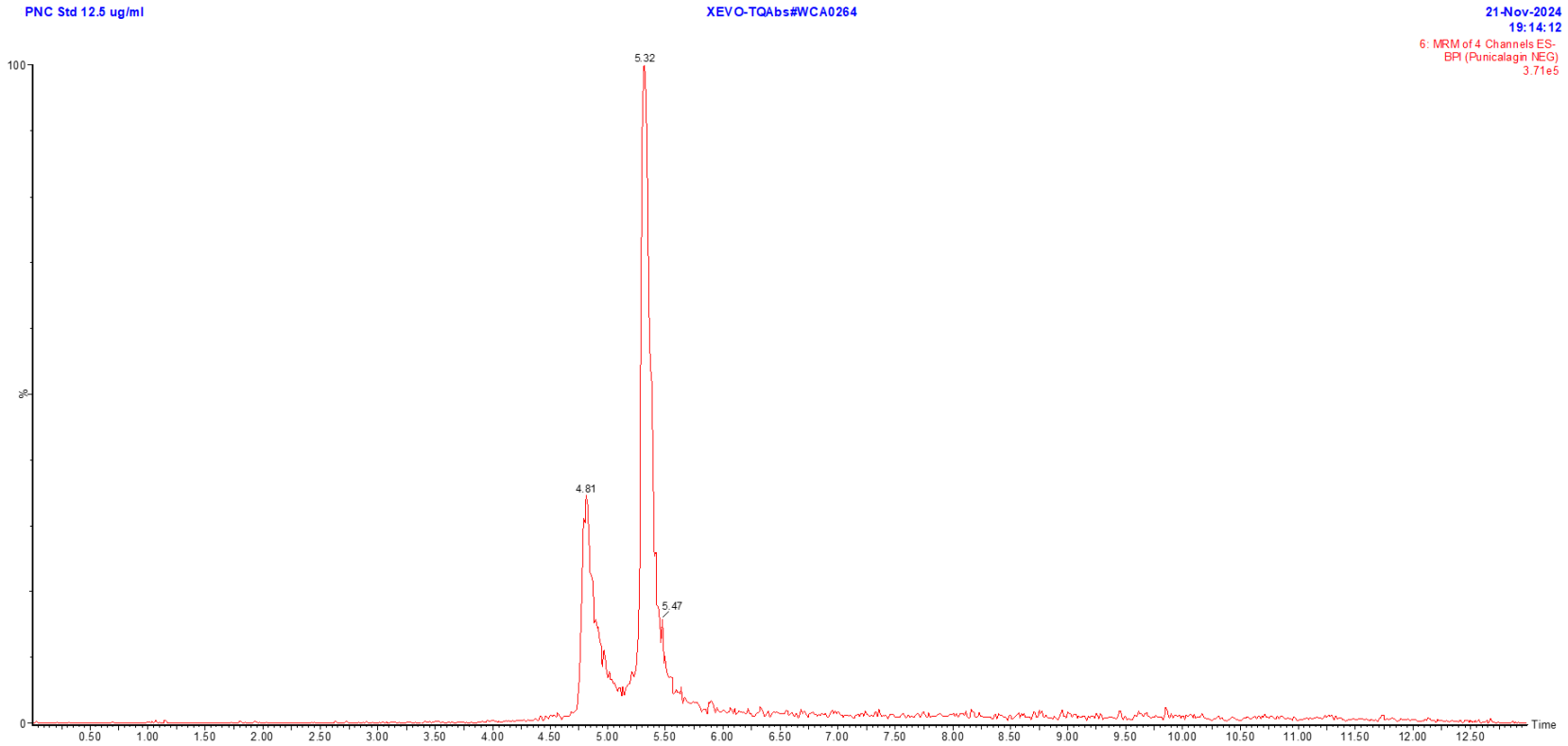
[a] CI = confidence interval.

Appendix 2

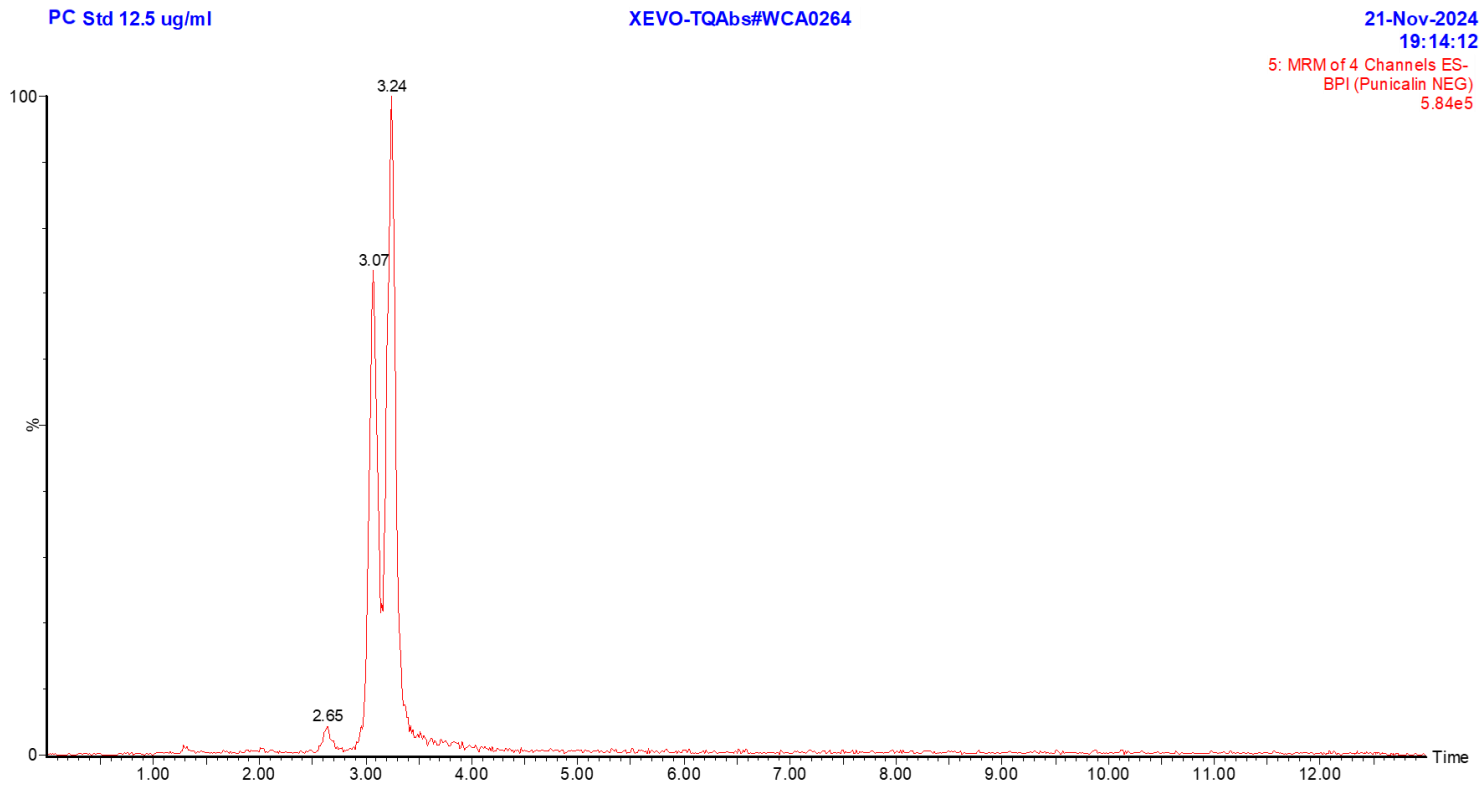
The following supplementary information are with reference to **Chapter 3**.



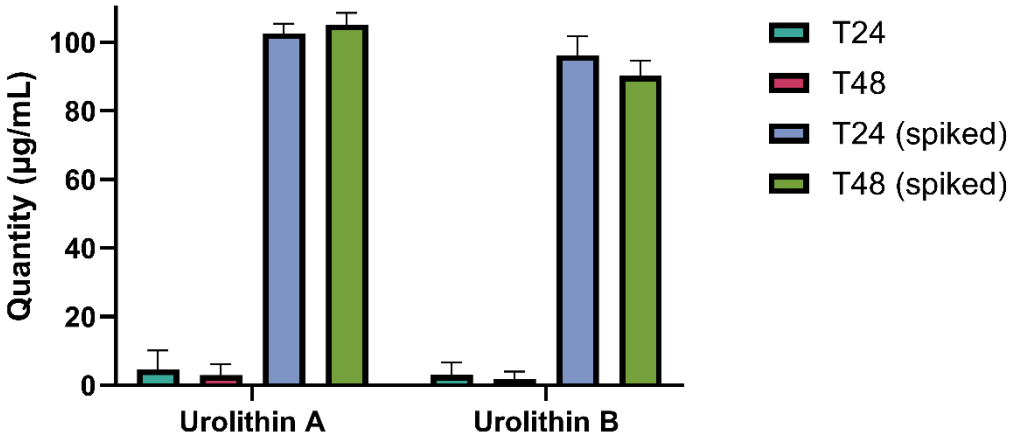
Supplementary Figure S4. Chromatogram of the pomegranate extract. *Chromatogram showing the diode array detection (DAD) data with a range of 210-500 nm (top) and. the Base Peak Intensity (BPI) data (bottom). The pomegranate extract was dissolved in 70% aqueous methanol (v/v) and shaken for one hour before analysis. Polyphenols were identified using the Waters Synapt G2-Si Q-TOF-MS. The mobile phase consisted of distilled water with 1% formic acid (solution A) and acetonitrile with 1% formic acid (solution B). Samples were run through an Acquity HSS T3 C18 1.8 µm, 100 x 2.1 column (Waters). 1 µL of each sample was run through a Luna Omega Polar C18 100A column (100 x 2.1 mm; particle size 1.6 µm) at 35 °C and a flow rate of 400 µL/min. The gradient was 3% solvent B for 5 minutes, after which solvent B was increased to 20% for 4 minutes, and then further increased to 50% for 2 minutes, lastly solvent B was increased to 95% for 1 minute before it was re-equilibrated to 3% for 2 minutes. In total, each sample ran for 15 minutes.*



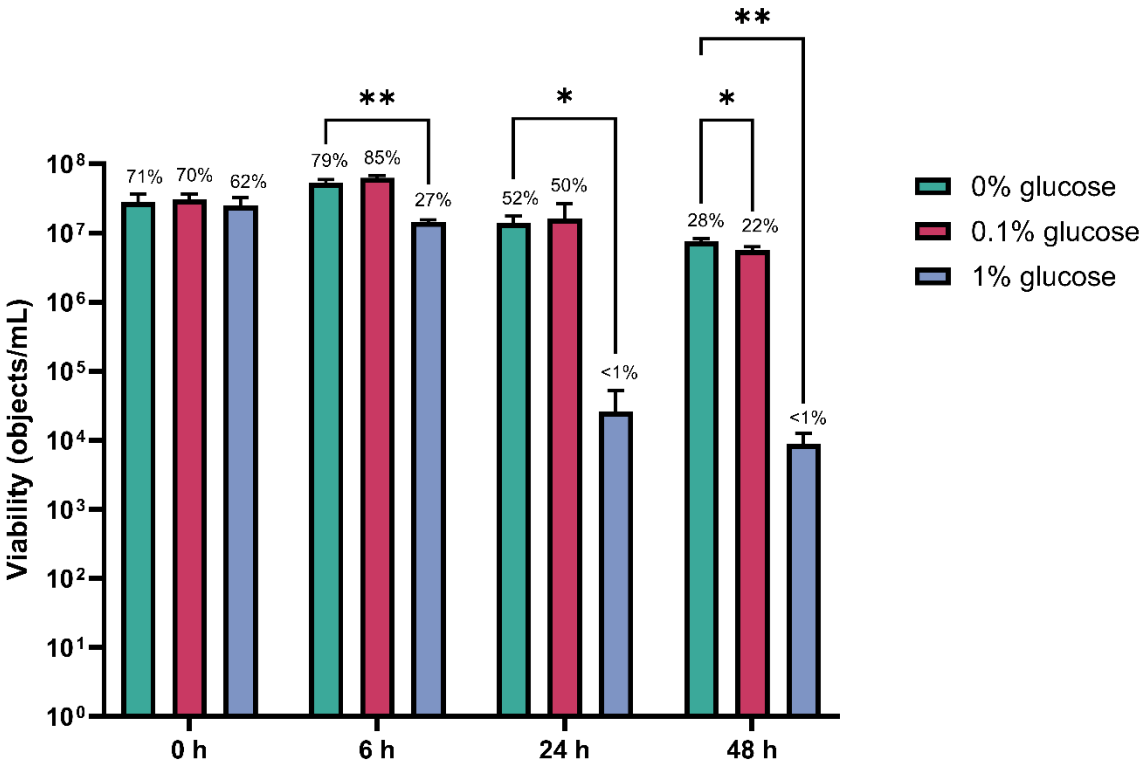
Supplementary Figure S5. LC-MS total ion current (TIC) of a punicalagin standard at 12.5 µg/mL. *Punicalagin was dissolved in 50% aqueous methanol (v/v), filtered, and analysed using LC-MS/MS. Two peaks were identified, corresponding to the alpha and beta isomers of punicalagin. Polyphenols were identified using a Waters TQ Absolute (Wilmslow, UK) system. The mobile phase consisted of distilled water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic (solution B). 1 µL of the punicalagin standard was run through a Luna Omega Polar C18 100A column (100 x 2.1 mm; particle size 1.6 µm) at 35 °C and a flow rate of 400 µL/min. The gradient was 3% eluent B for 5 minutes, after which eluent B was increased to 20% for 4 minutes, and then further increased to 50% for 2 minutes, lastly eluent B was increased to 95% for 1 minute before it was re-equilibrated to 3% for 2 minutes.*



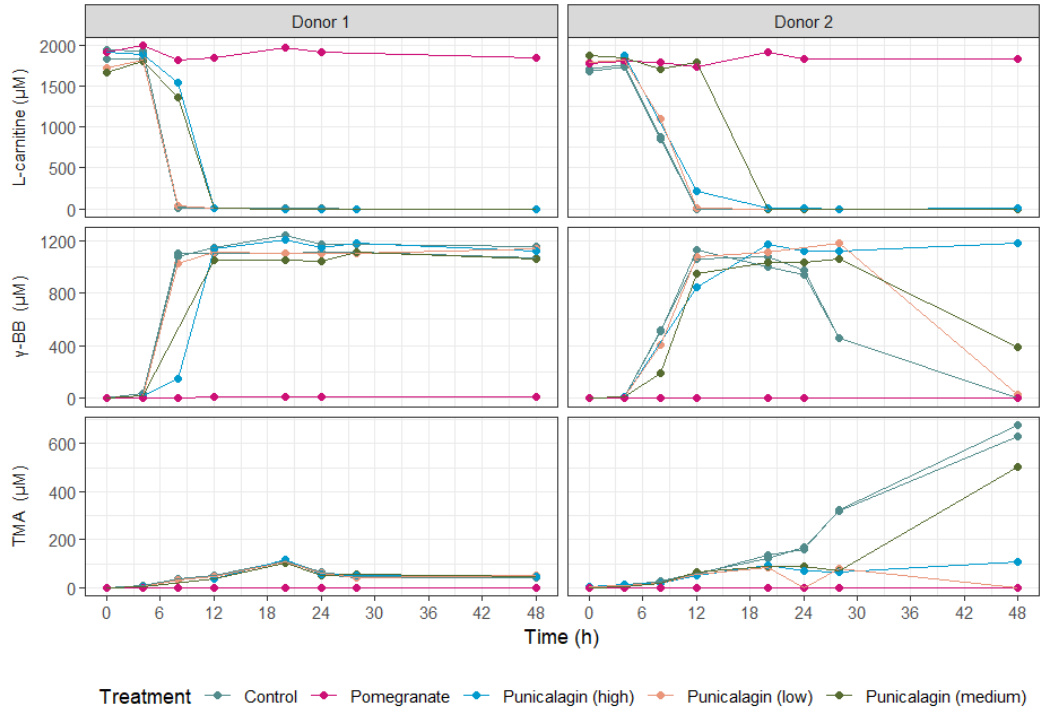
Supplementary Figure S6. LC-MS total ion current (TIC) of a punicalin standard at 12.5 µg/mL. *Punicalin was dissolved in 50% aqueous methanol (v/v), filtered, and analysed using LC-MS/MS. A split peak was identified, indicating the presence of a mixture of alpha and beta isomers. Polyphenols were identified using a Waters TQ Absolute (Wilmslow, UK) system. The mobile phase consisted of distilled water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic (solution B). 1 µL of the punicalagin standard was run through a Luna Omega Polar C18 100A column (100 x 2.1 mm; particle size 1.6 µm) at 35 °C and a flow rate of 400 µL/min. The gradient was 3% eluent B for 5 minutes, after which eluent B was increased to 20% for 4 minutes, and then further increased to 50% for 2 minutes, lastly eluent B was increased to 95% for 1 minute before it was re-equilibrated to 3% for 2 minutes.*



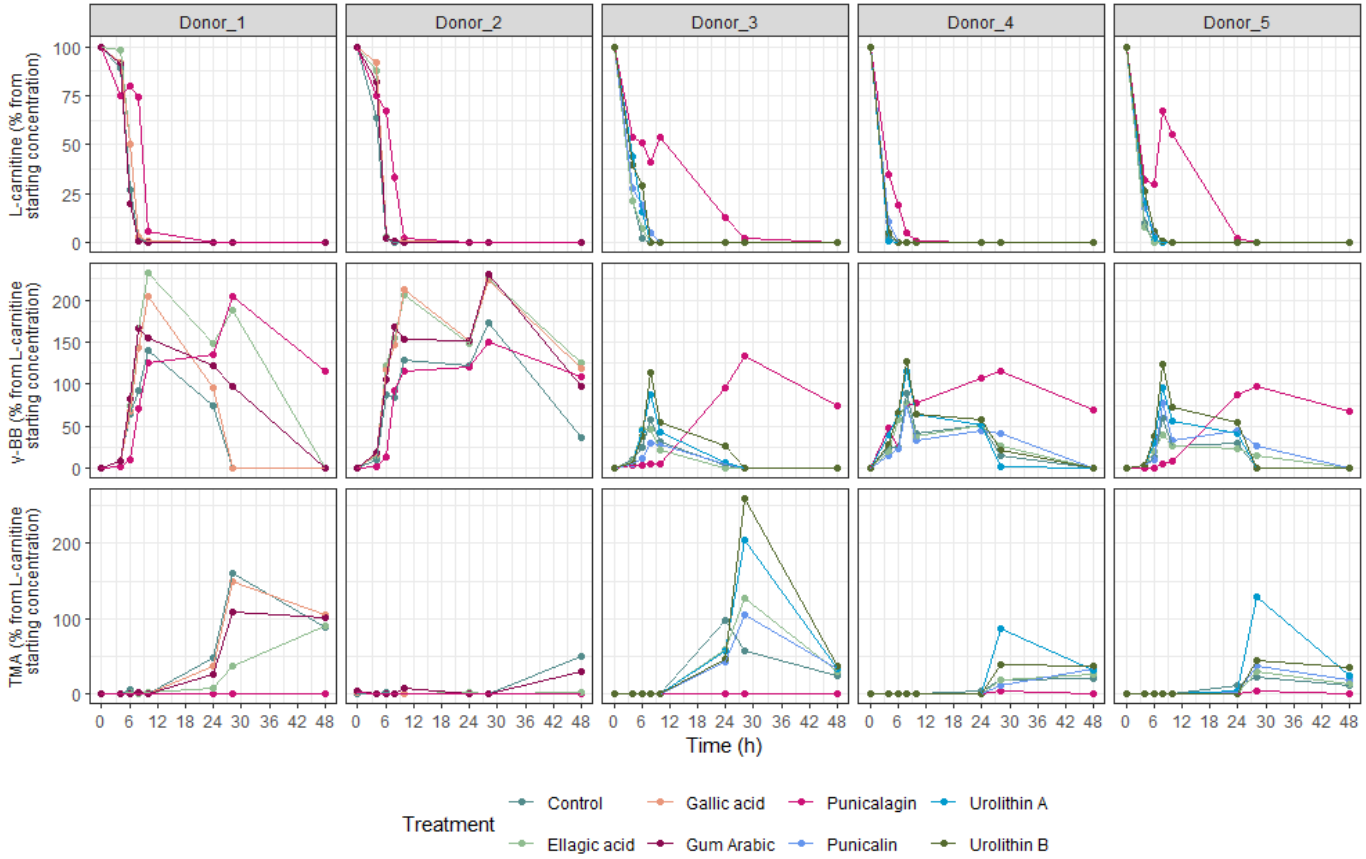
Supplementary Figure S7. Appearance of urolithin A and B from the pomegranate extract in the in vitro batch colon models compared with samples spiked with urolithins at 100 ppm. *Data are presented as mean ± SD from two donors. In vitro colon models were inoculated with the pomegranate extract (22.8 mg/mL), 2 mM L-carnitine, and 1% faecal inoculum from a healthy donor. Samples were collected at 24 and 48 hours and were stored at -80 °C until the LC-MS/MS analysis. Prior to LC-MS/MS analysis, samples were split, and half was treated with urolithin A and B at 100 parts per million (ppm) while the other half remained untreated.*



