Modulating Microbial Production of Trimethylamine from Choline with Probiotics and Polyphenols

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ABSTRACT

Background: The gut microbiota is essential in metabolising dietary components like choline and carnitine, producing trimethylamine (TMA) in the colon which is then converted into trimethylamine N-oxide (TMAO) in the liver. TMAO has been linked to cardiovascular disease (CVD) risk and development of atherosclerosis. Despite debates on the use of TMAO as a disease marker, due to its prognostic properties in CVD pathogenesis it is paramount to identify effective treatments for at-risk individuals.

Objective: This thesis aimed to investigate the capacity of human gut microbiota to produce TMA and develop strategies for modulating microbial TMA production from choline using probiotics and polyphenols.

Approaches: *In-vivo* TMAO levels were assessed in plasma and urine and their relationship with the *in-vitro* capacity to produce TMA from choline was investigated for correlations with the structure and function of the gut microbiota using shotgun metagenomics approach. A mixed-strain probiotic supplement and its components were tested using *in-vitro* colon models for their effect on TMA production in faecal samples and TMA-producing strains, while polyphenols were screened for inhibitory effects on TMA production from choline.

Results: *In-vivo* TMAO status did not correlate with TMA production capacity measured *in-vitro,* but significant correlations were found between these variables and gut microbiota composition and function. Mixed probiotic supplement minimally affected TMA production, yet its individual strains showed promise in faecal matrices. Polyphenols, particularly more complex structures, demonstrated inhibitory effects on choline metabolism though different mechanisms of action.

Conclusion: This work deepened our understanding of the role of the gut microbiota in TMA production and its relationship with *in-vivo* TMAO status. By investigating the role of probiotics and polyphenols in reducing TMA production from choline, targeted strategies for mitigating risks associated with elevated TMAO levels can be established in the future. These insights may guide the development of dietary interventions to modulate the gut microbiota-mediated and reduce elevated TMAO levels.

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ABBREVIATIONS

3-4HPPA	3-(4-hydroxyphenyl)propinoic acid	MeOH	methanol
ABB	Anaerobe basal broth	MFC	Microbiota-free control
ADP	adenosine diphosphate	MI	myocardial infarction
ANOVA	Analysis of variance	MRS	De Man–Rogosa–Sharpe
ApoE(-/-)	Apolipoprotein E deficient	MTTB	methyltransferase family of proteins
ATCC	American type culture collection	MW	molecular weight
AUC	Area under curve	NAFLD	non-alcoholic fatty liver disease
BA	bile acid	NASH	Non-alcoholic steatohepatitis
BB	butyrobetaine	NCBI	National centre for biotechnology information
BCCT	Betaine/choline/carnitine transporter	NCTC	National collection of type cultures
BHI	Brain heart infusion	NEC	Necrotising enterocolitis
CA	chlorogenic acid	NF	nuclear factor
CAD	coronary artery disease	NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
CFU	colony forming units	OCCT	Oral carnitine/choline challenge test
CHD	coronary heart disease	CH3	methyl group
CH_3	methyl group	OD	optical density
CI	confidence interval	OH	hydroxyl group
CKD	chronic kidney disease	PAD	peripheral artery disease
CMB	batch colon model	PATRIC	Pathosystems Resource Integration Center
CMBB	batch-batch colon model	PBS	phosphate-buffered saline
CMHT	high throughput colon model	PC	phosphatidylcholine
CPM	copies per million	PCR	polymerase chain reaction
CTR	control	Pfam	Protein family domain
CV	coefficient of variation	PM	Proteus mirabilis
CVD	cardiovascular disease	PMS	*P. mirabilis* specific medium
DMB	3,3-dimethyl-1-butanol	PP	polyphenol
DMSO	dimethylsulfoxide	PRX	probiotics
DNA	deoxyribonucleic acid	PXN	Protexin
DSM	German Collection of Microorganisms and Cell Cultures	QIB	Quadram Institute Bioscience
EC	Enzyme commission	QQ-plot	probability plot
EDTA	Ethylenediaminetetraacetic acid	RR	relative risk or risk ratio
ESI	electron spray ionisation	RT	retention time or room temperature
EtOH	ethanol	RXN	Reaction annotation of MetaCyc
FMO	flavin monooxygenase	SAM	S-adenosylmethionine (superfamily)

FOS	fructooligosaccharides	SCFA	short-chain fatty acids
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	SD	standard deviation
GC	nucleotides guanine and cytosine	SDI	Shannon diversity index
GI	gastrointestinal	SEM	standard error of means
GOS	galactooligosaccharides	SHIME	Simulator of the Human Intestinal Microbial Ecosystem
GS	glycerol stock	STEMI	ST-segment elevation myocardial infarction
HDL	high density lipoprotein	T2DM	type 2 diabetes mellitus
HF	high fat	TAG	triacyl glycerides
HFBA	heptafluorobutyric acid	TCA	trichloroacetic acid or the citric acid (cycle)
HS	Qubit High Sensitivity	TE	Tris EDTA (buffer)
IL	interleukin	TIM-1/TIM-2	TNO Gastrointestinal model
JNK	c-Jun N-terminal kinase	TMA	trimethylamine
KEGG	Kyoto Encyclopedia of Genes and Genomes	TMAO	trimethylamine-N-oxide
KO	KEGG Orthology	TNF-α	tumour necrosis factor
LC-MS/MS	liquid chromatography tandem mass spectrometry	UPLC	ultra performance liquid chromatography
LDL	low density lipoprotein	UV	ultraviolet
LRGG	lactobacillus rhamnosus GG	VC	Vehicle control
MACE	major adverse cardiovascular event	WCH	Wilkins Chalgren agar
MAG	metagenome assembled genome	WGS	whole genome sequencing
MAPK	mitogen-activated protein kinase	WHO	world health organisation
MCC	microcrystalline cellulose	WMD	weighted mean difference

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisory team, my primary supervisor Dr Paul Kroon, secondary supervisor Prof Arjan Narbad and the infinitely invaluable support from Dr Priscilla Day-Walsh. Their guidance, encouragement, and insightful conversations throughout my doctoral research were integral to my progress. Their expertise and resourcefulness have been instrumental in shaping this thesis and facilitating my growth as a researcher. I am profoundly grateful for their patience and dedication. Thank you to other members of my supervisory team, Dr Laura Lehtovirta-Morley and my industry supervisor Dr Richard Day for widening the opportunities and knowledge that were accessible to me during my PhD.

I extend heartfelt thanks to all members of the Kroon Group, past and present, whose collaborative drive and supportive spirit eased the difficult times during my research journey. The stimulating discussions, sharing experiences in the lab and beyond, and camaraderie within the group created the moments of my PhD I will cherish forever. Special thanks to Dr Emad Shehata, Dr Jasmine Percival, Dr Gemma Beasy, Ms Julia Haarhuis, Dr Olla Al-Jaibaji, Dr Mariam Gamal-El-Din and Ms Marianthi Firoglani-Moschi for their never-ending support, assistance and friendships made during this process.

I am also grateful to the Narbad Group for sharing their knowledge, time and resources that enabled me to learn new skills and troubleshoot, with the guidance from such outstanding scientists like Dr Melinda Mayer, Dr Lizbeth Sayavedra, Dr Lee Kellingray and Dr Jacob Scadden. My dear friends Dr Jade Davies and Dr Bushra Schuitemaker kept me sane during endless experiments and shared with me many of their excellent protocols, tips and tricks that saved me hours of work and contributed to my development as a researcher but also helped me become a better friend.

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established in the Warren Group and helped me troubleshoot optimisation of that method. Thank you all and to other members of the Quadram Institute that helped me progress to the next stage, be it with their acts of kindness, helping me with sampling late at night or simply allowing me to pick their brain. It has been said that it takes a village to raise a child, I would say it takes a research community to finish a PhD.

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I owe this thesis to my writing buddies Dr Bushra Schuitemaker, Ms Jennifer Robinson, Ms Prachi Hejib and the facilitators of the writing retreats at UEA that held me accountable and provided companionship during the hardest part of my PhD, your enthusiasm and perspective gave me so many ideas and kept me going during this difficult period of my journey. I am grateful to my friends outside of my PhD that did not question the radio silence for the past many months and understood that there were no spoons left for conversations – to Dr Ellie Hassan, Ms Geraldine Mynors, Ms Megan Mortimer, Ms Eerika Haajanen, and my best friend Ms Natalie Kollegova, I would not even be doing this PhD if it was not for your presence in my life and all the love that you have shown me over the years.

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DEDICATION

I wanted to dedicate this thesis to my grandmother that passed away earlier this year after a long time struggling with terminal illness. I know she would have been so proud of me, and I vow to do as much good in my life as she did.

Božena Filáková

(22.12.1946 – 15.1.2024)

CONTRIBUTIONS

This thesis highlights original research done by the author. Some aspects of this research stemmed from work done by other scientist; therefore, their contribution is outlined below.

Chapter 2 describes the methodology used for experimental work which was already established at the Quadram Institute prior to commencement of this project.

- Dr Priscilla Day-Walsh and Dr Emad Shehata optimised methods for the *in-vitro* fermentation of methylated amines (4) and polyphenol compounds using the batch colon model system that was used for experimental work described in Chapter 3 and Chapter 4.
- Dr Shikha Saha and Dr Priscilla Day-Walsh developed a method for quantification of methylated amines in urine, plasma, and fermentation cultures (4, 5) which was used in all chapters of this thesis.
- Dr Emad Shehata developed the methodology for analysis of phenolic compounds in colonic fermentation cultures of anthocyanin degradation (6) and helped the author expand the methodology to qualitatively assess the breakdown of other polyphenols which forms a section of Chapter 5.
- Dr Fred Warren and Dr Hannah Harris together with Dr Jennifer Ahn-Jarvis optimised the *in-vitro* batch-batch colon model system (7, 8) that was used for experimental work in Chapter 4 and Chapter 5.
- Bacterial strains used for the development of the single-strain model of TMA-producing bacteria were obtained from the Narbad group culture collection and were isolated by Dr Lizbeth Sayavedra (*Desulfovibrio desulfuricans* QI0028) published in 2021 (9) and Dr Fatma Cebeci (*Citrobacter freundii* FC50) published in her thesis (10).
- Majority of experimental work was conducted using samples from the BERI study. The contributions to the study were as follows, quoted from doctoral thesis of Dr Jasmine Percival (11): "*Contributions to the human study were as follows. Dr Paul Kroon, Wendy Hollands and Dr Hassan Aboufarrag conceptualised the study and organised the trial. The recruitment process was managed by Wendy Hollands. Participant study days were managed by Wendy Hollands and Dr Hassan Aboufarrag. Participant health screening and blood samples were taken by Aliceon Blair. Encapsulation of bilberry and black rice extracts was carried out by Wendy Hollands and Natalia Perez. Data was collected by Dr Hassan Aboufarrag, Dr Priscilla Day-Walsh, Mark Philo and Dr Jasmine Percival.*" (3, 11, 12). The study period was from November 2017 to September 2018. The study was conducted in the Human Nutrition Unit at the Quadram Institute

Bioscience (QIB), Norwich, UK and all procedures were approved by both the QIB Human Research Governance Committee and the West Midlands – South Birmingham Research Ethics Committee (ref no: 17/WM/0154). Each participant gave written informed consent prior to taking part in the study. The study was registered with clinicaltrials.gov (Ref: NCT03213288).

Chapter 3 investigates human urine and plasma samples from the BERI study for their levels of methylated amines and their relationship with the faecal microbiota's form and function.

- Plasma and urine samples were quantified by Dr Priscilla Day-Walsh for their methylated amine levels and compiled in a dataset that was then further characterised and analysed by the author for correlations with individual's capacity to produce TMA.
- Faecal samples from the BERI study were used for preparation of glycerol stocks by Dr Jasmine Percival which were investigated for their ability to produce TMA from choline in the *in-vitro* colon model. This experimental work, sample processing, data analysis and interpretation was done by the author.
- Faecal samples from the BERI study were also used for DNA extraction, library preparation and submission for shotgun metagenomics sequencing by Dr Jasmine Percival. Further analysis of the faecal DNA for abundance of major archaea was performed by Dr Nicholas Morley. Samples were sent to Novogene for sequencing, and metagenomics analysis was performed by Dr Perla Rey with Dr Jasmine Percival. Further analysis of the dataset for presence of pathways associated with methylated amine metabolism, data visualisation and correlations with *in-vivo* and *in-vitro* metabolomics was performed by the author.

Chapter 4 investigates the effect of probiotics on TMA production from choline by the gut microbiota using *in-vitro* colon models and interactions of potential probiotic strains with TMA-producing bacteria.

- The CASE partner and partial funder of the research, ADM Protexin, had no role in the design, collection, management, analysis, and interpretation of the data in this chapter. Dr Richard Day, MBBS and other members of the Medical Affairs team at ADM Protexin provided feedback on the experimental design of work conducted during a remote placement with the partner. ADM Protexin provided a random subsection of freeze-dried probiotic strains that form the Bio-Kult probiotic supplement. Bio-Kult supplement used for *in-vitro* colon models was purchased from a high-street retailer by the author.

- *In-vitro* fermentations were performed using BERI study glycerol stocks due to restrictions on usage of fresh faecal samples due to COVID-19. Glycerol stocks from faecal samples obtained from participants recruited onto the QIB Colon Model study were collected prior to March 2020. The study was approved by the Quadram Institute Bioscience Human Research Governance Committee (IFR01/2015), and London-Westminster Research Ethics Committee (15/LO/2169). The informed consent of all participating subjects was obtained, and the trial is registered at http://www.clinicaltrials.gov (NCT02653001).
- All experimental work, data collection, analysis, visualisation, and interpretation were done by the author under supervision from Dr Paul Kroon, Professor Arjan Narbad and Dr Priscilla Day-Walsh.

Chapter 5 describes screening of polyphenols for their effect on TMA production from choline by the gut microbiota using *in-vitro* colon models and interaction of selected polyphenols with TMA-producing bacteria.

- Experimental design, data collection, analysis, visualisation, and interpretation of polyphenol effect on TMA production was completed by the author.
- Quantification of polyphenol metabolites in fermentation cultures was carried out under guidance of Dr Mariam Gamal-El-Din and Dr Emad Shehata using methodology developed by Dr Emad Shehata (6, 13), however, the data analysis, visualisation and interpretation was performed by the author.

Chapter 1

General introduction

INTRODUCTION OUTLINE

The introduction section is split into two main parts. Firstly, part one is laying out the scientific background to aid the reader in understanding the production of bacterial metabolites from dietary precursors, conversion of TMA into its oxidised liver metabolite TMAO and the associations of this gut microbial metabolite with disease. Secondly, part two introduces the strategies for modulation of TMA production by the gut microbiota, sets out the project aims, objectives and hypotheses, highlighting the approaches taken to test the set hypotheses.

LITERATURE REVIEW

1.1 The gut microbiota in health and disease

The gut microbiota is formed by a diverse community of microorganisms that reside in the gastrointestinal (GI) tract of humans. This intricate ecosystem is formed by bacteria, archaea, fungi, viruses, and other microorganisms, playing a fundamental role in a multitude of physiological processes, granting its ability to impact human health and disease (14-20). So far, scientists have not reached a consensus about the structure and function of a healthy microbiota. However, the idea that the stability, balance and diversity of the microbial communities contributes to the homeostasis of the gut microbiota has been accepted as an important determinant of health (21-23). When a microbial community becomes unstable and imbalanced, this is referred to as dysbiosis (24-26), which has been associated with various pathologies, including cardiometabolic diseases (27-31). Microbial metabolites are the key players of the gut microbiota, influencing host physiology, metabolism, and immune function. The microbes in our gut possess a diverse plethora of genes encoding enzymes used for catabolism of carbohydrates, production of short-chain fatty acids, vitamin synthesis, protein metabolism and many other processes (16, 32, 33). The activity of the gut microbiota increases the host ability to harvest nutrients from food after initial digestion and absorption in the upper GI tract and improves bioavailability of potentially beneficial compounds that would be otherwise excreted without realising their health effects. Some gut metabolites have been associated with gut homeostasis and improved health outcomes such as short chain fatty acids (SCFAs), bile acids (BAs) and antimicrobial peptides. However, others have been recognised as markers of gut microbiota dysbiosis and potential contributory factors in the development of disease (27, 29, 34-36). One of these potentially harmful products of microbial metabolism is trimethylamine *N*-oxide (TMAO) which has been linked to cardiovascular disease (CVD) risk and has been reported as an important contributory factor in the development of atherosclerosis and hypertension, which are two major CVD risk factors (37).

1.2 The formation of TMAO

TMAO is an abundant methylated amine playing a number of different roles in the systems of various organisms, in the environment but also in human physiology (38). In humans, TMAO is formed in the liver by flavin-containing monooxygenases (FMOs) by oxidising a gut microbiota-derived metabolite called trimethylamine (TMA) (39). TMA is generated from dietary precursors by microorganisms residing in the gut that can produce enzymes able to degrade these substrates. Choline, phosphatidylcholine (PC), L-carnitine, betaine, and even dietary TMAO are some of the contributors to microbial TMA production. These compounds are present in a number of dietary sources, commonly found in animal products such as meat, fish, eggs and dairy (40) but also in some plant-based sources like legumes, cruciferous vegetables and in some dietary supplements, with both choline and betaine being considered essential nutrients (41). When digested and absorbed in the upper GI tract from their dietary sources, these compounds perform numerous functions in the human body, outlined in **Table 1-1**. If they are not absorbed into circulation from the small intestine (42-45), these compounds can be degraded in the colon by different enzymes produced by the gut microbiota. These enzymes and the metabolites that they produce from dietary precursors are displayed in **Figure 1-1.**

Table 1-1 Dietary sources of TMA precursors and their functions in the human body and in other organisms.

Substrate	Function	Sources
PC and choline (40)	Synthesis of phospholipids vital for cell membrane function, production of acetylcholine, early brain development (46)	Meat, poultry, fish, dairy products, eggs, cruciferous vegetables, soy beans, whole grains (47)
L-carnitine	Critical role in energy production and long-chain fatty acid transport (48), also used as a sport performance supplement	Beef, whey, fish (49)
Betaine (50)	Used in sport performance enhancement, and important for methyl group metabolism	Beetroot, spinach, whole grains
TMAO (51)	Osmolyte in fish, the function in mammals is unknown	Crustaceans, fish (high in sharks and rays)

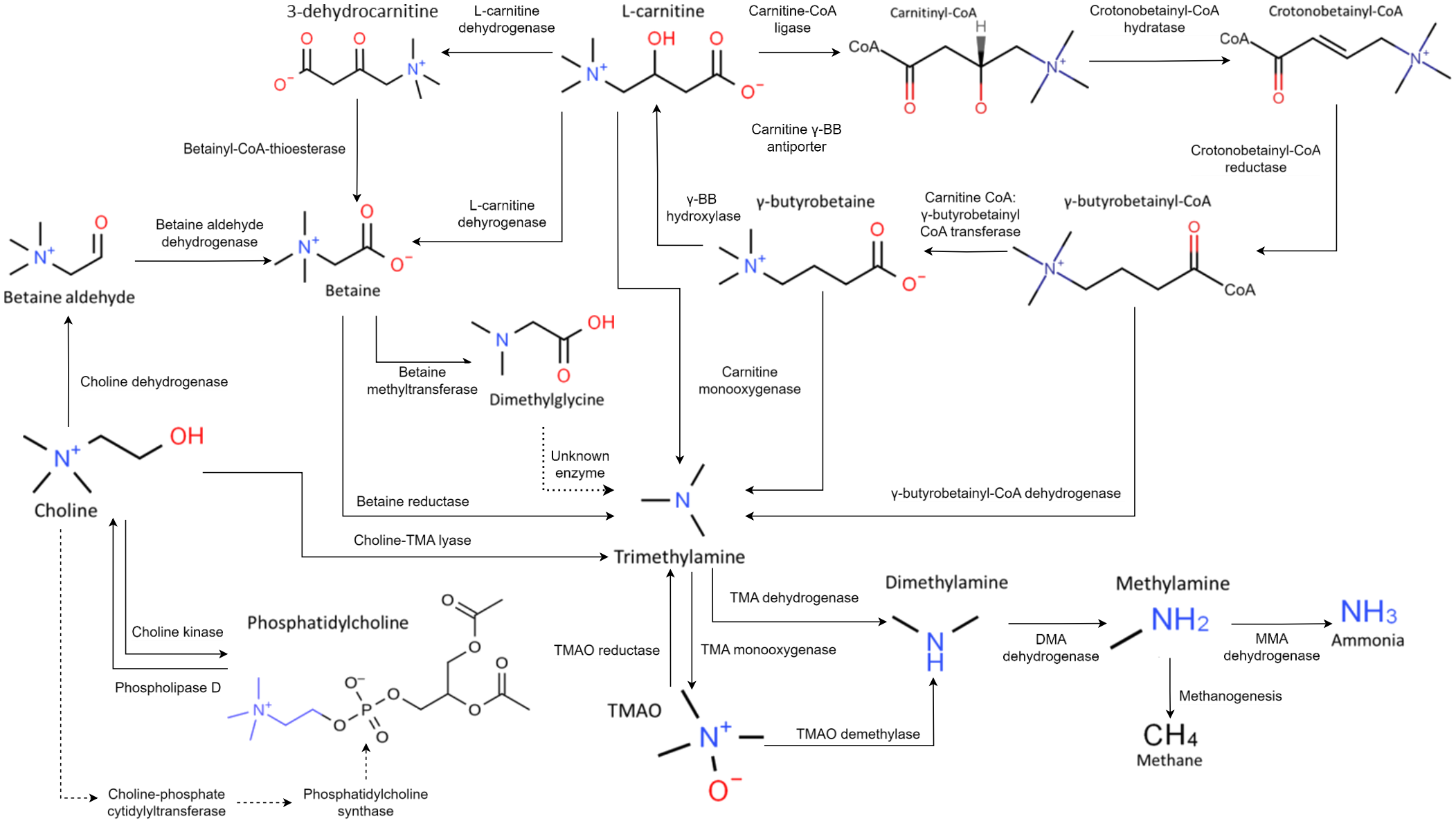

Figure 1-1 Microbial pathways contributing to TMA production from dietary precursors and TMA utilisation and degradation. Figure created based on MetaCyc pathways.

The role of the gut microbiota in TMA production became apparent in 2011 with Wang *et al.* (27) reporting an obligatory role of gut bacteria in metabolism of PC. Since then, multiple studies have been undertaken to investigate the main microbial contributors to TMA production and the metabolic pathways that result in substrate metabolism (2, 52-57). Human and animal studies brought more insight into the main families of bacteria that are involved in TMA/TMAO production. With the use of metabolomics and genome sequencing techniques, substrate metabolism was attributed to bacterial catabolism in the large intestine, which was quantifiable with gut metabolites and bacterial enzyme activity. Specifically for choline metabolism, Craciun and Balskus (56, 57) discovered choline utilisation (*cut*) gene cluster in *Desulfovibrio desulfuricans.* They identified a candidate gene for choline degradation by reporting activity of choline TMA-lyase involving a radical C-N cleavage, generating TMA and acetaldehyde (58). One of the genes of the cluster encoding this activity is *cutC* and its activator *cutD* which encodes a glycyl radical activating enzyme. The microbial metabolism of choline into TMA and TMAO is summarised in **Figure 1-2**.

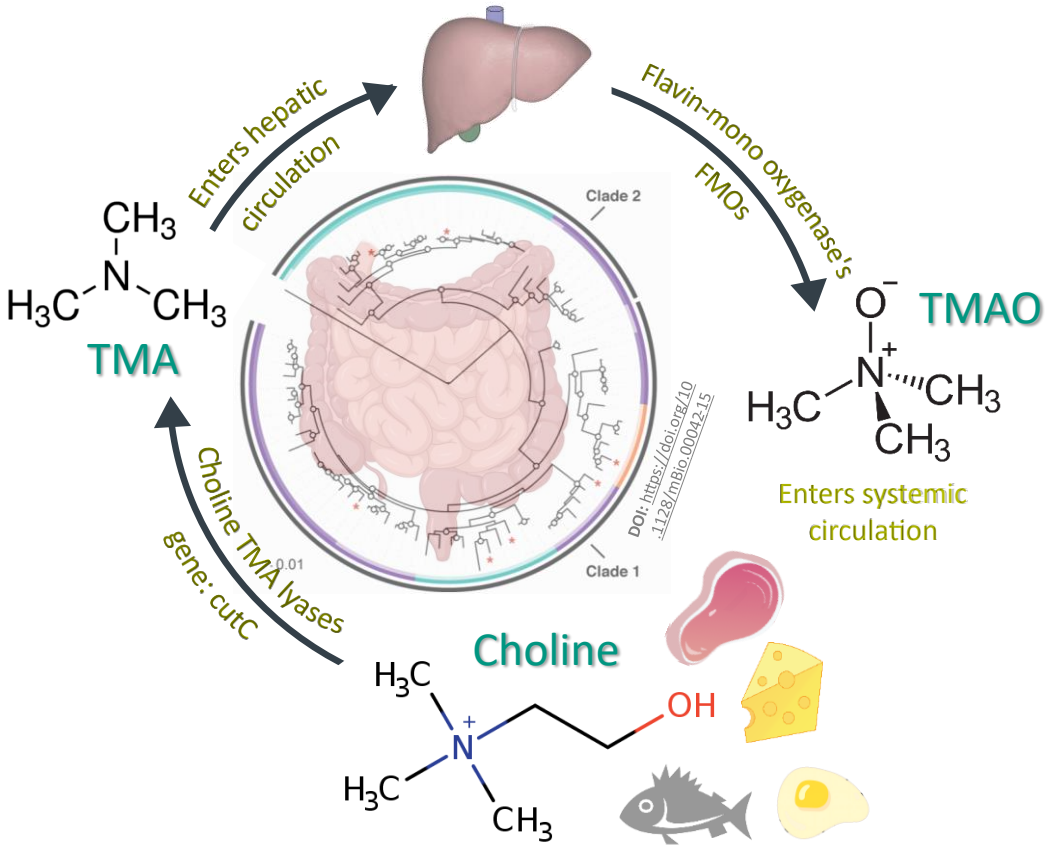

Figure 1-2 Overview of microbial metabolism of choline into TMA in the gut and its oxidation into TMAO in the liver. CutC phylogenetic tree in figure was taken from Martinez-del Campo et al, 2015 (2)

Previous publications reported that the organisation of the *cut* gene cluster has been proposed to play a role in TMA production but studies reported that the organisation is not always related to phylogeny. Different strains of the same species may not have the same ordering of the *cut* gene cluster or they may differ in the length of the *cutC* gene (59). This suggests that the choline utilisation cluster might be a strain-specific metabolic trait that was likely acquired by lateral gene transfer (59). This implies that phylogeny might be a poor predictor of microbial production of TMA and further insight into the metabolic potential of certain bacterial populations is needed. In later studies, bacteria that are able to produce choline-TMA lyases have been identified in human faeces and despite their relatively low abundance, this pathway is deemed to be quite ubiquitous within anaerobic bacteria residing in the human GI tract (60). In that study, Romano and colleagues isolated commensal bacteria from faeces and tested them for their ability to utilise choline *in-vitro* under anaerobic conditions. They identified nine strains of eight species representing two different phyla (previously referred to as Firmicutes and Proteobacteria) and six genera that showed significant choline consumption and TMA production: *Anaerococcus hydrogenalis, Clostridium asparagiforme, C. hathewayi, C. sporogenes, Escherichia fergusonii, Proteus penneri, Providencia rettgeri,* and *Edwardsiella tarda*.

Kalnins *et al.* (58) then further described the structure and function of the *cutC* choline-TMA lyase from *Klebsiella pneumoniae.* They reported that both *cutC* and its activator *cutD* are essential for the reaction. Craciun, Marks and Balskus (56) established that *cutC* is an exclusively choline-binding glycyl radical enzyme and is widely distributed across then known Firmicutes and Proteobacteria phyla.

Other genes encoding enzymes linked to substrate metabolism are the *cntA/cntB* pathway of L-carnitine catabolism, and *yeaW/yeaX* pathway reported by Koeth (61). They noted that the presence of choline, L-carnitine or γ-butyrobetaine can induce changes in the gut microbiota and increase proportions of *Allobaculum,* but decrease proportions of *Candidatus Arthromitus* or *Lachnospiraceae*, which were associated with both lower TMAO levels and an anti-thrombotic phenotype. Wu *et al.* (47) reported that individuals with regular consumption of carnitine (omnivores) had a higher TMAO production capacity than people with low habitual exposure to carnitine (vegetarians/vegans). However, they did not find an association of increased TMAO levels after oral carnitine challenge test with high Firmicutes/Bacteroidetes ratio, which has been previously reported in the literature as an indication of gut dysbiosis (49).