Supplementary Figure S8. Viability per treatment (objects/mL) on a log_{10} scale and percentage viable cells of total cells within the sample. *Viability was established using the ImageStreamX (Amnis) flow cytometer. Data are presented as mean ± SD of three biological replicates from one donor. Statistical analysis was performed using Two-Way ANOVA with post-hoc pairwise comparisons between each glucose treatment and the control (0% glucose) at each time point (* P < 0.05, ** P < 0.01). High-throughput in vitro colon models were inoculated with 1% faecal inoculum from a healthy donor and different concentrations of glucose (0, 0.1, 1%). Samples were collected at multiple time points over 48 hours. Directly after collection, colon model samples were filtered through a pluriStrainer Mini 40 µm. A Guava® Bacterial Count & Viability Kit (Luminex) dye working solution was prepared, in which samples were diluted 1/10. Prior to analysis, samples were vortexed and incubated at 37 °C for 30 minutes.*



Supplementary Figure S9. Effects of the pomegranate extract and punicalagin at increasing doses on L-carnitine metabolism in vitro, stratified by individual donors. *Average concentrations of L-carnitine, γ-butyrobetaine (γ-BB), and trimethylamine (TMA) are shown per donor over 48 hours. High-throughput in vitro colon models were inoculated with 1% faecal inoculum from a healthy donor, 2 mM L-carnitine, and a pomegranate extract (22.8 mg/mL) or punicalagin (0.4, 0.8, 1.6 mg/mL) or no treatment (control). Samples were collected at multiple time points over 24 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*



Supplementary Figure S10. Effects of the pomegranate polyphenols and urolithins on L-carnitine metabolism in vitro, stratified by individual donors. *Average percentages of L-carnitine, γ-butyrobetaine (γ-BB), and trimethylamine (TMA) from L-carnitine starting concentration are shown per donor over 48 hours, with 3-4 replicates per donor. High-throughput in vitro colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine. Samples were collected at multiple time points over 24 hours and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*

Appendix 3

Pomegranate (Dermogranate®) extract Certificate of Analysis (CoA) and Technical Data Sheet (TDS) obtained from Medinutrex (Sicily, Italy).

CERTIFICATE OF ANALYSIS

medinutrex
mediterranean nutraceutical extracts

Registered office: VIA VINCENZO GIUFFRIDA, 202
95128 CATANIA – ITALY
Production site: CONTRADA GIROTTA, SNC
98051 BARCELLONA POZZO DI GOTTO (ME) – ITALY

VAT NUMBER: 05333560877
www.medinutrex.com

E-mail: info@medinutrex.com

Product:

DERMOGRANATE™

Compilation date: 05.06.2023
Last review: 09.06.2023

Batch n°	2/23
Manufacture date	05.06.2023
Expiry date	05.06.2026
Storage	Keep container unopened in cool, dry place
Country of origin	Italy
Botanical name	Punica granatum L.
Plant part used	fruit

PHYSICAL/CHEMICAL TEST

Analysis	Specification	Results
Total polyphenols (w/w)	> = 20	25.70
Ellagic acid and derivatives (w/w)	> = 10	11.10
Punicalagins (w/w)	> = 7	8.20
Form	Powder	Conform
Color	Brown	Conform
Taste	Typical of pomegranate	Conform
Smell	Typical of pomegranate	Conform
pH	> 3.0	Conform
Solubility	Moderately soluble in water	Conform

Lead (Pb)	< 3ppm	Conform
Arsenic (As)	< 3ppm	Conform
Cadmium (Cd)	< 1ppm	Conform
Mercury (Hg)	< 0.1ppm	Conform
Pesticides	Conform to Reg CE 396/2005	Conform
PAH	Conform to Reg CE 1881/2006	Conform
Benzo(a)pyrene	< 10 µg/kg	Conform
Sum of Benzo(a)pyrene, Benz(a)anthracene, Benzo(b)fluoranthene and Chrysene	< 50 µg/kg	Conform
Residual solvent (ethanol)	< 0.2%	Conform
Total plate count (CFU/g)	< 10000	Conform
Yeast and Mould (CFU/g)	< 100	Conform
Enterobacteriaceae (CFU/g)	< 100	Conform
Escherichia coli	Absent	Conform
Pseudomonas aeruginosa	Absent	Conform
Staphylococcus aureus	Absent	Conform
Salmonella specie	Absent	Conform
Particle size	More than 90% pass 300 µm	Conform

GMO: product no- GMO (Reg. 1829/2003-1830/2003 EC).

Irradiations: this product has not been irradiated.

TECHNICAL DATASHEET

medinutrex
mediterranean nutraceutical extracts

Registered office: VIA VINCENZO GIUFFRIDA, 202
95128 CATANIA – ITALY
Production site: CONTRADA GIROTTA, SNC
98051 BARCELLONA POZZO DI GOTTO (ME) – ITALY

VAT NUMBER: 05333560877
www.medinutrex.com

E-mail: info@medinutrex.com

Product:

DERMOGRANATE™

Compilation date: 05.06.2023
Last review: 09.06.2023

PART 1: GENERAL INFORMATION	
Product name	**Dermogranate**
Botanical name	*Punica granatum* L.
INCI name	Punica Granatum Fruit Extract
CAS number	84961-57-9
Extraction solvent	ethanol / water
DER	up to stated assay
Excipients quantity	arabic gum max 20.0%
Botanical family	Punicaceae
Origin of the raw material used for this product	Italy, Sicily
Growing condition	cultivated
Vegetative period	at maturity
Collection period	September - November
Part of plant used	fruit
Preparation type	dry extract
Particle size	not less than 90% through 300 microns
Intended Use	Raw material for food and cosmetics.
Active substances of the plant	polyphenols, ellagic acid and derivatives, punicalagins
Biological marker	ellagic acid

Radioactivity	< 600 Bq/kg
Contra-indications, warnings	ellagic acid (as a tannin) could cause precipitation of proteins
Eventual particular notes	none
Nutritional values	Carbohydrates: 90-95% - Fat: 0-1% - Protein: 0-1% - Minerals: 3-5% Energy value (Kcal/100 gr): 409 Energy value (KJ/100 gr): 1711
Preservatives	absent
Antioxidants	absent
Storage conditions	store in a well closed container away from moisture and direct sun light
Retest date	three years

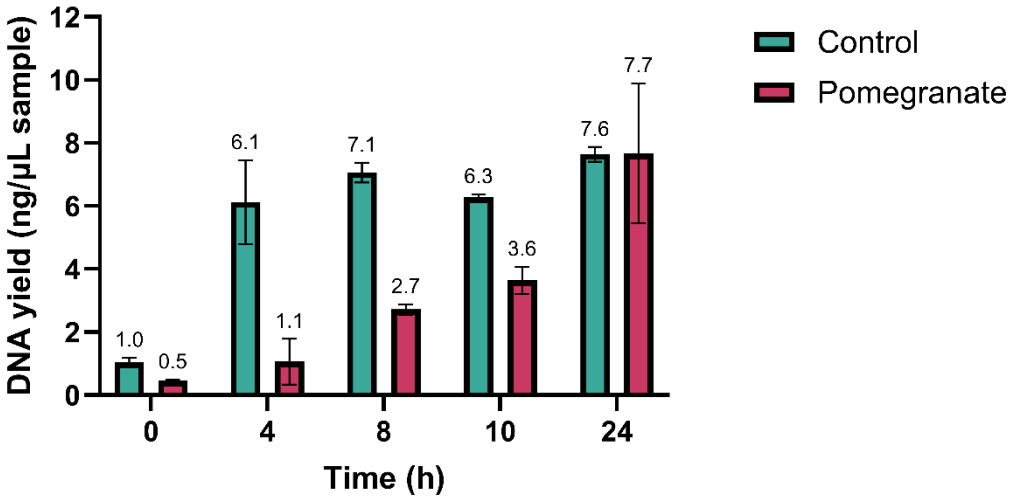
PART 2: TECHNICAL SPECIFICATIONS	
Product	Dermogranate
Code	2/23
Description	hygroscopic powder
Colour	Brown
Odor	characteristic
Taste	characteristic
Assay: Total Polyphenols (met. Spectrophotometric)	>= 20.00 % w/w
Assay: Ellagic acid and derivatives (met. HPLC)	>= 10.00 % w/w
Assay: Punicalagins (met. HPLC)	>= 7.00 % w/w
Bulk density	450 - 650 g/l
Loss on drying	<= 5.0 % w/w
pH	3.0 – 5.0
Hydrosolubility	partially watersoluble
Heavy metals	< 20 ppm (method C Ph. Eur. current edition)
Lead (ref. Reg. (EC) 1881/2006)	< = 3.0 ppm*
Cadmium (ref. Reg. (EC) 1881/2006)	< = 1.0 ppm*

Mercury (rif. Reg. (EC) 1881/2006)	< = 0.1 ppm*
Residual solvents	complies to Ph. Eur. current edition and Directive 2009/32/EC*
Pesticides	complies to Ph. Eur. current edition and Reg. 2005/396/EC and amendments concerning pesticides residues searched (with reference to E/D ratio)* (conformity reported in the certificate of analysis if available and/or upon request)
Aflatoxins	Aflatoxin B1: < 2 ppb* Aflatoxin B1,B2,G1,G2: < 4 ppb*
Benzo(a)pyrene (Reg. (EC) 1881/2006)	< = 10.0 ppb*
Sum of Benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene (Reg. (EC) 1881/2006)	< = 50.0 ppb*
Microbiological quality (Ref. Ph. Eur. current edition depending on the intended use)	
- Bacterial count (TAMC: ref. 5.1.8, cat. B oral use)	< = 10.000 ufc/g
- Yeasts and Moulds (TYMC: ref. 5.1.8, cat. B oral use)	< = 100 ufc/g
- Pathogens (ref. 5.1.8, cat. B oral use)	Salmonella: absent in 25 g* Escherichia coli: absent in 1 g*
Bile-tolerant gram-negative bacteria (ref. 5.1.8, cat. B oral use)	< = 100 ufc/g*

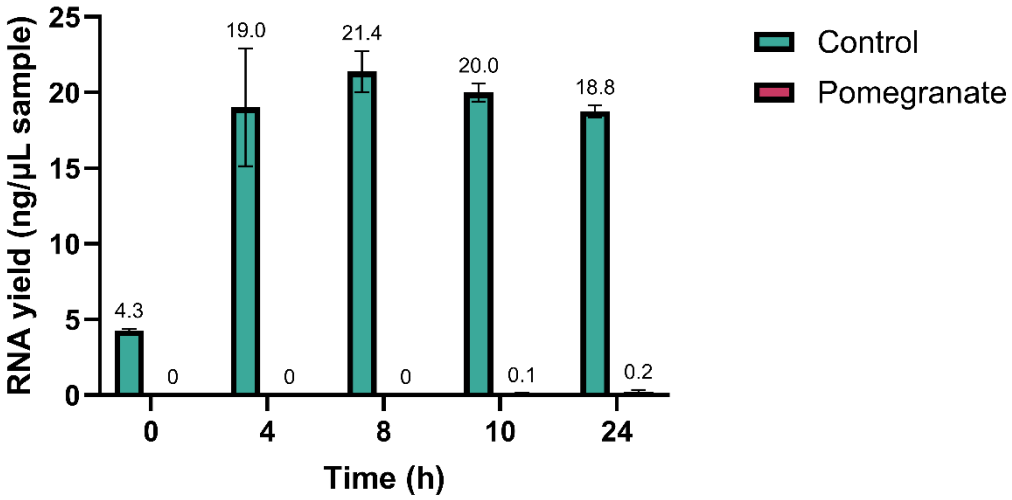
NOTE
GMO: Free from GMO (Reg. (EC) 1829/2003 and 1830/2003) BSE/TSE FREE - GLUTEN FREE ALLERGENS: Free from substances or products causing allergies or intolerances NANOMATERIALS: Free from engineered nanomaterials (Reg. (EU) 1169/2011 Annex II) Melamine Free (Reg. (EU) 594/2012)
THIS PRODUCT AND RAW MATERIAL, FROM WHICH IT IS OBTAINED, SHOULD NOT BE IRRADIATED
THIS PRODUCT IS SUITABLE FOR OVO-LACTO-VEGETARIANS AND VEGANS
The data reported in this Technical Data Sheet (excluding analytical ones) are taken from literature, among which (if applicable) Italian ministerial guidelines for physiological effects and CosIng (European Commission database for information on cosmetic substances). In any case this information will not discharge you from duty to identify and to monitor the product according to the use to which it is intended and the current legislation in the Country of use. (*) analysis performed on basis of specific self-control plan - The drug extract ratio (DER) is intended as drug (final) extract ratio, included any excipient, as per Ph. Eur. current edition, monograph n. 52300 "Monographs on herbal drug extracts" - Herbal extracts with assay are intended as standardised extract, unless otherwise specified - In standardised extract the excipient is added for adjust the content of constituent(s) (assay), in extracts based on DER to guarantee the final DER

Appendix 4

The following supplementary information are with reference to **Chapter 4**.



Supplementary Figure S11. The DNA yield (ng/µL sample) in pomegranate-treated and control colon model samples at 0, 4, 8, 10, and 24 hours of fermentation. *Data are shown as mean ± SD of two donors. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine, with or without a pomegranate extract (22.8 mg/mL). Collected samples were directly stored at -80 °C until DNA extractions were carried out using the RNeasy PowerFecal Pro Kit (Qiagen). DNA yield was estimated using a Qubit™ dsDNA High Sensitivity Assay Kit.*



Supplementary Figure S12. The RNA yield (ng/µL sample) in pomegranate-treated and control colon model samples at 0, 4, 8, 10, and 24 hours of fermentation. *Data are shown as mean ± SD of two donors. In vitro batch colon models were inoculated with 1% faecal inoculum from a healthy donor and 2 mM L-carnitine, with or without a pomegranate extract (22.8 mg/mL).* Collected samples *were first resuspended in RNAlater and left at 4 °C for 24 hours before being stored at -80 °C until* RNA *extractions were carried out using the RNeasy PowerFecal Pro Kit (Qiagen). RNA yield* was *estimated using a Qubit™ RNA HS High Sensitivity Assay Kit, respectively.*

Appendix 5

TESSA Study Protocol, version 7 (15 November 2024).

Quadram Institute Science Health Food Innovation

NIHR | National Institute for Health Research

NHS Norfolk and Norwich University Hospitals NHS Foundation Trust

TMAO-reducing Effects of a pomegranate Supplement Simultaneously Administered with L-carnitine

Quadram Institute Bioscience Chief Investigator:

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Funder:
Wellcome Trust

None of the study team members declare any conflicts of interest.

This document contains confidential information that must not be disclosed to anyone other than the Sponsor, the Investigator Team, the HRA, the host organisation, and members of the Research Ethics Committee, unless authorised to do so.

wellcome

PROTOCOL SIGNATURE PAGE

Study Title: The trimethylamine N-oxide (TMAO)-reducing effects of a pomegranate supplement simultaneously administered with L-carnitine (TESSA)

Version 7; 15 November 2024

Sponsor's Approval:

This protocol has been approved by The Quadram Institute Bioscience Human Research Governance Committee (HRGC)

Signature 

Name Dr Antonietta Hayhoe

Role Head of Human Study Team

Date 17 February 2025

I have fully discussed the objectives of this trial and the contents of this protocol with the Sponsor's representative. I understand that the information in this protocol is confidential and should not be disclosed other than to those directly involved in the execution or ethical review of the trial.

I agree to conduct this trial according to this protocol and to comply with its requirements, subject to ethical and safety considerations and guidelines, and to conduct the trial in accordance with International Conference on Harmonisation (ICH) guidelines on Good Clinical Practices (GCP) and with the applicable regulatory requirements.

Chief Investigator name and professional address:

Paul Antony Kroon

Quadram Institute Bioscience, Norwich Research Park, Norwich, Norfolk, NR4 7UQ, UK.

Signature: 

Date:

15 November 2024

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Executive Summary

High plasma levels of trimethylamine N-oxide (TMAO) are positively associated with incidence of major adverse cardiovascular events (MACE), which include heart failure and risk factors for cardiovascular disease (CVD), such as atherosclerosis, hypertension, arrhythmia, and coronary artery disease [1]. Moreover, high plasma levels of TMAO are also associated with higher all-cause mortality [2]. Elevated levels of TMAO have been shown to promote endothelial dysfunction, increase platelet reactivity, adversely affect lipid metabolism, and cause inflammatory responses, all of which underlie the significance of this compound in the progression of atherosclerosis. L-Carnitine is a major dietary precursor of TMAO, which is metabolised by the human gut microbiota to γ-butyrobetaine (γ-BB) and finally to trimethylamine (TMA)[3]. In the liver, TMA is converted into TMAO. Currently, no effective, low-risk treatments are available to reduce TMA and, subsequently, TMAO levels. Therefore, we screened several polyphenols and extracts for their effectiveness in reducing TMA production in an *in vitro* model of the human colon. Using this model, we discovered that a polyphenol-rich pomegranate extract can significantly delay and reduce TMA production from L-carnitine in a dose-dependent manner. Following these *in vitro* observations, we propose this human trial to investigate the effects of a pomegranate extract (PE) on plasma TMAO accumulation from an oral carnitine response test (OCRT) in healthy adults. The central hypothesis is that PE will inhibit the appearance of plasma TMAO from the OCRT compared with the placebo control.

This is a two-phased intervention trial. We will aim to recruit 40 participants onto the TMAO Status Phase (Phase I), during which participants undergo an OCRT to assess their TMAO response status. Those who are deemed high TMAO producers and who also meet the eligibility criteria will be invited to the Intervention Phase (Phase II). We aim to have 16 participants complete Phase II. Phase I will start prior to phase II and will run in parallel until 16 participants have completed Phase II. Phase II is an 18-day double-blind, randomised, placebo-controlled, crossover study and

will consist of two 48-hour pharmacokinetic studies, separated by a 10-day washout period. A two-day run-in period precedes each pharmacokinetic study. In Phase II, participants will be randomly allocated to one of the two interventions for the first pharmacokinetic study and, after the washout period, they will crossover onto the other intervention for their second pharmacokinetic study. One intervention involves an OCRT (1.5 g L-carnitine) co-administered with a pomegranate extract (1.6 g) whereas the other intervention involves an OCRT (1.5 g L-carnitine) co-administered with microcrystalline cellulose as the placebo control.