1.3 The relationship between elevated plasma TMAO levels and disease

Several studies found associations of elevated TMAO concentrations in plasma and multiple disease outcomes, such as cardiovascular disease (1, 44, 62, 63), chronic kidney disease (41, 64-66), inflammation (67-71) and neurodegenerative disorders (72, 73). Advancements in research on TMAO and its associations with disease have been dominated by the Cleveland Clinic research group, attributing the increased risk of CVD in cardiac patients to elevated plasma TMAO levels (27). Their group as well as other researchers described the underlying mechanisms of TMAO and disease and attributed its detrimental effects on health to promotion of atherosclerotic phenotype (27, 43, 44, 61, 74). TMAO played a role in altered lipid metabolism and bile acid pool size (75-78), platelet hyperreactivity and a thrombotic phenotype (79-82), together with endothelial dysfunction and inflammation (69-71, 83-86). **Figure 1-3** from a publication by Tang and Hazen, 2017 (1) illustrates some of the mechanisms and meta-organismal pathways of TMAO associations with disease.

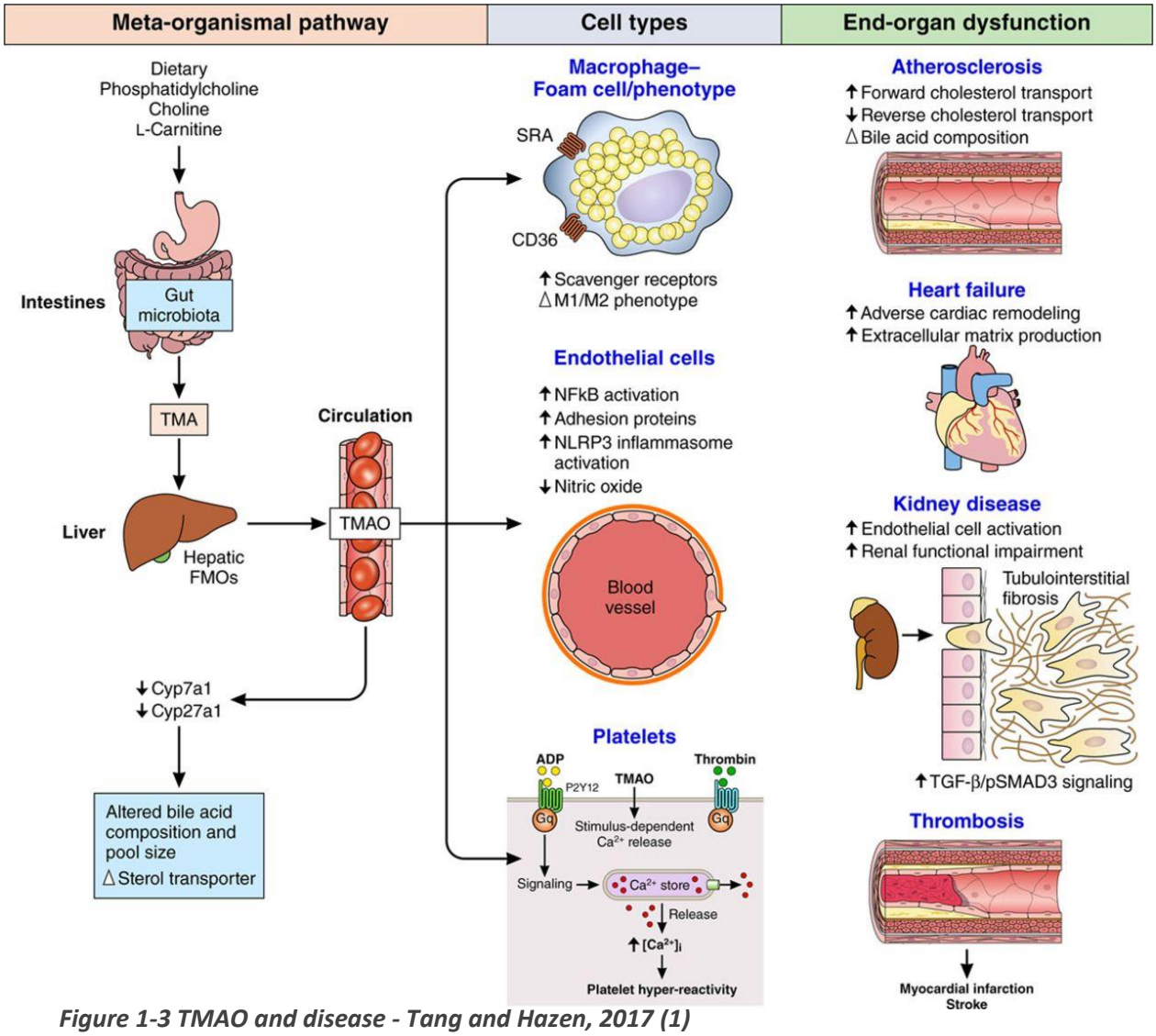

Figure 1-3 TMAO and disease - Tang and Hazen, 2017 (1)

1.3.1 Evidence from observational and prospective cohort studies

These findings led to investigations of the long-term effect of TMAO accumulation in tissues and plasma and the association with disease in prospective cohorts. Tang *et al.* (44) conducted a 3-year follow-up study of 4,000 patients undergoing elective coronary angiography. They examined the relationship between fasting plasma TMAO and major adverse cardiovascular events (MACE) such as heart failure, myocardial infarction, or stroke. In this cohort, patients in the highest quartile of plasma TMAO levels showed 2.5-fold increased risk of MACE compared to the patients in the lowest quartile. They also claim to predict the risk of incident MACE with higher plasma levels independent of the presence or extent of coronary artery disease and other conventional CVD risk factors.

Other studies reported an association of higher plasma concentrations of TMAO in coronary heart disease (CHD) cohorts compared to those with normal coronary arteries (87). Dong *et al*. (88) reported elevated plasma TMAO levels in patients with type 2 diabetes mellitus (T2DM), however, they claim that plasma TMAO was an independent predictor in CHD patients with or without T2DM. In a cohort of 275 CHD patients, Yu *et al.* (70) reported that urinary TMAO levels, but not its precursors (choline, carnitine, betaine), were linked to potential acceleration of development of CHD and its risk. Sheng *et al.* (89) claimed that elevated plasma TMAO levels were associated with higher coronary atherosclerotic load in patients with ST-segment elevation myocardial infarction (STEMI) compared to healthy controls.

More evidence from studies based in the US and Europe was collated by Heianza *et al.* (90) in their systematic review and meta-analysis of prospective studies assessing the risk of MACE and death in over 19,000 participants across 22 studies and 19 data points. The pooled relative risk (RR) of elevated plasma TMAO levels for the development of MACE when compared to low TMAO levels using a fixed-effect model was 1.62 (95% CI, 1.45, 1.80; P<0.001). When omitting data published by the same group, the RR was 1.73 (95% CI, 1.5, 2.00). From the available studies (n=9), they also established a dose response relationship of 1 µmol/L increment of plasma TMAO accounting for unadjusted pooled RR of 1.05 (95% CI, 1.01, 1.07). Their results also indicated that associations of elevated plasma TMAO levels with incidence of MACE and all-cause death were independent of conventional risk factors such as kidney dysfunction, T2DM and obesity. Moreover, they quantified the relationship of the TMAO precursor choline, L-carnitine and betaine and the risk of death or MACE. They found that elevated concentrations of the substrates resulted in 1.3-1.4 higher risk of MACE compared to individuals with lower concentrations. Further evidence supporting the associations between TMAO levels and incidence of disease is available elsewhere (91-94). There have been some further reports of correlations between CVD and plasma TMAO levels (95, 96) and further

research has been conducted into to the characterisation of the gut microbial patterns associated with cardiovascular disease (49, 97-101).

1.3.2 TMAO and atherosclerosis

The Cleveland Clinic research group has published several studies in high impact factor journals, suggesting an association between elevated concentration of plasma TMAO and atherosclerosis, a risk factor of CVD. They demonstrate that the gut microbiota is imperative to TMAO formation from dietary phosphatidylcholine (PC). In their animal study, mice that received broad spectrum antibiotics for 3 weeks prior to ingestion of isotopically labelled d9-PC showed no appearance of d9-TMAO in plasma. They elucidated these results by describing the pathways of TMAO production from PC. They then established a dose-dependent association of fasting plasma TMAO, choline and betaine levels and CVD phenotypes, including peripheral artery disease (PAD), coronary artery disease (CAD) and history of myocardial infarction (MI) in a large independent clinical cohort of subjects undergoing elective cardiac evaluations.

To explore this further, this group conducted a 20-week mice-feeding study to measure the atherosclerotic lesion growth on choline or TMAO supplemented diet. They found a positive relationship between plasma TMAO levels and aortic lesion area as well as a correlation between the liver enzyme flavin-monooxygenase 3 (FMO3) expression with both TMAO levels and aortic lesions. This suggests a role of FMO3 and other flavin-monooxygenase-expressing genes in promotion of atherogenesis. They investigated a proposed mechanism of atherogenesis by exploring the mRNA and surface protein levels of macrophage scavenger receptors connected to atherosclerosis – CD36 and SR-A1. These are indicators of endogenous formation of cholesterol-laden macrophage foam cells. In mice fed 1% choline diet compared to 0.1% choline diet, higher dose choline supplementation augmented the atherosclerosis at 20 weeks of age nearly three-fold compared to mice that were fed high-choline diet with antibiotics. This confirms the role of gut microbes in metabolism of phosphatidylcholine, subsequent TMA production, conversion to TMAO with FMOs in the liver, leading to atherosclerotic lesion formation in mice (27).

Geng *et al.* (84) supported this finding with mechanistic study of TMAO-induced atherosclerotic lesion formation via the CD36/MAPK/JNK pathway. However, Collins *et al.* (102) found that different TMAO concentration failed to increase aortic lesions in ApoE(-/-) mice. The Cleveland research group then published a confirmatory study of this animal model - the associations of acute egg yolk ingestion and the effect of oral antibiotics on plasma and urine TMAO levels (44). Other substrates have been investigated for their link to

atherosclerosis promotion by Koeth and colleagues who focused on the metabolism of L-carnitine by gut microbiota (43) and reported gamma-butyrobetaine as an intermediate metabolite from L-carnitine and other dietary sources (61). However, the mechanisms behind the atherosclerosis promotion needed further investigation and motivated many more studies in the upcoming years (98, 100, 103-106).

1.3.3 TMAO and platelet hyperreactivity

Another possible pathway of the associations with CVD is the role of TMAO in platelet hyperreactivity and thrombosis risk. Zhu and colleagues (79) compared germ-free mice (C57BL/6J) and their regular mice counterparts for their reaction to choline and TMAO supplemented diets. Conventional mice displayed increased thrombosis potential when fed choline (1% of chow) for 6 weeks, compared to germ-free mice on the same diet. They performed an *in-vivo* thrombosis assay that simulates a carotid artery injury model with $FeCl_3$ and measured time to cessation of blood flow. They found that choline-fed conventional mice accrued shorter time to cessation of blood flow, as well as higher percentage of platelet aggregation, and established a pro-thrombotic phenotype.

These were the first studies reporting a direct causative effect of TMAO on cardiovascular disease in animal model studies, but the true effect in humans was yet to be explored back then. Zhu *et al.* (82) observed the effect of daily choline supplementation (450 mg/day) for 2 months in healthy vegans/vegetarians compared to omnivores. They reported a 10-fold increase in plasma TMAO levels at 1 and 2 months in both groups that was strongly correlated with enhanced platelet aggregation responses to submaximal adenosine diphosphate (ADP, 5 μM) and demonstrated a dose-response of plasma TMAO levels and platelet function. However, it should be mentioned that they only measured TMAO levels once at each time point after an overnight fast without recording dietary intake prior to the test day.

1.3.4 TMAO and lipid metabolism

Researchers elsewhere explored other potential mechanisms and established the contribution of TMAO to lipid metabolism imbalance (76). Studies explored the relationship of FMO3 and its capacity to reduce reverse cholesterol transport, alter sterol metabolism and induce changes to bile acid (BA) composition and pool size. This way, the microbiota could also impact the cholesterol levels, and through the FMO3/TMAO pathway further contribute to the atherogenic role of the gut metabolite (43, 78). However, some consideration needs to be given to the lack of unified methodology across studies investigating the links between FMO3 expression, TMAO levels and atherogenic phenotypes, as that could create bias and mistake

associations with causations. Nevertheless, the high plasma TMAO levels seem to be predicted by a complex network of metabolic pathways with causal links to atherosclerosis (75).

1.3.5 TMAO and inflammation

Another pathway of TMAO contribution to atherosclerosis and other pathologies is through inflammation. Studies explored the mechanisms behind activation of NF-kappa B (NF-κB) pathway which was induced by elevated TMAO levels and resulted in the increase of inflammatory cytokines, chemokines, and other pro-inflammatory genes (69). It is thought that TMAO also triggers oxidative stress and activation of NLRP3 inflammasome, which is crucial for development of atherosclerosis as well as induction of endothelial dysfunction (49). This can lead to reduced endothelial cell repair, decreased circulating endothelial precursors and higher severity of cardiovascular events (86). This phenomenon has been further investigated to elucidate the potential mechanisms of the effect of TMAO on endothelial cell function and inflammation by others (68, 83, 107). Several detailed review articles and meta-analyses have been published, further clarifying the involvement of TMAO with disease (38, 49, 98, 108-116).

1.4 Measuring TMAO and establishing TMAO status

Despite the strong associations shown in all the evidence presented in this section, some limitations of these studies should be noted. Most cohorts and study participants were from a clinical setting and very limited data was available from the general population. Moreover, most studies were conducted with patients already at risk of CVD, therefore we cannot be certain of the translation of the results to a lower-risk population. Due to the character of observational studies, there can be a potential of residual confounding by unmeasured variables that were not adjusted for. Similarly, since the gut microbiota and TMAO levels are influenced by environmental factors, such as dietary intake, there is a need for detailed assessment of eating habits and consumption of substrates prior to test days to investigate the role of diet in the associations of gut metabolites and the measured outcomes (117-119). And finally, the studies were compared based on their own definition of ‘elevated’ TMAO levels and more standardised terminology and methodology needs to be established to allow easier assessment of the effects across different studies. In most studies, TMAO levels were only measured on one occasion (1 time-point) which might not reflect the true value and long-term representation of the plasma levels of the gut metabolite (49, 90). Most mentioned studies suggest the use of TMAO as a marker of CVD and a prognostic value of the incidence and severity of MACE, however, caution should be taken in extrapolating these finding to a wider population. There are many unanswered questions that still need to be considered. Is TMAO reliable enough to be used as a marker of disease? Is TMAO the main culprit of the gut-

metabolite/heart axis? Or is TMAO just a bystander that was caught in the crossfire as discussed by Cho *et al.* (120)?

The associations of TMAO and increased RR of MACE (stroke, MI or death) (44), acute coronary syndromes (121, 122), CAD, heart failure (62) and PAD (85) have led to the use of TMAO as both short and long-term predictor of these conditions and 5-year mortality risk. Furthermore, TMAO improved risk stratification for all-cause mortality or reinfarction at 6 months after MI (123). However, there are multiple studies that showed no association between TMAO and incident CV events, CHD and CVD (93, 124-126). According to these studies, TMAO levels increase with decreasing function of kidneys and have been associated with mortality in patients with chronic kidney disease (CKD). The influence of kidney function on plasma TMAO levels sparked an interest in many researchers as well as hesitation to classify TMAO as a marker of CVD due to its potential to be influenced by other factors.

1.4.1 Kidney disease as a confounding factor

Missailidis *et al.* (64) measured plasma TMAO in 179 CKD patients and compared them to 80 healthy controls. They reported that TMAO levels were strongly associated with the degree of renal function. Stubbs *et al.*, Mafune *et al.* and Bain *et al.* (127-129) all showed that TMAO levels were higher in patients with later stages of CKD and that there was a significant reduction (up to 60%) in plasma TMAO and TMA levels after a single haemodialysis session and that the levels of post-dialysis plasma TMAO were comparable to healthy controls. Moreover, successful kidney transplantation resulted in significant reductions of plasma TMAO concentrations (127). These associations are not surprising as TMAO is filtered from the blood by the kidneys. The association is attenuated when adjusted for age, sex and race, however, still remains mostly significant (130). Despite this evidence, it is unclear whether increased levels of TMAO are the main contributors to the nearly 2.8-fold increase in all-cause mortality risk in CKD patients, and if they directly cause damage in kidneys as suggested in a study by Tang *et al.* (65). This relationship of CKD, renal function and TMAO levels complicates the use of TMAO as a marker of CVD as renal function would have to also be tested to determine the true predictive effect of TMAO levels.

1.4.2 TMAO can be influenced by dietary intake

Several studies suggest that diet modulates the plasma levels of TMAO (131, 132). Plasma TMAO levels in humans increase after choline, betaine and L-carnitine ingestion as well as shortly after consumption (within 15 min) of TMAO and TMA rich products, suggesting that these do not need to be metabolised by the gut bacteria like the rest of the substrates. The disposal of TMAO occurs through urination, sweating and exhalation and an impairment of any

of these pathways may result in accumulation of TMAO in tissues and blood. As previously mentioned, some claim that the associations between TMAO and risk of CVD are a direct tie to the impact of diet on the incidence of disease (34).

For decades, CVD has been linked to various dietary components such as red meat, saturated fats, cholesterol and other substrates. So how can we be certain that TMAO is not just a bystander in the causative effects of diet on CVD and other factors contributing to increased risk of all-cause mortality? Moreover, TMAO precursors choline and betaine are also essential substrates for optimal functionality of physiological processes (50), as choline has many beneficial effects and is recommended in increased doses during pregnancy due to its impact on cognitive function of infants (46). TMAO is also present in fish in relatively high concentrations, but consumption of fish and adoption of the Mediterranean diet showed negative correlation with CVD risk which may imply that TMAO can also have some protective properties or be outweighed by the beneficial effects of fish consumption. Nevertheless, the impact of diet on TMAO levels is notable and contributes to the variation of plasma levels and potential hinderance of the use of TMAO as a reliable marker of disease (133, 134).

1.4.3 Variation in TMAO levels

Plasma TMAO levels exhibit vast inter- and intra-individual differences. They can be influenced acutely by diet as mentioned above, by the presence of other diseases (such as diabetes and CKD), by the level of FMO3 expression and hormonal changes (135). FMO3 is a flavin-containing monooxygenase active in the liver that rapidly converts TMA in blood into TMAO. The level of expression of this enzyme is also associated with differences in TMAO levels as reported in the prevalence of diseases (since it influences lipid metabolism and cholesterol transport) and seems to be a part of a complex diet-gene regulation-liver-microbiota homeostasis (41, 52, 77, 78, 119, 136). Furthermore, as opposed to plasma choline and betaine levels, TMAO was more strongly affected by variation overtime in a 1-year follow up study by Kuhn *et al.* (137). More literature related to TMAO as a marker of CVD and other disease is available in the following studies (31, 138, 139). Overall, the case for TMAO as a stable marker of disease should be further explored and all the potential confounding factors should be measured and accounted for when translating the results from observational studies.

1.4.4 Determination of TMAO status beyond assessment of TMAO levels

Rath *et al.* (140) investigated multiple strains containing *cutC/D* and *cntA/B* and their ability to produce TMA. They developed a gene-targeted assay to examine the TMA-producing potential of bacterial communities and to further characterise the composition of the functional

community in more detail. They screened over 67,000 genomes from the Pathosystems Resource Integration Center (PATRIC) and found 545 dereplicated protein sequences from 1107 genomes containing *cutC* genes. Most of these were from the phyla Pseudomonadota (specifically classes Gammaproteobacteria and what used to be classified as *Deltaproteobacteria*, now being a separate phylum Thermodesulfobacteriota) and Bacillota (Clostridia and Bacilli). These showed synteny with *cutD* (with a few exceptions) and contained the gene-characteristic conserved amino acid residues that were previously reported in the literature (2, 57). They discovered high diversities of the *cutC* genes when performing metagenomics analysis of faecal samples, however, majority of sequences were similar to genes of *Clostridium* XIVa or *Eubacterium* sp. AB3007, as discovered with their gene-targeted assay. The *cutC*/*D* genes were present in 71% of their samples, however only in small concentrations - on average, 0.11% of all bacterial communities. The observation of highly diverse genes might in the future drive the development of type-specific intervention strategies to target whole consortia rather than single taxa, to restrict specific members of the TMA-producing community.

1.5 Approaches for changing TMAO status

Based on these associations, scientists have attempted developing strategies to alter plasma TMAO levels through reduction of TMA produced in the gut with modulating the gut microbiota. An effective way of changing the gut microbial population is providing alternative substrates resulting in production of beneficial microbial metabolites such as SCFAs and anti-microbial peptides (141-144). This in turn could reduce the abundance of bacteria that use choline as their substrate (145, 146) or employ some other mechanisms resulting in lower TMA production. Modulating plasma levels of TMAO and identifying effective strategies has become an increasingly researched topic. There are number of interventions aiming to change the TMAO status – dietary restriction strategies, supplementation interventions or using pharmacological agents to modulate circulating TMAO levels in diseased population. A systematic review of 13 studies including dietary supplementation and pharmaceutical agents for their effect on TMAO reported that despite some successful attempts of decreasing TMAO with meldonium or metformin, majority of dietary supplementation strategies failed to elicit an effect (147). The review highlighted the need for placebo-controlled, crossover studies with baseline TMAO measures to determine the effectiveness of treatments, together with investigation of microbiota changes to understand the modes of action. In their review, they urge researchers to focus on three main strategies to change TMAO levels:

- Reducing consumption of TMAO precursors

- Blocking TMAO formation via TMA–lyase enzyme inhibition
- Suppressing production of TMA by modulating the gut microbiota

1.5.1 Dietary interventions reducing intake of TMAO precursors

Number of dietary interventions have been investigated for their feasibility to reduce plasma TMAO levels (131, 148-151), as an alternative to treatment by antibiotics as shown by Tang *et al.* (44, 74). There is a need for sustainable methods and long-term solutions to alleviate the burden of the adverse effects of TMAO. Considering that the main source of TMA-producing precursors are animal products (96, 152-155), a seemingly non-invasive method would be to limit dietary intake of meat, fish or dairy (17, 156). However, total elimination would not be recommended as these contain some vital compounds (such as choline) that are necessary for optimal function of the body (40, 41, 50, 54, 117, 157). Moreover, studies investigating the direct link between choline and TMAO rich diet did not always show a clear correlation between the consumption of substrates and increase in CVD incidence and other diseases (34, 75, 125, 131, 158). Therefore, the more likely culprit might be the TMA/TMAO themselves, hence more strategies for reduction of TMA without excluding its precursors are needed.

1.5.2 Inhibition of enzymes participating in substrate metabolism

Enzyme inhibitors can work in several ways - directly blocking the active site to prevent the binding of substrate, changing the affinity of the enzyme to take up substrate by altering pH or increasing abundance of other compounds that could be used instead of substrate. Altering the structure/function of the enzyme by denaturing proteins or affecting gene expression and translation are other possible strategies. The inhibition of TMA producing enzymes was a strategy adopted by Wang *et al.* (159) who were the first ones to demonstrate that a structural analogue of choline, 3,3-dimethyl-1-butanol (DMB) that is found in human diet, could effectively inhibit the production of TMA without being lethal to the bacteria. They reported that DMB was effective at targeting multiple substrates and being also compatible with *cntA/B* and *yeaW/X*. Later, they modified the DMB to target specifically the *cutC/cutD* pathway and fed a single bolus dose to mice to reduce TMAO levels, which was effective for up to 3 days (160). DMB has been further tested in animal models to reverse TMAO induced endothelial dysfunction (161). However, the efficacy, pharmacodynamics and safety of this product still need to be investigated in humans (162). These results suggest that targeting the gut microbial production of TMA via enzyme inhibition may be a potential therapeutic target (163, 164). Beyond structural analogues, other compounds have been identified to have an impact on enzyme inhibition, some of which are plant polyphenols and their bioactive compounds.

Plant polyphenols and bioactives

Plant polyphenols are secondary metabolites of plants that provide them with protection against pathogens, pigmentation for UV blocking and facilitate growth (165). Structurally, they have one or more hydroxyl groups attached to a benzene ring and are categorised based on the number of benzene rings and other structural properties. There is an extensive amount of literature focusing on the effects of plant polyphenols, their derivatives and conjugates on various disease outcomes such as cardiometabolic syndrome (166, 167), T2DM (150), inflammation (168) and cancer (144). To narrow down the available evidence, only polyphenols with a direct effect on TMAO production are discussed in this thesis, however, some useful resources discussing the general impact of polyphenols on the gut microbiota are available elsewhere (169-173). Some of the polyphenols most used for TMA regulation belong to the subclasses called anthocyanins, flavanols and stilbenes (174). Large proportion of these polyphenols escape small intestinal absorption and are fermented in the large intestine by the gut microbiota (142). There, they can act as promoters of bacterial growth or inhibitors of the pathogenic effects of other bacterial groups. Moreover, some polyphenols have been linked to anti-inflammatory properties and reduction in the secretion of TNF-α, IL-1b and IL-6, that also play role in cardiovascular disease (175, 176). Resveratrol, a member of the stilbenes group, was effective at remodelling the gut microbiota in ApoE-/- mice and inhibiting TMA production (177). Grape pomace polyphenol extract, also containing resveratrol, reduced TMAO serum levels in a randomised placebo-controlled crossover study, suggesting that it could be an effective nutraceutical formulation for attenuating the effects of TMAO (178). However, this study did not determine whether the act of TMAO reduction was due to gut microbiota modulation or enzyme inhibition. More research needs to focus on the mechanisms behind this effect.

Studies investigating the use of polyphenol-rich food sources and their individual compound components showed that there are differences in TMA-lowering response based on the diet of the donors of faecal samples used in *in-vitro* fermentation, considering if they are omnivorous or vegetarian (96). For instance, Bresciani *et al.* (179) showed that during a 24-h incubation of choline in vegetarian faecal starter together with blonde grapefruit juice and pink grapefruit juice, TMA concentration was reduced by 91-94% compared to the non-supplemented control. When using omnivorous faecal starter, TMA was reduced by 57-64%. For other fruit juices, opposite effect was found, where omnivorous faecal starters elicited higher reduction of TMA (79% and 91%, respectively) when pomegranate and blood orange juice were used, respectively, compared to no reduction of TMA in vegetarian faecal starter.

Their study then explored the most abundant compounds found in orange juice such as hesperidin, narirutin and ferulic acid, as well as cyanidin-3-glucoside found in blood oranges. They reported no effect of any of the polyphenolic compounds tested on choline degradation into TMA by 24 hours of incubation, attributing the main effect of the fruit juices to their carbohydrate content. They tested different sugar doses (mixture of sucrose, fructose, and glucose) and found a TMA reduction between 83-92% in vegetarian faecal starters and highly variable reaction in omnivorous faecal starters, between 10-74%. They later discussed that the differences in the reduction of TMA concentration in omnivorous faecal starters but not vegetarian ones with fruit juices might be influenced by the microbial composition and the pH differences of faecal samples. They attributed some of the reduction in TMA with blood orange and pomegranate juices to the colonic catabolism of their polyphenol compounds such as anthocyanins, that are often converted into phenolic acids. Phenolic acids have been previously linked to antimicrobial activity which could be a possible mechanism of action. However, the fact that the effects of fruit juices were not translated when individual components were used points towards favourable metabolic pathways where carbohydrates are utilised instead of choline, rather than any real effect of the phenolic components.

A comprehensive review about the use of dietary phytochemicals for the inhibition of TMAO was published by Iglesias-Carres *et al.* (180). They summarised a big portion of the available literature connected to the health associations of plasma and urinary TMAO and TMA from multiple study designs from observational studies to clinical trials and dietary intervention studies. Another major review article (181) was published in 2021 summarising the evidence of the use of phytochemicals and bioactive compounds on TMAO production. From these reviews, it has become apparent that most studies used whole foods, powders, PP extracts and mixtures to decrease TMAO production or elicit cardioprotective effects. This approach does not elucidate which polyphenol compounds in these ingredients, mixtures and extracts could be responsible for the reported effect.

There is a gap in evidence supporting the notion that some types of polyphenols would be more likely to inhibit TMA production than others. This is due to studies testing various ingredients that contain multiple classes of polyphenols as well as studies not being reproduced in different models or by multiple research groups. For instance, resveratrol is a reported inhibitor of TMAO production in animal studies and in clinical setting (177, 182). However, other compounds also originating from grapes have not been explored to the same extent.

From these two reviews and other published literature, changes to TMAO levels are proposed due to two main mechanisms that can work alone or in synergy. Firstly, TMAO levels decrease

with addition of phytochemical treatment due to inhibition of choline TMA-lyases and other enzymes produced by gut bacteria. Secondly, TMAO levels decrease with phytochemical supplementation due to modulation of gut microbiota signatures harbouring abundance of TMA-producing bacteria.