The primary aim of this study is to compare the plasma TMAO response (area under the curve, AUC) from L-carnitine co-administered with PE versus L-carnitine co-administered with a placebo control. The secondary aims are to estimate the effects of PE on the concentration of L-carnitine and L-carnitine metabolites (e.g., γ-BB, TMA) in the blood plasma, urine and faeces. PE metabolites will be quantified in urine collected over a 48 hour period following each intervention. Faecal samples will be collected at baseline and 48 hours of each pharmacokinetic study to investigate changes in gut microbiome profile induced by PE. Participants will also complete a food frequency questionnaire (FFQ), to estimate if a greater TMAO response is correlated with a higher habitual consumption of L-carnitine-rich foods. Omnivorous (animal-based) diets compared to vegan/vegetarian diets tend to be higher in protein quantity and quality. Low protein diets have shown to impact the function of peripheral mononuclear cells (PBMCs). PBMC's will be isolated from blood, their RNA sequenced, and their phagocytic activity will be assessed and compared to PBMC's collected from those following a vegan/vegetarian.

This study is funded by the Wellcome Trust through the EDESIA: Plants, Food, and Health PhD programme. This study aligns with the central aims of the EDESIA programme by serving to expand our current understanding of the impact of plant-based foods on human health and the gut microbiome. This study is innovative in that it is the first to gain mechanistic understanding of how a pomegranate extract may reduce TMAO production from L-carnitine supplements in human subjects.

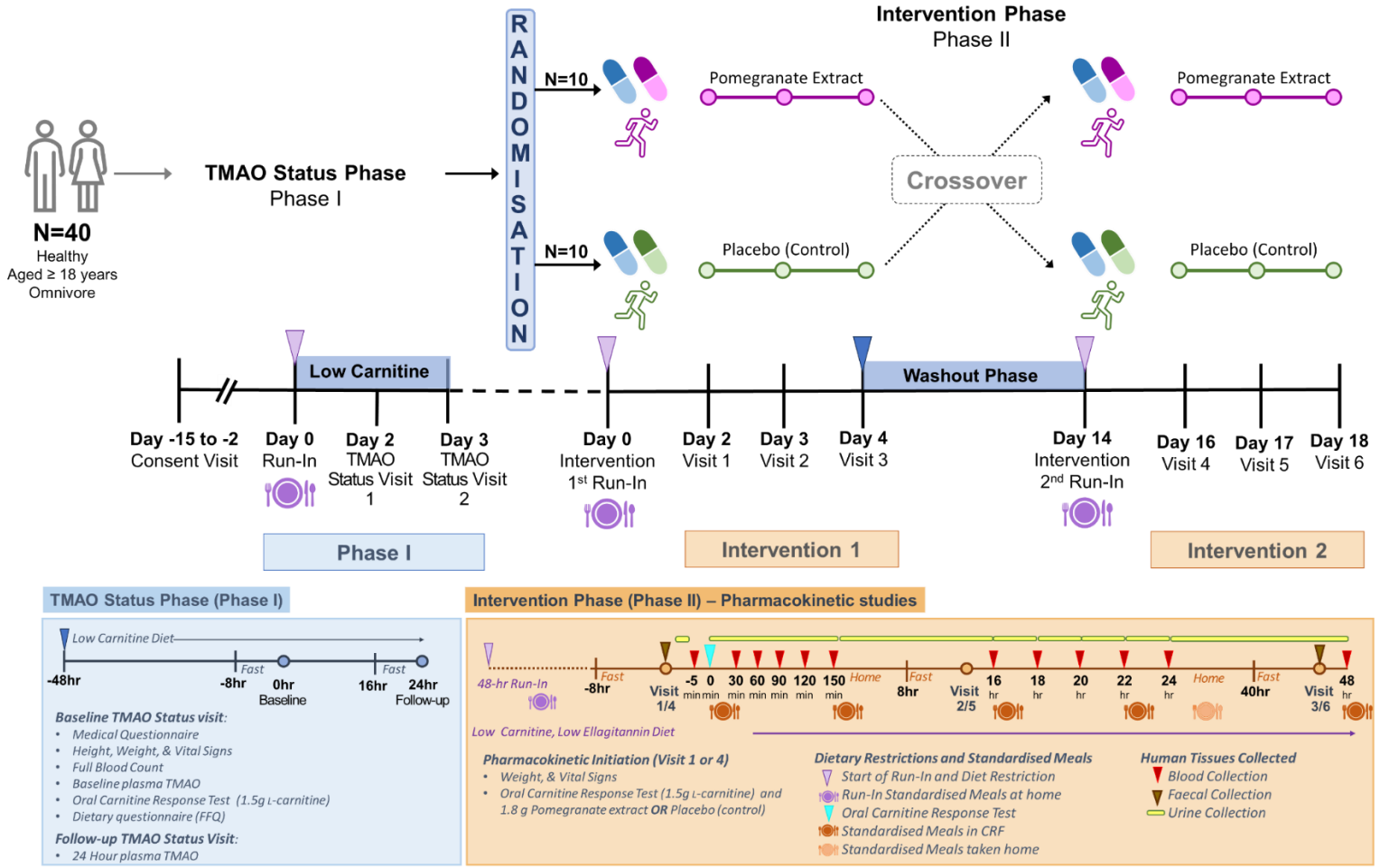

Figure 1. Study design schematic of the TESSA study which is a two-phased study, including an 18-day double blinded randomised crossover trial consisting of two 48-hour run-in and two 48-hour pharmacokinetic studies.

Table 1. Study details.

Trial Title	The trimethylamine N-oxide (TMAO)-reducing effects of a pomegranate supplement simultaneously administered with L-carnitine	
Internal ref. no. (or short title)	TESSA	
Clinical Phase	Not relevant (non-CTIMP)	
Trial Design	A double-blind, randomised, placebo-controlled, crossover trial	
Trial Participants	Healthy adults, aged 18≥ years, habitually consuming an omnivorous diet.	
Planned Sample Size	16 participants to complete the Intervention Phase	
Dietary intervention duration	18 days involving two dietary interventions. Each intervention consists of a 2-day run-in followed by a 2-day pharmacokinetic study starting with the administration of the dietary intervention (L-carnitine with a pomegranate extract or placebo control). The two interventions are separated by a 10-day washout period.	
Planned Trial Period	36 months (3 years)	
	Aims	**Outcome Measures**
Primary	To estimate the effects of a pomegranate extract (PE), simultaneously administered with L-carnitine, on the area under the curve (AUC) of circulating TMAO over 48 hours after administration.	Area Under the Curve (AUC), generated from pharmacokinetic curves for TMAO concentrations in blood plasma.
Secondary	To estimate the pharmacokinetic effects of PE, simultaneously administered with L-carnitine, on its metabolism in blood plasma samples.	AUC, generated from pharmacokinetic curves for L-carnitine, γ-BB, and TMA in blood plasma, as well as TMAO C_{MAX}, TMAO $T_{1/2}$, and TMAO T_{MAX}.
	To estimate the pharmacokinetic effects of PE simultaneously administered with L-carnitine on the excretion of L-carnitine and its metabolites.	AUC, generated from TMA, TMAO, γ-BB and L-carnitine in urine and faecal samples.
	To assess the correlation between L-carnitine metabolism and the metabolism of PE polyphenols (punicalagin, ellagic acid, urolithins), quantified in urine.	Quantify the main PE polyphenols and their gut microbiota metabolites (punicalagin, ellagic acid, urolithins) in urine at the different time points.
	To compare the change in gut microbiome profiles between the	Determine the changes in indices of diversity and taxonomic abundances (from

	PE and placebo intervention.	shotgun metagenomics data) following administration with the PE and placebo intervention.
	To measure the abundance and expression of L-carnitine-metabolising (bacterial) genes after intervention with PE, compared to intervention with a placebo control.	Compare absolute abundance of L-carnitine metabolising genes and their transcripts in faecal samples using specific qPCR and qRT-PCR assays.
	To assess the correlation between TMAO production and habitual consumption of L-carnitine-rich foods.	Correlate the FFQ scores for L-carnitine intake with the increase in plasma TMAO concentration from baseline to 24 hours.
	To understand the relationship of omnivore diets (protein quantity) on PBMCs and macrophage activity	Correlate FFQ estimates for protein intake with PBMC's and macrophage activity.
Description of the Dietary Intervention	For the experimental intervention, participants consume capsules containing 1.5 g L-carnitine and 1.6 g of a pomegranate extract along with their standardised control meal. For the placebo (control) intervention, participants consume capsules containing 1.5 g L-carnitine and microcrystalline cellulose (MCC) along with their standardised control meal.	

1. Scientific background

1.1 Gut microbiota, TMAO, and cardiometabolic health

The human gastro-intestinal tract is home to complex bacterial communities called the gut microbiota. Gut microbial stability (homeostasis) is essential for maintaining human health [4, 5], whilst gut microbial instability (dysbiosis) contributes to the development of a variety of metabolic diseases, including cardiovascular disease (CVD) [6]. Indeed, gut microbiota dysbiosis has been recognised as an important contributory factor in the development of atherosclerosis and hypertension, two major CVD risk factors [7].

A newly recognised metabolite that is linked with CVD is trimethylamine N-oxide (TMAO), which needs the gut microbiota to be generated. TMAO has been linked to various cardiometabolic diseases and all-cause mortality risk [1, 2, 8]. For instance, the combined data from 36 observational studies showed that high levels of TMAO were associated with a 74% greater risk for a major adverse cardiovascular event (MACE) and data from 12 studies showed an association with a 50% greater risk of cardiovascular diseases (CVD) [1]. Elevated levels of TMAO in mechanistic studies have been shown to promote endothelial dysfunction [9], increase platelet reactivity and consequently the propensity for thrombosis [10], adversely affect lipid metabolism [11] and inflammatory responses [12], all of which underlies the significance of this compound in the progression of atherosclerosis. Thus, the gut microbiota has been put forward as a key player in the pathogenesis of TMAO-induced atherosclerosis and, therefore could be a potential target in the prevention and treatment of CVD [2].

1.2 The formation of TMAO

TMAO is formed in the liver by flavin-containing monooxygenases (FMOs) from a gut microbiota-derived metabolite called trimethylamine (TMA) [13]. TMA is generated from dietary precursors including L-carnitine, choline, phosphatidylcholine and betaine solely by gut microbial enzymes that reside in the colon. Among these, L-carnitine is a major contributor to microbial TMA production, and is mostly found in meat, fish and dairy products but also in dietary supplements and some energy drinks [14]. L-Carnitine is first metabolised by the human gut microbiota to γ-butyrobetaine (γ-BB) and finally to trimethylamine (TMA) [3]. In the liver, TMA is converted to TMAO by FMOs, particularly FMO3 (Figure 2).

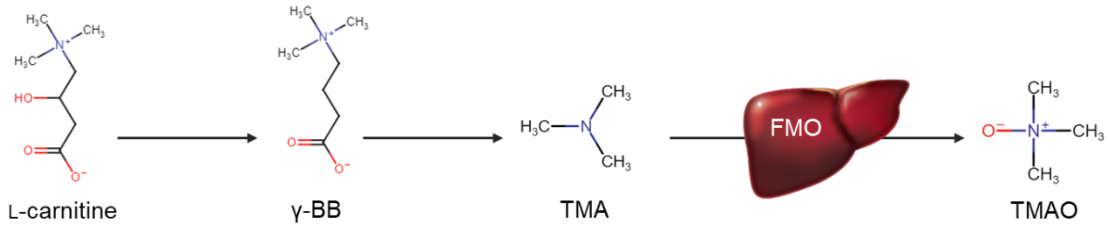

Figure 2. The metabolic pathway of L-carnitine to TMAO. Gut microbiota are responsible for the metabolism of L-carnitine to γ-butyrobetaine (γ-BB) and trimethylamine (TMA). Flavin-dependent monooxygenases (FMOs) are responsible for the conversion of TMA to trimethylamine N-oxide (TMAO).

1.3 Treatments to inhibit microbial TMA production *in vitro*

Interventions that have been suggested to reduce the microbial production of TMA include broad-spectrum antibiotics [15] and the choline analogue 3,3-dimethyl-1-butanol (DMB) [16]. However, there are significant risks associated with the long-term use of antibiotics including the loss of the beneficial gut microbiota and selecting for antimicrobial resistance, and the effectiveness of broad-spectrum antibiotics wears off after 6 months [15]. Regarding DMB, its suppressing effects could not be replicated in later studies [17]. Therefore, there is some interest in exploring low-risk treatments and their mechanism of action to reduce TMA production and TMAO exposure.

There are several reports providing evidence that dietary polyphenols and polyphenol-rich foods can reduce TMA production and host TMAO levels [18]. Polyphenols form an abundant group of phytochemicals that are found in many food and medicinal plants. Polyphenols have been shown to modulate enzymatic activity and to affect cell receptors and transporters [19], and such effects may plausibly underlie the ability of polyphenols to impact on TMA production in the colon.

The Kroon group at QIB has recently screened a broad range of different polyphenols including various flavonoids, phenolics and stilbenoids and some polyphenol-rich plant extracts for their ability to inhibit gut microbial production of TMA from the precursors L-carnitine and choline using an *in vitro* model of the human colon [17]. The standout effective treatment was a pomegranate extract (PE) which substantially delayed the metabolism of L-carnitine into the intermediate metabolite, γ-BB, in a dose-dependent manner. Significantly higher L-carnitine concentrations were observed at 10 and 12 hours after inoculation for all PE doses ($p < 0.001$) and at 20 hours for the highest dose ($p < 0.01$) (Figure 3A). The appearance of γ-BB reflected the metabolism of L-carnitine, with significantly lower concentrations at 10, 12, and, for the high PE dose, at 20 hours after inoculation (Figure 3B). At 28 hours after inoculation, the high PE dose significantly inhibited the TMA production by 186.1 μM at 28 hours after inoculation ([95CI 6.8 to 365.4], $p = 0.0398$). At 48 hours after inoculation

the inhibition of TMA production was statistically significant ($p < 0.01$) for all three PE doses (Figure 3C).

We observed large inter-individual variation in TMA production from L-carnitine (Figure 4). A previous human study in which participants were subjected to an oral L-carnitine response test (OCRT) reported that omnivores show a >20-fold greater d3-TMAO response from d3-L-carnitine than vegetarian participants [20]. Another human study involving an OCRT also reported high inter-individual variation in plasma TMAO concentrations [21]. The large variations in TMAO productivity can be explained by the highly interpersonal gut microbiota of the participants. It is likely that omnivores are exposed to higher levels of L-carnitine (from meat) and therefore have a gut microbiome that is more efficient in the production of TMA, causing a greater TMAO response.

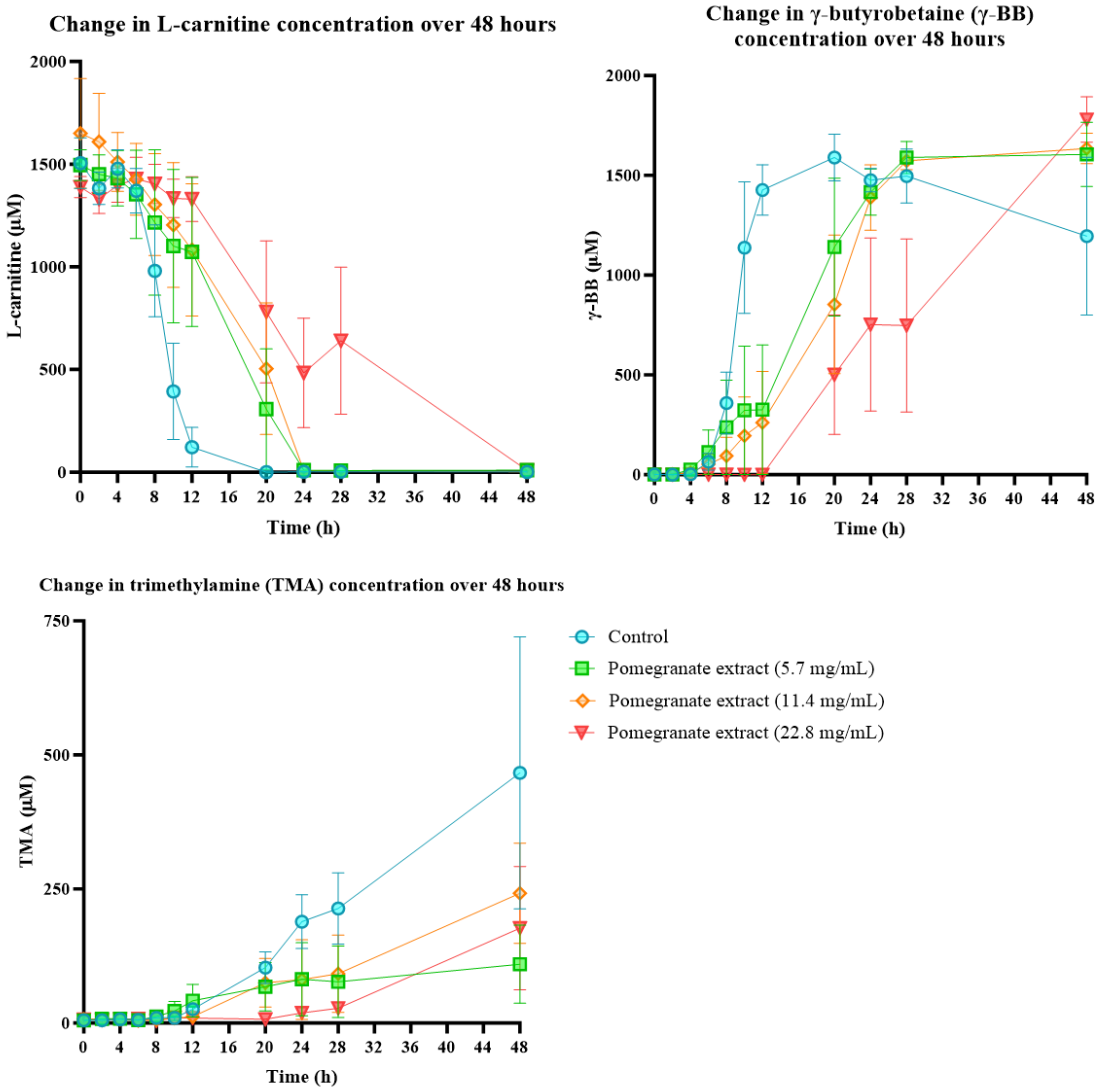

Figure 3. The dose-dependent effects of the pomegranate extract (PE) in an *in vitro* model of the human colon, on (A) L-carnitine metabolism to (B) γ-butyrobetaine (γ-BB), and finally (C) trimethylamine (TMA). Data presented as mean ± SEM with n = 4 donors.

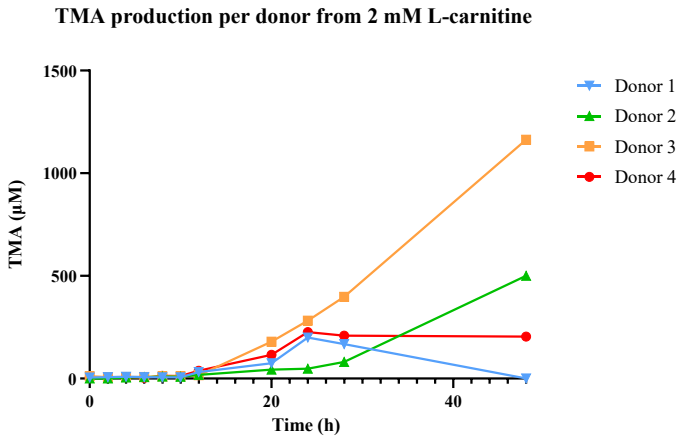

Figure 4. Substantial inter-individual variation between donors in the production of TMA from 2 mM L-carnitine under untreated conditions. The TMA production over 48 hours is shown per donor.

1.4 Polyphenols inhibit microbial TMAO production *in vivo*

To date, there are no published human intervention trials that have investigated the effects of the consumption of polyphenol-rich pomegranate products on TMAO production. However, evidence from a mice study showed that resveratrol, a stilbene polyphenol, could reduce TMAO-induced atherosclerosis in mice [22]. A stilbene-rich grape extract was also studied in a human intervention study. Participants consumed the grape extract daily for 28 days, resulting in significantly reduced plasma TMAO levels [23].

1.5 The bioavailability of polyphenols

Polyphenols differ in bioavailability [19]. Some polyphenols, such as phenolic acids and isoflavones, can be made bioavailable by intestinal enzymes, while other polyphenols, including anthocyanins and flavanones, are very poorly bioavailable and reach the colonic microbiota [24]. Moreover, the bioavailability of polyphenols differs between individuals. For instance, Larrosa *et al.* (2012) reported the large interindividual variation in the bioavailability of the pomegranate polyphenols, ellagitannins and ellagic acid [25]. Hence, it is hypothesised that the reduced plasma TMAO levels that were observed in the *in vivo* studies are a result of the inhibiting effect of polyphenols on the TMA production by colonic microbiota.

1.6 Gaining insights into peripheral blood mononuclear cells in omnivore diets

Habitual diets have an important role in dictating the quantity of protein consumed. Specifically in Western adult diets, there is a proclivity of high protein to be associated with meat-eaters and protein content decrease with lacto-ovo-vegetarian to vegan diets [26]. From 1800 to the 1950s, meat consumption steadily increased in the Western diet [27], but to feed

the steadily increasing human population, developed economies are promoting a shift towards plant-based proteins [28]. There is conflicting evidence on the health outcomes of consuming a low or high-protein diet. A recent animal study at QIB has shown that a low protein diet has protective effects and could lead to beneficial effects on immune responses and overall health [29].

Peripheral Blood Mononuclear Cells (PBMCs) are a type of white blood cells that play an important role in the immune system. Vegan and vegetarian diets compared to omnivore diets tend to be less protein-dense and may lack certain essential amino acids. Moreover, vegan/vegetarian diets compared to omnivore diet may be low in certain nutrients, specifically iron. Plant-based iron (non-haem iron) is less readily absorbed than animal-based iron (haem iron) [30]. Iron and macrophages share a crucial interdependence. Iron regulates macrophage function whilst macrophages facilitate iron recycling. However, the impact of dietary intake of protein quantity in omnivore diet on innate immunity in humans is largely unknown.

1.7 Summary

Altogether, a small body of *in vivo* evidence can substantiate the *in vitro* findings and support the hypothesis that polyphenol-rich products inhibit microbial TMA formation from L-carnitine and choline. However, to date no human studies have tested the effect of polyphenols or polyphenol-rich products on TMAO production after an OCRT. Furthermore, the effects of many other polyphenols, such as ellagitannins, remains unknown. Hence, in this randomised, placebo-controlled, crossover, pharmacokinetics study, we will investigate the effect of a polyphenol-rich (ellagitannins and ellagic acid) PE on plasma TMAO levels. Given the evidence from the available literature and our own *in vitro* data supporting the notion that PE can inhibit L-carnitine metabolism, we will estimate the ability of PE to reduce the microbial conversion of L-carnitine to γ-BB and then to TMA and TMAO in healthy, omnivorous participants.

2. Hypothesis, Aims and Objectives

2.1 Hypothesis

The main hypothesis addressed by this proof-of-concept study is that the co-administration of 1.6 g PE with 1.5 g L-carnitine will significantly reduce plasma TMAO compared with the co-administration of the placebo control with L-carnitine. We further hypothesise that supplementation with L-carnitine and PE will alter the gut microbiota composition compared to the L-carnitine with a placebo control, and specifically change the relative abundance of L-carnitine metabolising genes within the gut microbiome.

2.2 Primary aim

To estimate the effect of PE, simultaneously administered with L-carnitine, on the area under the curve (AUC) of TMAO in blood plasma over 48 hours after administration in healthy adults.

2.3 Secondary aims

- To estimate the pharmacokinetic effects of PE when simultaneously administered with L-carnitine on its metabolites (L-carnitine, γ-BB, and TMA) in blood plasma samples.
- To estimate the pharmacokinetic effects of PE when simultaneously administered with L-carnitine on the excretion of L-carnitine and its metabolites (γ-BB, TMA, TMAO) in urine and faecal samples.
- To estimate the correlation between L-carnitine metabolism and the metabolism of PE polyphenols (punicalagin, ellagic acid, urolithins), quantified in urine.
- To estimate the effect of L-carnitine supplemented with PE on the gut microbiome profile (species diversity and abundance) compared with L-carnitine and a placebo control.
- To measure the abundance and expression of L-carnitine-metabolising (bacterial) genes after the co-supplementation of L-carnitine with PE, compared to co-supplementation with a placebo control.
- To assess the correlation between the TMAO response to the OCRT and the habitual consumption (during the past month) of L-carnitine-rich foods, as estimated by a food frequency questionnaire (FFQ).
- To understand the relationship of protein content in omnivore diets on PBMCs and macrophage activity

2.4 Outcome measures

This study has been designed in order to provide information on the ability of PE to reduce the conversion of L-carnitine to TMAO in healthy, omnivorous participants. Additionally, the study will provide an understanding of the gut microbial changes induced by supplementation with L-carnitine as well as PE.

Table 2. Objectives, outcome measures and sample description of the TESSA study.

Objectives	Outcome Measures	Sample Description
Primary Objective To compare the effect of a single dose of 1.6 g PE versus a placebo control on plasma TMAO levels from 1.5 g L-carnitine in healthy adults.	The area under the curve (AUC), generated from pharmacokinetic curves for TMAO concentrations in blood plasma.	For each pharmacokinetic study, blood plasma will be taken (cannula) at 0, 0.5, 1, 1.5, 2, 2.5, 16, 18, 20, 22, 24, and 48 hours after the ingestion of the capsules.
Secondary Objectives To compare the effect of a single dose of 1.6 g PE versus a placebo control on L-carnitine induced plasma TMAO pharmacokinetics.	The maximum plasma concentration (C_{max}), half-life ($t_{1/2}$), time to reach the C_{max} (T_{max}), generated from pharmacokinetic curves for TMAO levels in plasma.	Blood plasma at 0, 0.5, 1, 1.5, 2, 2.5, 16, 18, 20, 22, 24, and 48 hours after the ingestion of the capsules.
To measure the pharmacokinetic changes in L-carnitine and its metabolites in blood plasma samples.	C_{max}, $t_{1/2}$, AUC, T_{max} generated from pharmacokinetic curves for L-carnitine, γ-BB, TMA in blood plasma.	Urine will be collected over 0-2.5, 16-18, 20-22, 22-24, 24-48 hours after the ingestion of the capsules.
To quantify the excretion of L-carnitine and its metabolites.	Quantify TMA, TMAO, γ-BB, L-carnitine in urine and faecal samples.	Faecal samples will be collected at baseline and between 24-48 hours after the supplementation.
To assess the correlation between L-carnitine metabolism and the metabolism of PE polyphenols (punicalagin, ellagic acid, urolithins).	Quantify the main PE polyphenols and their gut microbiota metabolites (punicalagin, ellagic acid, urolithins) in urine.	Urine will be collected at baseline, 0-2.5, 2.5-16, 16-18, 20-22, 22-24, and 24-48 hours.
To estimate how co-supplementation of L-carnitine with PE affects the gut microbiota composition compared to co-supplementation with a placebo control.	Compare shotgun metagenomics data before and after supplementation as well as between PE and placebo, to determine changes in indices of diversity and in taxonomic abundances.	Bacterial DNA will be sequenced from faecal samples collected at baseline and between 24-48 hours after the start of each of the two interventions.
To estimate how co-supplementation of L-carnitine with PE affects the abundance of L-carnitine-metabolising genes and their expression compared to co-supplementation with a placebo control.	Compare absolute abundance of L-carnitine metabolising genes and their transcripts in faecal samples using specific qPCR and RT-qPCR assays.	Bacterial DNA and RNA will be extracted from faecal samples collected at baseline and between 24-48 hours after each of the two interventions.
To assess if there is a correlation between greater TMAO production and greater habitual consumption of L-carnitine-rich foods.	Correlate the dietary intake of L-carnitine, measured by the FFQ, with the increase in plasma TMAO concentration from baseline to 24 hours.	All participants in Phase I will complete an FFQ about their dietary intake of the 30 days prior to Phase I.
To estimate the effect of omnivore diets (protein quantity) on PBMCs and macrophage activity	Correlate FFQ estimates for protein intake with PBMC's and macrophage activity	Phase I FFQ dietary protein intake in the 30 days prior to Phase I.

3. Study design

3.1 Study Team

The study will be conducted and managed at the Quadram Institute (QI), led by Dr Paul Kroon (Chief Investigator), who is a group leader in the Food, Microbiome and Health Programme at the Quadram Institute Bioscience (QIB). Julia Haarhuis, who is a postgraduate student at QIB, will act as the Principal Investigator. Dr Jennifer Ahn-Jarvis (Food Health Human Studies Lead) will provide expertise in formulation and preparation of the PE and placebo capsules as well as assist with coordinating the study, and will provide guidance in ethics protocol development as well as support during study visit days as needed. Clare Ferns (NHS) will be the NHS collaborator and will provide practical insights into delivering/coordinating nursing activties. Dr Shikha Saha and Mark Philo (QIB Analytical Chemists) will provide asistance with the metabolite analyses on this study. Dr Christopher Quince, group Leader at the Earlham Institute (EI) and QIB, along with Dr Robert James (postdoctoral researcher at EI and QIB) will provide support with the metagenomic analyses. PBMCs will be isolated and phagocytic activity will be assessed by Dr Naiara Beraza. Dr George Savva (QIB statistician) will provide support with the statistical analyses and randomisation of the study agents. In rare instances where additional help in covering study visits is needed, the QIB human studies support team will assist. A delegation log will be used for recording the roles and responsibilities of the local research team and the authorisation of the Chief Investigator (CI). Dr Priscilla Day-Walsh will provide additional expertise in methylamine metabolism.

The study will be carried out in collaboration with the NIHR Norfolk Clinical Research Facility (CRF). NIHR Norfolk CRF is housed within the Quadram Institute (QI) and is accredited by the National Institute for Health and Care Research and hosted by the Norfolk and Norwich University Hospital (NNUH) NHS Foundation Trust. All analyses of human tissues will be carried out at QIB. The shotgun metagenomic sequencing of faecal DNA samples will be carried out at the Norwich Bioscience Institutes (NBI) or by a commercial supplier, using DNA extracts and sequencing libraries prepared with the support from the QIB Sequencing Team or other team members. All data analyses will be carried out by members of the study team at QIB and EI. All clinical aspects of the study will be carried out by the CRF team. The team will follow NNUH standard operating procedures. The clinical procedures on visit days will be carried out by two members of the CRF team, including a healthcare professional trained in NNUH emergency procedures and a designated member of staff to provide support.

3.2 Study Summary

This is a two-phased intervention trial. We will aim to recruit 40 participants onto the TMAO Status Phase (Phase I). Those who are deemed high TMAO producers (>5 μM and >50% increase between baseline and 24-hour follow-up) and who also meet the eligibility criteria will be invited to the Intervention Phase (Phase II). Participants will be randomised when they enter the Intervention Phase. We aim to have 16 participants complete the Intervention Phase. Phase I will start prior to phase II and will run in parallel until 16 participants have completed the Intervention Phase.

Participants who have completed an expression of interest (EOI) form will be contacted to schedule a study talk. The study talk will be provided by phone, virtual, or face to face meeting. Participants who meet the eligibility criteria, except for TMAO producing status (which will be measured during Phase I), will be provided an electronic copy of the informed consent and allowed a minimum of 24 hours before they can schedule their consent visit. The informed consent will be reviewed with the participant, and participants will have an opportunity to ask questions. Once participants have signed the informed consent form (ICF), they will be scheduled for the Phase I visits. Prior to their Phase I visits, participants will be asked to follow a restricted (low-carnitine and low-ellagitannin) diet for 48 hours. Phase I is comprised of two visits within a 24-hour period where high TMAO producing participants will be identified. At the first of the two visits, fasting blood will be collected, after which participants will be given three capsules containing a total of 1.5 g L-carnitine (oral carnitine response test, OCRT). The participants are then asked to return the next day (after 24 hours) for another blood collection. The change in the TMAO concentration in the blood plasma between baseline and 24-hour follow-up will be used to determine TMAO response status of the participants. This, and a completed medical questionnaire, will be used to determine whether the participant will be invited for the Phase II. Participants will be informed about whether they are invited for Phase II wihtin four weeks after completing Phase I. We aim to have participants start Phase II (starting with the Run-in) within three months after the invite to Phase II.

For the Intervention Phase, participants will participate in a total of 6 study visits over the course of 18 ± 1 days. Each intervention period consists of a 2-day run-in, intervention administration on Study Visits 1 or 4, which is followed by a 48-hour pharmacokinetic study (OCRT) on Study Visits 2 and 3 or Study Visits 5 and 6. Participants will be randomly allocated into one of the two arms (L-carnitine + PE or L-carnitine + placebo) for the first pharmacokinetic study and then, after the 10-day washout, participants will crossover onto the other arm for their second pharmacokinetic study. Within each intervention, participants will be asked to fast prior to their first study visit (Study Visits 1 and 4) for 8 hours. Blood, urine, and faecal

samples will be collected at specified time points throughout the 48-hour pharmacokinetic study. Therefore, for each pharmacokinetic study, 12 blood samples, six urine samples, and two faecal samples will be collected. For the duration of the study (except during the washout), participants will be asked to follow a low-carnitine and low-ellagitannin diet and consume the standardised meals provided at prescribed time periods during the pharmacokinetic studies.

3.3 Study Flow Chart

1. Study talk (phone/virtual/face-to-face)
    a. A study team member will discuss the Participant Information Sheet (PIS) with the participant and answers any questions.
2. Informed consent (virtual/face-to-face)
    a. A study team member will explain the study details and answer any further questions.
    b. The participant will complete the Informed Consent Form (Annex 2) and the Diet and Meal Specification Sheet (Annex 16) on REDCap (eConsent).
    c. The Medical Questionnaire (Annex 3) will be completed where possible, for initial review by the study team and, if deemed necessary by the study team, by the medical advisor.
    d. The participant will be provided with instructions for Phase I (Annex 19).
3. Run-in for Phase I (at home) – Phase I Day 0
    a. Participants will be asked to restrict their diet for 48 hours before their Phase I baseline visit at the CRF.
4. Phase I Baseline visit (at the CRF) – Phase I Day 2
    a. Participants are asked to arrive at the CRF in the morning after an overnight fast (≥8 hours) and following a 48-hour restricted diet.
    b. Height, weight, blood pressure and pulse will be measured.
    c. Participants will review and complete the Medical Questionnaire with the CRF nurse.
    d. Blood is collected for a full blood count (FBC), serum creatinine and Blood Urea Nitrogen (BUN) levels, and baseline plasma TMAO levels.
    e. The participant will undergo an OCRT by consuming 1.5 g L-carnitine and will be instructed to continue the restricted diet for the next 24 hours.
    f. A complimentary, light breakfast will be provided at the end of the visit.
5. Phase I Follow-up visit (at the CRF) – Phase I Day 3
    a. Participants are asked to arrive to the CRF in the morning after an overnight fast (≥8 hours) and following a 24-hour restricted diet.
    b. Blood is collected at 24±0.5 hours after L-carnitine adminstration to determine the 24-hour plasma TMAO production status (see 4. Eligibility Criteria).
    c. Participants will complete an FFQ to assess their diet over the past 30 days.
    d. Participants have the option to take home a faecal collection kit and a urine collection pot to collect their baseline samples, and their standardised breakfast for Phase II.
    e. A complimentary, light breakfast will be provided at the end of the visit.
6. Run-in for intervention period 1 (at home) – Phase II Day 0 and 1

a. Participants will be asked to follow a restricted diet and abstain from alcohol consumption for 48 hours.
b. A standardised breakfast will be provided to be consumed in the morning of their intervention visit. Participants will be asked to complete a dietary checklist (Annex 15a-f), retain any uneaten portion, and bring it along to their intervention visit.
c. The participant collects their baseline faecal sample within 24 hours of their first intervention visit.

7. Study Visit 1 (0-2.5 hours after supplementation) – Phase II Day 2
   a. Before this visit, participants will be asked to consume the provided standardised breakfast and begin their 8-hour fast.
   b. Participants will be asked to arrive to the CRF between 14.30-16.00, after their 8-hour fast.
   c. Participants will submit a faecal sample, collected within the 24 hours prior to the visit, and a baseline urine collection, collected on the morning of the visit. If the participant forgot to collect a baseline urine sample, they will have the opportunity to collect it at the CRF.
   d. A study team member completes the Study Visit Assessment Checklist (Annex 14b).
   e. Weight, blood pressure and pulse will be measured.
   f. After cannula placement, fasting blood samples (time -5 minutes) will be collected to measure baseline TMAO, L-carnitine, and other L-carnitine metabolites.
   g. Participants will undergo an OCRT by consuming 1.5 g L-carnitine (3 capsules) and randomised to receive either 4 capsules of PE or the placebo control (time 0).
   h. Participants will be instructed to consume the provided standardised lunch immediately following L-carnitine ingestion.
   i. A CRF nurse will continue to collect blood at 0.5, 1, 1.5, 2, and 2.5 hours after the supplementation, using the indwelling cannula.
   j. Urine samples are collected between 0-2.5 hours after supplementation. A study team member will aliquot and store urine on the day of the visit.
   k. At the end of the visit, the cannula will be removed, and participants will be provided supplies to collect their urine between 2.5-16 hours at home.
   l. A complimentary dinner will be provided (low-carnitine, low-ellagitannin) before the participant is sent home. The participant is asked to consume the dinner at 18.30 (T2.5) and the evening snack between 20.00-22.00 in the evening (T4-T6).

8. Study Visit 2 (16-24 hours after supplementation) – Phase II Day 3
   a. Participants will be asked to arrive to the CRF in the morning.
   b. Participants will submit their 2.5-16-hour urine collection to a study team member. On the day of the visit a study team member will aliquot and store the urine.
   c. The CRF nurse will place a cannula to continue blood collections for the following time points: 16, 18, 20, 22, and 24 hours after supplementation.
   d. Meals (low-carnitine, low-choline, and low-ellagitannin) will be provided throughout the day **after** blood is collected at the following times (times may vary slightly dependent on the exact time of T0):
      - Breakfast at 8.00 (T16)
      - Morning snack at 10.00 (T18)
      - Lunch at 12.00 (T20)

- Afternoon snack at 16.00 (T24)
- Dinner and evening snack will be provided for the participant to take home and consume the dinner at 18.00 (T26) and the evening snack between 20.00-22.00 (T28-T30)

e. During the visit, participants will be encouraged to provide urine samples at these time intervals 16-18, 18-20, 20-22, 22-24 hours in separate urine pots.
f. At the end of the visit, participant will be provided a urine collection pot to collect their urine for 12 hours (24-48-hour urine collection) and a faecal collection kit to collect their post-intervention faecal sample.

9. Study Visit 3 (48 hours after supplementation) – Day 4
   a. Participants will consume the provided meals at home at the following times (may vary slightly dependent on the exact time of T0):
      - Breakfast at 8.00 (T40)
      - Morning snack at 10.00 (T42)
      - Lunch at 12.00 (T44)
   b. Participants arrive at the CRF between 15.00-16.00.
   c. Participants will submit their 24-48-hour urine collection and their post-intervention faecal sample to a study team member. On the day of the visit, a study team member will aliquot and store urine and faecal samples. If the participant is unable to provide a faecal sample, they will be asked to deliver it the next day.
   d. A CRF nurse takes a 48-hour blood sample.
   e. A snack will be provided at the end of the visit.
   f. Participants have the option to take home a faecal collection kit and urine collection pot for their faecal and urine samples to be collected during the run-in period of intervention 2.
10. Washout Phase – Day 5 to Day 13

    Participants will return to their normal diet.
11. Run-in for intervention period 2 (at home) – Day 14 and 15

    Same as described in Run-in for intervention period 1.
12. Study Visit 4 (0-2.5 hours after supplementation) – Day 16

    Same as described in Study Visit 1
13. Study Visit 5 (16-24 hours after supplementation) – Day 17

    Same as described in Study Visit 2
14. Study Visit 6 (48 hours after supplementation) – Day 18

    Same as described in Study Visit 3 except participant will not be given any additional study supplies at the end of the visit.