1.5.3 Change of gut microbiota composition

The involvement of gut microbiome in a vast number of metabolic processes has been extensively studied (33, 35, 183-186). Some of the physiological and systemic processes include energy metabolism and the impact of gut microbiota on obesity, type 2 diabetes and cardiometabolic disorders (187). Gut microbiota can be modulated by dietary composition and eating habits, contributing to the development, management, or prevention of non-communicable diseases. Despite rapid modification of gut microbiota within 24 h of dietary interventions, a longer-term feeding studies failed to show substantial changes to the compositional features of gut microbiota (188, 189). Furthermore, dietary interventions are notoriously difficult to control, and often, the substitution or addition of dietary components influence a multitude of systemic changes, complicating the investigation of mechanism behind the intervention effect (187, 190). Nevertheless, there are multiple strategies that could impact gut microbiota composition, and with that improve the TMAO status.

Firstly, an increase in proportions of beneficial gut bacteria (such as *Bifidobacteria* and *Lactobacillus* genera) to offset the detrimental effects of TMA/TMAO (30). This could be achieved through supplementation with probiotics lacking TMA-lyase encoding genes. Secondly, increasing the abundance of TMA metabolising bacteria and archaea to minimise the amount of TMA available for conversion to TMAO could be another possible approach (191-194). Thirdly, decreasing the abundance of the TMA-producing bacteria with bioactive compounds derived from plant polyphenols and other sources could result in lower TMA production (147). And last but not least, providing prebiotics for production of beneficial metabolites like SCFAs produced from catabolism of fibre have been linked to decreased incidence of disease and cardiovascular disease prevention (25, 49, 52). To understand these strategies further, their use in diseased population and modelling their effects in pre-clinical studies are discussed in more detail.

Probiotics

Probiotics can be defined as ‘live microorganisms that, when administered in adequate amounts, confer a health benefit to the host’ – Hill *et al.* 2014, p506 (195). To align with this definition, microorganisms must be alive in an adequate number when administered and strains must be genetically identified, classified, and denoted by numbers, letters, or names.

The probiotic effect of the strains should also be supported by studies performed on the intended recipient host. Strains showing benefit for one condition may not be a probiotic for another condition. Therefore, only tested strains in the target host may be referred to as probiotics as per the scientific definition (195). However, numerous organisms with a potential therapeutic effect have been discovered, with some exhibiting immunomodulatory activity, modulation of the gut-liver or gut-brain axis, inhibition of pathogenic microbes of the gut, urogenital tract, and skin. The use of probiotics for altering the gut microbiota composition has been previously explored in animal models and human subjects in relation to prevention of disease (196-198). However, the actual health benefits of probiotics have not been supported by any approved health claims to date as majority of studies have been underpowered or not successfully reproduced. Nevertheless, scientists have tested the effectiveness of probiotic supplements to offset the detrimental effects of cardiovascular disease (103, 199), through improving blood pressure, blood glycaemic response and lipid profiles.

Lactobacillus plantarum 299v supplemented to men with stable coronary artery disease reduced inflammation, oxidative stress and improved lipid profiles despite TMAO levels remaining unchanged (200). Qiu *et al.* (201) experimented with single strain supplementation in mice to offset choline-induced TMA production in the cecum, with *Lactobacillus plantarum* ZDY04 and later with *Enterobacter aerogenes* ZDY01 (202). In rats fed high-fat diet, *Enterococcus faecium* WEFA23 decreased TMAO production and improved the diversity of the gut microbiota (66, 118). There is only a handful of studies with human subjects that investigate the interactions of TMAO and probiotics (203, 204). Tenore *et al.* (205) demonstrated that Lactofermented Annurca apple puree effectively controlled plasma TMAO levels and other CVD risk factors, however, functional foods potentially operate by different mechanisms than single strains or probiotic mixtures. Boutagy *et al.* (158) showed that a multi-strain probiotic VSL #3 did not manage to lower plasma TMAO levels in subjects on a high-fat diet, however these subjects were healthy and prior to randomisation to the high-fat diet their TMAO levels were very low. In general, the application of probiotic supplementation in healthy population remains poorly understood (188, 189, 206, 207). Sohouli and colleagues (208) reviewed the effects of probiotic supplementation on TMAO levels in clinical trials. They identified 8 interventions with 270 participants reporting that probiotic supplementation has no significant effect on TMAO levels compared to the control group (weighted mean difference (WMD) − 0.08 μmol/L; 95% CI − 0.76 to 0.60, P = 0.813).

Another systematic review reported that the probiotic effect on TMAO levels is strain-specific and only one probiotic strain, *Lactobacillus rhamnosus* GG, showed an effect both in animal models and human studies (209). Another systematic review and meta-analysis reported no

effect of microbiota-driven treatments (probiotics, prebiotics and symbiotics) on TMAO levels (210). However, all of these studies claim that the small sample size posed a substantial limitation of the observations (190, 211), together with other shortcomings of probiotic studies like their clinical efficacy compared to lasting gut colonisation and the different mechanisms of activity. Others argue that the effectiveness of probiotics in modulating the gut microbiota is dependent on the existing basal microbiome prior to supplementation (212). Despite limited evidence supporting the persistence of supplemented probiotics in the GI tract of human subjects, probiotics have conferred a myriad of beneficial effects through improving clinical outcomes in diseased populations. The mechanisms behind this effect and the possible impact in healthy subjects need to be further explored and more focused analysis of the mechanistic actions of individual strains or mixtures and their capacity to alter TMA production is necessary. Exploring the interactions of probiotics and the gut microbiota in a controlled environment of simulated human colon enables investigation of interplay between dietary components, microorganisms from the host and probiotic supplements as has been demonstrated in several studies (213-217).

Prebiotics, symbiotic and postbiotics

Both probiotics and polyphenols produce metabolites that can play a role in host health. Whether that would be through directly affecting the systemic processes in the host or acting as an endocrine organ and maintaining the homeostasis in the gut (215). They can also work together to alter the gut microbiota composition with inhibition of certain genera and promotion of others. These complex interactions give rise to changes in microbial diversity and promote production of SCFAs, which have been linked to anti-atherogenic effects (218) and other health benefits. SCFAs are products of colonic fermentation of various substrates, such as polyphenols or non-digestible carbohydrates. In plant-based food, phenolic compounds and dietary (and other types of) fibre are closely interlinked and can be classified as prebiotics – ‘nutrients or dietary constituents of nonmicrobial composition that provide a growth advantage of beneficial bacteria’ (219). Prebiotics have been previously effective in shifting the Bacteroidetes to Firmicutes ratio in insulin resistant mice (122, 220) as well as normalising *Akkermansia muciniphila* which has been associated with control of HF diet-induced metabolic disorders (141, 221). Common prebiotics such as fructans, galactooligosaccharides (GOS), fructooligosaccharides (FOS), resistant starch and β-glucans have been explored for their ability to increase *Bifidobacteria* in the human gut microbiota.

Many plant polyphenols found in fruits, vegetables, cocoa, teas and other plant-based foods can also fall into the category of prebiotics. Some have been linked to improvement of digestion, brain function, and blood sugar levels, as well as protection against blood clots,

heart disease, and certain cancers (140, 143, 178, 179). They can also produce compounds with anti-microbial properties that can act in the gut and decrease abundance of undesirable bacteria and with that reduce the risk of disease (181). For instance, anthocyanins have been used to modulate the gut microbiota and contribute to proliferation of some beneficial bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. (86) and simultaneously inhibit some potentially harmful bacteria like *Clostridium histolyticum*. Combining probiotics and prebiotics in the form of polyphenols and fibres might serve as an effective therapeutic approach for reducing TMA production (222).

Other gut microbiota mediated approaches - archaea, phage and FMT

Majority of publications that focused on the TMA/TMAO pathway and how to reduce their production have explored the bacterial interactions with the substrates in detail. However, other components of the gut microbiota should not be overlooked. Methanogenic archaea have been proposed as an alternative approach to reducing TMAO production since they utilise TMA produced in the gut as substrate for methane generation (223). These methanogens are present in the human gut, however, often in small proportions and not in all individuals. Methane producing individuals often harbour *Methanobrevibacter smithii* or the less common *Methanosphaera stadtmanae.* Three unusual methanogens were discovered in 2008 - *Methanomassiliicoccus luminyensis, Candidatus Methanomethylophilus alvus* and *Candidatus Methanomassiliicoccus intestinalis* – that can also effectively reduce TMA. This finding led to a proposal to use these pure archaeal cultures as 'archaebiotics' (224). Of course, there are many challenges of this approach – how to administer these oxygen-sensitive microorganisms, what is the optimal dose to achieve TMA depletion and will this approach translate to changes in TMAO levels, just to name a few. However, it is a promising, potentially sustainable way to reduce TMAO production *in-vivo* and should be further explored (193, 194).

Moissl-Eichinger and colleagues (225) who piloted a more effective method of DNA extraction for archaea were able to detect their diversity in the human body. These findings could potentially explain intra- and inter-individual differences in TMA production that cannot be attributed to bacterial signatures. Similarly, bacteriophage are somewhat less explored members of the microbiota capable of modulating the gut microbial composition (226). The use of bacteriophage as an alternative approach to pre- and probiotic supplementation to reduce TMA production has not been well explored. However, it could be a potential substitution for antibiotic treatment of specific species of bacteria producing TMA. A more widely researched strategy for changing the gut microbiota is faecal microbiota transplant (FMT). This technique has been successfully used for improving clinical outcomes in patients

infected with *C. difficile* (103, 227) and has a potential to impact the gut microbiota composition with persistent effects.

PROJECT OBJECTIVES AND APPROACHES

From the presented evidence, there is a clear gap in the published literature pointing towards a lack of a sustainable strategy for reducing TMA production from dietary precursors. Experimental work needs to be carried out to find an effective way of modulating the gut microbiota or inhibiting the enzymes responsible for TMA production. Due to the complexity of the gut microbiota interactions with the host, some of these principles should first be investigated *in-vitro*, to limit the number of confounding variables in order to understand the underlying mechanisms. Combining multiple techniques such as metabolomics, microbiology and molecular biology with *in-vitro* fermentation assays, a dietary strategy to reduce TMA production can be established.

Therefore, the overall aim is to investigate the relationships between TMAO levels *in-vivo*, capacity to produce TMA *in-vitro*, how these relate to the structure and function of the gut microbiota, and how they can be altered with dietary strategies like polyphenols and probiotics. With that, the main hypothesis is that *in-vivo* TMAO status and *in-vitro* capacity to produce TMA from choline are correlated and dependent on the gut microbiota structure and function. Furthermore, the hypothesis is that *in-vitro* capacity to produce TMA from choline can be modulated with probiotic and polyphenol supplementation.

To reach the main aim, the following objectives were set to answer the main questions of this thesis with proposed approaches.

Question 1 – How do TMAO levels in human participants relate to the capacity for TMA production from choline by the gut microbiota?

Objective 1: Investigate the relationship of plasma and urinary TMAO levels measured on multiple different occasions in human subjects with their ability to produce TMA determined through *in-vitro* fermentations of faecal samples supplemented with choline.

> **Approach**: Using available metabolomics data from the BERI cohort and determining TMAO status of each participant based on the six independent measures of plasma and urinary TMAO levels. Using glycerol stock faecal slurries from the BERI study for *in-vitro* fermentation experiments to establish outcome measures that would describe the *in-vitro* capacity to produce TMA from choline.

Objective 2: Determine the abundance of TMA-producing bacterial pathways in the human faecal samples.

Approach: Using a metagenomics dataset from the BERI human study for relative abundance of TMA-producing and TMA-utilising pathways for determining the abundance of TMA-producing enzymes in the KEGG orthology database, MetaCyc database, Pfam domains and taxonomic profile.

Objective 3: Relate metagenomics findings to plasma and urinary TMAO levels, together with the capacity to produce TMA from choline *in-vitro*.

Approach: Calculating correlations of *in-vivo*, *in-vitro* and *in-silico* analyses from the BERI cohort to determine relationships of these variables.

Question 2 – How can the ability of faecal microbiota to metabolise choline into TMA be changed with probiotic or polyphenol supplementation?

Objective 1: Test the effectiveness of Bio-Kult multi-strain probiotic supplement and its components to reduce TMA production from choline-supplemented faecal cultures.

Approach: Using individual and pooled faecal samples to investigate the effect of mixed probiotic or individual strains on choline metabolism and TMA production.

Objective 2: Screen polyphenols for their effect on inhibition/reduction of TMA production from choline

Approach: Identifying potentially effective compounds from published literature and investigating the impacts of structural properties on the polyphenol ability to inhibit TMA production

Question 3 – What are the mechanisms responsible for reducing TMA production from choline?

Objective 1: Investigate the possible modes of action by utilising a single-strain model of TMA-producing bacterium.

Approach: Using microbiology and molecular biology techniques to determine possible mechanism behind the effective compounds and/or probiotic supplementation.

THESIS STRUCTURE

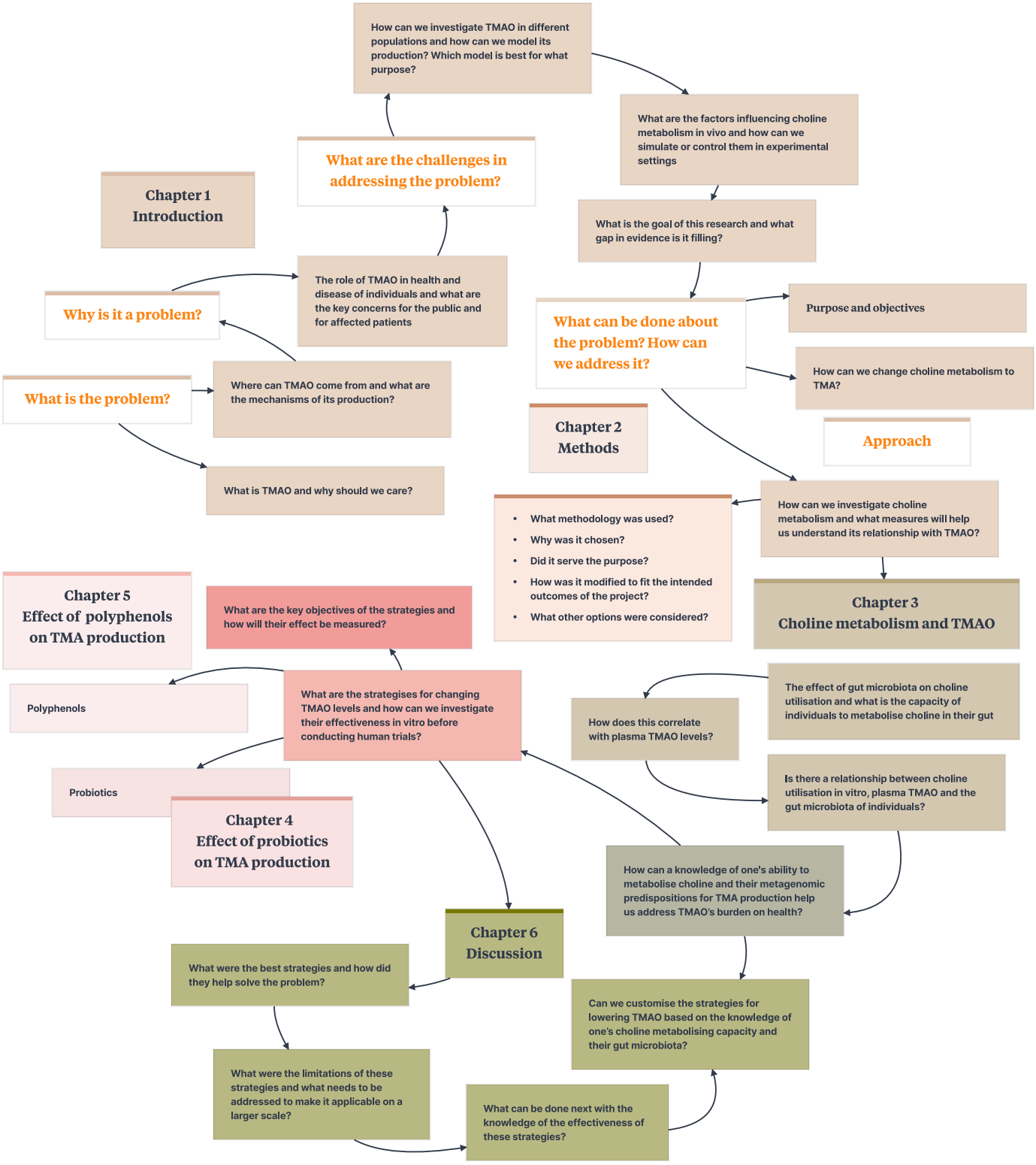

Figure 1-4 Thesis structure – mind map

Chapter 2

Materials and methods

THE USE OF *IN-VITRO* FERMENTATION MODELS TO INVESTIGATE MICROBIAL METABOLISM OF SUPPLEMENTED SUBSTRATES

In-vitro fermentation models play a crucial role in exploring microbial metabolism of various substrates in a controlled environment. These models can be supplemented with complex microbial communities from *ex-vivo* samples (GI tract contents, saliva, skin swabs, environmental samples, food samples and many others) or consortia of microbes characteristic of an environmental niche, animal, body site or diseased state. The fermentation models used in this thesis were predominantly chosen for their established methodology for fermentation of faecal samples from humans, and their use for simulating microbial metabolism in the colon.

2.1 Batch fermentation colon model - CMB

This method has an established operating procedure for investigation of batch culture bacterial metabolism and is used widely across the institute. The model colon was set up to mimic processes in the distal colon using 1% faecal inoculum in nutritive culture media, incubated for 24-48 hours under continuous nitrogen supply to maintain anaerobic atmosphere and liquid addition of acid and base to maintain pH within the range of 6.6-7.0, representative of the distal colon. All media were pre-reduced in the anaerobic cabinet for a minimum of 24 hours up to 48 hours and held at 37 °C to be ready on the day of inoculation if not sparged with nitrogen.

2.1.1 Media preparation

Nutritive culture medium was prepared according to the instructions in **Table 2-1** and as described by Day-Walsh and colleagues (4). Deionised water was mixed using a magnetic stirrer plate and a flea during addition of compounds listed in **Table 2-1**, topped up to 1 L and then autoclaved. This medium could be stored at 4°C for up to 2 weeks. Before using the medium for *in-vitro* fermentations, anhydrous D-glucose (10 g) was gradually added to 50 mL of medium aliquoted in a glass beaker with a magnetic stirrer flea and mixed until fully dissolved. Glucose enriched medium solution was filtered through a 0.45 µm syringe filter into the autoclaved media flask under laminal flow in Class II microbiological safety cabinet, capped and mixed with a magnetic stirrer.

Table 2-1 Composition of CMB Nutritive medium with notes for preparation

Compound	Amount	Unit	Notes for preparation
peptone water	2	g	Wait until fully dissolved
yeast extract	2	g	Granules can get ‘stuck’ in the vortex – wait until fully

			dissolved
$NaHCO_3$	2	g	
NaCl	0.1	g	Use balance with up to 4 decimal places of a gram
K_2HPO_4	0.04	g	Use small weighing boat, de-ionisation function balance
KH_2PO_4	0.04	g	Use de-ionisation function balance
$MgSO_4 \cdot 7H_2O$	0.01	g	Rinse weighing boat with prepared medium to avoid loss
$CaCl_2 \cdot 6H_2O$	0.01	g	Use new weighing boat to avoid sticking
Tween80	2	mL	Very viscous, aliquot with a serological pipette and rinse with prepared medium
vitamin K	10	µL	Prepare 1:1 solution with 99% ethanol for solubility - then add 20 µL of the mixture and rinse pipette tip
L-cysteine·HCl	0.5	g	Weigh using ventilated balance station
bile salts	0.5	g	
hemin	0.02	g	Add after dissolving in a few drops of 0.5 M NaOH in an Eppendorf tube

A portion of the medium was supplemented with 1 M choline solution (dissolved in ultrapure water and filter sterilised) to make 10 mM choline enriched medium (2 mL of 1 M choline added to 198 mL of medium). This was an alternative to addition of 1 M choline solution (200 µL per 100 mL vessel) dissolved in PBS directly into colon model vessels with a sterile pipette tip, possibly contaminating the opening of the glass vessel with the body of the pipette or running the risk of the small volume not being added directly to the vessel and compromising the starting concentration of supplemented choline.

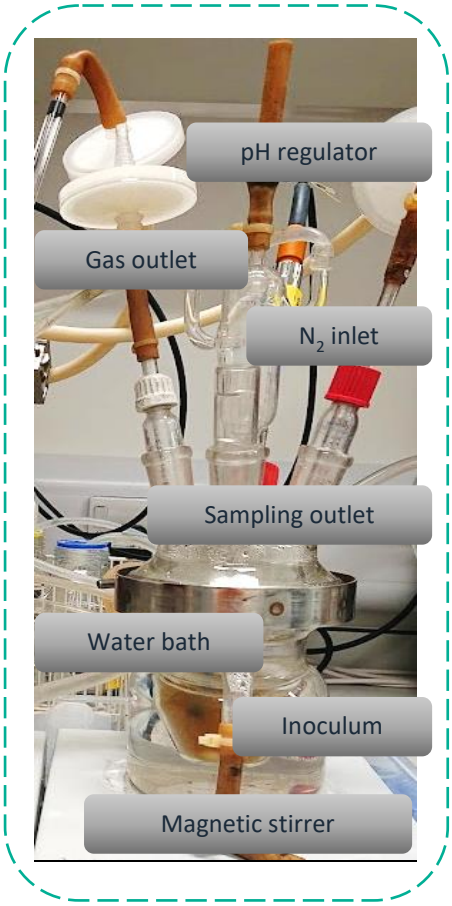

Figure 2-1 Colon model glass vessel

2.1.2 Vessel set-up

Glass vessels for model colon (100 mL or 300 mL) were set up as displayed in **Figure 2-1**. All outlets and inlets were covered with aluminium foil together with the reusable filters and vessels were autoclaved and dried prior to use (usually for multiple days in the drying ovens). One day prior to inoculation, vessels were connected to a water bath system to prepare for incubating cultures at 37 °C. Sterile medium was transferred into vessels via sampling inlet (using a sterile measuring cylinder), with the volume dependent on vessel size and volume of other compounds added to inoculum.

Medium was bubbled with oxygen-free N_2 via the filtered inlet reaching the culture level and mixed with magnetic

stirrers to establish an anaerobic environment overnight or for at least 8 hours. On the day of inoculation, pH probes were introduced to the vessels and the medium was adjusted to pH between 6.6-7.0 using Fermac 260 pH control units connected to 0.5 M NaOH and 0.5 M HCl. The pH unit was calibrated and primed using standard solutions of pH 4.0 and pH 7.0 for calibrating pH meters before inoculation and units were set to the minimum drip volume for pH adjustment.

2.1.3 Inoculum preparation from the BERI study samples

Preparation of glycerol stocks from faecal samples acquired as part of the BERI study is described in detail in section 2.23. Prior to inoculation, glycerol stock tubes were thawed inside an anaerobic cabinet at 37°C for at least 1 hour to revive frozen cultures.

2.1.4 Acquisition of faecal samples from the QIB Colon Model Study

Some faecal samples used for initial optimisation of methodology and for establishing usability of colon model for fermentation of substrates using frozen glycerol stocks were obtained from participants enrolled in the QIB Colon Model Study. To be eligible, study participants had to be aged 18 or over, live or work within 10 miles of the Norwich Research Park, have a normal bowel habit including bowel movement between three time as day to three times a week, an average stool type of 3–5 on the Bristol Stool Chart, and no diagnosed chronic gastrointestinal health problems such as irritable bowel syndrome, inflammatory bowel disease, or coeliac disease. Enrolled participants were asked to confirm prior to stool donation that they did not ingest antibiotics for at least 4 weeks before giving faecal sample, were not currently pregnant or breast-feeding, had not recently had an operation requiring a general anaesthetic, were not taking multivitamin supplements, did not experience a gastrointestinal complaint such as vomiting or diarrhoea within the last 72 h or did not test positive COVID-19 using lateral flow test (last point applicable to participants donating samples after 2020). The study was approved by the QIB Human Research Governance committee (IFR01/2015), and London-Westminster Research Ethics Committee (15/LO/2169). The informed consent of all participating subjects was obtained, and the trial is registered at http://www.clinicaltrials.gov (NCT02653001).

2.1.5 Fresh faecal inoculum preparation

Fresh faecal samples were deposited by donors at the Quadram Institute Clinical Research Facility and were collected promptly on the day of the experiment. A portion of sample was weighed into a double-layer mesh-filter stomacher bag using a wooden tongue depressor inside a Class II microbiological safety cabinet. Sample was diluted into a 10% faecal slurry (1/10, w/v) with autoclaved 0.01 M phosphate-buffered saline (PBS) which was pre-reduced

inside the anaerobic cabinet for at least 24 h, hand homogenised to mix sample into the liquid and then homogenized in a stomacher (Seward Stomacher 400 Circulator) for 45 s at 230 rpm. Stomacher bag was returned into the safety cabinet and mesh filter was discarded prior to transferring faecal slurry into sterile 50 mL Falcon tubes. Tubes were capped and kept at 37°C prior to use if used within one hour. Alternatively, in the event of samples not being used immediately after being deposited at the Quadram Institute Clinical Research Facility, solid faecal samples contained in a plastic bag inside a white plastic bucket were preserved using an anaerobic sachet to generate oxygen-free atmosphere and kept inside the air-tight cooler at 4 °C for no longer than 12 h prior to use. Faecal slurries were then prepared as described above with pre-warmed, sterile PBS and used immediately for inoculation of colon model vessels.

2.1.6 Storage of faecal samples from the QIB Colon Model Study

For long term storage of samples from the Colon Model Study, glycerol stocks with final faecal concentration of 37.6% (w/v) were prepared in the same way as the BERI human study, with 50 g of fresh faecal sample mixed with 50 g of autoclaved, pre-reduced, potassium phosphate buffer (50 mM concentration and pH 7 - to prepare 200 mL of phosphate buffer at pH 7, 934 mg K_2HPO_4 and 631 mg KH_2PO_4 was added to distilled water with 100 mg L-cysteine hydrochloride). The mixture was homogenised in the stomacher for 30-45 s at 230 rpm and filtrate was aliquoted into sterile 50 mL falcon tubes. Autoclaved, pre-reduced glycerol was added to slurries based on the volume of yielded filtrate (if 50 g buffer was added into 50 g faecal sample but after homogenisation only 76 mL of filtrate was aspired from the stomacher bag, then only 19 mL of glycerol was added) to make a 25% glycerol stock (v/v). Glycerol stocks were thoroughly mixed in the Falcon tube by inversion and light vortexing and aliquoted into sterile 15 mL centrifuge tubes for storage at -80 °C for up to 4 years (samples were collected mostly before the COVID-19 pandemic in late 2019 and early 2020 and used throughout the project from 2019-2023). Prior to inoculation, glycerol stock tubes were thawed inside an anaerobic cabinet at 37°C for at least 1 hour to revive frozen cultures.

2.1.7 Fermentation conditions

On the day of the experiment, glycerol stocks were defrosted, and the temperature of medium was warmed to 37 °C. If fresh faecal slurries were used, faecal sample was processed as described above and stored at room temperature until inoculation. Pre-reduced choline enriched medium was added with a serological pipette to the treatment vessel to a final concentration of 2 mM. Faecal slurries or glycerol stocks were inoculated into warm media to a final 1% faecal concentration and were mixed throughout the experiment with a magnetic stirrer and a flea. Culture pH was measured and adjusted using the pH pump with acid and alkali. Samples were taken at 0, 4, 8, 12, 24, 30, 36 and 48-hour time points (unless otherwise

stated), taking 1 mL aliquot for LC-MS analysis and 0.5 mL aliquot in a sterile tube for bacterial plating and optical density measurements, if those were measured outcomes of the experiment.

2.1.8 Sample storage and processing

Colon model samples of fermentation cultures collected throughout the experiment were stored in plastic tubes and transferred into a -20 °C freezer immediately after collection. Samples were defrosted in preparation for optical density measurements if separate aliquots were not collected, and samples were then centrifuged and filtered using a 0.45 µm syringe filter into a sterile Eppendorf to be ready for metabolomics analysis. Samples remained defrosted at room temperature for up to 6 hours whilst being processed (although mostly placed at 4 °C if not used immediately) and then transferred to -80 °C for long-term storage. The study design diagram is shown in **Figure 2-2**. Optimisation steps were carried out to determine the difference in peak areas between filtered and non-filtered (centrifuged only) culture samples, and with a negligeable difference between the centrifuged-only supernatant and filtered supernatant, most samples were used without the filtering step (data not shown here).