4. Eligibility Criteria

4.1 Inclusion criteria for TMAO Status Phase (Phase I)

- At least 18 years of age.
- BMI between 18.5 and 30 kg/m^2. Those with a BMI outside the reference range may have an altered metabolism.
- On an omnivorous diet, consuming at least 4 portions (1 portion = 100 g raw or 75 g cooked) of meat (e.g., beef, pork, lamb, chicken, and turkey) per week for at least 2 months prior to enrolment in the study.

- Live within 40 miles from the Norwich Research Park.

4.2 Inclusion criteria for Intervention Phase (Phase II)

- TMAO producer (plasma TMAO increase of >5 μM and >50% following OCRT)

4.3 Exclusion criteria for Phase I and II

- Follow a vegan, vegetarian, pescatarian, or another diet in which meat is not regularly consumed within the last two months.
- No access to a freezer to store several of the provided meals and ice packs.
- Those unwilling to follow the dietary restrictions and consume the standardised diet that will be provided during the study.
- Those allergic or intolerant to any of the ingredients of the intervention or standardised meals (e.g., wheat, eggs, milk, nuts, soybeans).
- Bowel movements less than 5 times per week.
- Current smokers (or stopped smoking for less than 3 months) as this may affect metabolism.
- Alcohol consumption > 14 units per week as this may affect metabolism.
- Those with anaemia or any conditions or medications that hinder blood clotting. This will be assessed on a case-by-case basis by the CRF medical advisor.
- Abnormally high or low blood pressure (i.e., ≤90/60 or ≥160/100 mmHg).
- Kidney function outside of the normal reference range (creatinine ≤59 or ≥104 μmol/L for men, ≤45 or ≥84 μmol/L for women; BUN ≤2.5 or ≥ 7.8 mmol/L).
- Medical conditions that are judged to affect the primary outcome measure for this study or which may compromise the well-being of the participant e.g., diabetes, non-alcoholic fatty liver disease (NAFLD), or fish odour syndrome. This will be assessed on a case-by-case basis.
- Regular or recent use of colonic irrigation or other bowel cleansing techniques (e.g., the use of laxatives).
- Any gastrointestinal disorders (e.g., Crohn’s disease, ulcerative colitis) or surgeries such as short bowel syndrome, gastric bypass, or banding.
- Have an active infection or received antibiotics within the last month.
- Current use of prescribed and non-prescribed medications that may affect the primary outcome measure for this study or might put participants at risk (this will be assessed on a case-by-case basis).
- Women who are pregnant or breastfeeding.

- Current use of dietary supplements judged to affect the study data (e.g., fish oil, L-carnitine), unless the participant is willing to discontinue them for the duration of the study (this will be assessed on a case-by-case basis as some supplements may not affect the study). Specifically, individuals who use vitamin D3 supplements will be asked to continue their supplementation. Participants who are on prebiotic supplementation will be asked to discontinue their supplementation for at minimum one month prior to their participation. For all other dietary supplementation, participants will be asked to discontinue supplementation for at least two weeks prior to their participation.
- Parallel participation in another research project that involves a dietary intervention or blood sampling within the last four months.
- Intends donating blood within 16 weeks prior to, during or after the study period.
- Are currently undergoing active treatment for cancer or heart disease.
- Are not willing or able to ingest four size 0 capsules and three L-carnitine capsules consecutively within a time span of ten minutes.
- Participants who have any difficulty swallowing (dysphagia).
- Any person related to or living with any member of the study team or part of the management/supervisory structure of the Chief Investigator.
- Lack capacity to provide informed consent.
- Unable to provide your GP contact details.
- Have symptoms of COVID-19, been asked to self-isolate, or have been diagnosed with COVID-19 in the last 14 days.

5. Study procedures

5.1 Recruitment Procedures

5.1.1 Recruitment Strategy

The study team aims to recruit 40 participants onto Phase I. Those who are deemed high TMAO producers will be invited for Phase II within four weeks of completing Phase I. The aim is to have 16 participants complete Phase II. Potential participants will be identified and recruited by the study team through the following methods:

i) The Quadram Institute Bioscience (QIB) participant database, containing the minimum amount of information (name, contact details, age, smoking status, gender) of people who previously registered an interest in volunteering for human studies at QIB. They have previously consented to register their contact details onto the database. Identification of participants from the database is carried out by the QIB database manager. Letters of invitation

(Annex 6a) and the PIS (Annex 1) will be sent to individuals who may fit the eligibility criteria.

ii) Poster/e-mail advertisements (Annex 5a), which will be placed/sent across the Norwich Research Park, including the University of East Anglia (UEA), John Innes Centre (JIC), The Earlham Institute (EI), Norfolk and Norwich University Hospital (NNUH), Quadram Institute (QI), The Sainsbury Laboratory (TSL), and at other suitable locations such as social clubs and supermarkets, within the vicinity of QI (approx. 40 mile radius), inviting anyone who is interested in receiving information about the study to contact the study team.

iii) Social media platforms (Facebook, Instagram, LinkedIn, X), which will display the study poster (Annex 5a) or the TESSA social media captions (Annex 5b).

iv) Local media (e.g., newspapers, magazines, and the QI news webpage), which will publish a press release about the TESSA study (Annex 5c).

The posters advertising this study (Annex 5a) will have tear-off contact slips as well as a QR code to direct participants to the study website, to facilitate participant recruitment. A member of the study team will send interested responders by email an electronic copy of the letter of invitation (Annex 6b) and a copy of the PIS (Annex 1) directing them to the online expression of interest (EOI) form on the QIB human studies website. For those who prefer correspondence by post and those identified from the QIB participant database will have hard copies of the letter of invitation and the PIS with a freepost self-addressed envelope addressed to the study team will be sent. Participants who complete the online EOI form or have sent the EOI form by post have agreed to be contacted by the study scientists. Therefore, the study team will contact interested participants by telephone or email to schedule a study talk.

5.1.2 Study Talk – Information Exchange

If the participant believes that they meet the criteria for the study after reading through the PIS, they will have three routes for expressing their interest in participating in the study. Participants can return the EOI form included in the PIS (Annex 1) using the provided freepost code to QIB, complete the online EOI form on the QI human studies webpage, or contact the study team by the phone number or email address on the PIS. The study team will schedule a study talk with all participants that complete the EOI form. A study team member will review the details of the study, discuss the study eligibility criteria, and answer any questions the potential participant may have. Participants will be made aware that their participation is voluntary and that they can withdraw from the study at any time. Participants will be given a ‘consideration period’ of 24 hours whereby they will not be contacted. After this period, participants can contact the study team and arrange for an informed consent visit. Once the consent visit has been scheduled, participants will be provided a copy

of the ICF (Annex 2) by email or by post and asked to read through the consent prior to the consent visit.

5.1.3 Informed Consent Process

Individuals who indicate they would like to take part in the study will be invited for an informed consent visit. Participants have the option of a virtual or face-to-face meeting to complete their informed consent. The following are the details of the two routes for informed consent:

- Face-to-face meetings to conduct the informed consent visit will be at the CRF. However, if a room is not available then a private meeting room in QIB will be used. For the face-to-face meeting, the study scientist will first ask participants to verify their name and address then the scientist will go through line by line the ICF (Annex 2) and participants will be encouraged to ask any questions during the consenting procedure. Participants will provide informed consent using the eConsent survey format of the ICF in REDCap (Research Electronic Data Capture) software. Participants will be asked to use the provided secure link or personalised QR code to access the REDCap eConsent. The study scientist will review the ICF and participants will be encouraged to ask any questions during the consenting procedure. Participants will initial the textbox and using their finger, stylus, or mouse to sign a copy of the ICF eConsent. As part of the eConsent, participants will also sign the Diet and Meal Specification Sheet (Annex 16). An electronic copy of the signed ICF and Diet and Meal Specification Sheet will be provided.
- Virtual meetings to complete the informed consent will be conducted using Microsoft Teams which has end-to-end encryption for data security. Informed consent will be obtained using the guidelines of the NNUH SOP 317 (Obtaining remote consent from Competent Adults) and using the eConsent survey format of the ICF in REDCap software. It is required that the participant has access to a computer or tablet and the internet in order to use the provided secure link or personalised QR code to access the REDCap eConsent. Once they have accessed the eConsent, participants will be asked if they have any additional questions and informed that recording of the meeting will begin. Participant will be asked to state their full name and address. The study scientist will review the ICF and participants will be encouraged to ask any questions during the consenting procedure. Participants will initial the textbox and using their finger, stylus, or mouse to sign a copy of the ICF eConsent. Participants will also sign the Diet and Meal Specification Sheet (Annex 16). An electronic copy of the signed ICF and Diet and Meal Specification Sheet will be provided.

Copies of the ICF and Diet and Meal Specification Sheet will be kept in a secure, locked file cabinet only accessible by the PI and study scientists. The signed forms will be stored separately from the case study folder so that there is no means to link the participant identity to their alphanumeric participant ID number. The participant's GP will be informed by letter (Annex 7) of their patients' participation in the study. The study scientists who will be taking informed consent must have completed the QIB human studies training program, possess a current Good Clinical Practice certificate, NHS research passport, and completed the NIHR informed consent training.

After the participant has completed the informed consent, a study team member will walk them through the Medical Questionnaire (on REDCap) to complete the questions that can be answered already. These will then be reviewed by the study team and, if deemed necessary by the study team, by the medical advisor prior to Phase I. During Phase I, the questionnaire will be reviewed and completed by a CRF research nurse. Lastly, the participants will be reminded of the procedures for the two Phase I visits. They will be provided instructions detailing the low-carnitine and low-ellagitannin diet (Annex 19, Phase I Instructions Sheet) that they will follow 48 hours before their first Phase I visit and 24 hours between their first and second Phase I visits. The instructions will detail the foods to avoid (foods high in L-carnitine and ellagitannins) and contain suggestions for replacement foods. Before the conclusion of this visit, participants will be asked for their date of birth and NHS number and to schedule their Phase I visits.

5.2 TMAO Status Phase (Phase I) – Two Visits

All participants will undergo a medical questionnaire, clinical measures (height, weight, BMI, blood pressure, pulse, full blood count, serum creatinine), and an OCRT (plasma TMAO). The OCRT is used to identify a participant's TMAO producing status. Participants may be invited for the Intervention Phase of the study if they meet the TMAO response cut-off of >5 μM and >50% increase between the Phase I Baseline Visit and the Follow-up Visit. Additionally, the CRF medical advisor will determine if participants will be invited for Phase II of the study using the forementioned assessments. There will be two Phase I visits spread across a 24-hour period and each visit will last approximately 30-60 minutes.

Phase I Baseline Visit

- Participants arrive after following a low-carnitine and low-ellagitannin diet for 48 hours and have fasted for 8 hours prior to this visit. Participants are asked to avoid vigorous exercise during the 12 hours prior to their first Phase I visit.
- **Medical Questionnaire** (Annex 2): CRF research nurse will review and complete the medical questionnaire. The questionnaire

includes any medications they are taking and informs us about their general health.

- **Anthropometry and vital signs** (Annex 14a, Phase I Assessment Checklist): Height, weight, BMI, blood pressure, and pulse will be measured by the CRF research nurse.
- **Caloric requirement**: BMI, gender, age, and activity level (reported by participant) will be used to estimate the caloric requirement for each participant, such that the right calorie option can be selected for the Phase II standardised meals (2,000, 2,500, or 3,000 kcal/day). If a participant's caloric requirement exceeds 3,000 kcal/day they will be informed about this and then given the option to withdraw from the study or proceed with the 3,000 kcal/day meal plan.
- **Cannulation accessibility assessment**: CRF research nurse will check the veins of the participant to test if the participant would be suitable for cannulation on two consecutive days.
- **Blood collection**: A trained CRF research nurse will collect blood by venepuncture. A maximum of 23 mL will be collected for baseline plasma TMAO levels, full blood count (FBC), serum creatinine and Blood Urea Nitrogen (BUN) (to calculate the BUN/Creatinine ratio as a measure for kidney function). The FBC and BUN/Creatinine ratio will be analysed by the Norfolk & Norwich University Hospital (NNUH) laboratory, while the plasma TMAO will be measured in-house at QIB. We will send copies of the medical test results from the Phase I visit to the participant's GP (Annex 8).
- **Oral carnitine response test** (OCRT): participant will be given 1.5 g L-carnitine (3 capsules) to consume. The CRF nurse or study scientist will record the amount and time the carnitine was consumed in the Phase I Assessment Checklist (Annex 14a). Participant will be reminded to continue to follow a low-carnitine and low-ellagitannin diet for the next 24 hours.
- **Breakfast**: At the end of visit, participant will be provided a small breakfast. This breakfast will be low-carnitine and low-ellagitannins.

Phase I Follow-up Visit

- Participants arrive after following the restricted diet for 24 hours and being fasted for 8 hours prior to this visit.
- **Blood sampling**: Again, a trained CRF research nurse will collect blood by venepuncture. A maximum of 6 mL will be collected 24-hours after carnitine ingestion. To be invited for Phase II, the increase in plasma TMAO between baseline and the 24-hour follow-up should be at least 5 μM in combination with an increase of at least 50%.

NB: In a previous study, 33 omnivores (and 23 vegetarians) received an OCRT of 1.5 g L-carnitine, after which their plasma TMAO levels at 24 hours ($TMAO_{MAX}$) were measured [21]. The authors categorised participants as high TMAO producers and low TMAO producers, using a cut-off of plasma $TMAO_{MAX}$ > 10 μM. 57.6% of the omnivores met the cut-off criterium to be categorised as a high TMAO producer [21]. Based on this study, we anticipate that approximately 50% of the participants in Phase I will meet our cut-off criteria. Therefore, we expect that we will need 40 participants to enrol in Phase I to achieve 20 participants to enroll in Phase II (with a 20% drop-out rate we expect that at least 16 participants will complete the Intervention).

- **VioScreen Food Frequency Questionnaire (FFQ)**: Participants will complete an online FFQ (VioScreen™, VioCare®) during Phase I to assess their usual intake of L-carnitine over the past 30 days (Annex 18). This will be used to correlate the habitual dietary intake of L-carnitine with the TMAO response from baseline to 24 hours after taking L-carnitine.
- **Faecal collection kit and urine collection pot**: Participants will be provided with instructions for collection of a baseline faecal sample and a baseline urine collection. The materials needed for the collections will be provided prior to their start of Phase II visit 1 (either by post or handover at the CRF).
  NB: Participants that are found to be not eligible and already have received their collection kits will be asked to return the unused collection kits either in person or by mail.
- **Breakfast**: At the end of visit, participant will be provided a small breakfast. Participants will also be provided with the standardised breakfast for their first visit of Phase II.

5.3 Randomisation Procedures of the Intervention

5.3.1 Randomisation

Participants will be randomised for the order in which they receive the two dietary interventions (L-carnitine + PE and L-carnitine + placebo) will be conducted by a suitably qualified person independent of the study team. Block randomisation will be employed to ensure that groups are balanced in number. Participants will be randomised into one random permuted block with a size of 20. Block allocation sequences will be generated by the biostatistician.

5.3.2 Blinding Procedure

Phase II is a double blind, placebo-controlled, crossover study. The Chief Investigator, Principial Investigator and Study Scientists will remain blinded during the allocation, throughout the study, and during the analyses of data from participants, to ensure an unbiased approach throughout the

evaluation of results. However, the code may be broken in the event of a medical emergency as seen as necessary and appropriate by the CRF Research Nurse.

The PE capsules, and placebo capsules will be prepared, packaged, and labelled with a 3-digit alpha or numeric code at the CRF kitchen in a hygienic and food-safe manner, and stored in a food grade, climate-controlled, food storage room. Both interventions will utilise the same obscure, capsule material and will look identical in colour and packaged in matching packaging.

Participants will be blinded to the results of their levels of TMAO, L-carnitine, and its metabolites until analysis is complete. Likewise, all samples will be batch analysed for TMAO, L-carnitine, and its metabolites as well the analysis for pomegranate polyphenols and its metabolites at the end of the study to prevent any accidental unblinding of the study team. Participants will be able to contact their GP surgeries for the blood test results after the study has completed. If the full blood count is found to be abnormal by the study medical advisor, the participant will be contacted by the study team and advised to contact their GP and potentially asked to withdraw from the study, however this will be assessed on an individual basis.

5.3.3 Allocation Concealment

During the entire duration of the study and data analysis phase, participants and study team members will remain blinded to the allocation of the intervention. To ensure that the allocation remains concealed throughout the study, the capsules (PE and placebo) will be randomised, and order of the capsules will be assigned to the participant. Dr Natalia Perez-Moral (QIB research scientist) who is not part of the study team, nor has the CI of this study as her line manager, will replace the existing coded labels on the capsule bottles with new labels with the participant ID and Intervention 1 for the capsules allocated for the first intervention and likewise for those capsules that have been randomised to be allocated for the second intervention will have the participant ID and Intervention 2. Dr Perez-Moral will possess the list which includes the original codes of the capsules that identify the identity of the placebos and their assigned participant ID and allocation order. In the event that the study team needs to be unblinded for a participant, Dr Perez-Moral and Dr George Savva will be notified.

5.4 Details of the Intervention Study Visits

For the 18-day intervention study, there are two 48-hour pharmacokinetic studies, and each study is separated by a 10-day washout phase. A 2-day run-in period precedes each pharmacokinetic study. In each pharmacokinetic study, there are three study visits conducted at the CRF

and the visits range in duration from 0.5 to 9 hours. Participants will be asked to restrict their diets during the entire duration of each intervention, which includes the two run-in days, to reduce the effects of variable background diet on the metabolism involving L-carnitine and PE polyphenols. Only a single dose of PE or placebo is given at each intervention period. The duration of each pharmacokinetic study is 48 hours, such that the entire TMAO response, including the return to TMAO baseline levels, can be captured.

5.4.1 Run-in Period

At the start of each intervention period, there is a 2-day run-in period, participants are advised to abstain from alcohol and follow a low-carnitine and low-ellagitannin diet for the full duration of the run-in period. Additionally, the participants are asked to not consume caffeine and undertake any vigorous exercise during the 12 hours prior to the first study visit. Participants will be given instructions on activities that need to be completed during the run-in period.

- **Dietary restriction**: The instructions will detail a low-carnitine and low-ellagitannin diet by providing a list of foods to avoid, which are high in L-carnitine and ellagitannins, and suggested replacement foods (Annexes 20-21, Phase I Instructions Sheet and Study Visits Instructions Booklet).
- **Standardised Breakfast**: In the morning of Study Visit 1 and 4, participants will consume a standardised breakfast at home, which will be provided and is described in the Diet and Meal Specification Sheet (Annex 16). Participants can collect the meal at the CRF prior to the visit or it can be delivered to them.
- **Dietary Adherence**: Participants will be asked to complete a dietary checklist indicating the adherence to the standardised diet (Annex 15a-f) and they will be instructed to bring their unfinished portion of food(s) to their study visit.
- **Faecal Sample Baseline Collection**: The participant will be instructed to collect a baseline faecal sample within the 24 hours preceding the pharmacokinetic studies. They will be asked to keep the stool sample in a provided anaerobic bag and refrigerated in a provided cool box. Instructions on how to collect the faecal sample will be provided in the Study Visits Instructions Booklet (Annex 20). If the participant is unable to provide a faecal sample before the start of the intervention, Study Visit 1 will be rescheduled until they are able to provide a faecal sample.
- **Urine Baseline Spot Collection**: The participant will be instructed to collect a baseline urine sample on the morning of Study Visit 1 and 4. A urine collection pot and instructions (Annex 20) will be provided.