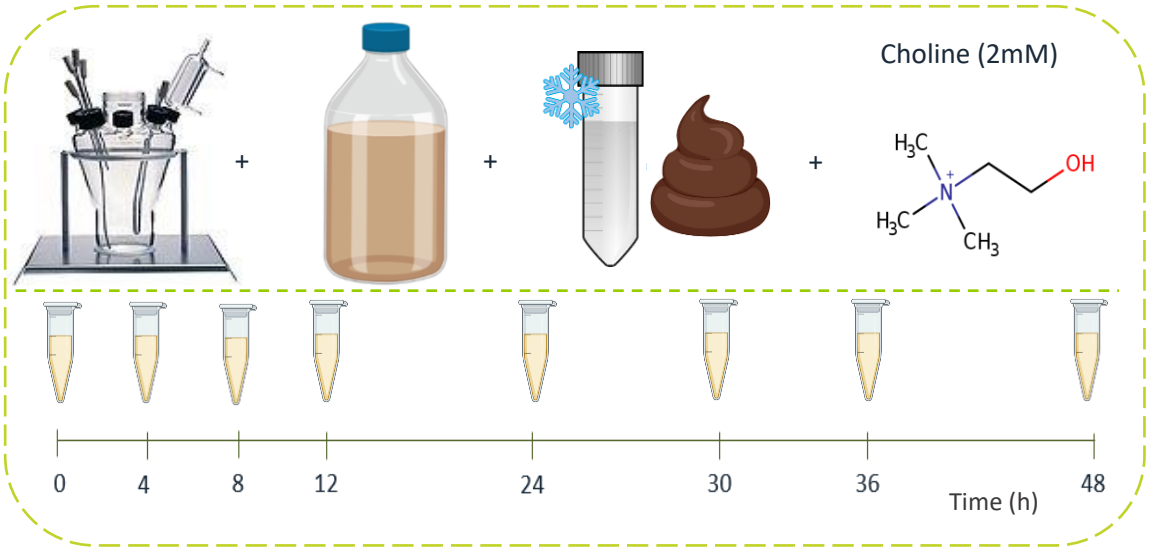

Figure 2-2 Diagram of colon model experimental design.

2.2 Use of batch colon model to measure capacity of BERI study individuals to produce TMA from choline

The BERI study samples were used to determine the individual capacity to produce TMA from supplemented choline using the *in-vitro* batch colon model. The fermentation of faecal glycerol stocks from participants in the BERI study was carried out using their representative Day 1 glycerol stocks (n = 23, collected usually on their first visit), with results of this analysis presented in Chapter 3. A further 30 glycerol stocks were available for 10 participants from

their faecal samples collected on Day 29 of each treatment (A, B or C). These were used to investigate the intra-individual variation of their capacity to produce TMA. Due to the limited availability of colon model vessels, the majority of Day 1 samples were inoculated in a single replicate (only 6-8 vessels were available for each colon model run, with only ~2 colon model runs available per month). Some Day 1 samples were inoculated in more than one replicate in future analyses (used for investigation of the probiotic effect on TMA production in Chapter 4) and these results for control vessels were then collated with the initial analysis of Day 1 samples from Chapter 3. For all BERI participants that donated stool samples, all available findings were used to determine their choline metabolism capacity (μM/h), TMA production capacity (mol%/h), maximum TMA produced from choline (mol%), final TMA (mol%) and abundance of choline at 48 h timepoint (final choline mol%). These variables were calculated for each colon model vessel and then averaged per participant to form a characteristic variable that was used for statistical analyses and correlation with other variables. More detailed description of how these variables were established is outlined in Chapter 3.

2.3 Use of batch colon model to investigate the effects of probiotics on TMA production from choline

2.3.1 Fermentation conditions

The batch fermentation colon model was also used for investigating the impact of a probiotic supplement on the microbial ability to produce TMA from choline. For this analysis, faecal glycerol stocks were used due to the restrictions on use of fresh faecal samples because of the presence of SARS-CoV2 in faeces and the potential risks for contracting COVID-19. Therefore, some glycerol stocks prepared prior to March 2020 were used for this analysis, together with glycerol stocks from the BERI human study. The fermentation conditions used for the main analysis using 7 faecal donors for their response to supplementing 1.7 x 10^7 CFU/mL equivalent of probiotic supplement Bio-Kult were as described in section 2.2. The probiotic supplement was compared against a controlled condition where 2 mM final concentration of choline supplemented to 1% faecal culture was incubated in the batch colon model and monitored over 48 h of incubation through frequent sampling at 0, 10, 12, 20, 24, 30, 36 and 48 h.

2.3.2 Measuring the viability of probiotic strains in colon model

The viability of probiotic supplement was investigated using the batch colon model (pH controlled) and using CMB medium without pH control (cultures grown in anaerobic cabinet) without the presence of faecal microbiota. For this analysis, different probiotic doses were tested in the batch colon model and the anaerobic cabinet was used for comparison between the use of the whole capsule of probiotic supplement and using the capsule contents only. For

the batch colon model, the pH of pre-reduced batch media (at 37 °C) was adjusted using acid and alkali. Different doses of probiotic supplement were measured in sterile environment into plastic 15 mL Falcon tubes, and then transferred into glass vessels by resuspending supplement with a portion of media from the glass vessel using a serological pipette. The inoculum was mixed throughout the experiment with a magnetic stirrer and a flea. Samples were taken at 0, 12, 16, 20, 24, 36 and 48-h time points for bacterial enumeration using the Miles and Misra method on Wilkins Chalgren agar for estimation of total anaerobes.

2.3.3 Viability of probiotics in non-pH-controlled environment

For analysis of probiotic viability in a non-pH-controlled environment and for investigating the number of bacteria available from enclosed supplement capsule versus the capsule contents, universal glass bottles with 20 mL of CMB medium were used (~10^8 CFU/mL), incubated statically at 37 °C inside an anaerobic cabinet, with samples taken using sterile pipette tips and sterile tubes at 0, 12, 16, 20, 24, 36 and 48-h time points for bacterial enumeration using the Miles and Misra method on Wilkins Chalgren agar for estimation of total anaerobes.

2.3.4 Measuring the viability of probiotic strains in colon model inoculated with 1% faecal sample

The total anaerobe count was determined in samples supplemented with probiotic strains and 1% faecal microbiota in a pH-controlled environment (pH 6.6-7.2), incubated in the batch colon model. Fermentation conditions were as described above, and samples were collected to quantify the changes in microbial density in probiotic-supplemented vessels compared to 1% faecal inoculum only inoculated vessels. The same analysis was performed in the anaerobic cabinet with non-pH-controlled samples, where one vial was inoculated with the whole probiotic capsule and 1% faecal inoculum, the other with the contents of the probiotic capsule and 1% faecal inoculum, compared to 1% faecal inoculum only.

Some of the conditions from the colon model (pH-controlled) environment and the anaerobic cabinet non-pH-controlled environment were also investigated for their abundance of lactic acid producing bacteria by plating on MRS agar at 0, 12, 24 and 48 h, to measure the growth of these species in the probiotic supplement with or without the contribution of faecal microbiota. These were compared to the number of bacteria enumerated on MRS in 1% faecal-only inoculated vessels/vials. During these experiments, the pH was measured in the anaerobic cabinet (whilst the colon model had a controlled pH range of 6.6.-7.2) using litmus paper, to investigate if the viability of bacteria was related to the pH of cultures.

2.3.5 Testing the homogeneity of weighed doses of probiotic supplement

After testing the ability of the Bio-Kult supplement capsule to release freeze-dried bacteria into media, doses were weighed in sterile environment as described above to deliver an estimated dose of 1.7 x 10^7 CFU/mL equivalent of probiotic supplement Bio-Kult into colon model vessel. To ensure that weighed supplement inoculated the same dose of probiotics, a smaller scale experiment was carried out in the anaerobic cabinet using CMB medium to explore the variation of inoculation load across different vials (n = 12). The survival of the supplemented strains at the end of the experiment was also measured to see if bacterial viability was similar across the replicates. Colony forming units (CFU/mL) were enumerated on Wilkins-Chalgren agar at 0 h and 24 h of incubation.

2.3.6 Pre-incubation of Bio-Kult probiotic supplement with faecal samples before supplementation with choline

To investigate if revived probiotic strains had an impact on TMA production from choline, an alternative fermentation method was utilised. Bio-Kult supplement dose was added to 1% faecal inoculum inside colon model vessels with batch media and allowed to grow for 12 h before the addition of choline to investigate their effect on TMA production. After 12 h (overnight) incubation of glycerol stocks and probiotic supplement, a portion of culture (12 ml or 1/5 of overall volume) was removed and replaced with choline-enriched pre-reduced media that was added using serological pipettes and taken as the start (0 h) of the experiment. This brought about a slight dilution of original inoculum; however, the inoculum was expected to have grown over the 12 h of pre-incubation, mitigating the dilution factor. Samples were collected at 0, 4, 12 and 24 h using inoculum from 3 separate donors and the changes in area under curve (AUC) for choline metabolism and TMA production kinetic curves were compared between this fermentation model and the model used for the main analysis of 7 participants described in the first paragraph of this section.

2.4 High-throughput *In-vitro* Fermentation System for the Investigation of Choline Metabolism to TMA – CMHT (high-throughput colon model)

Due to the limited availability of colon model vessels, a high-throughput method needed to be adopted for study of the inhibitory effects of phytochemicals and other dietary strategies for reducing TMA formation. A 96-well batch fermentation model using nutritive culture medium (referred to as CMHT – High-throughput colon model) with 2% faecal inoculum using frozen faecal glycerol stocks and no other carbohydrate substrate was used as described by Iglesias-Carres and colleagues in 2021 (228). They recommended these conditions with 5 mM of

chlorogenic acid or gallic acid as effective inhibitors of TMA production by the faecal microbiota, compared to DMB that inhibited TMA production by around 50%.

2.4.1 Media preparation

CMHT nutritive culture medium was adapted from the original recipe by increasing potassium phosphate buffering, due to pH-lowering properties of some phytochemicals and probiotics used for these experiments. The medium ingredients are listed in **Table 2-2**, with two separate solutions that were mixed and diluted prior to experiments. The solution A was prepared in a glass bottle placed on a stirring block with a magnetic flea, filled with 450 mL deionized water. The individual ingredients were added in the order as listed in the table, ensuring each was fully dissolved before adding the next. When fully dissolved, the solution was made up to 500 mL, and the pH was measured using a pH meter and adjusted to 6.8 with concentrated HCl or NaOH solutions. Solution B was prepared by adding individual ingredients to 450 mL boiled deionised water in a 1 L glass Duran bottle placed on a stirring block with a magnetic flea. After all ingredients were fully dissolved, the solution was made up to 500 mL with room temperature deionised water and the pH was adjusted as in solution A. Solution B was autoclaved for 15 min at 121 °C and Solution A was filter sterilised using a 0.22-µm sterile filtering system or with a large syringe. Cooled solutions were stored sealed and covered with foil at 4 °C until use. A few days before the experiment, solutions A and B were combined, diluted to 1 X concentration (e.g. 25 mL of A, 25 mL of B with 50 mL of sterile ultrapure water), and pre-reduced inside an anaerobic cabinet.

Table 2-2 CMHT Nutritive culture medium

CMHT nutritive culture medium	Solution A 2X in 500 mL (g)	Solution B 2X in 500 mL (g)
peptone water	2	2
yeast extract	2	2
NaCl	0.1	0.1
Na_2HPO_4	0.04	0.04
K_2HPO_4	3.978	3.978
KH_2PO_4	3.697	3.697
$MgSO_4 \cdot 7H_2O$	0.01	0.01
$CaCl_2 \cdot 6H_2O$	0.01	0.01
$NaHCO_3$	2	2
hemin	0.05	0.05
bile salts	0.5	0.5
Tween 80	2 mL	2 mL
vitamin K1	10 µL	10 µL
resazurin	0	0.001
L-cysteine ·HCl	0	0.5

2.4.2 Inoculum preparation

For inoculation of this model, glycerol stocks prepared according to the method in 2.1 were utilised instead of the recommended 35.5% faecal concentration in 12.5% glycerol stock as stated by the authors of the published method. Faecal glycerol stocks were defrosted for at least 1 h prior to inoculation inside an anaerobic cabinet at 37 °C. For some assays, 10% fresh faecal slurries made with anaerobic PBS were used instead of glycerol stocks to reduce the incubation time and because fresh faecal donations were available after adjustments to the QIB Colon Model Study to mitigate risks of contracting COVID-19. Fresh faecal slurries were prepared as mentioned in the batch-fermentation section and aliquots were transferred to the anaerobic cabinet prior to inoculation.

2.4.3 Fermentation conditions

A 96-deep-well plate that was autoclaved and pre-reduced with all the consumables was used for the assay. The fermentation model was carried out in an anaerobic cabinet, and all culture components were made anaerobic before inoculation. In a 1.1 mL 96-well plate, 405 µL of nutritive culture medium was dispensed into each well using a sterile reservoir and a multi-channel pipette. In another sterile reservoir, 45 µL choline stock solution (10 mM in PBS 1X) was aspired and added to appropriate wells with media using a multichannel pipette to reach final 500 µM concentrations of supplemented choline. Using a sterile 1000 µL pipette tip, 180 µL of fresh faecal slurry was added to appropriate wells to reach 2% final faecal concentration. Any treatment compounds were added using methanol dissolved stocks, volumes were calculated based on desired concentration. Culture was made up to 900 µL final volume with pre-reduced sterile PBS using a multichannel pipette where possible. The same pipette tips were then used for mixing the contents of wells by pipetting up and down at least 10 times, avoiding spillages into neighbouring wells. A diagram of the model is displayed in **Figure 2-3**.

2.4.4 Sample collection, storage, and processing

Samples were taken after inoculation using multichannel pipettes and transferred into a storage 96-well plate that was covered with adhesive seal, labelled, and stored at -20 °C until analysis. The 96-well plate with fermentation culture was sealed with a sterile plastic adhesive seal (usually used for PCR assays) to prevent evaporation. Samples were then taken at different timepoints after mixing the culture with pipetting up and down and a new seal was used to re-cover the 96-well plate to prevent cross-contamination. After 24 h of incubation, the plate with remaining culture was removed from the anaerobic conditions and stored sealed at -20 °C until analysis.

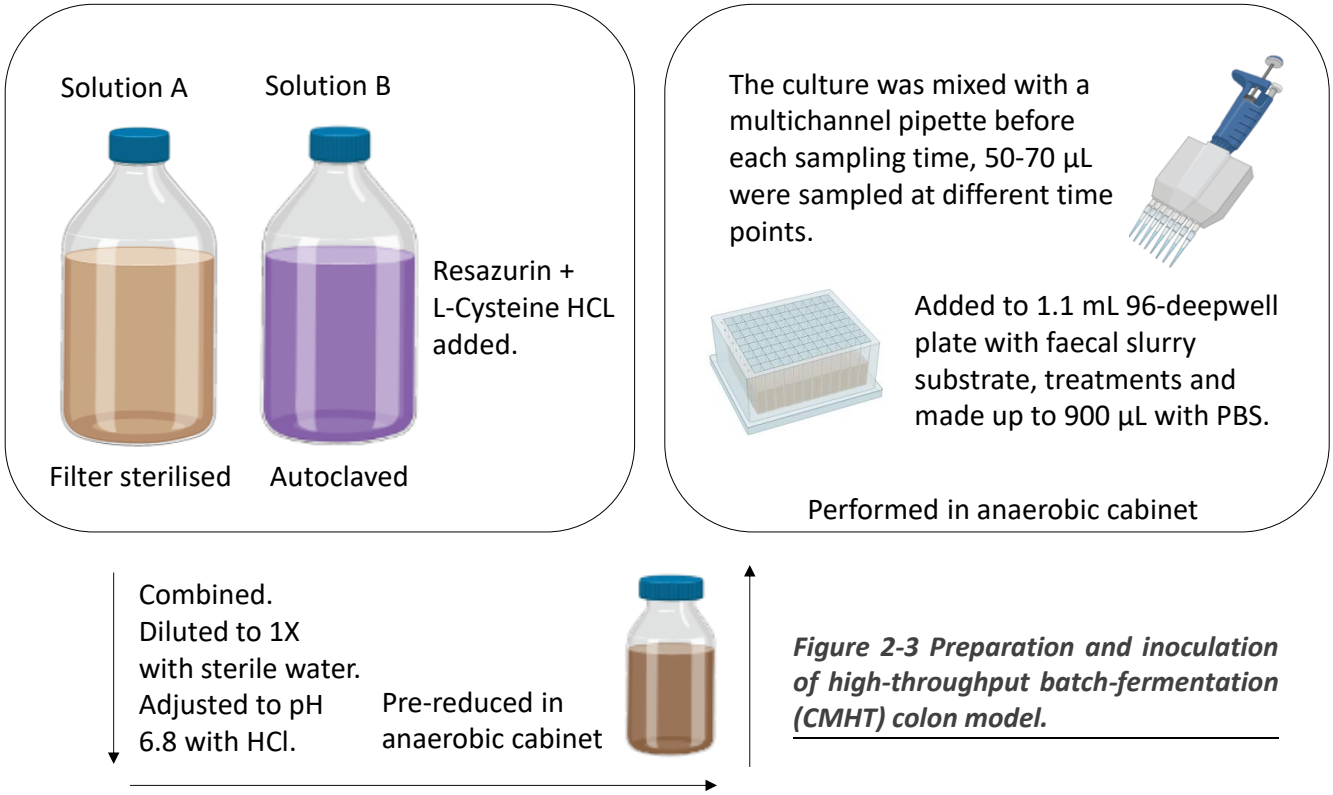

Figure 2-3 Preparation and inoculation of high-throughput batch-fermentation (CMHT) colon model.

2.5 Use of CMHT for assessing feasibility of using chlorogenic acid as a positive control for inhibition of TMA production

Chlorogenic acid and gallic acid were previously reported to be very effective inhibitors (228) of TMA production from choline at 100 µM concentration when supplemented into a high-throughput colon model incubated with 2% faecal inoculum. Having a positive control for inhibition experiments using phenolic compounds was identified as an important aspect of study design for the planned experiments. Therefore, the original experiment by the Neilson group (229) was replicated to investigate if chlorogenic acid can be used as a positive control when screening for polyphenols that inhibit TMA from choline. This method was replicated with choline only as a control (n=30 wells), 10 mM DMB with choline (n=10 wells) and 5 mM CA with choline (n=20 wells). Fresh 10% faecal slurry was used for 2% final faecal inoculum. The 96-well plate was incubated in the anaerobic cabinet and sealed with plate PCR film between sampling for 24 h. Samples were taken at 0, 4, 8, 10, 16, 20, 24 and 24 h.

2.6 Use of CMHT for screening of polyphenols with ability to inhibit TMA production from choline

After initial optimisation steps of the CMHT using chlorogenic acid and different formulations of faecal inoculum, 2% final faecal concentration achieved by inoculation of pooled 10% slurry from two donors was used for investigating the ability of phenolic compounds (28 non-

flavonoids, 5 flavonoids and 1 stilbene) to modify TMA production. All wells were supplemented with 45 µL of 10 mM sterile choline solution in PBS to make a 500 µM final choline concentration, added to 1.1 mL 96-well plate with 405 µL of pre-reduced CMHT medium according to **Table 2-3**. Phenolic compounds that were selected for screening of non-flavonoids were dissolved in 50% methanol and sterile PBS to make 360 mM concentration stock (final concentration of methanol in fermentation culture was 0.3%). The phenolic stocks were added to appropriate wells in six replicates (5 µL per well) to reach a final concentration of 2 mM, and 180.2 µL of 10% faecal slurry were added to all wells. Control wells with choline were inoculated in 6 replicates and additional methanol control of choline had 5 µL of 50% methanol mixed with sterile PBS added to account for any inhibitory effects of the solvent (6 replicates). Positive control had 5 mM final concentration of chlorogenic acid (6 replicates). For flavonoid screening, compounds were dissolved in 50% methanol with 4% formic acid to prevent spontaneous degradation and diluted to 180 mM stock concentration.

Table 2-3 Experimental design for screening of non-flavonoid and flavonoid compounds using CMHT.

Screening of non-flavonoid compounds								
Wells	**Condition**	**Choline (10 mM)**	**Medium**	**10% slurry**	**non-flavonoid compound (360 mM)**	**50% methanol**	**PBS**	**Total Volume**
		(µL)	(µL)	(µL)	(µL)	(µL)	(µL)	(µl)
A1-6	500 µM choline x6	45	405	180.2	0	5	264.8	900
H7-12	500 µM choline + 5 mM chlorogenic acid x6 (positive control)	45	405	180.2	5	0	264.8	900
A7-H6	500 µM choline + 2 mM non-flavonoid compounds x28 x6	45	405	180.2	5	0	264.8	900
Screening of flavonoid compounds								
Wells	**Condition**	**Choline (10 mM)**	**Medium**	**10% slurry**	**flavonoid compound (180 mM)**	**50% methanol**	**PBS**	**Total volume**
		(µL)	(µL)	(µL)	(µL)	(µL)	(µL)	(µl)
A1-6	500 µM choline x6	45	405	180.2	0	10	259.8	900
A7-12	500 µM choline + 5 mM chlorogenic acid x6 (positive control)	45	405	180.2	12.5 (360 mM stock)	0	257.3	900
B1-6	500 µM choline + 2 mM of (+)-Catechin x6	45	405	180.2	10	0	259.8	900

B7-12	500 µM choline + 2 mM of Eriodictyol x6	45	405	180.2	10	0	259.8	900
C1-6	500 µM choline + 2 mM of Luteolin x6	45	405	180.2	10	0	259.8	900
C7-12	500 µM choline + 2 mM of Quercetin x6	45	405	180.2	10	0	259.8	900
D1-6	500 µM choline + 2 mM of Cyanidin x6	45	405	180.2	10	0	259.8	900
D7-12	500 µM choline + 2 mM of 3'-hydroxydaidzein x6	45	405	180.2	10	0	259.8	900
E1-6	500 µM choline + 2 mM of Resveratrol x6	45	405	180.2	10	0	259.8	900

Sampling was carried out as described above with 50 µL fermentation culture taken at 0, 6, 8, 10, 12 and 24 h using multichannel pipette with sterile tips. Some phenolic compounds turned into suspensions when mixed with nutritive culture medium or throughout the assay, therefore, thorough mixing was carried out every sampling time. Samples were stored in 96-well sealed plates at -20 °C until analysis.

2.7 Use of CMHT for confirmation of polyphenol effect on TMA production

A subset of phenolic compounds was re-tested in CMHT (also CMBB – see later) model using the same pooled faecal sample that was inoculated as 10% faecal slurry, prepared as previously described. For this experiment, 6 compounds were explored alongside CA positive control, choline control, microbiota free-control and methanol/DMSO control of different percentages to mimic the solvent content of phenolic conditions. To avoid large variation in supplemented phenolics in each replicate well of the 96-well plate, phenolic conditions were prepared in batches using sterile pre-reduced Falcon tubes containing 7.87 mL of medium, 20 µL of sterile 1 M choline solution, 111 µL of phenolic compound (180 mM stock) and 2 mL of 10% faecal slurry. Chlorogenic acid (CA) control had 139 µL of 360 mM CA stock added. Microbiota-free-control had 2 mL pre-reduced PBS added instead of faecal slurry. Methanol and DMSO solutions in different proportions were prepared in sterile tubes and 111 µL was added to Falcon tube with 7.87 mL media (4 conditions were tested; MeOH 30% + DMSO 10%, MeOH 25% + DMSO 25%, MeOH 50% + DMSO 50% and DMSO at 100%) supplemented with 20 µL of choline stock and 2 mL of faecal slurry. All conditions were made up to 10 mL with pre-reduced sterile PBS and thoroughly mixed by inverting tubes until all components were fully

incorporated. Homogenised culture was dispensed into a deep 96-well plate (1 mL per well) according to the template in **Figure 2-4**.

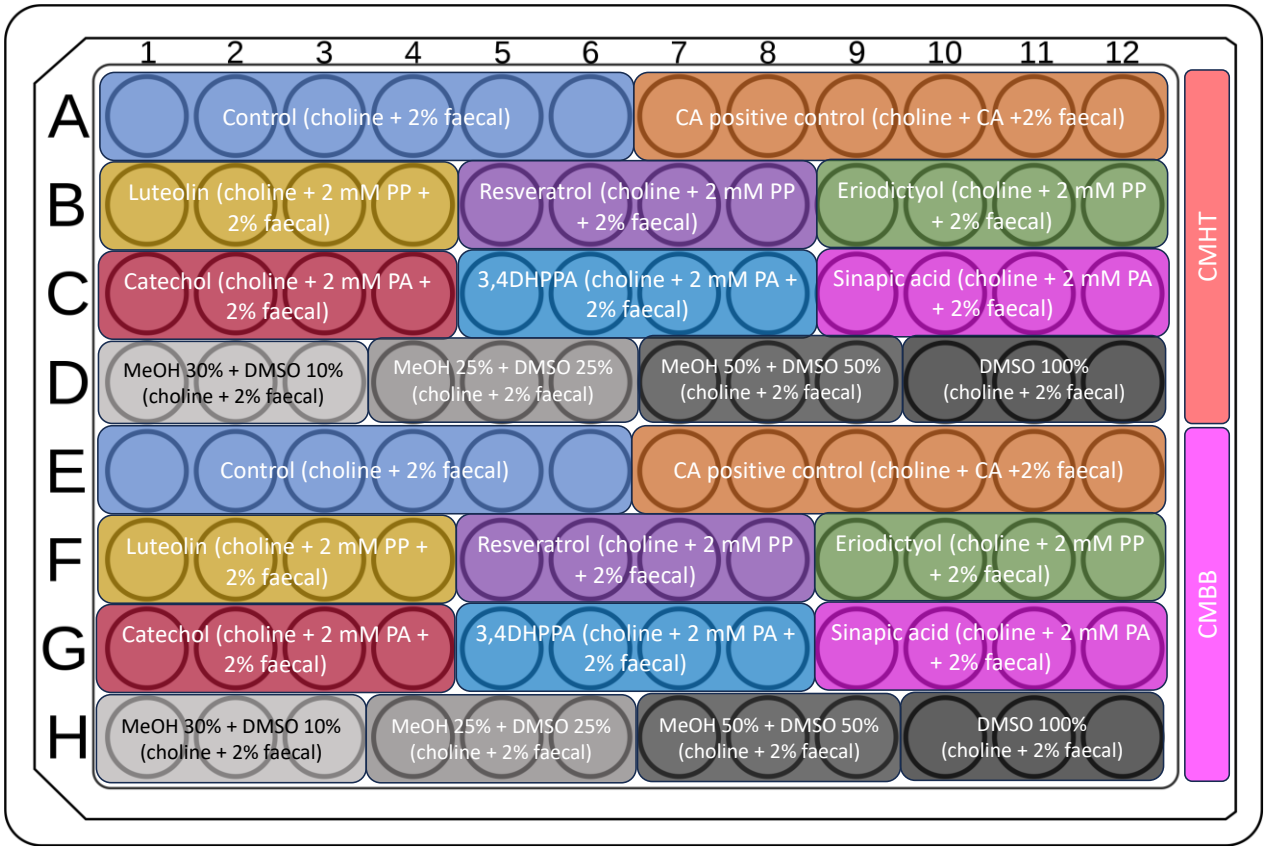

Figure 2-4 96-well plate sample allocation and experimental design for confirmation of phenolic effect on TMA production from choline.

Samples were taken at 0, 2, 4, 6, 8, 10 and 12 h using a multichannel pipette after mixing well contents at least 10x by pipetting up and down. Samples were collected into sterile 96-well plate and sealed with an adhesive film. Samples were frozen immediately after collection and stored at -20 °C until analysis. After sampling at 0 h, 125 µL of prepared culture was transferred into a sterile pre-reduced 96-well plate filled with 125 µL of pre-reduced sterile PBS for measurement of optical density using spectrophotometry as a proxy for bacterial growth in fermentation cultures. Multi-well plates were covered with a gas permeable membrane and incubated in an anaerobic plate reader. Additionally, viable counts were determined with cultures plated (using the Miles and Misra method) on Wilkins-Chalgren agar after 8 h of incubation to compare the toxicity of phenolic compounds and different solvents against control. Finally, pH was measured in one replicate of each condition (taken from original batch tubes) at 0, 8 and 12 h. Phenolic compound stocks were prepared as described in

Table 2-4.

Table 2-4 Preparation of phenolic compounds for determination of effect on TMA production from choline

Code	Name	MW	Weight for 180 mM stock in 300 µL (mg)	Solvent and percentage
F1	Luteolin	286.24	15.5	100% DMSO
F2	Resveratrol	228.24	12.3	50% MeOH
F3	Eriodyctiol	288.25	15.6	25%MeOH/25%DMSO
NF1	Catechol	110.04	5.94	50% MeOH
NF2	3-(4-hydroxyphenyl) propionic acid	166.06	8.96	50% MeOH
NF3	Sinapic acid	224.07	12.1	50%MeOH/50%DMSO
CA	Chlorogenic acid (360 mM stock)	354.31	38.26	50% MeOH

2.8 Use of CMHT to investigate the survival of probiotic strains in different types of batch media

For analysis of the viability of probiotic strains from the Bio-Kult supplement in different fermentation media, diluted stocks of bacterial pellets harvested after 12-18 h of probiotic strain growth in their recommended media were inoculated into 10 mL CMB, CMBB and CMHT media in sterile glass bottles incubated static at 37 °C inside an anaerobic cabinet. This was to determine which fermentation medium was most suitable for investigating the effect of individual probiotic strains on TMA production by the faecal microbiota. The viable count was determined after 24 h of incubation by plating cultures using the Miles and Misra method on MRS agar. The pH stability of fermentation cultures was also measured at two timepoints - 8 and 24 h - to compare the pH of grown cultures, as that formed one of the criteria for a suitable fermentation system due to the inhibition of choline metabolism at lower pH. In previous analyses, the pH of CMB decreased substantially due to glucose fermentation (1% glucose) compared to CMBB (0.1% glucose) and CMHT (no glucose). Samples were collected into 1.5 mL sterile plastic tubes and the pH was measured outside of the anaerobic cabinet after samples reached room temperature using a micro-probe and a pH meter.

2.9 High-throughput *In-vitro* Fermentation System for the Investigation of Choline Metabolism to TMA – CMBB (batch-batch colon model)

This method, referred to as batch-batch colon model (CMBB) with its media formulation first described by Williams *et al.* (230), is a scalable model able to accommodate a high-throughput batch fermentation of methylated amines and investigate the feasibility of using dietary

strategies such as probiotics and polyphenols for reducing TMA production. CMBB is a non-pH controlled static model of human colon that has been used for faecal fermentations of carbohydrates and proteins. It is a sealed anaerobic model composed of autoclaved basal media, vitamin phosphate solution and reducing agent that was pre-reduced with CO_2 prior to inoculation with faecal sample and substrate. The model is fully anaerobic throughout the experiment due to self-sealing butyl rubber septa sealing the contents of serum bottles after serological sampling with needle and syringe. Therefore, anaerobic cabinets or continuous bubbling of gas are not required to maintain an anoxic atmosphere.

2.9.1 Media preparation

The medium is comprised of multiple solutions that were combined to prepare the basal solution that was reduced with CO_2, aliquoted in serum bottles and then autoclaved ahead of the experiment. Bottles with basal solution could be stored at 4 °C for ~2 weeks. Additional solutions sensitive to heat and oxygen were added/prepared on the day of the experiment before inoculation.

Figure 2-5 displays the preparation of medium ready for inoculation. The solutions used for preparation of medium were prepared as follows.

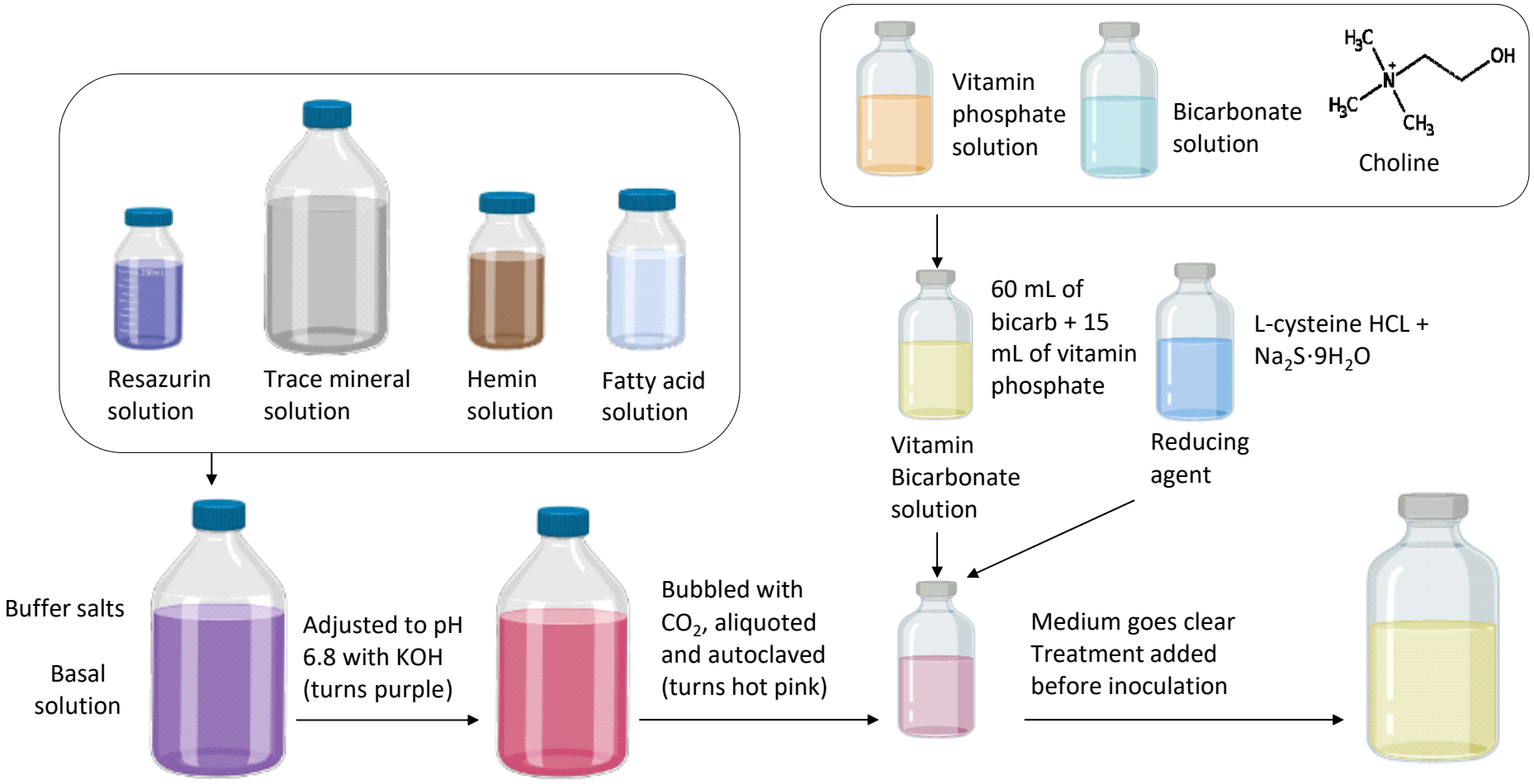

Figure 2-5 Batch-batch media preparation – figure was generated using Biorender.

2.9.2 Preparation of solutions used for making basal solution

The trace mineral solution was prepared in a glass bottle placed on a stirring block with a magnetic flea, filled with 800 mL 0.02 M HCl. The trace minerals (listed in medium ingredients in **Table 2-5**) were added in the order as listed in the table, ensuring each was fully dissolved before adding the next. The best method is to pre-weigh exact amount of individual trace minerals needed into a clean plastic Eppendorf (1.5 mL) and rinse the tube with diluted HCl from the glass bottle to ensure all of compound was transferred to the solution. This was repeated with all trace minerals and the solution was made up to 1 L with 0.02 M HCl and stored at 4 °C for up to 6 months in a sealed bottle.

The fatty acids solution was prepared in a fume cupboard by first preparing 100 mL of 0.2 M NaOH in a Duran bottle. The acids listed in **Table 2-5** were added by a serological pipette after first wetting the plastic of the pipette by aspiring liquid up and down a few times. After all acids were added, the solution was stored at 4 °C in a sealed bottle for up to ~3 weeks. Resazurin solution was made by mixing 0.05 g resazurin in 50 mL of deionised water, mixed well with a magnetic stirrer and a flea, then stored in a sealed bottle covered with foil at 4 °C. Haemin solution was made by adding 0.05 g of haemin to 25 mL of 0.05 M NaOH in a 500 mL volumetric flask. This solution was made up to 500 mL with boiled deionised water, cooled down in room temperature and stored in a foil-wrapped bottle at 4°C. A concentrated solution of 6 M KOH was made by dissolving 33.6 g KOH in deionised water and made up to 100 mL with deionised water and stored in a sealed Duran bottle in room temperature. This solution was used for adjusting pH of basal solution.

2.9.3 Preparation of solutions added to autoclaved basal solution

A carbonate solution was made by adding 41 g Na_2CO_3 (sodium carbonate anhydrous) to 500 mL boiled deionised water. The solution was bubbled with CO_2 for ~30 min whilst cooling down, using a pipette tip secured to the gas tubing and sealing top of bottle with cotton wool to prevent excessive evaporation and precipitation. When cooled down, final volume was checked and topped up to 500 mL with more deionised boiling water (to prevent re-introduction of oxygen). This solution was aliquoted into serum bottles (60 mL each bottle), capped with rubber stopper, crimped, and autoclaved at 121 °C for 15 min. Bottles were stored at room temperature and checked for precipitation before use. Vitamin phosphate solution was prepared in a beaker by dissolving 27.35 g KH_2PO_4 in 500 mL deionised water warmed up to 30 °C to aid dissolving of vitamins in **Table 2-5**. These were prepared similarly to the trace mineral solution ingredients and weighed out into individual microtubes, suspended with phosphate buffer, and then pipetted into the Duran bottle to ensure all of compound was added. When all ingredients were incorporated, the solution was filter-sterilised into sterilised

serum bottles (there would be loss of colour if all compounds were not dissolved and filter would get blocked), sealed with rubber stoppers, capped, crimped, and stored at 4 °C covered with aluminium foil until use.

2.9.4 Preparation of basal solution

Once all these solutions were prepared, all dry ingredients in the recipe for basal solution in **Table 2-5** were weighed out into a large Duran bottle with volume markings (2-5 L). Boiling deionised water was used to fully dissolve all compounds (around 500 mL) whilst mixing with a magnetic stirrer and a flea. Then, the prepared solutions were added to the Duran bottle with a serological pipette according to the recipe in **Table 2-5**. Using a pH meter, the medium was adjusted to 6.8 with the concentrated KOH solution (with pH probe that can withstand the higher temperature and adjust pH based on temperature readings), turning the medium from pink to deep purple. Prepared solution was made up to desired volume with boiling deionised water. After this step, the pH was re-checked. The medium was pre-reduced with CO_2 that was bubbled through the solution until cooled and dark pink, approximately 1-2 h per L. When cooled, 76 mL basal medium was dispensed into clean and autoclaved serum bottles using a multi-dispenser, sealed with butyl rubber stoppers, crimped using aluminium caps ensuring caps are tightly secured, and then autoclaved at 121 °C for 15 min. Autoclaved serum bottles can be stored at 4°C until used.

2.9.5 Preparation of vitamin carbonate with supplements and addition into autoclaved basal solution

On the day of the experiment, two final solutions were prepared - vitamin carbonate solution and reducing agent solution. These were not prepared more than one day prior to inoculation. For vitamin carbonate, 15 mL of vitamin/phosphate solution was added into bottles that contained 60 mL autoclaved sodium carbonate solution by injection through the septum with a sterile needle (pressure relieved in carbonate solution first by injecting a sterile needle through the septum). The original formulation for this solution was modified by withdrawing 5.74 mL of vitamin carbonate solution to allow supplementation with 40% glucose (3.19 mL in 75 mL of vitamin carbonate was 1.7% glucose solution that was diluted to 0.1% concentration in final 85 mL of culture) and 1 M choline (2.55 mL added of 1 M solution in 75 mL vitamin carbonate was 34 mM choline concentration that was diluted to 2 mM final concentration in 85 mL culture). The choline-glucose-enriched vitamin carbonate solution was added to the basal solution filled serum bottles (5 mL each) and conditions without choline had glucose-enriched vitamin carbonate added instead. Basal solution turned hot pink and cloudy after addition of vitamin carbonate. Finally, a reducing solution was made by combining 0.5 g L-Cysteine ·HCl and 0.5 g $Na_2S{\cdot}9H_2O$ weighed into a small glass Duran bottle and dissolved in 25 mL boiled deionised

water. The pH was measured using pH paper and adjusted to pH 10 with concentrated NaOH (3 M). A small volume (1 mL) of reducing solution was added per basal solution bottle.

Table 2-5 Batch-batch medium ingredients and solutions

Media components	**Amount**
Trace Mineral Solution (store at 4°C) - Stable for 6 months	
Hydrochloric acid HCl (0.02M, 20 mL of 1M HCL into 1L) Add minerals to make 500 mL	1000 mL
Manganese chloride $MnCl_2 \cdot 4H_2O$	25 mg
Ferrous Sulphate $FeSO_4 \cdot 7H_2O$	20 mg
Zinc chloride $ZnCl_2$	25 mg
Copper chloride $CuCl \cdot 2H_2O$	25 mg
Cobalt chloride $CoCl_2 \cdot 6H_2O$	50 mg
Selenium dioxide SeO_2	50 mg
Nickel chloride $NiCl_2 \cdot 6H_2O$	250 mg
Sodium molybdate $Na_2MoO_4 \cdot 2H_2O$	250 mg
Sodium metavanadate $NaVO_3$ (most difficult to dissolve)	31.4 mg
Boric acid (H_3BO_3)	250 mg
Fatty Acid Solution (store at 4°C) - Stable for 2-3 weeks	
NaOH (0.2M) or 800mg in 100 mL	200 mL
Acetic acid	1.37 mL
Propionic acid	0.6 mL
Butyric acid	0.368 mL
Isobutyric acid	0.094 mL
2-Methylbutyric acid	0.11 mL
Valeric Acid	0.11 mL
Isovaleric acid	0.11 mL
Resazurin (store covered at 4°C)	
Resazurin	50 mg
Deionized water	To 50 mL
KOH (concentrated, store at RT)	
KOH	33.6 g
Deionized water	To 100 mL
Haemin (store covered at 4°C), stable for 12 months	
Haemin	50 mg
NaOH (0.05 M)	25 mL
Deionized water at 100°C	425 mL
Sodium Carbonate (store at RT)	
Na_2CO_3	20.5 g
Deionized water at 100°C	250 mL
Vitamin-Phosphate (store covered at 4°C)	
Potassium phosphate monobasic KH_2PO_4 (27.35g in 500 mL in 30°C)	500 mL
Biotin	10.2 mg
Folic acid	10.3 mg
Calcium pantothenate (D-L-pantothenic acid hemi calcium salt)	82 mg

Nicotinamide	82 mg
Riboflavin	82 mg
Thiamine HCl	82 mg
Pyridoxine HCl	82 mg
Para-amino benzoic acid	10.2 mg
Cyanocobalamin (Vitamin B12)	10.3 mg
Reducing Agent (stable ~ 1 day)	
Deionized water at 100°C	25 mL
L-Cysteine HCl	0.5 g
$Na_2S•9H_2O$	0.5 g
NaOH – concentrated (use to adjust pH to 10)	
Basal Solution (2L recipe)	
Deionized water (Make sure all chemicals are dissolved in 500 mL before reagents)	500 mL
KCl	1.4268 g
NaCl	1.4268 g
$CaCl_2•2H_2O$	0.4756 g
$MgSO_4•7H_2O$	1.189 g
PIPES buffer - (increased buffering capacity to make 25 mM in final solution)	13.4 g
NH_4Cl	1.284 g
Trypticase Peptone	2.378 g
Reagents	
Resazurin solution	2.348 mL
Trace Mineral solution	23.781 mL
Haemin solution	23.781 mL
Fatty Acid solution	23.781 mL
KOH– concentrated (use to adjust pH to 6.8)	
Deionized water at 100°C	~1.4 L
Overall, each medium bottle contains:	Total 82 mL
Basal solution	76 mL
Vitamin carbonate solution enriched with choline and glucose	5 mL
Reducing agent	1 mL

2.9.6 Preparation of media for inoculation

One prepared medium bottle was opened to measure pH with an electrode and pH meter. The pH of the media was adjusted using concentrated KOH or HCl to 6.8-7.0, adding small amounts with sterile pipette tips and noting down the volume required for the desired pH. This volume was then added to all prepared media bottles using a needle and syringe to change pH to 6.8-7.0. Ideally, the colour of the medium after pH adjustment changed to light pink and nearly colourless. Finally, the serum bottles containing the pH-adjusted experimental media had their headspace sparged with CO_2 for 2-3 min using plastic tubing with sterile needles piercing the septum and outlet needle displacing any oxygen present in the headspace. If media remained cloudy, sterile gassing needle was lightly submerged into media and bubbled until colourless.

The bottles were incubated at 37 °C until reaching the desired temperature or stored in the incubator overnight, ready for inoculation with faecal samples in the morning.

2.9.7 Inoculum preparation

For preparing faecal slurries, sterile PBS was pre-reduced for 12-24 h (depending on volume) in an anaerobic cabinet. Pooled stool sample from 2 donors was slowly defrosted at 4 °C (usually overnight or over 4-8 h). Once defrosted, the stool was weighed using a tongue depressor into a stomacher bag in a Class II microbiological safety cabinet (at least 5 g) and a 10% slurry was prepared as previously described in section 2.1.

Pre-warmed serum bottles were inoculated with 3 mL of 10% faecal slurry using a 19G needle and syringe. The needle was reused for bottles with the same condition and replaced when blunt to prevent spillages or accidents. Conditions without faecal microbiota (microbiota-free control, MFC) were instead inoculated with 3 mL pre-reduced PBS.

2.9.8 Fermentation conditions

The colon model was incubated at 37°C for 24-48 h and samples were taken aseptically at various timepoints with a sterile 21-23G needle and syringe inside a microbiology safety cabinet after resuspending contents by mixing, relieving gas build up with a sterile needle, fitting a syringe into the needle top, inverting the serum bottle, withdrawing liquid culture from the bottle, and then expelling aspired volume into sterile Eppendorf tubes placed in a rack. Samples were stored at -20 °C freezer until analysis.

2.10 Use of CMBB for determination of dose response of probiotics on TMA production from choline

The CMBB medium was prepared as described above until all serum bottles had all components added apart from faecal inoculum. Probiotic supplement capsules were decanted into a UV sterilised weighing boat inside a microbiology safety cabinet, and doses of $1.7x10^6$, $8.4x10^6$, $1.7x10^7$, $3.3x10^7$ and $8.4x10^7$ CFU/mL equivalent were weighed out into empty sterile serum bottles. Prepared medium was decanted into probiotic-containing serum bottles and resealed with butyl rubber stoppers and crimped aluminium caps. The headspace of bottles was sparged with CO_2 until media turned colourless again (resazurin reacted with oxygen whilst media was poured into new serum bottles) as described before. Probiotic enriched bottles were transferred into an incubator to be pre-incubated for 12 hours in CMBB medium without faecal samples to allow the rehydration of the supplement and growth of strains in pH 6.6-7.2 at 37 °C.

At the beginning of the experiment, gas generated during the 12 h fermentation was released from the serum bottles by piercing septa with a sterile needle prior to addition of inoculum (to release pressure and avoid spillages) and all other control conditions were inoculated with pooled faecal 10% slurry from two donors according to experimental design displayed in **Table 2-6** to reach a final faecal concentration of 0.35 %. Faecal inoculation was considered the start of the experiment (0 h) from which point choline metabolism and TMA production were observed in Bio-Kult (PRX) supplemented bottles compared to control, MFC, and MFC+PRX that had $1.7x10^7$ CFU/mL dose of probiotic without addition of faecal sample. Supplement filler control was supplemented with autoclaved microcrystalline cellulose (MCC) to quantify the interactions of the bulking agent with microbial matrix. Samples were collected aseptically using 21G needles and syringes at 0, 4, 8, 12, 24, 26, 30, 36 and 48 h and stored at -20 °C until analysis.

Table 2-6 Experimental design of probiotic dose response with pooled faecal sample from 2 donors.

Code	Condition	PRX dose (mg)	CFU added	Final CFU conc. In 85 mL	CMBB medium	10% slurry	PBS	Total volume
					(µL)	(µL)	(µL)	(µL)
PRX10	PRX $1.7x10^6$ + faecal x3	14.2	1.42×10^8	$1.67x10^6$	81000	3000	985	85000
PRX50	PRX $8.4x10^6$ + faecal x3	70.5	7.10×10^8	$8.35x10^6$	81000	3000	929	85000
PRX100	PRX $1.7x10^7$ + faecal x3	142	1.42×10^9	$1.67x10^7$	81000	3000	858	85000
PRX200	PRX $3.3x10^7$ + faecal x3	284	2.84×10^9	$3.29x10^7$	81000	3000	716	85000
PRX500	PRX $8.4x10^7$ + faecal x3	710	7.10×10^9	$8.35x10^7$	81000	3000	290	85000
C	Choline (2mM) + faecal (control) x3	0	0	0	82000	3000	0	85000
MCC	Choline (2mM) + faecal + MCC x3	142	0	0	82000	3000	840	85000
MFC+PRX	PRX $1.7x10^7$ + no faecal x3	142	$1.42x10^9$	$1.67x10^7$	82000	0	2858	85000
MFC	Choline (2mM) + no faecal x3	0	0	0	82000	0	3000	85000

2.11 Use of CMBB for investigating the ability of individual probiotic strains from Bio-Kult supplement to survive in different batch fermentation media

This experimental methodology was described in the CMHT colon model section 2.8. CMBB medium was prepared as described previously without the addition of a faecal slurry. Instead, sealed CMBB serum bottles ready for inoculation were transferred into the anaerobic cabinet, uncapped and aliquoted into glass universal vials (10 mL each, measured by serological pipette controller). This medium was then inoculated with the individual probiotic stocks and fermentation was carried out as described in CMHT method section.

2.12 Use of CMBB for investigating the effect of individual probiotic strains from Bio-Kult supplement on microbial capacity to produce TMA from choline

After exploration of probiotic strain viability in **section 2.8** and the section above, CMBB was selected to explore the role of single probiotic strains in modulating TMA production in faecal samples supplemented with choline. This model was optimised for inoculation with fresh faecal samples with final concentration of 0.35% from 10% faecal slurry made with PBS (w/v). Where fresh samples were not available, snap frozen faecal samples slowly defrosted at 4°C in anaerobic atmosphere were used for slurry preparation. In the control condition and for inoculation of faecal enriched cultures, 10 µL of 1 M choline solution was added in a batch vial supplemented with faecal slurry, thoroughly mixed by inverting, and homogenised culture was aliquoted into a 96-well plate in triplicate for all conditions containing faecal microbiota. Diluted stocks of probiotic bacteria were prepared as described in **section 2.16** and strains were inoculated into CMBB medium in a deep 96-well plate as monocultures or added to faecal enriched wells. Cultures were mixed by pipetting and samples were taken at 0, 4, 8, 16, 20, 24 and 30 h using multichannel pipette, and sterile 96-well plates were used for sample storage. Potion of fermentation culture was transferred into a clear sterile 96-well plate to be used for measuring growth of Bio-Kult (PRX) monocultures and faecal enriched cultures by spectrophotometry at 600 nm inside an anaerobic cabinet set at 37 °C.

2.13 Use of CMBB for determination of polyphenol effect on TMA production from choline

This experimental methodology was described in CMHT model **section 2.7**. CMBB medium was prepared as described previously without the addition of a faecal slurry. Instead, sealed CMBB serum bottles ready for inoculation were transferred into the anaerobic cabinet, uncapped and aliquoted into 15 mL Falcon tubes according to the instruction in the CMHT section (measured by serological pipette controller).

SINGLE-STRAIN MODEL OF TMA-PRODUCING BACTERIUM

To understand the effects of different dietary strategies that may reduce TMA production, a more simplified model allowing direct observations of the interactions between substrates and bacteria outside of the complexities of faecal matrix was utilised. For this reason, a single-strain model of a TMA-producing bacterium was developed to understand the processes driving changes in TMA production from choline observed *in-vitro*. Therefore, published literature was searched for bacterial strains containing *cutC/D* genes that have been reported to produce TMA from choline using *in-vitro* fermentation assays (2, 56, 57, 59, 60, 140, 157, 231).

From the available literature, strains listed in **Table 2-7** were chosen due to their published genomes and availability in culture collections. Strains from the Narbad culture collection (QIB, Norwich) were considered for exploration of their ability to metabolise choline into TMA. Two of the available strains had their genome sequenced, *Desulfovibrio desulfuricans* QI0028 and *Citrobacter freundii* FC50, and were screened for presence of *cutC* genes prior to analysis of TMA production capacity.

Table 2-7 TMA-producing strains considered for the single-strain TMA production model

Species	**Strain**	**NCBI taxon ID**
Strains available in publicly accessible culture collections		
Oleidesulfovibrio alaskensis	G20	207559
Proteus mirabilis	HI4320	529507
Desulfovibrio desulfuricans	ATCC 27774	525146
Escherichia fergusonii	UMN026	564
Proteus mirabilis	ATCC 29906	525369
Proteus penneri	ATCC 35198	471881
Escherichia coli	MS 200-1	749550
Escherichia coli	MS 69-1	749531
Klebsiella sp.	MS 92-3	749535
Halodesulfovibrio aestuarii	ATCC 29578	1121444
Proteus mirabilis	BB2000	1266738
Citrobacter freundii	NBRC 12681	1006003
Proteus vulgaris	NCTC 10376	585
Strains available from the Narbad group culture collection		
Clostridium sporogenes	n/a	n/a
Enterococcus hirae	n/a	n/a
Citrobacter freundii	FC50	n/a
Desulfovibrio desulfuricans	L1-7b (QI0028)	n/a
Desulfovibrio desulfuricans	E3-2 (QI0029)	n/a

This list was then narrowed down to four strains, two from the Narbad culture collection (*C. freundii* FC50 and *D. desulfuricans* QI10028) that had their genomes sequenced and two strains

available in public culture collections. *P. mirabilis* ATCC 29906 was ordered from the DSM culture collection under accession number 4479. It was isolated from the urinary tract of humans and was previously shown to produce TMA from choline. A second strain (*H. aestuarii* ATCC 29578) was also ordered from the DSM culture collection under accession number 17919 and chosen due to its low abundance in humans and its origin in marine ecosystems, to understand the ability of the strain to produce TMA from choline in different environments. These four strains were assessed for multiple criteria to investigate their suitability for use as a single-strain model of TMA-producing bacteria. The criteria were as follows:

i) Ability to consistently grow anaerobically to sufficient cell density in its recommended medium

ii) Ability to grow in batch fermentation media (CMBB or CMHT)

iii) Ability to metabolise supplemented choline to TMA in anaerobic conditions when grown in their recommended medium and in batch fermentation media

iv) Ability to grow on agar plates to calculate estimated OD_{600} value at 10^8 CFU/mL from linear regression of optical density vs viable count (CFU/mL)

All candidate strains were tested and were eliminated from being suitable candidates if they did not meet those criteria. The media preparation and fermentation conditions for these tests are described in the following sections. All media and culture vessels were maintained under anaerobic conditions using an anaerobic cabinet (Don Whitley, UK) with materials pre-reduced prior to use for at least 12 h in an atmosphere of 5% CO_2, 10% H_2 in N_2 at 37˚ C. Anaerobic media were also prepared and maintained using the Hungate tube method where specified. Methodology and results for strains *C. freundii* FC50 and *H. aestuarii* DSM 17919 are detailed in Appendix I.

2.14 Desulfovibrio desulfuricans QI0028

D. desulfuricans strain QI0028 was obtained from the Narbad Group culture collection (isolated by Dr Tianqi Li) and grown anaerobically, static, using Hungate tubes with Postgate C medium and inoculated with a liquid culture by Dr Melinda Mayer in an anaerobic cabinet set to 37 °C. Strain was grown for 3-5 days and when fully grown (turbidity about 0.8), it was stored at 4 °C and used as 'mother culture' for inoculation due to its sensitivity to freezing and thawing, and oxygen exposure.

2.14.1 Preparation of glycerol stocks

The 'Mother culture' was subcultured into fresh Postgate C medium in Hungate tubes and grown into exponential phase inside anaerobic cabinet at 37 °C (~20 hours) after which it was

transferred into sterile pre-reduced Falcon tubes and centrifuged for 10 min at 4000 × g at 4 °C. Supernatant was decanted and the pellet was resuspended in glycerol solution from an orange Cryobead tube and transferred onto the beads. Sealed tube was mixed by inverting and glycerol stock was snap frozen using dry ice and stored at -80 °C.

2.14.2 Culture preparation

For the first few experiments exploring growth and choline metabolism, the 'mother culture' was used for inoculating of fresh Postgate C medium at 2% and the exponential phase of this culture was used for inoculation of experiments. The growth of first passage from the 'mother culture' was highly variable and could take between 1-6 days. Therefore, frozen glycerol stock was sometimes used, whilst being kept on dry ice, same as for inoculation of other strains. When accelerated growth was needed due to time constrains, up to 6% of the 'mother culture' or previous 1st passages of *D. desulfuricans* were used for inoculation.