5.4.2 Pharmacokinetic Initiation – Study Visit 1 (Day 2) and 4 (Day 16)

The pharmacokinetic initiation visit (Visits 1 and 4) intends to capture the pharmacokinetic response of the first 2.5 hours after supplementation (L-carnitine + PE or L-carnitine + placebo) and will examine L-carnitine absorption and its early metabolism.

- **Standardised Breakfast**: Participants will be reminded to consume the provided standardised breakfast and then start their 8-hour fast prior to their study visit.
  NB: If participants have not fasted then they will need to reschedule their visit to another day.
- **Arrival**: Participants will arrive to the CRF between 14:30-16:00 and will be walked to their clinic room. Participants will submit their baseline faecal and urine collections and the dietary checklist that they received at Phase I. If the participant forgot to collect a baseline urine sample, they will have the opportunity to collect this at the CRF.
- **Study Visit Assessment Checklist** (Annex 14b): A CRF research nurse or study scientist will complete the checklist. The nurse will document any changes in the participant's health or medication. The nurse will measure and record height, weight, blood pressure, and pulse.
- **Cannulation and Blood collection** (time -5 min): A trained CRF research nurse will proceed to place a cannula into a vein in the participant's forearm or hand and a maximum of 14 mL of blood will be collected for a baseline measure. The cannula will be flushed with sterile saline to keep the cannula patent according to the CRF SOP for indwelling cannulas. The cannula will be kept in place during the duration of this visit and removed after the last blood sample (2.5 hours after supplementation) has been collected. A maximum of three attempts will be permitted. If the cannulation fails, the participant will be invited to come back on another day (after a minimum of four days).
- **Oral carnitine response test** (OCRT): After blood is collected, a study team member will provide the participant 1.5 g L-carnitine (3 capsules) to consume and 4 capsules of PE or 4 placebo capsules (Time 0). The supplementation will be taken with 150 mL water. Participants will be given 10 minutes to consume all the capsules. Timing of blood collection will start once participant has ingested all capsules (Time 0) and lunch will be served immediately afterwards. Blood plasma samples will be obtained by the CRF research nurse through the cannula at 0.5, 1, 1.5, 2, and 2.5 hours after the supplementation. At each time point a maximum of 6 mL blood will

be collected, and a dead volume of 3 mL will be discarded at each time point after the first collection; hence a total of 59 mL blood will be collected at this visit. The time when the participant has consumed the capsules, as well as the blood collection times, will be recorded on the on the Study Visit Assessment Checklist (Annex 14b). If the cannula becomes occluded or the participant complains about pain at the site, the nurse can use their discretion to remove the cannula and replace the cannula or collect the blood by venepuncture.

- **Urine Collection** (Time 0 to 2.5 hours): Participants will be encouraged to provide a urine sample during their stay. This urine sample will be 0-2.5 hours after the supplementation. Participants will be provided a urine collection pot and instructions how to collect the urine during their stay.
- **Home Urine Collection** (Time 2.5 to 16 hours): Materials will be provided for the participant. Participants will be instructed to collect all their urine at home. This urine collection will reflect 2.5-16 hours after the supplementation.
- **Meals**: A standardised dinner and an evening snack will be provided at the end of the visit (low-carnitine, low-choline and low-ellagitannin) to take home.

5.4.3 Pharmacokinetic Midpoint – Study Visit 2 (Day 3) and 5 (Day 17)

This pharmacokinetic midpoint visit (Visits 2 and 5) intends to capture the pharmacokinetic response of the period from 16 to 24 hours after the intervention.

- **Arrival**: Participants will be asked to arrive to the CRF between 7:00-9:00. The participant will be walked through to a clinical room, where they will hand the urine collection pot and the dietary checklist that they received on the day before, along with any uneaten portions of their provided meals.
- **Study Visit Assessment Checklist** (Annex 14b): CRF research nurse or study scientist will complete the checklist. Nurse will document any changes in the participant's health or medication overnight. Nurse will measure and record blood pressure, and pulse.
- **Cannulation and Blood collection**: A trained CRF research nurse will proceed to place a cannula into a vein in the participant's forearm and blood collection will resume for the pharmacokinetic study beginning at 16 hours after supplementation and followed by blood collections at 18, 20, 22, and 24 hours after the supplementation. At each time point, a maximum of 6 mL blood will be collected, and a dead volume of 3 mL will be discarded at each time point after the first collection; hence a total of up to 42 mL of

blood will be collected at this visit. A maximum of three attempts will be permitted. If the cannulation fails, the participant will be given the option to withdraw or to restart the pharmacokinetic study (after a minimum of four days).

- **Standardised Meals**: Participants will be provided standardised (low-carnitine, low-choline, and low-ellagitannin) meals during their visit at the following specified times and after has been collected for that timepoint:
    - Breakfast 16 hours after the intervention.
    - Morning snack 18 hours after the intervention.
    - Lunch 20 hours after the intervention.
    - Afternoon snack 24 hours after the intervention.
    - Dinner, evening snack and breakfast for the next day will be taken home.
- **Multiple Urine Collections**: Participants will be encouraged to provide a urine sample at 4 different time intervals during this visit. The times are 16-18, 18-20, 20-22, and 22-24 hours after the supplementation. A separate urine collection pot for each of the time intervals will be provided during the study visit. Urine will only be collected if the participant produces a sample. Additionally, participants will be provided a urine collection pot to collect the 24-48-hour urine after the supplementation at home.
- **Home Faecal Sample Collection**: The participant will be instructed to collect a post-intervention faecal sample at home between 24 and 48 hours after they consumed the L-carnitine. If the participant is unable to provide a sample before the next visit, they will be asked to provide the sample once they are able to. This may be on another day after the Study Visit 3 or 6. The participant will be given the option to bring in the sample or for a courier to be hired to deliver the sample to the CRF. Instructions on how to collect faecal samples will be provided in the Study Visits Instructions Booklet (Annex 20).

5.4.4 Pharmacokinetic End – Study Visit 3 (Day 4) and 6 (Day 18)

This short 30-60-minute visit (Visit 3 and 6) marks the end of the pharmacokinetic study and the end of the intervention period.

- **Arriva**l: Participants will be asked to arrive to the CRF between 14:30-16:00 (not fasted) and will be walked to their clinic room. The CRF nurse or study scientist will collect the completed urine collection for 24-48 hours after supplementation, faecal collection kit, and dietary checklist that they received on the day before, along with any uneaten portion of the provided meals.

- **Blood collection**: A trained CRF research nurse will collect blood by venepuncture. A total of up to 6 mL will be collected for the 48-hour time point after the supplementation.
- **Complimentary snack/tea**: Participants will be provided snack before they are sent home.
  NB: After the first intervention period, participants are provided the option to take home a faecal and urine collection kit for their baseline faecal and urine samples to be collected during the second intervention run-in period. They will also receive their standardised breakfast for visit 4.

5.4.5 Washout Phase

After the completion of the first intervention period, participants will be asked to return to their normal diet for 10 days before entering the second intervention period.

5.5 Completion of the study

Participants who have completed the full study have completed all six Phase II study visits, completed the study questionnaires, and submitted the prescribed urine and faecal samples. Upon completion of the study, participants will be provided with the general findings of the study and a basic summary of the intervention. All participant information will be pseudo-anonymised, and no individual data or results that link participants to the study findings will be reported.

5.6 Withdrawal from the study

Participants will be withdrawn from the study if they lose the capacity to consent during the study and/or any medical conditions or medications change that make them ineligible for the study. Moreover, if the CRF nursing staff has any difficulties with cannulation leading to a maximum of three failed cannulation attempts or failed blood collection, participants will be given the option to withdraw from the study or will be asked to reschedule the pharmacokinetic initiation study visit. For subsequent visits after the pharmacokinetic initiation visit where cannulation has failed and blood collection is not possible, participants will be given the option to withdraw or restart their pharmacokinetic study. Participants will be withdrawn after two failed completions of the first intervention period. Participants will be reminded that their participation is voluntary and that they are free to withdraw from the study at any time. They may do so without giving a reason and their clinical care and participation in future studies at QIB will not be affected. In the event a participant is withdrawn or withdraws from the study, they will be sent a withdrawal letter (Annex 12). The withdrawal letter elaborates that any sample or data collected up to the point of withdrawal will be kept and may be used in the study and participants will not be contacted again. The participant's GP will be informed that they are no longer taking part in the study (Annex 9).

Participants will be replaced to maintain the balance of the block randomisation until 16 participants have completed the study.

6. Dietary Intervention and Dietary Control

6.1 Phase I Diet

Participants will be asked to follow a low-carnitine and low-ellagitannin diet starting 48 hours prior to the first Phase I visit, until they have completed the Phase I follow-up visit. The instructions for the low-carnitine and low-ellagitannin diet are provided in the Phase I Instructions Sheet (Annex 19). Before their baseline and follow-up Phase I visits, participants are asked to be fasted for at least 8 hours. The TMAO from L-carnitine is still detectable in the plasma until 48 hours after the intake, therefore the dietary restriction is 48 hours. The dietary restrictions allow for a low L-carnitine and choline intake from the diet, with the intake of both nutrients not exceeding 200 mg.

6.2 Phase II Run-In Diet and Meal

During the 48-hour run-in period participants will follow a diet low in carnitine and ellagitannins, as outlined in the Study Visits Instructions Booklet (Annex 20). Similar to Phase I, the dietary restrictions for Phase II allow for an intake of maximum 200 mg L-carnitine and choline from the diet. As part of the run-in diet, a breakfast will be provided on the morning of the first Study Visit (Visit 1 and 4). Standardised meals will be provided until their last Study Visit (Visit 3 and 6). The run-in breakfast and other standardised meals are described in the Diet and Meal Specification Sheet (Annex 16). The run-in breakfast will be provided at Phase I visit 2 (for intervention 1) and Phase II visit 3 (for intervention 2), or be sent to the participant from the supplier (Tesco Plc or another grocery store that provides a delivery option).

6.3 Dietary Intervention

Participants will be provided three carnitine capsules and four pomegranate or control capsules, along with 150 mL water. Directly after the first blood draw, they will receive a meal (as described in Diet and Meal Specification Sheet, Annex 16).

6.3.1 L-Carnitine Capsules

The L-carnitine capsules are marketed under the name Carnipure® and are obtained through VitaminExpress LCC. The supplement is provided with a Technical Data Sheet (TDS) (Annex 21) and Certificate of Analysis (CoA) (Annex 22). Three L-carnitine capsules, containing a total of 1.5 g L-carnitine (500 mg per capsule) will be taken as part of the OCRT. The dose is the same as the OCRT dose that was used and described by Wu *et al.* (2019); they provided 1.5 g of L-carnitine to be consumed by participants in their human study [31].

6.3.2 The Pomegranate Extract

The extract, marketed under the name Dermogranate®, is provided by Medinutrex (Catania, Italy). The extract has been microbiologically tested and is provided along with a TDS (Annex 23) and CoA (Annex 24). Participants will be asked to consume once 1.6 g PE, providing 320 mg polyphenols including 160 mg ellagic acid and 112 mg punicalagin, and once four identically looking placebo capsules. To determine a suitable dose of PE, we chose an amount that was no higher than doses that had been safely administered previously (as described in section 16.5 of this protocol), and would be expected to be effective based on the concentrations used in the *in vitro* experiments (section 1.3 of this protocol). The volume of the human colon has been estimated to hold an average volume of 561 mL (including the ascending, transverse, and descending colon) [32]. Considering that the PE capsule would only be in one part of the colon at a given time, we predicted it would be contained within a volume of approximately 187 mL (561 mL divided by 3 parts of the colon). Therefore, the dose of 1.6 g PE corresponds to 8.6 mg/mL, which is between the low (5.7 mg/mL) and medium (11.4 mg/mL) dose used in our *in vitro* experiments. A size 0 capsule can contain approximately 400 mg of PE, so four capsules make up a dose of 1.6 g PE.

6.3.3 Preparation of the Intervention Capsules

The PE and placebo capsules will be prepared hygienically and safely in the CRF by a member of the study team with formal Level 2 training in food safety and hygiene, the QIB in-house food hygiene training module (QIB Human Studies Training Programme) and in compliance with Environmental Health Guidelines and Food Standard Agency. Standard operating procedures (SOPs) for the preparation, storage, and administration of the capsules will be followed. A HACCP plan for low-risk food will be created for the study product; HACCP plans are implemented as part of GMP for food manufacturing and catering.

All ingredients are commercially available and safe for human consumption. Since the capsules will be combined at the CRF, a subset of capsules will be sent to ALS Laboratories (UKAS accredited laboratory) for microbiological testing to ensure microbiological safety. The capsules will be made close to the intervention start date after which the subset of capsules will be sent for microbiological testing. A list of the microbiological analysis that will be assessed can be found in Figure 5.

Analysis
TVC (30°C)
E.coli (presumptive)
Enterobacteriaceae (presumptive)
Bacillus cereus (presumptive)
Yeast & Mould (DRBCA)
Clostridium perfringens (presumptive)
Salmonella (per 25g)**
Coagulase Positive Staphylococci

Figure 5. The suite of microbiological analyses for the capsules.

Prior to administration, each capsule will be assigned a unique code to effectively blind the participants (see 5.2.2 Blinding procedure) and study team. Specification sheets of ingredients including lot number, packaging, labelling requirements, storage condition, stability and shelf-life testing will be requested when the capsules are prepared ready for distribution to the participants. The capsules will then be packaged in food grade recyclable plastic tamperproof containers with a lid and the labelling found in Figure 6. The capsules will be stored at CRF food grade storage room with climate and humidity controlled at 20 to 60% relative humidity until administration to the participants. SOPs and kitchen use plan will be used according to the CRF code of practice. An inventory log and administration record will be kept by the study team to keep track of the capsules administered by which study team member and the specific participant who consumed the capsules.

Figure 6. Opaque vial (26 mL) and example label that will be used for capsules packaging.

6.4 Dietary Control

Participants will be asked to follow a restricted diet and consume standardised meals for six days during Phase II of this study, to be consumed either during their study visit or at home. Participants will be provided with two menu options of which they will need to select a single option and maintain for the full duration of the study. It is possible to change an item on the meal plan if necessary, which will be discussed on a case-by-case basis. Participants are not required to finish the meals, as long as they report it in the Dietary Checklist (Annex 15a-f) and bring the uneaten portion to the CRF.

6.4.1 Dietary Restriction/Controlled Diet

Participants will follow a low-carnitine and low-ellagitannin diet (Annexes 20-21, Phase I Instructions Sheet and Study Visits Instructions Booklet), starting 48 hours before the intervention. Starting from 8 hours before the intervention, participants will be provided with standardised meals (Annex 16), which will be low-carnitine and low-ellagitannin, and several meals will also be low-choline. The restricted diet is in place to prevent interference by the diet on the study measurements, namely the formation of TMAO from other sources other than the L-carnitine supplementation. During the study visits, participants will be provided with water, up to 250 mL during the shorter visits (visit 1, 3, 4, 6) and up to 1 L for the long visits (visit 2 and 5). They are allowed to consume up to two cups of coffee and two cups of (black or green) tea during their visits. The meal plans are designed such that major sources of L-carnitine, choline, and ellagitannins are avoided, with a threshold of 200 mg/day from each choline and L-carnitine, based on concentrations reported by the United States Department of Agriculture (USDA) Foods Database and available literature [14, 33-36].

6.4.2 Standardised Meals

Participants will be provided with meals. They will receive four meals in the CRF on the long study visits (visit 2 and 5) and two meals at each of the shorter visits (visit 1, 3, 4, 6). The rest of the meals will be consumed at home. A full standardised diet is provided on all days of the study visits, six days in total. To meet individual caloric requirements, participants will be assessed for their caloric requirement during Phase I, based on their BMI, gender, age, and activity level (as reported by the participant). Three calorie options will be available: 2,000, 2,500, and 3,000 kcal/day. Participants will be told that they do not have to complete the meals, as long as they report on the Dietary Checklist (Annex 15a-f) what they have eaten. The standardised diets contain a daily average of 12% protein, 29% fat, and 56% carbohydrates (Diet and Meal Specification Sheet, Annex 16).

6.4.3 Food Safety of Standardised Meals

QIB has standard operating procedures for the storage and delivery of food to research participants. Those procedures will be adhered to when processing and handling our food items, in accordance with the Environmental Health Guidelines. Standardised meals will be ordered from a commercial supplier (Tesco Plc or another grocery store that provides a delivery option) and will be delivered directly to the address of the participants. Participants will be instructed to store the meals appropriately, either frozen or in the fridge. The meals that will be provided in the CRF will be stored at -20°C for frozen meals and between 4-8°C for fresh meals.

7. Experimental methods

7.1 Pomegranate Extract/Capsule Analysis

The content of the main pomegranate polyphenols (punicalagin, punicalin, ellagic acid, gallic acid) will be assessed prior to the start of the trial. Furthermore, the moisture content of the extract will be analysed upon preparation of the pomegranate extract into capsules, to ensure that all participants receive the same amount of the extract.

7.2 Collection and processing of blood, urine, and faecal samples

7.2.1 Blood samples

Whole blood will be collected into EDTA tubes and then immediately centrifuged at 3000×g for 15 mins to obtain plasma (2.0-2.5 mL). For each collected blood plasma sample, sub-samples of 200-1,000 µL plasma will be aliquoted into 5-10 separate storage tubes. All plasma will be stored at -80 °C until batch analysed by HPLC MS (see section 7.3). The serum sample and second whole blood sample that are taken on the Phase I baseline visit will be sent off to NNUH Pathology, where they will be tested for full blood count (FBC), as well as serum creatinine and BUN levels. The BUN/Creatinine ratio will be used to determine normal kidney function.

PBMC's will be collected from blood collected using the CPT Mononuclear Cell Preparation Tube (8mL). Once the plasma from the green and blue tubes are removed, the buffy coat that remains will be used for the exploratory PBMC studies which are detailed below.

7.2.2 Urine samples

Participants will be provided urine collection pots that have been pre-weighed and pre-conditioned with boric acid (0.5g/1L) as a preservative. Each complete urine collection will be weighed and then up to ten sub-samples of 1 mL will be aliquoted into separate vials before acidification with 20% HCL (to a final concentration of 1%) to reduce the pH. Samples will be stored at -80 °C until batch analysis.

7.2.3 Faecal samples

Participants will be provided with a faecal collection kit consisting of an anaerobic bag and clip, a sample catcher, and a cooler box. Up to ten aliquots (500 mg each) of fresh faecal matter will be placed into appropriate vials and stored at -80 °C until the analysis of DNA and metabolites.

7.3 Analysis of TMAO, L-carnitine, γ-BB, and TMA in plasma, urine, and faeces

The analysis of TMAO and its metabolites in plasma, urine and faeces will be performed using a published LC-MS/MS method [17]. Biological samples will be thawed at room temperature. For faecal samples, typically

500 mg of the samples will be weighed into a tube, combined with glass beads and homogenised. The supernatant from the processed faecal samples, or plasma and urine samples, will each be transferred into separate vials. The samples will be mixed with 50% trichloroacetic acid and incubated at 4 °C for 5 minutes. An internal standard mixture of isotopically labelled (d9-) TMAO, L-carnitine, γ-BB and TMA (in 0.2 M acetic acid) will be added to the samples and centrifuged for 10 minutes. The supernatants will be diluted in distilled water and analysed by LC-MS/MS (Agilent Technologies, USA). The LC-MS/MS conditions are described elsewhere [17].

7.4 Analysis of polyphenols and derivatives in the urine samples

The main pomegranate polyphenols and their metabolites (including gallic acid, punicalagin, ellagic acid, urolithins) present in the urine samples will be quantified using an established in-house extraction procedure and LC-MS technique. In brief, trifluoroacetic acid will be added to aliquots of plasma to precipitate the proteins. Post centrifugation (16,000 ×g; 4°C; 15 minutes) samples of plasma will be diluted 1:3 in distilled water and transferred into auto-sampler vials for HPLC analysis. The mobile phase will constitute 0.1% formic acid in distilled water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). 1 µL of each sample will be run through a Waters HSS T3 column (100 x 2.1 mm; particle size 1.8 µm) at 35°C and a flow rate of 0.4 mL/min. The gradient will start at 3% mobile phase B for 5 minutes, after which mobile phase B will be increased to 20% for 4 minutes, and then further increased to 50% for 2 minutes, lastly mobile phase B will be increased to 95% for 1 minute before it re-equilibrates to 3% for 2 minutes. In keeping with good analytical practice, samples will be run in a random order, but all the samples of one participant will be analysed on the same day.