2.14.3 Media preparation

Postgate C and Anaerobic Basal Broth (ABB) were prepared using the Hungate system by the QIB Media lab according to **Table 2-8**. Solid medium was prepared by adding 16 g/L of agar into liquid media inside a glass Duran bottle instead of the Hungate system and then autoclaved for 15 min at 121°C. Media was dispensed under laminal flow into sterile petri dishes and then placed in the anaerobic cabinet for 12-24 h to pre-reduce. CMBB medium was also prepared as described before and dispensed into sterile Falcon tubes (10 mL media each) inside the anaerobic cabinet to remain anoxic.

Table 2-8 Postgate C medium and Anaerobic basal broth

Postgate C medium	g/L
sodium lactate	6
sodium sulphate	4.5
ammonium chloride	1
yeast extract	1
potassium phosphate	0.5
sodium citrate tri basic	0.3
magnesium sulphate 7-hydrate	0.06
iron sulphate 7-hydrate	0.004
calcium chloride	0.04
L-cysteine hydrochloride	0.5
resazurin	0.0008
deionised water	Up to 1 L
Anaerobic basal broth (Oxoid CM0957)	g/L
Peptone	16.0
Yeast extract	7.0
Sodium chloride	5.0
Starch	1.0
Dextrose	1.0
Sodium pyruvate	1.0
Arginine	1.0
Sodium succinate	0.5
L-cysteine HCl	0.5
Sodium bicarbonate	0.4
Ferric pyrophosphate	0.5
Haemin	0.005
Vitamin K	0.0005
Sodium thioglycolate	0.5
Dithiothreitol	1.0
Deionised water	Up to 1 L
pH 6.8 ± 0.2 @ 25°C	

2.14.4 Growth and choline metabolism assays

Postgate C medium, ABB and CMBB were enriched with 1 M choline solution (20 µL added into uncapped tube using a sterile pipette tip) to make a final 2 mM choline concentration for investigating TMA production ability. Cultures were inoculated in triplicate with 2 % of 2nd passage of cells and incubated static for 36 h with turbidity readings taken at 0, 2, 4, 6, 8, 10, 12, 14, 22, 24, 30 and 36 h. For assessment of growth and choline metabolism in CMBB, due to low optical density readings and inconsistent growth in different vessels (Hungate tubes, Universal glass bottles, and plastic 15 mL Falcon tubes), viable count of *D. desulfuricans* incubated in CMBB with ~2 mM choline in triplicates were enumerated at inoculation, 4 and 8 h of incubation. Colonies were counted after 24 to 36-hour incubation on Postgate C medium solidified with 1.6% agar. In another experiment, optical density readings were taken with a

spectrophotometer set at 600 nm using a 96-well plate reader where growth of *D. desulfuricans* was compared between cultures incubated with or without 2 mM choline added to ABB with readings every 15 minutes after 30 seconds of shaking prior to OD measurements. This experiment was then replicated using ABB in Hungate tubes grown over 38 hours and measured by a turbidometer readings taken at 0, 12, 14, 18, 20, 24 and 38 h. Readings were taken inside anaerobic cabinet using sterile ABB as blank.

2.15 Proteus mirabilis DSM 4479

P. mirabilis DSM 4479 was purchased from the DSM collection in freeze-dried form. The strain was inoculated into aerobic Nutrient broth and grown into exponential phase (~1 day) at 37 °C incubator. A 2% subculture was grown inside an anaerobic cabinet to acclimatise the strain to anoxic conditions and slightly higher temperature (it is a mesophilic facultative anaerobe) and after reaching exponential phase (4-8 h), glycerol stock of the strain was prepared as described previously.

2.15.1 Culture preparation

For inoculation from frozen glycerol stock, dry ice was used same as for inoculation of the other strains. The second passage was used for inoculation of experiments and preparation of pellet stocks, unless otherwise stated. Some cultures had 2 mM choline concentration supplemented to 2nd passage culture to promote expression of choline-TMA lyases during growth and that inoculum was referred to as 'induced'.

2.15.2 Media preparation

Nutrient broth (**Table**) was prepared from powder using boiling water and autoclaved in a glass Duran bottle for 15 min at 121°C. Medium was dispensed under laminal flow into sterile glass universal bottles. Vials were placed in the anaerobic cabinet for 12-24 h to displace oxygen. For preparation of agar plates, nutrient broth was supplemented with 16 g/L of agar powder prior to autoclaving and when cooled down to pouring temperature was dispensed into sterile petri dishes. Occasionally, Wilkins-Chalgren agar, BHI agar (BHI with 1.5% agar) and MacConkey agar were used for viable count enumeration, prepared according to manufacturer's instructions (Oxoid CM0619, CM1135 and CM0007, respectively). CMBB was also prepared as described previously and dispensed into sterile glass universal bottles (10 mL media each) or sterile 15 mL Falcon tubes inside the anaerobic cabinet to remain anoxic. A specialised medium (PMS) previously described by Jameson and colleagues (232) was adapted to investigate factors that reduced the metabolism of choline to TMA by *P. mirabilis*. The medium was supplemented with specific amino acids that promoted swarming of *P. mirabilis*.

Table 2-9 Nutrient broth

Nutrient broth (Oxoid CM0001)	g/L
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
pH 7.4 ± 0.2 @ 25°C	

2.15.3 Solutions for preparation of *P. mirabilis* swarming media (PMS)

Micronutrient solution was prepared by adding individual amino acids and nicotinamide (see **Table 2-10**) into deionised water warmed up to 30 °C (with a few drops of concentrated HCl added to help dissolve the ingredients). When fully dissolved, the solution was filter sterilised into a sterile serum bottle, sealed with a butyl rubber stopper, and crimped with an aluminium cap. The solution was stored at 4 °C until use. Ferric citrate solution was prepared using 140 mg iron (III) ammonium citrate dissolved in 10 mL ultrapure water and then filter sterilised and stored in a 15 mL Falcon tube at 4 °C until use. Sodium fumarate was prepared by dissolving 40 g of sodium fumarate dibasic made up to 500 mL with deionised boiling water in a 1 L Duran bottle whilst being mixed on a stirrer plate. When fully dissolved, the solution was filtered using a 0.22 µm filtering system and stored in aliquots of 100 mL in sterile serum bottles and sealed with rubber stoppers. The solution was stored at room temperature until use. The ingredients for basal solution in **Table 2-10** were dissolved in deionised water and autoclaved for 15 min at 121°C in a 1 L Duran bottle.

2.15.4 Adding solutions to make PMS and preparing solid media

When the basal solution had cooled down, 100 mL fumarate (1 serum bottle was uncapped) was added to the Duran bottle under laminal flow, 5 mL of micronutrient was added with a needle and syringe, and ferric citrate solution was added using a sterile pipette tip. Sterile water was used to bring the volume to 500 mL to make a 2X medium. For liquid solution, the medium was mixed 1:1 with sterile water and dispensed into sterile universal bottles (10 mL each) and stored at 4 °C until use. For preparation of solid media, yeast extract solution was prepared by mixing 1 g of yeast extract and making it up to 100 mL with deionised water in a 200 mL glass Duran bottle which was autoclaved (1% w/v solution). Yeast extract solution was diluted to 0.05% by mixing 5 mL of 1% solution and making solution up to 100 mL with ultrapure sterile water. This was solidified with 3 g agarose that was melted using a microwave at 800 W in short 20 s burst, swirling contents each time until fully dissolved. When slightly cooled down, prepared agarose was mixed with swarming media in 1:1 ratio (v/v) and poured

into sterile petri dishes immediately (solidified very fast due to lower temperature). The final concentration of agarose was 1.5% (w/v) with 0.025% (w/v) yeast extract added to PMS.

Table 2-10 Proteus mirabilis Swarming medium

***P. mirabilis* Swarming medium** (PMS)	Amount	Unit
Basal solution (make up to)	350	mL
K_2HPO_4	7	g
KH_2PO_4	3	g
$MgSO_4 \cdot 7H_2O$	0.1	g
$(NH_4)_2SO_4$	1	g
HEPES buffer	5.96	g
PIPES buffer	7.56	g
Micronutrient solution (make up to)	100	mL
L-histidine	0.3	g
L-tryptophan	0.3	g
Nicotinamide	0.3	g
L-isoleucine	0.3	g
Conc. HCl	<1	mL
Ferric citrate solution (50 mM)	10	mL
Iron (III) ammonium citrate	140	mg
Fumarate solution (0.5 M)	500	mL
Sodium fumarate dibasic	40	g
***P. mirabilis* swarming medium** (PMS) 2X	500	mL
Basal solution	350	mL
Micronutrient solution	5	mL
Ferric citrate solution	1	mL
Fumarate solution	100	mL
Sterile ultrapure water	44	mL

2.15.5 Growth and choline metabolism assays

CMBB and Nutrient broth were supplemented with filter-sterilised 1 M choline solution (20 µL in 10 mL to make 2 mM final concentration unless otherwise stated) to investigate the ability of *P. mirabilis* to metabolise choline and produce TMA in different media. Cultures were inoculated in triplicate with 2 % of 2nd passage and incubated static for 24 h. Optical density readings were taken with a spectrophotometer at 600 nm using clean cuvettes at 0, 4, 6, 8 and 24 h of incubation.

The impact of inducing *P. mirabilis* cells with 2 mM choline during growth of 2nd passage was tested on the growth of 1% 2nd passage supplemented to CMBB enriched with 5 mM choline. The aim was to investigate if 'induced' cells grow to a greater extent in choline rich media compared to cells naïve to choline as a substrate. Optical density values were measured using an anaerobic plate reader at 600 nm every 15 min for 12 h with each condition incubated in 6 replicates.

2.15.6 Colony forming units and standard curve of diluted culture pellet

P. mirabilis stock of 10 mL culture was prepared as described before by harvesting cells after 8-12 h growth. Stock was serially diluted to 50%, 25% and 12.5% and plated on Nutrient agar. Optical density and viable count (CFU/mL) were measured and plotted to calculate a linear regression for determining optical density of 10^8 viable cells/mL of diluted stock. Due to the rapid growth of *P. mirabilis*, the number of cells harvested at different timepoints were enumerated by counting colonies on Nutrient agar after 4, 6 and 12 h of growth in Nutrient broth. Agar plates were incubated for 24 h before counting.

2.16 Using a single-strain model of TMA-producing bacteria for co-culture experiments with Bio-Kult strains

Previously, individual probiotic strains were investigated for their effect on choline metabolism and TMA production in the complex matrix of human faecal samples. To understand the interaction of probiotic strains with TMA-producing strains directly, strains were co-cultured together using the CMBB fermentation medium in a 96-well high-throughput format inside the anaerobic cabinet. But first, the characteristics of their growth and the best timepoint to harvest cells for inoculation needed to be established.

2.16.1 Growth of probiotic strains and preparation of pellet dilutions

A subset of strains from the Bio-Kult supplement (8 out of 14 strains that were selected based on availability at the time of investigation, supplied by ADM Protexin) was further explored by utilising their freeze-dried form for preparations of glycerol stocks after strains were fully grown in MRS medium, subcultured, grown to exponential phase, and preserved in cryovials as previously described for TMA-producing strains. A growth curve was established using MRS medium by measuring optical density of diluted culture (1:10 with PBS v/v) with a spectrophotometer and clean cuvettes at 0, 4, 6, 8, 10 and 12 h of incubation. Colony forming units of these cultures were also enumerated on MRS agar at 0, 4, 8 and 12 h of incubation, counted after 24-36 h of incubation. At 12 h, cultures were harvested by centrifugation as previously stated and resuspended with PBS made up to 1 mL (apart from strain 8 that did not grow sufficiently within that time and 18 h culture was used for stock preparation). Stock dilutions (100%, 50%, 25% and 12.5%) had their optical density measured and plotted against their respective viable counts plated on MRS agar. These resulted in linear regression equations that were used to calculate OD of 10^8 CFU/mL cultures (as for TMA-producing strains), which was used for streamlined inoculation of co-culture experiments with equal numbers a TMA-producing strain and a probiotic strain. The information about the individual strains, the media that they were grown in, and the time of harvesting cells are reported in

Table 2-11. MRS and BHI media were prepared per manufacturer's instructions (Oxoid: CM0359 and CM1135, respectively) and solidified with 1.6% agar.

Table 2-11 Probiotic strains from the Bio-Kult supplement and their fermentation conditions.

Strain number	Strain name	Protexin® ID	Medium	Time of harvest (h)
1	*Lactobacillus acidophilus* R20001	PXN® 35™	MRS	12
2	*Lactobacillus paracasei* R20022	PXN® 37™	MRS	12
3	*Lactobacillus rhamnosus* R20031	PXN® 54™	MRS	12
4	*Bifidobacterium longum* R20008	PXN® 30™	BHI	18
5	*Lactobacillus plantarum* R20125	PXN® 47™	MRS	12
6	*Bifidobacterium bifidum* R20032	PXN® 23™	BHI	18-20
7	*Bacillus subtilis* R20059	PXN® 21™	MRS	12
8	*Streptococcus thermophilus* R20035	PXN® 66™	MRS	16-18

2.16.2 Confirmation of inoculation load using bacterial plating

To confirm the inoculation concentration each time 10^8 CFU/mL diluted stocks of strains were prepared, stocks were serially diluted and plated using the Miles and Misra method on MRS or BHI agar to determine the viable count at the time of inoculation. The concentration of diluted stock served as a proxy of viable count from inoculation point as 1:10 or 1:100 dilutions were used for inoculation of experiments to reach a final concentration of 10^6 CFU/mL at inoculation (0h).

2.16.3 Media preparation and experimental design of co-culture experiments

CMBB was used for inoculations of co-culture experiments, supplemented with 2 mM final choline concentration in both co-culture conditions and in monocultures of TMA-producing strain and probiotic strains. *P. mirabilis* supplemented with choline was considered a control condition and probiotic strains with supplemented choline served as negative controls of choline metabolism as they do not contain *cutC/D* genes. A vehicle control (VC) was media only and microbiota-free control (MFC) had supplemented choline, but no strains were added. The experimental design is outlined in **Table 2-12** and all conditions were prepared as batch cultures in Falcon tubes and 1 mL of each condition was dispensed into a sterile deep 96-well plate in triplicate as shown in **Figure 2-6**. Original batch cultures in Falcon tubes were then used for enumeration of viable count (CFU/mL). Over the incubation period of 24 h, samples were taken at 0, 2, 4, 6, 8, 10 and 24 h after inoculation of strains into CMBB.

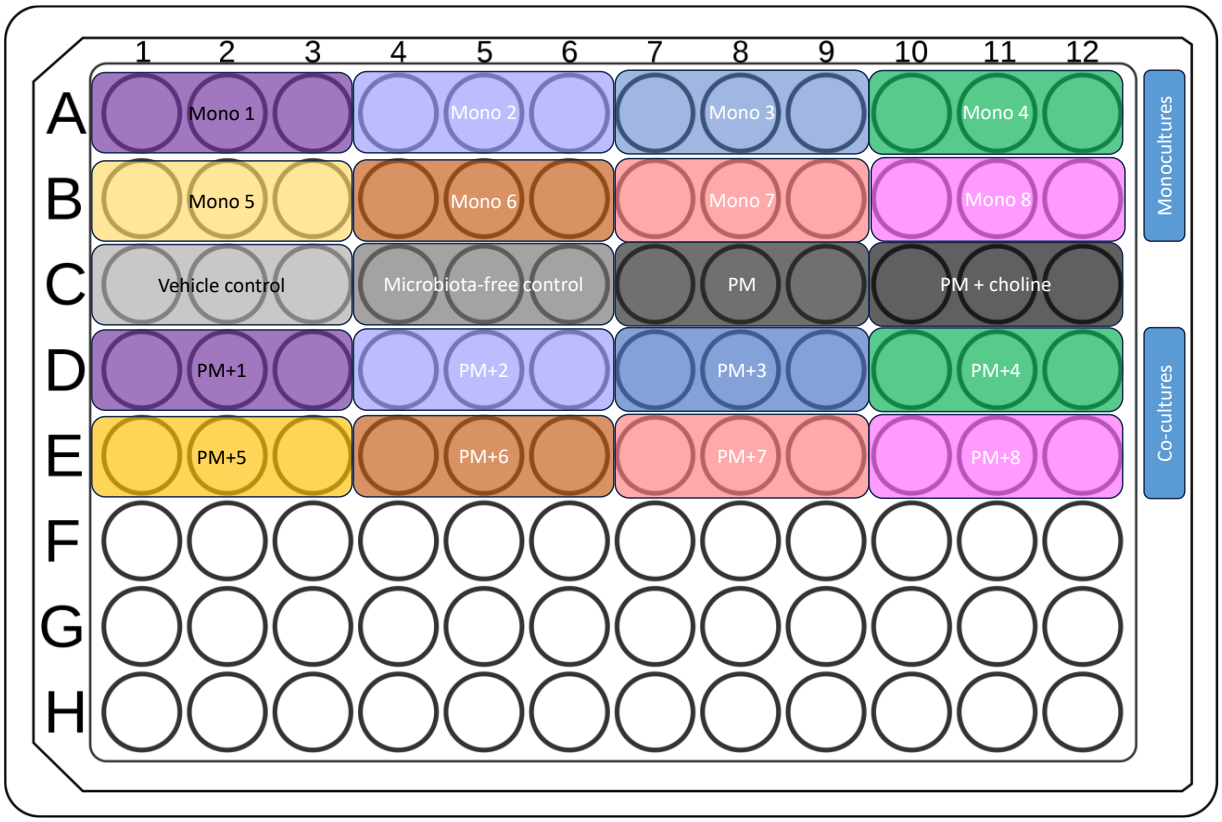

Figure 2-6 96-well template of co-culture experiment – PM + individual probiotic strains

Table 2-12 Experimental design of TMA-strain co-culture with individual probiotic strains

Type of culture	Vials	Probiotic	*P. mirabilis*	Choline
Monocultures	Mono 1	+	-	+
	Mono 2	+	-	+
	Mono 3	+	-	+
	Mono 4	+	-	+
	Mono 5	+	-	+
	Mono 6	+	-	+
	Mono 7	+	-	+
	Mono 8	+	-	+
	P. mirabilis (PM)	-	+	-
	P. mirabilis (PM) + choline	-	+	+
Control	VC	-	-	-
Control	MFC	-	-	+
Co-cultures	1 + PM	+	+	+
	2 + PM	+	+	+
	3 + PM	+	+	+
	4 + PM	+	+	+
	5 + PM	+	+	+
	6 + PM	+	+	+
	7 + PM	+	+	+
	8 + PM	+	+	+

During this experiment, multiple outcomes were measured:

- choline and TMA concentration in probiotic monocultures, co-cultures and *P. mirabilis* supplemented with choline (control) at 0, 2, 4 and 10 h using LC-MS/MS.
- bacterial growth in monocultures and co-cultures using optical density at 600 nm changes over 24 h in an anaerobic plate reader measured every 15 min after 30 s of shaking
- confirmation of inoculation load by viable count of diluted stocks of probiotic strains and *P. mirabilis* on BHI agar as previously described
- viable count of probiotic bacteria monocultures on MRS and BHI at 8 h
- viable count of probiotic strains in co-culture with *P. mirabilis* plated on BHI agar at 8 h
- viable count of *P. mirabilis* in co-culture with probiotic strains plated on BHI agar at 8 h
- viable count of all bacteria in co-culture plated on WCH agar at 8 h

From these measurements, the effect of probiotics on TMA production and growth of *P. mirabilis* could be established.

2.17 Investigating the effect of applying probiotic supernatant in a single strain model on choline metabolism and TMA production

To understand the impact of probiotics on TMA production and *P. mirabilis* growth without direct inter-strain interactions, cell-free supernatant from grown cultures of probiotic strains incubated in their recommended media (MRS or BHI) was supplemented into *P. mirabilis* specific swarming (PMS) medium. This was used to simulate a 'postbiotic' effect of probiotic-produced metabolites, as previously reported in literature (198, 213, 214, 233, 234). Moreover, when probiotics grew in MRS and BHI, substrate fermentation substantially decreased the culture pH. To what extent this happened in the co-culture experiments where only limited sources of simple carbohydrates were available was not clear, and importantly the mechanisms of probiotic effect on TMA production from choline by *P. mirabilis* needed to be investigated. Therefore, PMS medium was supplemented with probiotic supernatant in 1:2 ratio (1 part supernatant and 2 parts PMS) that was either adjusted to a neutral pH ~7.0 or non-adjusted with pH of supernatant ~ 3.0-4.0. The impact of supernatant pH on *P. mirabilis* growth and its ability to metabolise choline into TMA was investigated, and the number of copies of the choline-TMA lyase encoding gene (*cutC*) was measured using qPCR to check that TMA production was not affected by the number of *P. mirabilis* cells (measured as *cutC* copy number).

2.17.1 Preparation of probiotic cell-free supernatant

Probiotic strains were grown as described previously for harvesting pelleted cells used for inoculation of co-culture experiments. The cell-free supernatant was harvested, and filter sterilised in anaerobic environment into sterile Falcon tubes. For pH-adjusted conditions, the pH of supernatant was raised to ~7.0 (pH measured using litmus paper inside an anaerobic cabinet) prior to filter-sterilisation. To confirm that the supernatants were not contaminated, 10 µL of undiluted filtered supernatant was inoculated on MRS agar and incubated for 24 h.

2.17.2 Fermentation conditions

Supernatant was added into PMS medium in 1:2 ratio (5 mL filtered supernatant to 10 mL of PMS) and all vials were enriched with choline to a 2 mM final concentration. A final concentration of 10^6 cells/mL of *P. mirabilis* from 10^8 CFU/mL stock was inoculated into supernatant enriched PMS medium. Batch cultures were dispensed into a sterile deep 96-well plate in triplicates and incubated in an anaerobic environment for 24 h at 37 °C. Choline enriched PMS medium with *P. mirabilis* inoculum and anaerobic PBS added instead of supernatant was used as a control (*P. mirabilis* + choline). To simulate the conditions of the supernatant enrichment, MRS medium at neutral pH (*P. mirabilis* + MRS control) and MRS adjusted to pH matching grown probiotic cultures (pH at 12h growth ~3.7) were used as additional controls. A further condition with a pooled supernatant from all probiotic strains added was investigated to mimic the impact of supernatant from a mixed strain probiotic enrichment (*P. mirabilis* + S-mixed). The experimental design is outlined in **Table 2-13**

2.17.3 Outcome measures

To quantify the changes in growth, OD at 600 nm was measured using a 96-well plate reader inside anaerobic cabinet with readings taken every 15 min over 24 h. After incubation for 8 h in PMS medium, individual batch cultures (incubated in glass vials) were sampled and plated on WCH agar, with the number of colonies counted after incubation in an anaerobic cabinet for 24 h. All vials were diluted to 10^{-7}, and all dilutions were plated in triplicate using Miles and Misra method. To assess effects of supernatant on choline metabolism to TMA, samples were taken at 0, 2, 4, 6, 8, 10 and 24 h but only 0, 2, 4 and 10 h samples were analysed for methylated amine concentrations using LC-MS/MS. Finally, at 8 h timepoint, DNA was extracted from cultures to quantify the number of *cutC* genes in *P. mirabilis* cultures (as a proxy for viable count).

Table 2-13 Experimental design of supernatant supplementation to PMS medium inoculated with P. mirabilis

Culture	Condition	*P. mirabilis*	Supernatant	Choline
Supernatant treated	S1 + *P. mirabilis*	+	+	+
	S2 + *P. mirabilis*	+	+	+
	S3 + *P. mirabilis*	+	+	+
	S4 + *P. mirabilis*	+	+	+
	S5 + *P. mirabilis*	+	+	+
	S6 + *P. mirabilis*	+	+	+
	S7 + *P. mirabilis*	+	+	+
	S8 + *P. mirabilis*	+	+	+
Control	MRS control + *P. mirabilis*	+	-	+
Control	MRS low pH + *P. mirabilis*	+	-	+
S treated	S-mixed (neutral) + *P. mirabilis*	+	+	+
Control	*P. mirabilis* + choline	+	-	+

2.18 Using a single strain model of TMA-producing bacteria to investigate the effect of polyphenols on TMA production

Single strains *P. mirabilis* and *D. desulfuricans* were used to investigate the mechanisms behind inhibitory properties of 5 mM chlorogenic acid and 10 mM DMB.

2.18.1 Fermentation conditions

Nutrient broth and Anaerobic basal broth were used for *P. mirabilis* and *D. desulfuricans*, respectively. Media were inoculated with 2% final concentration of 2nd passage cells and 2 mM choline as described in previous sections. Phenolic-treated conditions were also compared to methanol only control that had the same percentage of methanol as polyphenol conditions, as methanol was used for dissolving of phenolic compounds.

2.18.2 Measuring growth by optical density and viable counts

This method was used to determine the population growth in bacterial cultures. Samples were diluted 1/10 (v/v) with PBS to reach 1 mL of solution (100 µL of sample to 900 µL PBS), vortexed and 1 mL was transferred into a clear cuvette to analyse in a spectrophotometer at 600 nm wavelength. Undiluted 1 mL of PBS served as a blank reference. Alternatively, samples were measured using a plate reader in a 96-well plate using 1/1 (v/v) dilution with PBS to reach 300 µL. Spectrophotometer was set at 600 nm wavelength inside an anaerobic cabinet. For enumeration of viable counts on solid media, fermentation samples were diluted 1/10 (v/v) in sterile anaerobic PBS (20 µL sample to 180 µL PBS), using a 96-well plate and serially diluted to 10^{-7}. The contents of the stock well (10^{-1} dilution) were mixed 15 times with a 200 µL multichannel pipette. Tips were discarded and 10 µL was taken and transferred into 10^{-2} well. Mixing was repeated and the process continued until 10^{-7} dilution was reached. Dilution droplets (5-10 µL) were dried, and plates were inverted throughout incubation. Colonies were

counted after 24 h incubation in the anaerobic cabinet and colony forming units (CFU) were back calculated to determine CFU/mL of cultures.

2.19 Quantification of gene copy numbers of *Proteus mirabilis*

This method was used to quantify the overall number of *cutC* gene copies from *P. mirabilis* to determine the effect of probiotic cell-free supernatant on *P. mirabilis*.

2.19.1 DNA extraction and quantification

Samples were collected after 8 h incubation of *P. mirabilis* with cell-free supernatant from Bio-Kult strains. Samples were snap frozen on dry ice and stored at -80 °C until used. DNA was extracted using the Maxwell RSC Cultured cells kit, as per manufacturer's instructions with the Maxwell RSC instrument from a pooled cell pellet from 3 technical replicates per condition. Pellets were resuspended in 300 µL TE buffer with 100 µL lysozyme (25 mg/mL) and mixed for 5 minutes by vortex. Samples were incubated at 37 °C for 30 min. After incubation, 20µl proteinase K and 10 µl RNAase A were added to samples and vortexed prior to starting the Maxwell workflow. DNA was eluted in 100 µL Elution Buffer and quantified using Qubit HS assay as per manufacturer's instructions. After quantification, samples were stored at -20 °C until analysis.

2.19.2 PCR of *P. mirabilis* to amplify fragment of *cutC* gene

To prepare a standard curve of *P. mirabilis cutC* gene, a PCR product of the *cutC* gene fragment was obtained by amplifying a portion of the sequence using previously published primers by Ramireddy *et al.* (235). To prepare template for the PCR assay, *P. mirabilis* DSM 4479 was grown in Nutrient broth enriched with 2 mM choline to exponential phase (6 hours), 500 µL of culture was centrifuged for 1 minute at 13,000 x g and supernatant was discarded. The pellet was washed with colony wash buffer, mixed thoroughly by a sterile pipette, and centrifuged again. Supernatant was discarded and pellet was resuspended with 10 µL sterile MilliQ water. Suspension was heated for 5 min at 95 °C using a heat block and 1 µL was used as template for PCR.

Table 2-14 PCR primers, reagents, and conditions

***cutC*_PM primers**	**Length (bp)**	**Sequence (5'-3')**	**Molecular Weight (kDa)**	**Fragment size (bp)**
Forward	21	CTGGCAGAACGTTTAGTTTCA	6.498	492
Reverse	19	TGGATTACCTTCCATTGCG	5.832	
Reagents			**For 1 sample (µL)**	**Final conc.**
2X GO TAQ G2 GREEN MASTER MIX			25	1X
Forward primer (10 µM stock – diluted from 100 µM)			1	0.2 µM

Reverse primer (10 µM stock – diluted from 100 µM)		1	0.2 µM
Nuclease free water		22	-
DNA template added to each well		1	150-200 ng
Step	**Cycles**	**Temperature °C**	**Time**
Activation	1	94	7 min
Denaturation	35	94	30 s
Annealing	35	58	30 s
Extension	35	72	30 s
Final	1	72	5 min
Fragment statistics			
GC content: 41.3 % Molecular weight: 303,997.36 Da Molarity of 1 µg/µl solution: 3.29 µM Number of molecules in 1 µg: 1.98 x 10^{12} Approx. 260 nm extinction coefficient: 7917610 $lmol^{-1}cm^{-1}$ A_{260} of 1 µg/µl solution after 100-fold dilution: 0.260			

2.19.3 Estimation of gene copy number in DNA fragment from PCR assay of *P. mirabilis*

For the PCR product quantification, product was cleaned up using Qiagen QiaQuick PCR purification kit, quantified with Nanodrop and confirmed with Qubit HS assay. Gene copy number in PCR product was calculated using an online DNA and gene copy number calculator according to the information in **Table 2-14**. The molecular weight per base pair was calculated by dividing the overall molecular weight by the number of base pairs in the amplified fragment (303,997.36/492 = 617.88 Da). PCR fragment was diluted to 10^9 which formed the highest concentration for the qPCR standard curve and was serially diluted to 100 gene copies. To quantify the absolute gene copy numbers of the unknown samples, a qPCR assay was used with new primers amplifying a portion of the PCR fragment in the standard curve. Primers, reagents and conditions are reported in **Table 2-15**.

Table 2-15 qPCR primers, reagents, and conditions

Primer name	**Sequence (5'-3')**	**Length (bp)**	**Product size (bp)**	**Reference**
Cons_*cutC*_PM_qPCR_F	CACAGCGAAGAAGAAGTGCG	20	99	created from *cutC*_PM PCR product
Cons_*cutC*_PM_qPCR_R	ACCTTGATTCACCACCAGCA	20		created from *cutC*_PM PCR product
GAPDH-F	TGACTGGTATGTCTTTCCGTG	21	140	Ramireddy 2021
GAPDH-R	CAGAACGCCTTTCAGTTCGC	20		Ramireddy 2021
Reagents				**For 1 sample (µL)**
GoTaq qPCR Master Mix				10

Forward primer (10 µM stock) (0.2 µM final)		0.4	
Reverse primer (10 µM stock) (0.2 µM final)		0.4	
Nuclease free water		5	
Total		16	
DNA template added to each well		4	
Step	**Cycles**	**Temp°C**	**Time**
Activation	1	95	7 min
Denaturation	35	95	30 s
Annealing	35	58-61*	30 s
Extension	35	72	30 s
Final	1	72	5 min
Melt curve (based on Rath *et al.* 2017)	1	95	5 s
	1	65	60 s
Ramp	7 acquisitions/s	65-97	5 s

Absolute quantification of gene copy numbers was performed by comparing unknown samples to calibration curve prepared with a known gene copy numbers to calculate the absolute cell counts per 1 mL of culture. All samples and standards were assayed in triplicate. Samples were analysed with DA2 tool provided by Thermo Fisher.

BERI STUDY INFORMATION, SAMPLE UTILISATION AND DATA ANALYSIS

2.20 Design, measurement outcomes, samples collected and storage

2.20.1 Study design

The BERI study was carried out by Dr Hassan Aboufarrag, Wendy Hollands, Dr Jasmine Percival, and Dr Paul Kroon, as mentioned in the **Contributions** section of this thesis. Details of the study have been published (3, 11, 12). Samples from the BERI study were made available for use in the work described in this thesis. The following details are adapted from the publication by Aboufarrag *et al.* (3) and the ethics application protocol published in their thesis (12).

The BERI study was a randomized, placebo-controlled, double-blind, three arm crossover human trial investigating the effects of consuming anthocyanin rich extracts from black rice and bilberry fruit on cardiometabolic markers of CVD. Both participants and organisers of the study were unaware which treatment was being consumed, and encapsulated supplements were labelled with A, B and C. Participants consumed 4 capsules each day in the morning for 28 days with a minimum of a 2-week washout period between arms. Participants were required to reduce their consumption of berries to a maximum of 3 portions a week, and these restrictions were established 2 weeks before starting the study and stood for the duration of the trial, including the washout periods. All outcome measures were assessed during a study visit on Day 1 and Day 29 of each arm. More information about the population characteristics and details about the study are available elsewhere (3, 11, 12).

2.20.2 Measurement outcomes

The outcome measures for this study were changes in concentrations of LDL-C, other blood lipids and lipoproteins (total and HDL-C, triglycerides, ApoA1, ApoB1, HDL3), bile acids, biomarkers of glycaemic control (glucose and fructosamine), and biomarkers of PON1 status. Additionally, plasma and urine samples were explored for phenolic metabolites, and their interactions with the gut microbiota were investigated using shotgun metagenomics. Finally, concentrations of TMAO and other methylated amines were assessed in plasma and urine. Faecal samples were assessed for the gut microbial capacity to produce TMA using *in-vitro* colon models, and the abundance of metabolic pathways involved in TMAO production or metabolism were assessed using the metagenomics dataset.

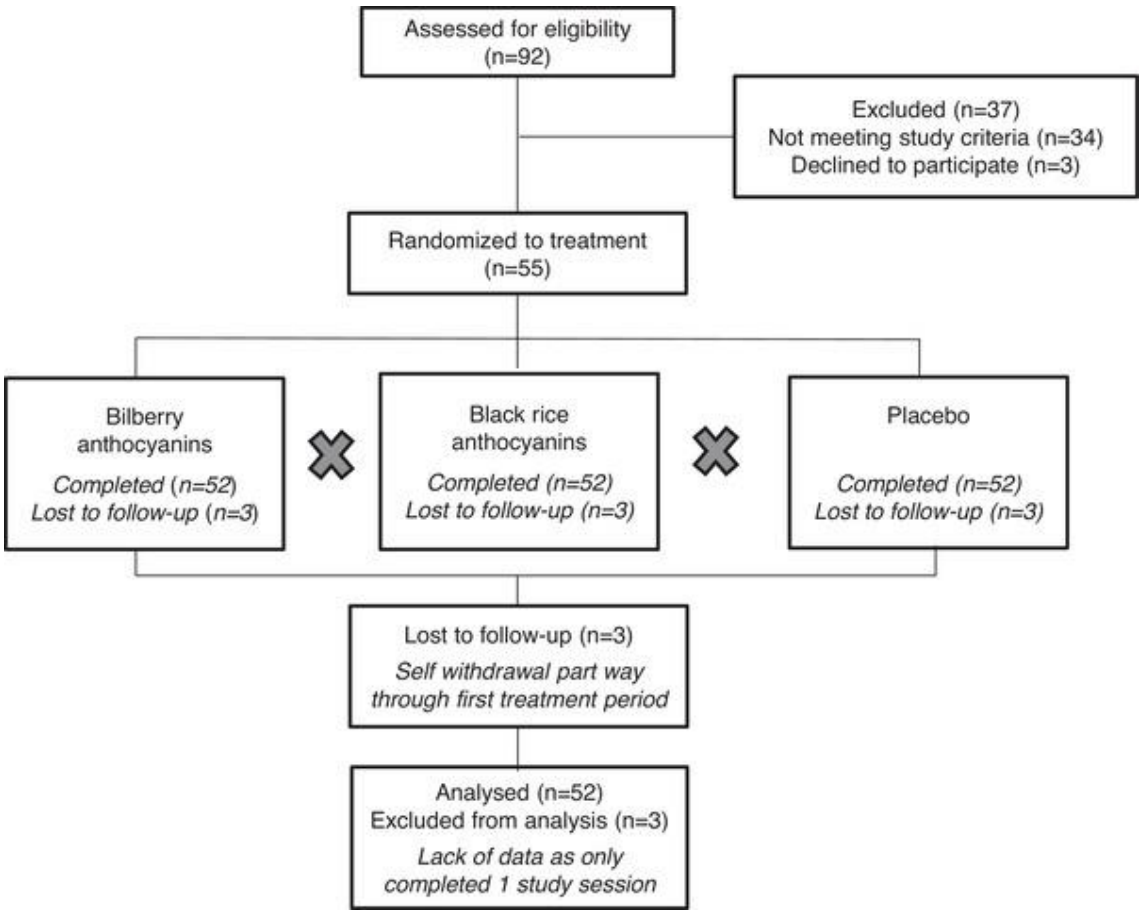

Figure 2-7 BERI study participant workflow from Aboufarrag et al (2022) - (3)

2.20.3 Stool sample collection and storage

Participants were given the option of providing a faecal sample within 3 days preceding the start of the treatment period (Day 1) or ± 3 days of finishing the treatment period (Day 29). Participants were provided with a faecal collection kit consisting of an insulated container, a plastic pot, bag and clip, and ice cube bags. Aliquots of fresh faecal samples were stored at -80 C until analysis. Portions of fresh faecal samples were also used to prepare faecal slurry glycerol stocks (see **Section 2.2** and **2.23**), and these were stored at -80 °C until used.

2.20.4 Urine and plasma sample collection and storage

Urine was collected in containers with ascorbic acid as previously described in the BERI study materials. Aliquots of 24 h urine collection were placed into appropriate vials and stored at -80°C until analysis. Metabolites in urine were determined using LC-MS methods as described in **Section 2.24**. Whole blood was collected into EDTA, and serum separating tubes to obtain plasma and serum as described previously (12). Samples were aliquoted into appropriate storage tubes, frozen on dry ice and then subsequently stored at -80 °C until analysis.

A diagram of samples that were utilised for investigating the relationships between *in-vivo* and *in-vitro* TMAO status and capacity to metabolise choline into TMA is displayed in **Figure 2-8.**

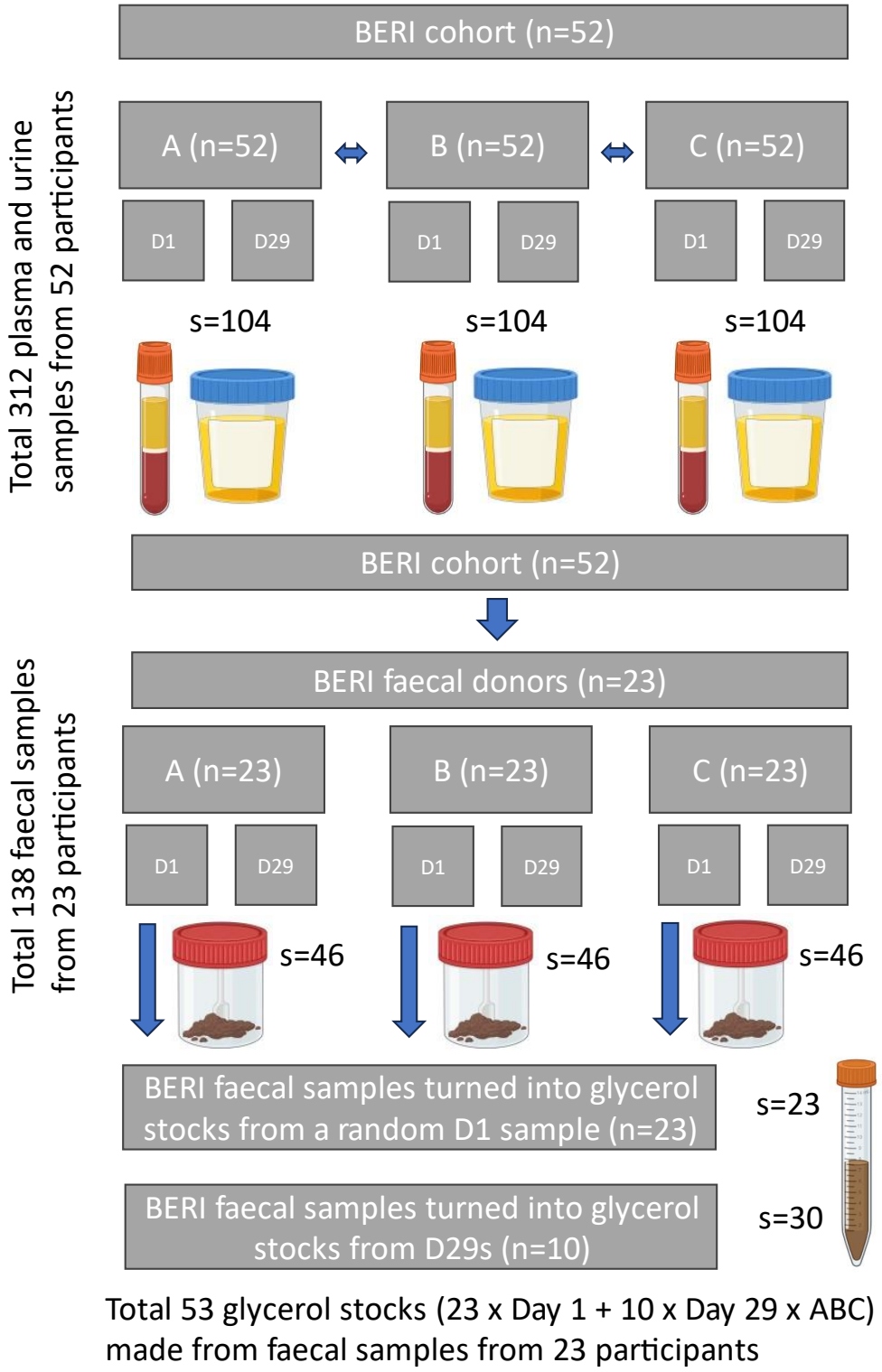

Figure 2-8 BERI study samples and their use in this thesis.

Pictures in figure provided by Biorender. Figure was drawn using MS PowerPoint.

2.21 DNA extractions of faecal samples

Preparation of DNA extracts was carried out by Dr Jasmine Percival. Faecal samples were thawed on ice and approximately 200 mg (± 5 mg) was weighed into a sterile tube and subjected to DNA extraction using the FastDNA Spin Kit for Soil according to manufacturer guidelines, with an additional bead beating step using FastPrep as previously described by Dr Jasmine Percival (11). DNA samples were then quantified using Qubit broad range reagents according to manufacturer guidelines, samples were kept on ice throughout DNA extraction and quantification protocols. Samples were then stored at -80°C until use.

2.22 Library preparation for sequencing

Library preparation from DNA extracts was carried out by Dr Jasmine Percival. DNA extracted from faecal samples was diluted to 0.5 ng/µl in DNAase-free water, a library was prepared by the QIB in-house sequencing department according to the method described by Foster-Nyarko *et al.* (236). Novogene performed shotgun-metagenomic sequencing at a sequencing depth of 10 Gb per sample using the Illumina Novaseq 6000 platform, producing raw paired-end reads.

2.23 Glycerol stock preparation for colon models

Faecal samples from the BERI study, used for experiments in Chapter 3 and Chapter 4, were provided by study volunteers within 6 hours of production. To preserve the microbial composition of faecal samples, a portion of sample was weighed into a double-layer mesh-filter stomacher bag using a wooden tongue depressor inside a Class II microbiological safety cabinet. The sample was then diluted 1:1 with 0.5 mM potassium phosphate buffer containing 0.05% L-cysteine hydrochloride at pH 6.6 which was then homogenised in a Stomacher at 230 rpm for 30 s. The filter insert was discarded, and faecal slurry was mixed with 99% glycerol based on the volume to give a final concentration of 25% glycerol. Slurry was thoroughly mixed and aliquoted into sterile 15 mL centrifuge tubes for storage at -80 °C for a period of 2-5 years (samples were collected in 2017-2018 and used throughout the project from 2019-2022).

2.24 Metabolomics analysis of methylated amines

Sample preparation and quantification of methylated amines from the BERI study was carried out by Dr Priscilla Day-Walsh. This section is adapted from our published article (4). Plasma and urine samples from the BERI study were thawed on ice, vortexed and then centrifuged for 10 min at 13000 × g and 4 °C. Ten µL of supernatant was added to 20 µL of 50% trichloroacetic acid (TCA) and refrigerated at 4°C for 5 min or cooled on ice to precipitate protein. Internal standard solution was prepared using stable isotope labelled compounds TMAO-d9, TMA-d9, choline-d9, L-carnitine-d9 diluted in 0.2 M acetic acid to reach a final concentration of 25 µM.

External standards for calibration curve were prepared using 100 mM solutions of TMAO, TMA, choline chloride, L-carnitine, betaine and γ-butyrobetaine. Ten µl of each solution were added to 440 µl of matrix (pooled urine or plasma). This stock solution was serially diluted to gain concentrations of 1000, 500, 250, 125, 62.5, 31.25 and 0 µM (matrix only). Ten µL of each standard dilution was added to 20 µL of TCA as seen in previous step of treating samples. All samples and standards were treated with 70 µL of internal standard solution, vortexed and then centrifuged for 10 min at 13000 × g. Chromacol vials (amber with inserts) (Thermal Fisher Scientific, UK) were filled with 95 µL milliQ water and 5 µL of supernatant from samples. Vials were crimped with aluminium caps, mixed by vortex and refrigerated at 4 °C until analysis.

For analysis, the Agilent 6490 MS/MS system was used, equipped with an electrospray ionization (ESI) source operated in positive-ion detection mode. Retention times and multiple reaction monitoring (MRM) were used to identify the metabolites:

Table 2-16 Methylated amines detected by LC-MS/MS, their retention times, and mass-to-charge ratios (m/z) as reported by Day-Walsh* et al. *(4)

Compound	**Retention time (min)**	**m/z**
Betaine	0.837	58.1
L-Carnitine	0.954	43.1
d_9- L-Carnitine	0.932	43.1
Choline	1.590	45.1
d_9-choline	1.588	49.2
TMA	1.699	44.1
d_9-TMA	1.677	49.2
TMAO	1.432	59.1/58.1
d_9-TMAO	1.451	66.1

Mobile phase A contained 10 mM ammonium acetate with 0.05% heptafluorobutyric acid (HFBA) in distilled water. Mobile phase B consisted of 10 mM ammonium acetate, 0.05% HFBA in 90% methanol. The gradient was started with 0% B, increased 3% B within 4 min, after washing for 2 min and equilibration was for another 2 min. The LC flow rate was 0.2 mL/min. The column used for this method was Acquity UPLC BEH C8 1.7 µm (50 x 2.1 mm) column. The column temperature and auto sampler were maintained at 40°C and 4°C, respectively. One µL was used for the injection volume. The quantification was done by the Agilent MassHunter quantitative software. The peak area ratio between the analyte/internal standard concentration was plotted for calibration curve. This protocol was developed by Dr Shikha Saha as previously mentioned.

2.25 Metagenomics analysis

Raw reads were trimmed and quality control was performed using fastp v0.20.1 with default options for paired-end data to remove adapter sequences and human sequences were

removed using Kraken2 v2.0.8, mapping the human reference genome (GRCh38.p12) as previously described in detail by Dr Jasmine Percival in her thesis (11). This analysis was carried out by Dr Perla Rey who used high quality reads to estimate the microbial taxonomic composition profiles using MetaPhlAn v3.0.2 and then performed functional profiling with HUMAnN3, which was used to estimate abundances of metabolic pathways determined by orthologous families from the KEGG Orthology database. Shannon diversity index was used to compare richness and evenness of individual samples. The assigned pathways from HUMAnN3 were then classified using EC numbers, Pfam domains and KO entries associated with TMA production and utilisation. Some other genes encoding enzymes that are homologs of choline-TMA lyases or are co-located in the wider *cut* cluster were also included. A table of included terms that were searched for in the HUMAnN3 abundance tables are available in supplementary information.

2.26 Data analysis, visualisation and statistics

Data was analysed and figures were generated using GraphPad Prism 10. Some analysis was carried out in MS Excel using pre-formatted formulae such as AVERAGE, STDEV, MIN, MAX, COUNT and others. Most figures and values show mean ± SD unless otherwise stated. Specific statistical analysis for each experiment was described in figure legends and all files are available for re-calculation/re-use in a Github repository.

SOPs, MATERIALS, EQUIPMENT, AND SOFTWARE

Other methodology that was used throughout the project is described below, with lists of reagents, materials, equipment and software used available in a database as part of supplementary information available in a Github repository:

(https://github.com/BarboraPeck/PhDthesis)

2.27 Quantification of methylated amines in *in-vitro* fermentation samples using LC-MS/MS

2.27.1 Preparation of reagents

Table 2-17 LC-MS reagent preparation

Reagent	Preparation	Storage	Use
0.2 M acetic acid	To make 250 mL, 2.871 mL of glacial acetic acid was added into 50 mL of dH_20 in 250 mL volumetric flask, swirled around and brought to 250 mL mark. Stock solution of glacial acetic acid was 17.416 M based on a density of 1.049 g/mL, a formula weight of 60.05 g/mol, and a concentration of 99.7% w/w.	Stored in room temperature.	Dilution of isotopically labelled compounds for internal standard solution
50% trichloroacetic acid (TCA)	To make 100 mL of 50% TCA, 49.8 g of >99.0% TCA (crystalline in room temperature) was weighed out in a fume hood on glass weighing boat and diluted to 100 mL in a volumetric flask. This number was calculated based on 50% concentration of 6.1 N trichloroacetic acid solution available for purchase	Stored at 4 °C	Precipitation of protein in culture supernatants
100 mM stock solutions for standard curves of methylated amines	Following compounds were weighed and diluted in 1 mL Milli-Q water to prepare stocks according to the following instructions: (g) = molecular weight (g/mol) * concentration (mol/L) * volume (L) -TMAO (mg) = 75.11 mg/mmol * 100 mmol/L * 0,001 L = 7.51 mg -TMA (mg) = 95.57 mg/mmol * 100 mmol/L * 0,001 L = 9.56 mg -Choline (mg) = 139.62 mg/mmol * 100 mmol/L * 0,001 L = 13.96 mg -L-carnitine (mg) = 197.66 mg/mmol * 100 mmol/L * 0,001 L = 19.77 mg -Betaine (mg)= 117.15 mg/mmol * 100 mmol/L * 0,001 L = 11.72 mg -γ-butyrobetaine = 181.66 mg/mmol * 100 mmol/L * 0,001 L = 18.16 mg	Can be kept in the freezer (-20°C) and defrosted each time before preparing a standard curve, however, some substrates degrade over time and quality control of peak areas was carried out.	Preparation of stocks of unlabelled compounds used for standard curve dilution of methylated amines
25 µM internal standard preparation (TMAO-d9, TMA-d9, choline-d9, L-carnitine-d9 and γ-butyrobetaine-d9)	Internal standard was prepared in 0.2 M acetic acid Volume of isotopically labelled compound was calculated based on volume of internal standard needed – each sample and standard curve dilution needed 70 µL of internal standard, therefore, if there were 94 total samples to be analysed, the required amount of internal standard was 6.6 mL (94x70=6580 µl). To allow for some error, 7 mL of internal standard was prepared according to the formula:	Used immediately each run or prepared in a batch of 100 mL and aliquoted into 15 mL falcon tubes for smaller runs and kept at -20°C.	Dilution of isotopically labelled methylated amine stocks stored at -20 and diluted to 25 µM final concentration in internal standard solution. Stock

	$V_1 = (c_2V_2) / c_1 = (25\ \mu M * 7\ 000\ \mu l) / c_1$ Where V_1 is the volume of labelled compound stock solution that was added to 0.2 M acetic acid (7 ml takeaway sum of added compound volume -> 7000 µl – 96.33 µl of compounds = 6903.7 µl of acetic acid) - TMAO-d9 (µL) = (25 µM * 7 000 µl) / 11 900 µM =14.7 µl - TMA-d9 (µL) = (25 µM * 7 000 µL) / 9 600 µM = 18.2 µL - Choline-d9 (µL) = (25 µM * 7 000 µL) / 13 400 µM = 13 µL - L-carnitine-d9 (µL) = (25 µM * 7 000 µL) / 4 900 µM = 35.7 µL - γ-Butyrobetaine-d9 (µL) = (25 µM * 7 000 µL) / 5 900 µM = 29.66 µL - Betaine-d3 = (25 µM * 7 000 µL) / 6 400 µM = 27.3 µL Compounds were added to acetic acid in 15 mL Falcon tube and vortexed thoroughly after each compound was added.		concentration was calculated from mg/mL reconstitution of isotopically labelled compounds based on available amount.
Faecal fermentation matrix for standard curve	Preparation of 1% faecal culture in batch colon model media for standard curve matrix. Matrix was prepared by dilution of 37% faecal glycerol stock (for 10 mL of matrix, prepare 9733 µL nutritive culture medium and 266 µL of faecal slurry) or by using 10% faecal slurry with PBS (1 mL of slurry and 9 mL of CMB medium). Prepared matrix was based on the faecal concentration in the fermentation samples. For batch-batch media (CMBB), faecal percentage was lower, therefore matrix was prepared by mixing 353 µL of 10% faecal slurry with CMBB. Suspension was mixed thoroughly and then centrifuged for 10 min at 4000 x g. Supernatant was filter sterilised using a 45um syringe filter, changing filters to prevent any spillages due to blockages or pressure build up.	Used immediately or stored at -20°C for re-analysis.	
Mobile phase A – 10 mM ammonium acetate with 0.05% heptafluorobutyric acid (HFBA) in water.	Handled in a fume hood. For 500 mL of Mobile phase A, 0.3854 g of ammonium acetate (HPLC grade) was added to 460 mL deionized water in a 500 mL volumetric flask, fully dissolved, then supplemented with 250 µL of heptafluorobutyric acid (HFBA) and brought up to 500 mL with deionized water.	Stored in room temperature	

Mobile phase B – 10 mM ammonium acetate with 0.05% heptafluorobutyric acid (HFBA) in 90% methanol.	Handled in a fume hood. For 500 mL of Mobile phase B, 0.3854 g of ammonium acetate (HPLC grade) was added to 50 mL deionized water in a 500 mL volumetric flask, fully dissolved, then supplemented with 250 µL of heptafluorobutyric acid (HFBA) and brought up to 500 mL with HPLC grade methanol. Solution was mixed and capped tightly to prevent evaporation of methanol.	Stored in room temperature, in a flammable cupboard between runs	
LC-MS wash buffer	25% water, 25% acetonitrile, 50% methanol.	Stored in room temperature, in a flammable cupboard between runs	Used during LC-MS runs to wash injection needle between samples.
External standard curve	A standard curve of methylated amines was prepared by adding 10 µL of each 100mM stock compound into 440 µL of filtered matrix. Highest concentration of standard curve should match the highest expected concentration in analysed samples. Serial dilution was carried out to the lowest expected concentration in analysed samples. A minimum of 6 standard curve points + matrix only samples were used for quantification. - 2000 µM = 10 µL of each 100 mM stock (6 compounds) + 440 µL filtered matrix - 1000 µM = 250 µL of 2000µM + 250 µL matrix - 500 µM = 250 µL of 1000 µM + 250 µL matrix - 250 µM = 250 µL of 500 µM + 250 µL matrix - 125 µM = 250 µL of 250 µM + 250 µL matrix - 62.5 µM = 250 µL of 125 µM + 250 µL matrix - 31.25 µM = 250 µL of 62,5 µM + 250 µL matrix - 15.625 µM = 250 µL of 31,25 µM + 250 µL matrix - 7.8125 µM = 250 µL of 15,625 µM + 250 µL matrix - 3.9 µM = 250 µL of 7,8125 µM + 250 µL matrix - 1.95 µM = 250 µL of 3,9 µM + 250 µL matrix - 0.98 µM = 250 µL of 1,95 µM + 250 µL matrix - 0 µM = only matrix	Used immediately or store at -20°C for later re-analysis	Used for quantification of methylated amines in analysed samples

2.27.2 Sample preparation for LC-MS/MS analysis

Samples were prepared as previously stated in **section 2.24** with minor modifications in sample preparations. Briefly, colon model samples were defrosted in room temperature, vortexed and then centrifuged for 10 min at 13000 × g and 4 °C. Optional filtration of supernatant was carried out if sampling volume was more than 1 mL by a syringe 0.22 µm filter. If samples were not filtered, caution was used to only pipette supernatant and no solid particles to prevent blocking of HPLC system and contamination with microbes. Five µL of supernatant was added to 25 µL of 50% trichloroacetic acid (TCA), vortexed and refrigerated at 4°C for 5 min or cooled on ice to precipitate protein. External standards for calibration curve were prepared as before and diluted in matrix to give concentrations of 2000, 1000, 500, 250, 125, 62.5, 31.25 and 0 µM (matrix only) of analytes. Matrix was prepared by matching percentage of faecal culture with culture media to establish background concentration of methylated amine that would be subtracted from external standard curve to matrix-match. Standard curve dilutions were mixed with TCA same as samples and all samples and standards were treated with 70 µL of internal standard solution (prepared as described before), vortexed and then centrifuged for 10 min at 13000 × g. Chromacol vials (amber with inserts) were filled with 95 µL milliQ water and 5 µL of supernatant from samples. Vials were crimped with aluminium caps, mixed by vortex and refrigerated at 4 °C until analysis. For some experiments where samples were collected in 96-well format, sample preparation was carried out as described but working in 96-well microplates (V-shape bottom) and samples were transferred using multi-channel pipettes into LC-MS instrument-compatible plate to prepare a final dilution with milliQ water in the same dilution factor (10 µL of supernatant with 190 µL milliQ water). Microplates were sealed with a tightly fitted mats, mixed thoroughly and briefly centrifuged for a few seconds to pool liquid. Plates were stored at 4 °C until analysis.