7.5 DNA extractions from faecal samples

DNA will be extracted from the faecal samples using FastDNA™ Spin Kit (MP Biomedicals) according to the manufacturer's instructions. The preparation of sequencing libraries will be carried out in-house at the NBI. From the extracted DNA a gut microbiota composition analysis will be carried out commercially or in-house at the NBI, using Illumina MiSeq technology. During this process, all human DNA will be filtered out of the samples. The DNA extracts will also be used to analyse the abundance of genes involved in L-carnitine metabolism by means of qPCR with primers designed to target the Cai and Bbu gene clusters. Additionally, RNA will be extracted using RNeasy PowerFecal Pro Kits (Qiagen). The extracted RNA samples will be used in RT-qPCR analyses to measure the expression of genes involved in L-carnitine metabolism (including Cai and Bbu gene clusters).

7.6 PBMCs isolation and macrophage activity assessment

Monocytes will be isolated from PBMCs using commercial kits and/or flow cytometry cell sorter. The RNA will be extracted from isolated monocytes for RNA sequencing, which will provide information about the function of these cells including expression of inflammatory cytokines and chemokines, expression of metabolic indicators or other cellular pathways. Alternatively, a fraction of the isolated monocytes will be exposed to LPS in vitro to determine their capacity to respond to bacterial-challenges. Inflammation-related gene expression will be analysed after stimulation and compared between diet-groups. Freshly isolated PBMCs may also be exposed to E coli-PhRhodo and analysed by flow cytometry. This technique will allow us to determine the phagocytic (bacteria-eating) capacity of monocytes.

7.7 Food Frequency Questionnaire (FFQ)

Participants will be asked to complete the VioScreen FFQ on the second Phase I visit. VioCare® is the company that will provide the VioScreen site for the FFQ. Habitual L-carnitine intake (the intake over the past 30 days) will be analysed using the nutrition analysis software based on food composition tables used in the USA. The FFQ has been validated in the USA, which reflects the Western diet and will therefore approximate the diet of the TESSA participants. Furthermore, this FFQ has been used in hundreds of human studies and it has standardised software for its analysis, which reduces any data-interpretation bias. The FFQ provides information about the participant's habitual diet through online questions to assess their intake of each food group. The time interval for assessment can be customised. For this study, we plan to use the FFQ over a 30-day interval. A completed VioScreen FFQ generates four clinical reports. The first report is a detailed personal health and nutrition summary detailing body measures, nutrition and food consumed. The second report contains healthy eating index (HEI) feedback, detailing current intake and recommended intake of food group areas. The third report contains the macronutrients and micronutrients from the top foods consumed. The fourth report is a dietary inflammation index (DII®) report, detailing areas of the diet that have inflammatory potential.

8. Statistical Analysis

8.1 Sample size calculation

The primary aim of this study is to provide a first estimate of the effect of PE on TMAO production *in vivo* as a proof of concept for future work. From our *in vitro* work, during which the TMA production rate is slightly slower, PE could reduce TMA production at 48 hours by 48.1-76.5%, dependent on the PE dose (Figure 3C, Scientific Background). In a study where participants consumed a polyphenol-rich grape extract daily for four weeks, habitual plasma TMAO concentrations were reduced by 63.6% compared with the baseline plasma TMAO levels, while the reduction was

only 0.54% for the control group [23]. In another study, in which participants took antibiotics for one week, TMAO production from L-carnitine was completely abolished at 24 hours, which is the reported time of maximal TMAO concentration (TMAO T_{max}) [20]. Since limited data is available on the study outcome, it is challenging to estimate the variance that is needed for an accurate sample size calculation.

Our aim is for at least 16 participants to complete the Phase II. Since limited prior data is available on the primary study outcome, it is challenging to estimate the variance that is needed for a precise power calculation. A study by Wu *et al.* (2020) [21] reported data suggesting a within-person standard deviation on a logarithmic scale of 1.178 for the maximum TMAO level over the measurement period. This would imply a power of 80% to detect a 60% reduction in TMAO production at $p < 0.05$. Our planned primary outcome is the AUC for TMAO, which is likely to be more precise, increasing the power to detect this effect. A smaller study with only six participants suggested a standard deviation for within-participant AUC TMAO of 0.694 on a natural log scale, implying much higher power for our current study, but this is imprecise.

Based on this and practical considerations, 16 participants will give us a reasonable chance to detect the effect and will provide better information on the likely effect size and the within-person standard deviation to inform future work on TMAO reduction.

8.2 Plasma TMAO cut-off used to determine participation in Phase II

It should be noted that not all omnivores produce significant levels of TMAO from L-carnitine. Therefore, we aim to invite only high TMAO producers for Phase II, who will be identified during Phase I as described in section '5.2 TMAO Status Phase (Phase I) – Two Visits'. For participants to be invited for Phase II, the increase in plasma TMAO levels between baseline and 24-hour follow-up should be at least 5 µM and more than 50% of the measured baseline value. This cut-off has been established based on the data from a study in 33 omnivores [21]. The participants in that study were subjected to an OCRT of 1.5 g L-carnitine, after which their plasma TMAO levels at 24 hours (TMAO T_{max}) were measured. The authors categorised participants as high TMAO producers and low TMAO producers, using a cut-off of plasma TMAO C_{max} >10 µM. At baseline, the TMAO levels of the 33 omnivores had a mean of 3.05 µM (± 0.98 S.E.M.). 19 out of 33 omnivores (57.6%) met the cut-off criterion to be categorised as a high TMAO producer [21].

9. Data analysis plan

A CONSORT diagram will be used to describe the flow of participants through the study, describing the numbers recruited, participating in Phase I, randomised into Phase II, and participating in each Phase II intervention.

Individual's results from Phase I will be used to invite high TMAO producing participants for Phase II, otherwise all data analyses will be conducted at the end of the study after 16 participants have completed Phase II. All collected data will be analysed *per protocol* (i.e., only participants who complete both interventions will be included in the final analysis), although the data of participants who do not complete both Phase II interventions may be used for *ad hoc* analyses. Participants will be excluded during the analysis if they present abnormal baseline TMAO values in one or both interventions, greater than >25 μM. This threshold is based on the average fasting plasma TMAO concentration of 33 omnivores, reported in a previous study [21], which was 3.05 μM (± 0.98 SEM). The value of 25 μM falls within the 99th centile for the log-normal distribution of data that has been previously reported.

For all data analyses described below, the observed effect will be considered statistically significant if $p < 0.05$. Confidence intervals of 95% will be reported for all estimates.

9.1 Primary outcomes

We will estimate the pharmacokinetic effects of PE on TMAO plasma concentrations by comparing the log AUC of TMAO between the PE and placebo interventions. A linear mixed model with the intervention and study period as fixed effects and the participant as a random effect will be used to estimate the difference in the TMAO log AUC between the two treatments. Baseline TMAO values will be used as a covariate in the analysis.

A log transformation has been chosen in the first instance based on limited data from previous studies, but alternative transformations or non-parametric tests may be used if this is unsuitable given the actual data.

The AUC will be calculated using the trapezium rule based on actual time points of measurements relative to the time of consumption of the L-carnitine supplement. Where up to two data points are missing in the curve (e.g., from technical issues, or if a participant cannot provide a sample) a curve will be estimated using imputed values for those points. Where more than two points are missing then the curve will be excluded from the primary analysis. This will be applied to the primary as well as the secondary outcomes.

9.2 Secondary outcomes

We will estimate the effects of PE on the concentration of L-carnitine, γ-BB, TMA, and other related metabolites in the blood plasma by comparing the log AUC of each of the compounds between the PE and placebo interventions. The log AUC of each of the two interventions will be compared within the individuals using linear mixed models.
The excretion of L-carnitine, γ-BB, TMA, TMAO, and other related metabolites will be analysed from the urine and faecal samples. The rate of excretion will be calculated as for each metabolite by dividing the metabolite concentration by the duration of collection (hours), i.e., metabolite (mmol/L) / hours. We will compare the difference in the rate of excretion between the PE and placebo interventions within individuals, using linear mixed models.

The main polyphenols present in PE (including gallic acid, punicalagin, punicalin, ellagic acid) and their metabolites (urolithins) will be quantified in the urine and the concentrations will be compared between the two interventions using linear mixed models.

The change in gut microbiome profiles, as obtained from shotgun metagenomics data, will be compared between the PE and placebo intervention to identify significant changes in the abundances of microbial species or genera. We will specifically look at species that are known to contain the *cai* and *gbu* gene clusters, which are responsible for the metabolism of L-carnitine and γ-BB, respectively. We have a specific interest in species containing the *gbu* gene cluster, which include *Emergencia timonensis* and *Ihubacter massiliensis* as, to our knowledge, these are the only organisms identified to date that contain this gene cluster.

The difference in the absolute abundance and expression of the bacterial genes involved in L-carnitine metabolism (including the *cai* and *gbu* gene clusters) between the interventions will be compared.

Finally, the data may also be explored for correlations between plasma TMAO log AUC and the excreted concentrations of L-carnitine, γ-BB, TMA, or TMAO. Also, from all participants that have participated in Phase I, we will explore the correlation between L-carnitine intake as measured by the FFQ and the increase in plasma TMAO concentration from baseline to 24 hours.

10. Data Protection and Participant Confidentiality

This is a QIB sponsored study based in the United Kingdom. For any (personal or medical) information collected from participants to undertake this study, QIB will act as the data controller. This means that QIB is responsible for ensuring that the data collected is stored in a secure manner throughout the course of the study. QIB will keep identifiable information about the participants for 15 years after the end of the study in a secure archive at the QIB or designated secure off-site location. The participants' rights to access, change or move their information are not affected, as we need to manage their information in specific ways for the research to be reliable and accurate. If the participant withdraws from the study, all personal and identifiable data will be removed, but pseudo-anonymised study data and samples already collected with consent will be retained and used in the study. No further data or samples will be collected, or any other research procedures carried out in relation to the participant.

We will follow Ethics and Research Governance and Good Clinical Practice (GCP) requirements. The collection, storage, processing, and disclosure of the study data will be managed by the study scientists (Julia Haarhuis and Jennifer Ahn-Jarvis) in adherence with UK General Data Protection Regulation (UK GDPR) and UK Data Protection Act 2018, with regards to the collection, storage, processing, and disclosure of personal information and will adhere to the GDPR and DPA core principles to maintain confidentiality. The legal basis used under the regulation that we employ to process the participant's personal information is for tasks carried out for public interest, which includes this study and its associated research. Participant's personal information will be stored in lockable filing cabinets at QIB. Suitable security measures and precautions are also taken for any confidential or personal data that are stored electronically. Participants' personally identifiable data such as their expression of interest (EOI) form and signed informed consent will be kept separately from the study site folder and securely stored in a locked cabinet or password protected electronic file that is only accessible by the study scientists. The data will be pseudo-anonymised with a unique, study-specific code which cannot be linked to the participant and is stored on a password-protected data file. All data collected during the study visits will be known only by this study specific participant code and kept separately from any personally identifiable data that can link the participant's identity to their participant ID number. Only the CI, PI, and study scientists will have access to the files linking personal data to the participants' unique code. Likewise, all correspondence and medical test results shared with the participant's GP will be kept strictly confidential.

The trial has a study specific email address (TESSA@quadram.ac.uk) and specific contact number (07584 760676) that the research team will use for recruitment purposes and for study-related correspondence with the research participants. The study email address will be a shared QIB account with restricted access to the research team only. We will include an automatic management (timely deletion) of emails and phone logs to ensure participant data protection. During the study, the participant will be asked to use external services to record study information. The data collected from the blood samples will be identifiable by the NNUH team at the CRF and will be transferred from NNUH Pathology department to QIB for analysis.

Informed consent (eConsent) will be collected during a remote or on-site visit using REDCap. The data protection impact process was reviewed and approved by the Norwich Research Park Data Protection Adviser (Annex 26 on Date 27/12/2023). Microsoft Teams will be used to record segments of the informed consent visit if held remotely. The recordings will be downloaded onto a QIB secure networked file folder that is accessible only to the CI and study scientists. The recordings will be automatically deleted from Microsoft Teams after 30 days. Microsoft data for European-based tenants is stored on servers in Europe. REDCap, which is a secure web application designed to build and administer online surveys, will be utilised in this instance to collect an electronic completed ICF. NBI (Norwich Biosciences Institute) data protection team led by Mr Bob Findlay and Pete Andrews (Senior IT Security Specialist) have assessed any security issues related to the use of Microsoft and REDCap software to collect this data. Participant specific links to the eConsent will be administered using REDCap software and has been readily used by over 120 countries including the UK. REDCap is GDPR compliant and the data collected from REDCap is stored onsite on the NRP on secure servers. They are managed and monitored by NBI data protection team.

The data collected from the FFQ will have no personal or identifiable information. The data will be transferred to a third party (outside of the EEA) to be analysed by the company VioCare®. All the data collected from the questionnaires will be kept anonymous (no personal identifying information), so the data will not need to be stored on an EU server and no data management agreements are required.

Participants will be informed in the consent that their name and address will be shared with a third-party vendor for delivery of the standardised meals (Tesco Plc or another grocery store that provides a delivery option) and when participants require a courier for collection of one or more faecal samples. Food delivery and courier services will only be made for participants who consented to share their address with the third-party vendors for study purposes.

11. Study Management Plan

A Trial Management Group including the CI (Dr Paul Kroon), PI (Julia Haarhuis) and Co-Investigator (Dr Jennifer Ahn-Jarvis) will be responsible for the day-to-day management of the trial. The PI will monitor all aspects of the conduct and progress of the trial, ensure that the protocol is adhered to and take appropriate action to safeguard participants and the quality of the trial itself. A delegation log will be used for recording the roles and responsibilities of the local research team and the authorisation of the PI for this (Staff signature and delegation log, HRA Template version 1.2, 2 January 2018). This log will include the main study activities that the PI can delegate to staff at the participating organisation. The task list and delegation log will be maintained as an up-to-date document throughout the duration of the study at the participating organisation.

12. Data Management Plan

12.1 Description of the data

Our data will include a medical questionnaire, an FFQ, and results from the analyses of biological samples taken (blood plasma, urine, faecal samples). The data will be managed using a spreadsheet format that will be stored in a networked file that is password protected and only accessible by the study team.

12.2 Specific management of personal data

Participants who will be successfully recruited onto the study will be assigned a unique code number which will be kept in a secure file. A lockable filing cabinet or cupboard will be used to keep paper documents that include the file linking the participant to the code and personal information. Manual files/folders will consist of separate named and numbered files for each participant. No data with the participants' name will be filed in the numbered file and vice versa. Only the study team will have access to the file linking personal data to the participants' unique code. All electronic data will be stored on a password protected shared data file. Data will be stored for at least 15 years after completion or discontinuation of the study in a confidential archive. Access to archived, electronic and confidential data will be limited to the study team. The quality assurance auditors may also be allowed access with the permission, and in the presence, of the CI. Any information collected prior consent via study-specific advertising material will be used solely for the purpose of the study and will be handled in compliance with the QIB Data Protection Policy to protect and respect participant's privacy. This process has reviewed and authorised by QIB Data Protection Officer. Data will be managed by the study team in compliance with UK General Data Protection Regulation (UK GDPR) and UK Data Protection Act 2018.

12.3 Specific management of samples

All biological specimens collected as part of the study will be known only by their code. All data collected will also be identified by code only. Laboratory results will be maintained in a spreadsheet form and will be in file formats that can be shared. Only pseudo-anonymised individual-level data will be shared within the study team.

12.4 Data collection and generation

Data will be collected by trained researchers onto study-specific forms and then uploaded into electronic data sets. Data will be collected by completing case report forms (CRFs) and will be used to build encrypted databases. Methods used to generate data from this study will be fully described in standard operating procedures (SOPs). The study record will include a detailed description of data collection and coding system.
All raw data will be collected on a continuous basis and supplemented with relevant additional information (e.g., identity of researcher collecting and entering data, date of collection). Most of the data will be in digital form; however, some data will originally be recorded in hardcopy-form and later transcribed to digital copies. Working copies of all datasets will be kept in an encrypted format on the institutional network (QIB) which has shared access with appropriately authorised research staff working on the project. We will ensure that clear audit trails link secondary processed information to primary data and will be audited in compliance with International Good Clinical Practice (GCP) standards.

12.5 Data sharing and access

The research protocols will be registered in a publicly accessible database after gaining favourable ethical opinion. Registration to ClinicalTrials.gov Protocol Registration and Results System (PRS) using QIB account will allow us to be transparent in our work. The study team will ensure fully compliance with the standards required for deposition of information in any relevant public databases. Pseudo-anonymised individual-level datasets and code will be published along with any publication on the study. Participants will provide informed consent using the eConsent survey format of the ICF in REDCap (Research Electronic Data Capture) software, as described in 5.1.3 Informed Consent Process. Consent forms clearly state the data sharing procedures for data generated from this study.

12.6 Relevant institutional policies on data sharing and data security

All data will be managed, protected, and shared in accordance with the requirements of the QIB Quality Code of Practice, the QIB Policy on

Safeguarding Good Scientific Practice and the Wellcome Trust Data Sharing Policy. All study collaborators will adhere to the same rigorous standards for data management.

13. Serious adverse events (SAEs)

13.1 Definition of serious adverse events (SAEs)

An SAE is defined as any untoward medical occurrence that:

- Results in death.
- Is life-threatening.
- Requires inpatient hospitalisation or prolongation of existing hospitalisation.
- Results in persistent or significant disability/incapacity.

Other ‘important medical events’ may also be considered serious if they jeopardise the participant or require an intervention to prevent one of the above consequences.
NOTE: The term "life-threatening" in the definition of "serious" refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

13.2 Reporting Procedures for Serious Adverse Events

A serious adverse event (SAE) occurring to a participant should be reported to the REC that gave a favourable opinion of the study where in the opinion of the Chief Investigator the event was ‘related’ (resulted from administration of any of the research procedures) and ‘unexpected’ in relation to those procedures. Reports of related and unexpected SAEs should be submitted within 15 working days of the Chief Investigator becoming aware of the event, using the HRA report of serious adverse event form (see HRA website https://www.hra.nhs.uk/approvals-amendments/managing-your-approval/safety-reporting/).

14. Definition of end of study

The end of study will be the date when all samples have been collected from all participants, questionnaires from all participants have been completed, and analysis of all biological (human tissues) samples have been completed. The participant will receive a summary of the results at the end of the study, and their personal (contact details) will be used for this purpose.

15. Participant payment and travel expenses

The participants will receive an inconvenience payment of £30 for completion of Phase I and an additional £320 upon completion of Phase II. If the participant withdraws or is excluded from the study, payment will be *pro rata. Pro rata* payments are as follows:

- Completion of Phase I: £ 30
- Completion of each Phase II intervention period (2x): £ 100
- Completion of the study: £ 80
- Submission of each faecal sample (4x): £ 10

Participants will also be reimbursed travel expenses to and from the hospital and car parking if required. This will be reimbursed at the QIB's current mileage rate or by reimbursing public transport costs on production of a ticket or receipt. In exceptional cases, participants may require transport by taxi, e.g., if they can't reach the CRF by car or public transport. In this case, a taxi will be arranged to be pre-paid.

16. Ethical and regulatory considerations

16.1 Good Clinical Practice

The Investigator will ensure that this study is conducted in accordance with the principles of the Declaration of Helsinki. The proposed research will be conducted in accordance with the conditions and principles of the International Conference on Harmonisation Good Clinical Practice (ICH GCP), and in compliance with national law. The research will meet the requirements of the UK General Data Protection Regulation (UK GDPR), UK Data Protection Act 2018 and relevant sponsor's policies.

16.2 Approvals

The protocol and annexes, including the ICF and PIS, will be submitted to a Research Ethics Committee (REC) and HRA for written approval. The study protocol and associated documents will be reviewed by the Human Research Governance Committee (HRGC) at QIB and approved by the QIB statistician prior to submission to the REC. The QIB HRGC adheres to the UK Policy Framework for Health and Social Care Research. Copy of the HRGC approval, and any correspondence with the committee will be available to the REC and HRA, if requested. The Investigator will submit and, where necessary, obtain approval from the above parties for all substantial amendments to the original approved documents.

16.3 Informed Consent

To ensure participants can make an informed decision as to whether they wish to take part in the study they will be provided with a PIS (Annex 1) and invited to an informal study talk by phone/online platform. Written consent will be obtained by a member of staff who is GCP trained and experienced in conducting human intervention trials. The participant must have the ability to make and communicate their decision, can give informed consent from the information given about the study, from the participants ability to understand the information, and exercise the right to choose to participate. All participants are free to withdraw at any time from the study without giving reasons.

16.4 Safety of the L-carnitine supplement

Three L-carnitine capsules, containing a total of 1.5 g L-carnitine (500 mg per capsule) will be taken as part of the OCRT. CRF nurses will be informed of rare side effects of L-carnitine ingestion. The side effects are not likely with the dose being used for this study but may be possible in those who are hypersensitive to L-carnitine. According to the Office of Dietary Supplements, US National Institutes of Health, the daily intake of L-carnitine in adults is estimated to be between 60 and 180 mg in omnivores and between 10-12 mg in vegans [37]. However, doses of up to 8 g have been used in dietary supplementation studies [38]. The doses we intend to feed in this study (1.5 g each time on two days separated by at least 11 days) fall well within the previously administered doses. For example, in a human intervention trial investigating the effects of various L-carnitine doses to treat peripheral artery diseases L-carnitine was fed at doses ranging between 1-8 g with no reported adverse effects over the 24-hour period [39]. In other pharmacokinetics studies using an OCRT, doses of 1.2-1.5 g L-carnitine were used [21, 31]. In another study, participants received an L-carnitine supplement of 500 mg daily for two months [20]. These studies did not report any side effects.

16.5 Safety of the pomegranate extract

Phase II participants will be asked to consume a total of 1.6 g PE, providing 320 mg polyphenols which includes 160 mg ellagic acid and 112 mg punicalagin, and once four identically looking, placebo controls. These amounts are considered safe based on evidence from other human trials [40]. In a clinical trial, 64 participants consumed between 710 and 1,420 mg per day of POMx, containing respectively 435 or 870 mg of gallic acid equivalents (GAEs), for 28 days [41]. The same research group also conducted a study in 70 participants who consumed 2,000 mg PE per day for up to 4 weeks [42]. No adverse effects were reported in these studies. In other clinical trials using PE or pomegranate juice with GAEs of up to

2,266 mg/day over a period ranging from 4 weeks to 3 years, no serious adverse effects have been reported [42-49].

16.6 Safety of the placebo capsules

Each placebo capsule will contain approximately 400 mg of combined pharmaceutical grade microcrystalline cellulose (MCC), gum Arabic (10%), and a brown edible food colour powder (1%). MCC is an inert substance commonly used in the food industry as a multipurpose food ingredient and in the pharmaceutical industry as an excipient in drug tablet formulations. It is the same material that we used successfully and without any adverse effects in the previous ethically approved BERI study (IRAS ID 223958). Gum Arabic is a dietary fibre that is often used to prepare food extracts (spray drying). It is present in the pomegranate extract and therefore we include it to better match the treatment and placebo control.

16.7 Procedures for any harm experienced by the participants

If, throughout the period of this study, a participant is harmed by taking part, there are no exclusive compensation privileges. If, due to negligence, harm is caused to the participant and there are grounds for legal action, the participant will likely have to pay for these legal costs. We appreciate that under specific circumstances, participants may still wish to file a complaint. If this is the case, a confidential service specially designed to support patients, relatives, and caretakers will be available to them. This is the Patient Advice and Liaison Service (PALS, http://www.nnuh.nhs.uk/patients-visitors/help-support/pals/). QIB has a liability insurance (Annex 25) covering research that involves human participants. QIB will not fund any legal costs arising from any action unless awarded by a court. Furthermore, as this study involves the CRF, which is an NHS facility, indemnity is provided through NHS schemes.

16.8 Participant wellbeing throughout the study

Throughout the study, study scientists will call the participant to check in on the intervention and discuss any issues or questions the participants have. If any participant becomes unwell at any stage of the study, the first action will be for them to see their GP or A&E. GPs will be informed about the participants' involvement in this study (Annex 7) by letter and will receive a copy of the PIS (Annex 1). The PIS will advise participants to contact the emergency service (via 999) in case of a medical emergency and ensure that the study team is contacted as soon as practically possible. The decision to exclude the participant from the study will be taken by the QI medical advisor.

16.9 Cannulation and blood sampling risk

The participants may experience discomfort from the placement of the cannula, during the blood collection, and administration of the saline to keep the cannula patent. Where the cannula has been placed, there may be a small bruise that develops but most bruises fade within 3-4 days. The blood sampling will be conducted by CRF research nurses who are fully trained and experienced in the placement of a cannula and collecting blood through a cannula. In very rare instances, infection, phlebitis and infiltration may occur. However, the CRF nursing staff are trained to troubleshoot if problems arise and can decide to discontinue the cannula when needed. This will minimise any risks associated with the procedure. If there are any issues regarding blood sampling on the day, the QI medical advisor can be contacted and can provide medical support, as the wellbeing of the participant is vital.

16.10 COVID-19 risk mitigation

QIB as research sponsor has been working with the NNUH to put in place appropriate measures to ensure the safety of research participants and staff in response to the COVID-19 outbreak. QIB researchers are asked to follow specific standard operating procedures (SOPs) for delivering face-to-face study appointments at the CRF. The following measures are in place:

- All participants will be given the option to decline attending their appointments if they are worried about increased risk of COVID-19 infection.
- Participants will be contacted within 7 days of any scheduled appointments at the CRF and asked not to attend their appointment if they have symptoms of COVID-19, been asked to self-isolate, or have been diagnosed with COVID-19 in the last 14 days.
- The research team will disinfect the common surfaces of the room where the participant will stay during their visit (chair, desk, door handles). Disinfectants will be provided by the CRF team in line with NNUH Infection control guideline.

16.11 Audits and inspections

The study may be subject to inspection and audit by QIB under their remit as Sponsor, and other regulatory bodies to ensure adherence to GCP and the UK Policy Framework for Health and Social Care Research.

16.12 Reporting

The CI shall submit once a year throughout the study, or on request, an Annual Progress report to the REC Committee, HRA (where required) host

organisation and Sponsor. In addition, an End of Study notification and final report will be submitted to the same parties.

17. Indemnity

QIB has £2.5 million commercial insurance for the TESSA study, including no fault liability insurance. The study will be covered by the current QIB Insurance as documented in Annex 25, which gives further details of QIB liability insurance. NHS bodies are legally liable for the negligent acts and omissions of their employees. If a participant is harmed whilst taking part in a clinical research study due to negligence on the part of a member of the study team this liability cover would apply. Non-negligent harm is not covered by the NHS indemnity scheme (Annex 25).

18. Finance

The study is sponsored by QIB and will be funded by the Wellcome Trust through the EDESIA: Plants, Food, and Health PhD programme (Annex 29).

19. Dissemination Policy

The research protocol will be registered to ClinicalTrials.gov Protocol Registration and Results System (PRS) using the QIB account, which is a publicly accessible database, after gaining favourable ethical opinion. Data arising from the study will be owned by QIB. Data will be disseminated in the form of scientific presentations and publication in open-access, peer-reviewed journals. The authors will acknowledge that the study was funded by the Wellcome Trust. Participants will also be informed of the outcome of the study. Participants' pseudo-anonymised research data collected during the study may be used to support other research in the future. In addition, we may collaborate with patient groups and professional groups to share the findings through multiple media channels including public engagement events and social media.

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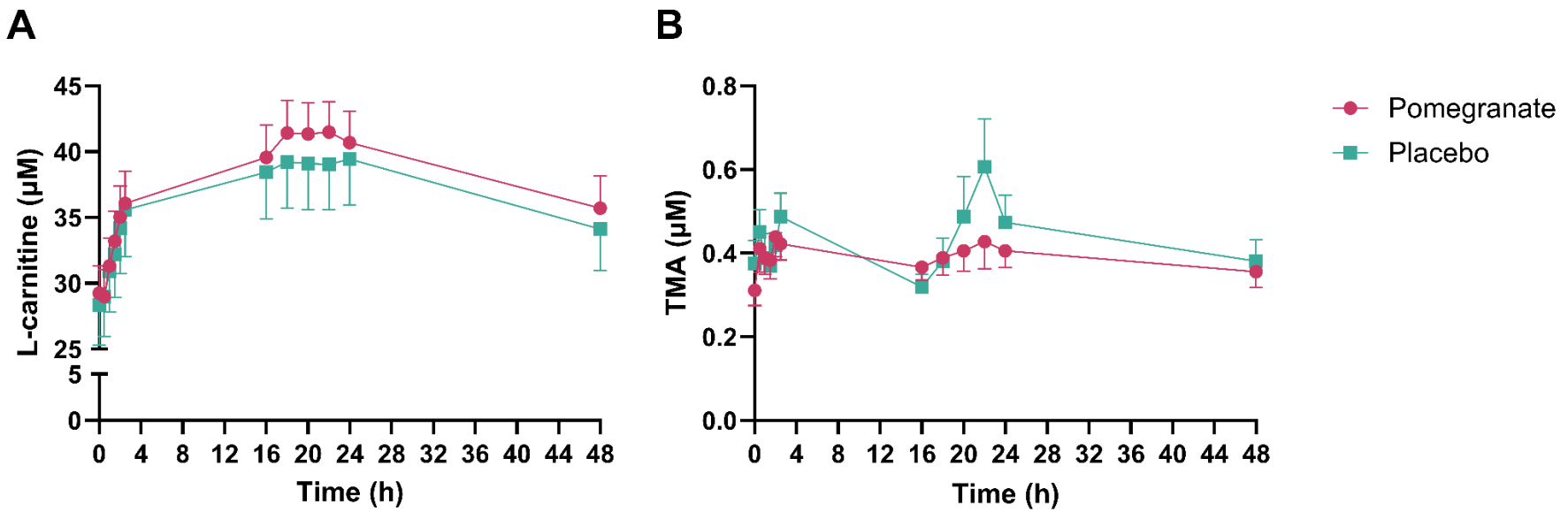
Appendix 6

TESSA Study Run-In Dietary Restrictions, version 3 (9 September 2024).

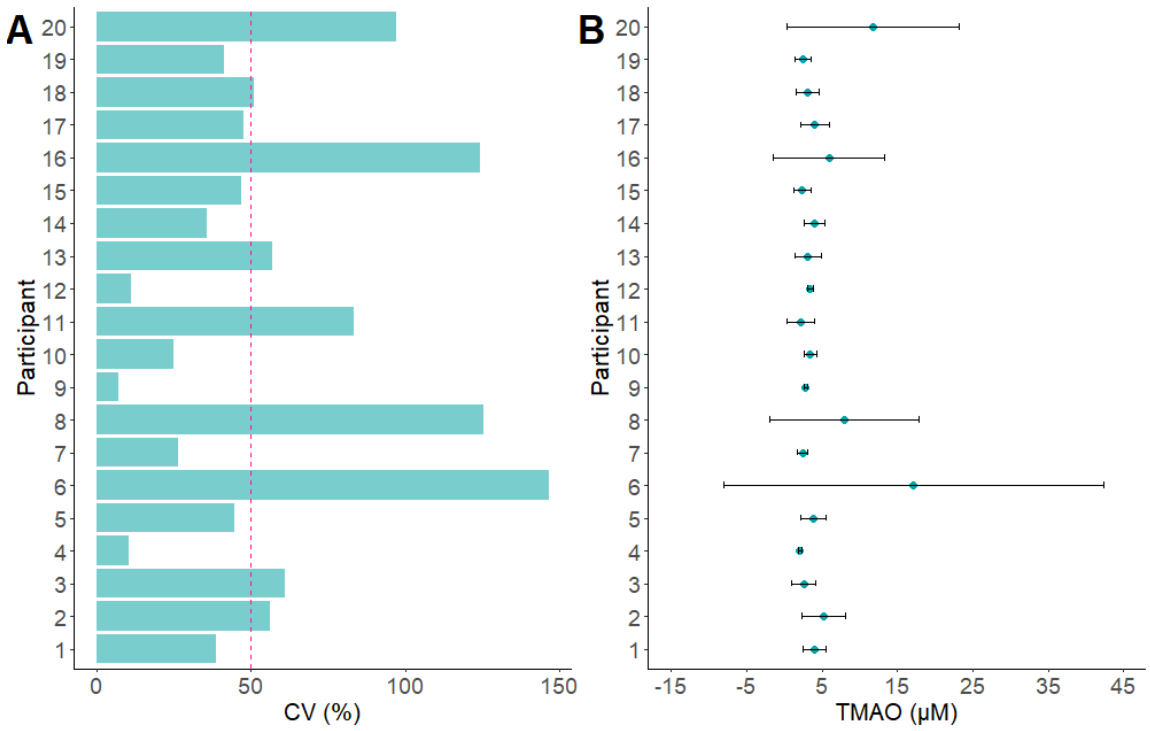
Food group	Restricted (avoid)
Meat products and fish (high in L-carnitine)	Chicken liver
	Chicken
	Turkey
	Beef (steak, ground beef, T-bone, tenderloin, ribs, liver)
	Pork (ham, shoulder, muscle, bacon)
	Tartar
	Lamb
	Duck
	Ribs
Dairy products and eggs (high in L-carnitine)	Yoghurt (0% fat)
	Milk (less than 2% fat)
	Goat cheese
	Munster cheese
	Camembert
	Saint Moret
	Comte
Fruit, including jams and juices (high in ellagitannins)	Raspberries
	Strawberries
	Blackberries
	Cloudberries
	Pomegranate
	Cherries
	Grapes
	Guava
	Blackcurrant
	Bilberries
	Marion berries
	Logan berries
	Elderberries
	Prunes
	Boysenberries
	Salmonberries
	Acai
	Goji berries
	Tayberries
	Chokeberries
	Blueberries
Nuts (high in ellagitannins)	Walnuts
	Pecan nuts
	Chestnuts
	Almonds
Beverages (high in ellagitannins)	Alcohol
	Berry juice
	Pomegranate juice
	Grape juice
	Blackcurrant juice
	Pomegranate or berry tea

Appendix 7

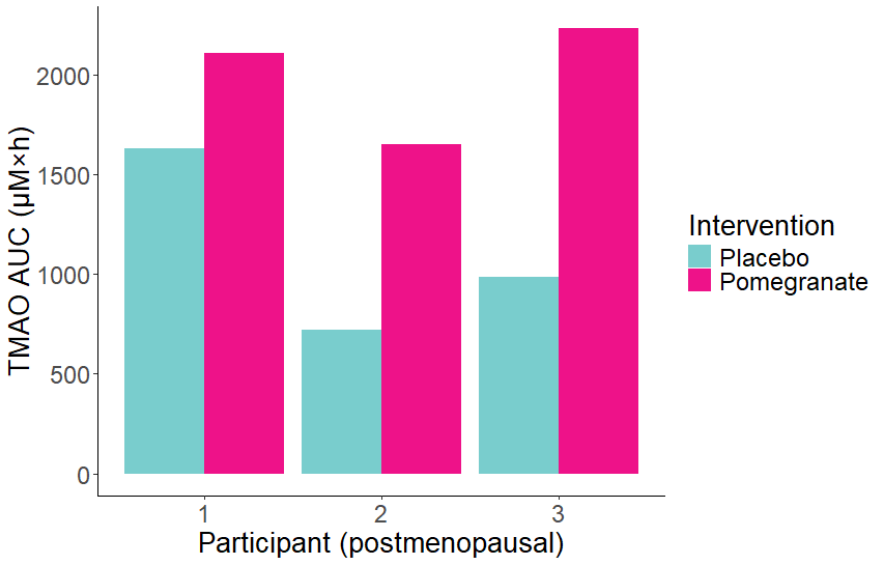
The following supplementary information are with reference to **Chapter 5**.



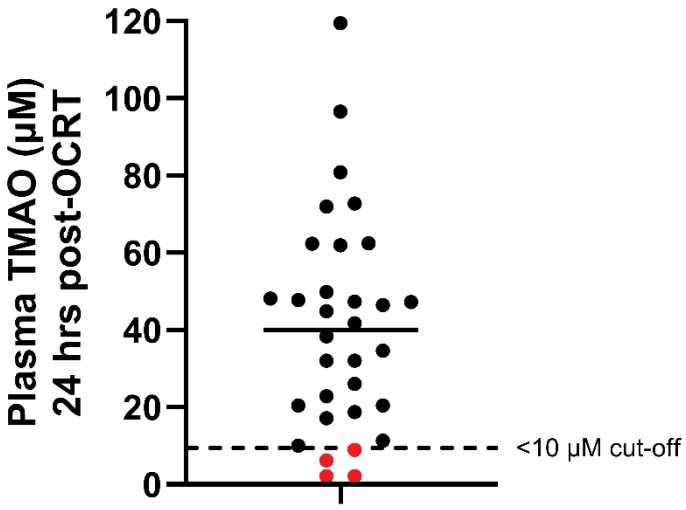
Supplementary Figure S13. L-Carnitine and trimethylamine (TMA) blood plasma concentrations in TESSA participants over 48 hours. *Data are shown as mean ± SEM across 16 participants. Participants underwent two oral carnitine response tests (OCRTs), consuming 1.5 g L-carnitine once with a placebo and once with a pomegranate extract. During 48 hours after the OCRT blood plasma was collected to estimate a pharmacokinetic curve for L-carnitine and TMA. Whole blood samples were centrifuged to obtain plasma and stored at -80 °C until LC-MS/MS quantification using isotope-labelled internal standards.*



Supplementary Figure S14. Variance of fasting baseline plasma trimethylamine N-oxide (TMAO) concentrations in TESSA participants who started Phase II. *(**A**) Coefficient of variation (CV) and (**B**) mean ± SD of baseline plasma TMAO measures per participant, for each of the 20 participants who started Phase II, with three measurements per participant except for participants 17-20 who only have two measurements. Baseline plasma TMAO was measured prior to undergoing the oral carnitine response tests (OCRTs). Whole blood samples were centrifuged to obtain plasma and stored at -80 °C until LC-MS/MS quantification using a d9-TMAO internal standard.*



Supplementary Figure S15. Trimethylamine N-oxide area under the curve (TMAO AUC) for the placebo and pomegranate intervention in "older" (postmenopausal) female TESSA participants. *Participants (n = 3) underwent two oral carnitine response tests (OCRTs), consuming 1.5 g L-carnitine once with a placebo and once with a pomegranate extract. During 48 hours after the OCRT, blood plasma was collected to estimate the TMAO AUC. Whole blood samples were centrifuged to obtain plasma and stored at -80 °C until LC-MS/MS quantification using a d9-TMAO internal standard.*



Supplementary Figure S16. Distribution of fasting blood plasma trimethylamine N-oxide (TMAO) levels 24 hours after the oral carnitine response test (OCRT), with <10 µM cut-off shown. *Data points represent individual Phase I participants (n = 32). The cut-off is based on a report by Wu et al. (2020) to categorise high- and low-TMAO producers. At 24 hours post-OCRT, 4 out of 32 TESSA participants showed TMAO levels <10 µM (indicated in red). Fasted blood samples were collected from TESSA participants prior to the OCRT in Phase I. Whole blood samples were centrifuged to obtain plasma and stored at -80 °C until LC-MS/MS quantification using a d9-TMAO internal standard.*