2.27.3 Integration and calculation of concentrations

The quantification was performed using the Agilent MassHunter quantitative software. Using the manual integration mode allows adjusting peak areas, if some are shouldered or absent. The peaks were quality checked to ensure they were within the retention time range. After checking the peaks for all relevant compounds, a table was exported into Excel. Files were saved as an Excel Workbook file to allow adding more tabs for downstream analysis. When manipulating Excel spreadsheets, documentation of formulae, calculations, slopes of equations and any changes that were made were noted on the spreadsheet.

The area under curve (AUC) is indicated as response (resp.). To calculate the final concentration, response of the 0 µM sample from the standard curve was subtracted from the response of each sample. Next, Peak Area Ratio (PAR) was calculated by dividing the AUC

(resp.) of the sample by the response of the internal standard. From these data, the standard curve was obtained by plotting the PAR of the external standards against expected concentrations in each sample of the standard curve. Linear trendline was added to the graph, forced intercept through zero and displayed equation. The slope of the equation of the standard curve was used to obtain absolute concentration (μM) for each of the samples: PAR of each sample was divided by the y of the standard curve equation.

2.27.4 Adjusting for volumes (CMB only)

The buffers used in the batch colon model dilute the metabolite concentrations over time. Therefore, it is important to note down the final volumes present in the vessels upon completing the experiment. The final volumes were used to adjust the LC-MS metabolite data for their dilution, using the following steps:

1. Calculate total volume: note final volume in the Excel sheet, create a new column "Total volume" and sum up the volume removed (e.g., 1 mL per time point would be 1* X time points) with the final volume, for each vessel
2. Calculate the difference in volume: create new column "Difference" and subtract the volume added at T0 from the total volume, for each vessel
3. Calculate volume change factor: create new column in which you divide the total volume by the volume added at T0, for each vessel
4. Calculate 1-fold change: create new column "1-fold change" and subtract 1 from volume change factor, for each vessel
5. Adjustment of volume per sample:
    - Take the concentration of time point X and multiply by (1+ "1-fold change") that matches the vessel
    - Multiply the obtained number by the time point divided by total time, e.g. 0/48 if adjusting for vessels at T0 and total time is 48 h
    - Confirm calculations: the data for the last time point times the volume change factor should match the adjusted volume for the last time point

These final concentration values were used for any downstream analysis.

Concentration values were converted into molar percentages of the concentration of supplemented choline and TMA produced from choline where choline abundance (mol%) indicates the percentage of highest molar concentration of choline measured in each fermenter as initial choline concentrations were not always the highest values due to possible entrapment of choline in faecal matrix upon supplementation or incomplete homogenisation of added choline in the sampled fermentation medium at 0 h. TMA from choline (mol%) is a molar equivalent of the highest measurement of supplemented choline that was converted into TMA during the incubation period, with background TMA or TMA arising from choline supplementation measured at 0 h subtracted from subsequent samples.

Chapter 3

The relationship between TMAO status and faecal capacity to produce TMA

ABSTRACT

Background

Trimethylamine N-oxide (TMAO) is one of the risk factors of cardiovascular disease mortality. Elevated concentrations of TMAO have been linked to increased chance of major adverse cardiovascular events due to promotion of atherosclerosis. There are several microbial metabolic pathways for TMAO production from dietary precursors. In particular, TMAO can be derived from choline (found in poultry, dairy and eggs) that is metabolised by the gut microbiota into trimethylamine (TMA), and then converted into TMAO in the liver.

Aims

The overall aim was to test the hypothesis that the capacity of individuals to convert choline to TMA (measured using *in-vitro* fermentation and metagenomic analysis of faecal samples) correlates with their plasma and urinary TMAO levels.

Approaches and Methods

Plasma and urinary TMAO levels were measured from participants in the BERI study to establish *in-vivo* TMAO status. *In-vitro* fermentations of faecal glycerol stocks from BERI study participants were undertaken in a batch colon model to investigate the metabolism of choline to TMA by the gut microbiota. Fermentation samples, urine, and plasma were analysed by LC-MS/MS method using isotopically labelled internal standards of methylated amines to measure concentration of TMAO, its precursors and metabolites. Faecal samples were subjected to shotgun metagenomics sequencing to establish their taxonomic and functional profile.

Results

In-vivo markers of TMAO status from the BERI study participants were generally stable with occasional samples having an elevated TMAO concentration. There was no relationship between *in-vitro* ability of the gut microbiota to metabolise choline to TMA and *in-vivo* TMAO status. Occasional high *in-vivo* TMAO samples were not associated with a change in capacity of gut microbiota to convert choline to TMA, and were associated with another factor, possibly diet. Metagenomic analysis revealed that the structure and function of the gut microbiota were similar for most members of the human cohort, indicating that similar genera/species and enzymic pathways were likely involved in TMA formation and degradation. The structure and function of the gut microbiota was different for one cohort participant (BERI 26), but this did not impact on *in-vivo* TMAO status or *in-vitro* metabolism of choline to TMA.

Conclusion

The hypothesis that the capacity of individuals to convert choline to TMA (measured using *in-vitro* fermentation and metagenomic analysis of faecal samples) correlates with their plasma and urinary TMAO levels was not supported. Most individuals had a similar *in-vitro* ability to metabolise choline to TMA, structure/function of the gut microbiota, and *in-vivo* TMAO status. Occasional high *in-vivo* TMAO samples were not associated with a change in the capacity of gut microbiota to convert choline to TMA nor in structure/function of the gut microbiota and may be diet-related. It would be interesting to retest the hypothesis with a cohort of individuals with a consistently low or high *in-vivo* TMAO concentration.

INTRODUCTION

Trimethylamine *N*-oxide (TMAO) is formed in the liver by flavin-containing monooxygenases (FMOs) into an oxidised product of gut microbiota-derived metabolite trimethylamine (TMA) (39). TMA is generated from dietary precursors including choline, phosphatidylcholine, L-carnitine, and betaine exclusively by microbial enzymes produced by organisms that reside in the human colon (52). Plasma TMAO concentrations have been linked to various cardiometabolic diseases and all-cause mortality risk (237-239). Elevated levels of TMAO in mechanistic studies have been reported to promote endothelial dysfunction (240), increase platelet reactivity and contributing to thrombosis (79), adversely affect lipid metabolism (241) and inflammatory responses (242), suggesting the involvement of this compound in the progression of atherosclerosis. However, the importance and surveillance of TMAO levels in healthy population have not been explored to the same extent and there is a lack of standardisation in methodological approaches to measure TMAO in human subjects (38). The gut microbiota and TMAO levels can be influenced by external factors such as dietary intake and substrate absorption, and there is a need for detailed assessment of eating habits and consumption of substrates prior to test days to investigate the role of diet in the production of TMAO (117-119). Furthermore, studies usually determine the association of TMAO with disease based on their own definition of 'elevated' TMAO levels and more standardised terminology and methodology needs to be established to allow easier comparison of effects across different cohorts and populations (95, 99). In most studies, TMAO levels were only measured on one occasion (1 timepoint) which might not be the best representation of plasma levels of gut metabolites (49, 90). The expression of FMO3 in the liver is also associated with differences in TMAO levels and seems to be a part of a complex diet-gene regulation-liver-microbiota homeostasis (41, 52, 77, 78, 119, 136). The availability of TMAO precursors in the colon is also dependent on the absorption of these compounds in the small intestine (35, 45, 139), with plasma choline and betaine levels showing stability over time, whereas TMAO levels were found to be more variable in a 1-year follow up study by Kuhn *et al.* (137). Regardless of these limitations, it is important to investigate the changes in plasma and urinary TMAO levels over time and how these can be used to establish TMAO status of participants.

It has been proposed that the gut microbial population and its metabolism of TMAO precursors affects TMAO levels in plasma and urine (117, 157, 243). With the use of metabolomics and genome sequencing techniques, substrate metabolism has been attributed to microbial pathways and reactions linked to enzyme commission (EC) numbers and the orthology of genes and genomes of participating microbes. Craciun and Balskus (56, 57) discovered a choline utilisation gene cluster in *Desulfovibrio desulfuricans* and identified an

enzyme choline-TMA lyase, generating TMA and acetaldehyde from choline (58). One of the genes of the cluster encoding this enzyme is *cutC* and its activator *cutD* which encodes a glycyl radical activating enzyme. Other genes encoding enzymes linked to substrate metabolism are the *cntA/cntB* pathway of L-carnitine catabolism, and *yeaW/yeaX* pathway reported by Koeth (61). With the improvement of sequencing techniques and establishment of databases collating information about the different metabolic pathways involved in substrate utilisation and metabolite production, the capacity of the gut microbiota to produce TMA from dietary precursors can now be investigated *in-silico*. However, this capacity needs to be tested *in-vitro* to establish the actual production of TMA by the human microbiota from different substrates. Substrate metabolism can be tested using *in-vitro* fermentation colon models inoculated with faecal samples or another microbiota-rich specimen from human subjects or by using a consortium of bacteria representative of the human gut population. Romano and colleagues (60) isolated commensal bacteria from the human gut and tested them for their ability to utilise choline *in-vitro* under anaerobic conditions. They identified nine strains (out of 79 isolated strains) from eight species representing six genera that showed significant choline consumption and TMA production: *Anaerococcus hydrogenalis, Clostridium asparagiforme, C. hathewayi, C. sporogenes, Escherichia fergusonii, Proteus penneri, Providencia rettgeri,* and *Edwardsiella tarda*. They also noted that some strains of the same species differed in their ability to metabolise choline, suggesting that the choline utilisation cluster might be a strain-specific metabolic trait that was likely acquired by lateral gene transfer. This suggests that phylogeny might be a poor predictor of microbial production of TMA and further insight into the metabolic potential of certain bacterial populations is needed. Therefore, by investigating the *in-vitro* capacity to produce TMA from precursors such as choline, its relationship with *in-vivo* TMAO status and *in-silico* predisposition for TMA production can be established.

3.1 Hypothesis and aim

The overall aim was to test the hypothesis that the capacity of individuals to convert choline to TMA (measured using *in-vitro* fermentation and metagenomic analysis of faecal samples) correlates with their plasma and urinary TMAO levels.

3.2 Objectives and approaches

The approaches used for fulfilling the objectives proposed to test the set hypothesis were:

Objective	**Approach**
Establish *in-vivo* TMAO status of participants from the BERI human study	Measure the plasma and urinary TMAO levels in 52 human participants in the BERI study
Determine participant capacity to metabolise choline into TMA using an *in-vitro* human colon model	Quantify the metabolism of added choline to TMA using an *in-vitro* colon model that simulates the human large intestine, enriched with faecal slurry from participants in the BERI study.
Determine the correlation between *in-vivo* TMAO status of participants with their *in-vitro* capacity to metabolise choline to TMA	Use appropriate statistical methods to establish the extent of correlation between participant plasma and urinary TMAO levels and their capacity to metabolise choline to TMA.
Determine the relationship between participant TMAO status and their gut microbiota structure and function.	Use a metagenomics approach to establish the dominant bacterial genera and species, and the presence of genes encoding enzymes involved in TMA formation in the gut microbiota of BERI study participants. Use appropriate statistical methods to establish the extent of correlation between the gut microbiota and (a) participant plasma and urinary TMAO levels, (b) *in-vitro* capacity to produce TMA from choline, and (c) the dominant bacteria and genes encoding enzymes involved in TMA formation detected in the gut microbiota.

RESULTS

The BERI study involved 52 participants undertaking a randomised placebo-controlled 3-way crossover 28-day intervention study investigating the effects of two anthocyanin treatments versus placebo on cardiometabolic markers including lipoprotein profiles and markers of insulin resistance in a hyperlipidaemic cohort (3). Plasma and urine samples were collected before and after each 28-day treatment, and faecal samples were collected at these timepoints from a subset of 23 participants. This study showed no effect on traditional markers of cardiovascular health. However, the effect of the anthocyanin treatments on TMAO status of the participants compared to placebo was not investigated. The purpose of this work was to determine the TMAO status of each participant before and after each treatment. An important precursor of TMAO is TMA formed by the gut microbiota, and the hypothesis was tested that the capacity of participant gut microbiota to form TMA correlates with their TMAO status.

TMAO STATUS IN THE STUDY POPULATION

Two markers of TMAO status were measured in human participants: plasma TMAO concentration (µM) and total urinary TMAO (µmol/24h). The results for plasma TMAO concentration for baseline (Day 1) and after 28 days of treatment (Day 29) are shown in **Figure 3-1** panel A. There did not appear to be any difference in TMAO plasma concentration between the different treatment (Day 1 and Day 29 for A, B and C) and the different Day 1 measures. For all treatments and timepoints, most values were clustered around 4 µM (median = 3.9 µM, range = 7.7 µM, n=52). However, all treatments and timepoints contained substantially higher values that skewed the overall distribution. The distribution was assessed with normality testing (D'Agostino & Pearson test) which confirmed that the data were not normally distributed as displayed in the QQ plot of actual values against values predicted via Gaussian distribution (Panel B). Values were better fitted in a lognormal QQ plot yet some timepoints did not pass the lognormal distribution test and deviated from the Gaussian ideal indicated as red dashed line (panel C), however, the plasma TMAO dataset represented as medians per participant passed the lognormal distribution (p=0.54). Therefore, lognormal testing was used to perform an ANOVA (Friedman test for non-parametric data with Dunn's multiple comparison test) with a heatmap of all values displayed in panel D. There were no significant differences between any of the treatments or timepoints (p=0.59). These data showed that neither timepoint nor treatment affected plasma TMAO concentration, and therefore the six measurements per participant (i.e. six separate timepoints) can be considered independent measures.

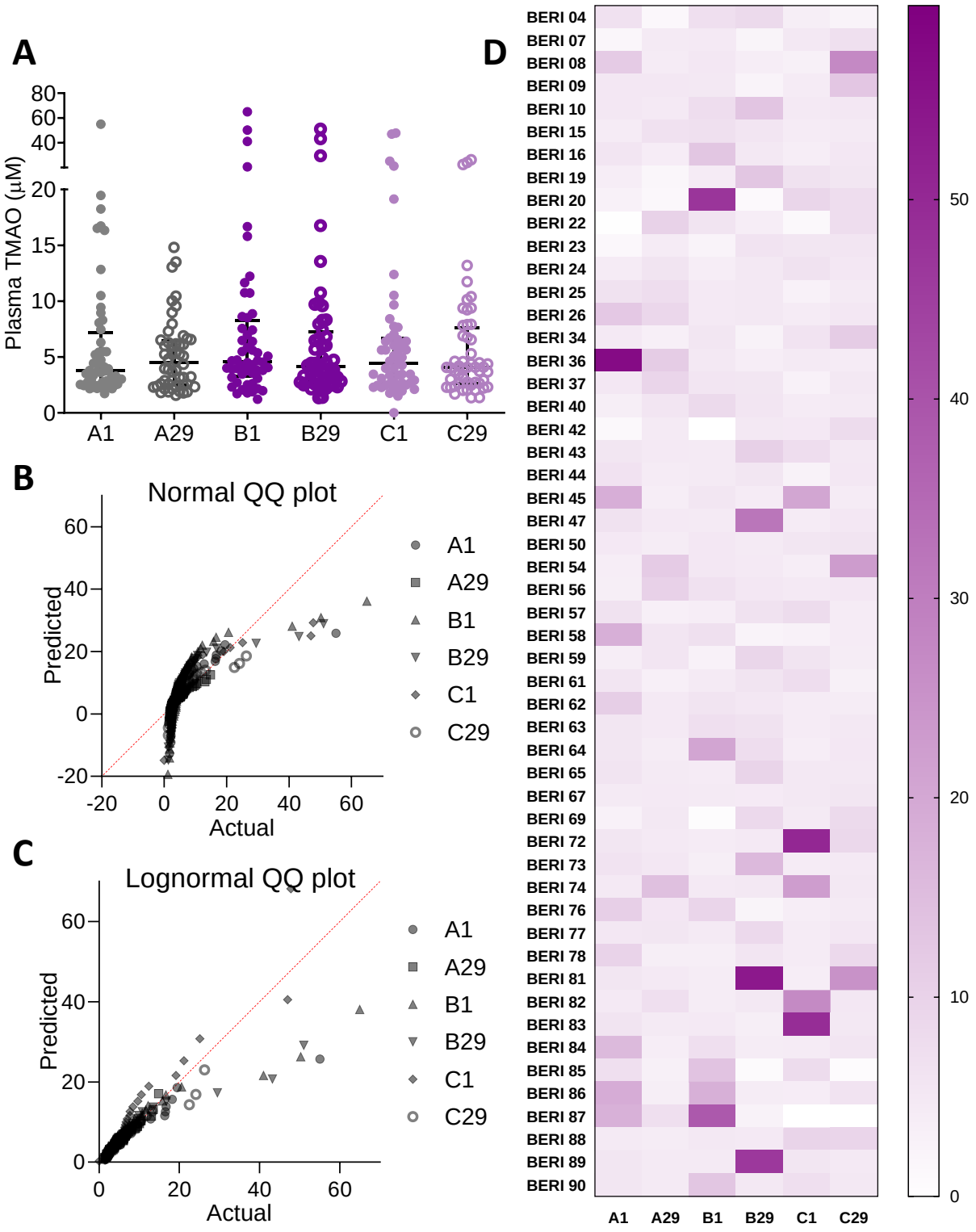

Figure 3-1 Plasma TMAO levels in the BERI study cohort (n=52)

A) Median and IQ range (all values displayed as symbols) plasma TMAO of 52 participants undertaking a randomised placebo-controlled 3-way crossover 28-day intervention study, values were measured before and after each 28-day treatment (Day 1 and Day 29 for treatment A, B and C).

B) QQ plot of Gaussian (normal) distribution of actual vs predicted values measured by D'Agostino & Pearson test.

C) QQ plot of lognormal distribution of actual vs predicted values measured by D'Agostino & Pearson test.

D) Heatmap of all values per participant from a Friedman test for non-parametric ANOVA with Dunn's multiple comparison test, there were no significant differences between any of the timepoints.

3.3 There was substantial intra-individual variation in plasma TMAO

Figure 3-1 panel D suggested a considerable intra-individual variation in the 6 independent measures of plasma TMAO concentration and this was assessed further by calculating the coefficients of variation (CV) of plasma TMAO concentration for each participant. The data in **Figure 3-2** shows the median and range of plasma TMAO concentration and the CV for each individual participant (n=52). This figure confirms that an individual's plasma TMAO values when measured on independent occasions are often highly variable. For example, only 3 CV were below 30%, whereas the CV of 12 individuals was > 100%. Nevertheless, there did appear to be participants whose plasma TMAO concentrations were less variable and those whose plasma TMAO concentrations were highly variable. Since it is possible that the variability of an individual's plasma TMAO concentration could be related to the other TMAO markers that are being assessed in this cohort, the participants were stratified according to their CV. A CV cutoff of 50% was used to establish two categories of participant: stable (< 50% CV; n=19) and changeable (> 50% CV; n=33). Participants in these categories and their individual TMAO values and CV are displayed in **Figure 3-3**.

Participants in the stable group showed median plasma TMAO concentration of 4.5 µM with ~15.0 µM range, showing only 51.3 % CV between all values for all members of this group. On the other hand, for the changeable group the median concentration was 3.7 µM with ~65.0 µM range and the overall CV of 138.7 %.

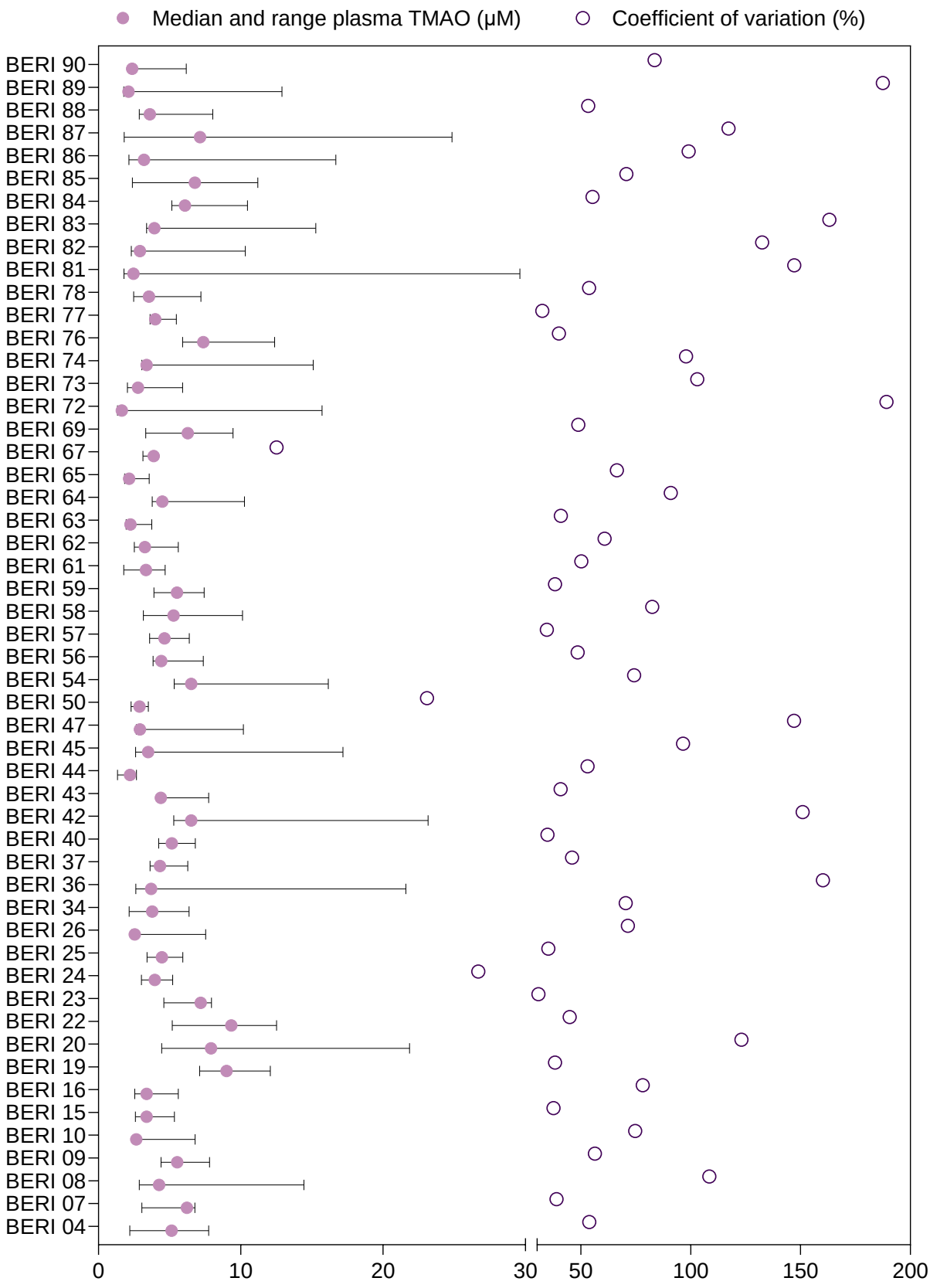

Figure 3-2 Variation in plasma TMAO levels of 52 participants

Median value and range of values of plasma TMAO in each participant measured at 6 separate timepoints in 52 participants (μM) and the coefficient of variation from these 6 timepoints (%). X axis is split into two segments with left side ranging from 0 – 30 to display the median plasma TMAO values (μM) and right side ranging from 30 – 200 to display the coefficient of variation (%).

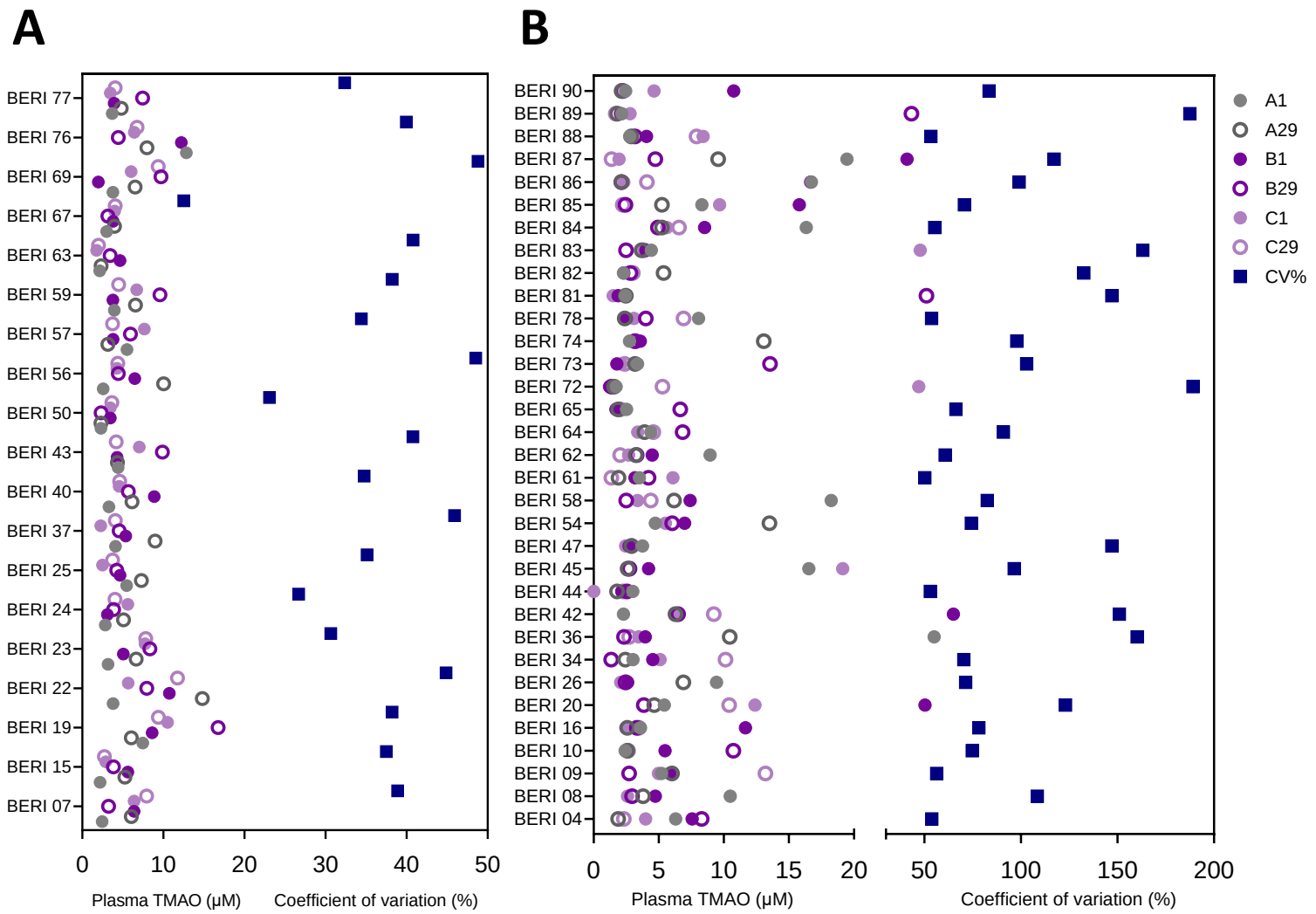

Figure 3-3 Stratification of participants based on their variability of plasma TMAO

Plasma TMAO concentration (μM) measured at 6 separate timepoints and the coefficient of variation (%) per participant. ***A****) participants classified as stable (< 50% CV) and* ***B****) participants categorised as Changeable (> 50% CV).*

3.4 Intra-individual variation was also apparent in urinary TMAO levels

The results for total urinary TMAO produced over 24 h (µmol/24h or µmol/day used interchangeably) at baseline (Day 1) and after 28 days of treatment (Day 29) are shown in **Figure 3-4** panel **A**. Similar to plasma TMAO, there did not appear to be any difference between the values measured at the 6 timepoints with median values of 426.9 µmol/day (5606 µmol/day range, n=52). The distribution of values was skewed similarly to plasma TMAO concentration with all treatments and timepoints containing substantially higher values compared to the median. The distribution was assessed with normality testing (D'Agostino & Pearson test) which confirmed that the data were not normally distributed (Panel B, left) and instead better fitted in a lognormal QQ plot (panel B, right).

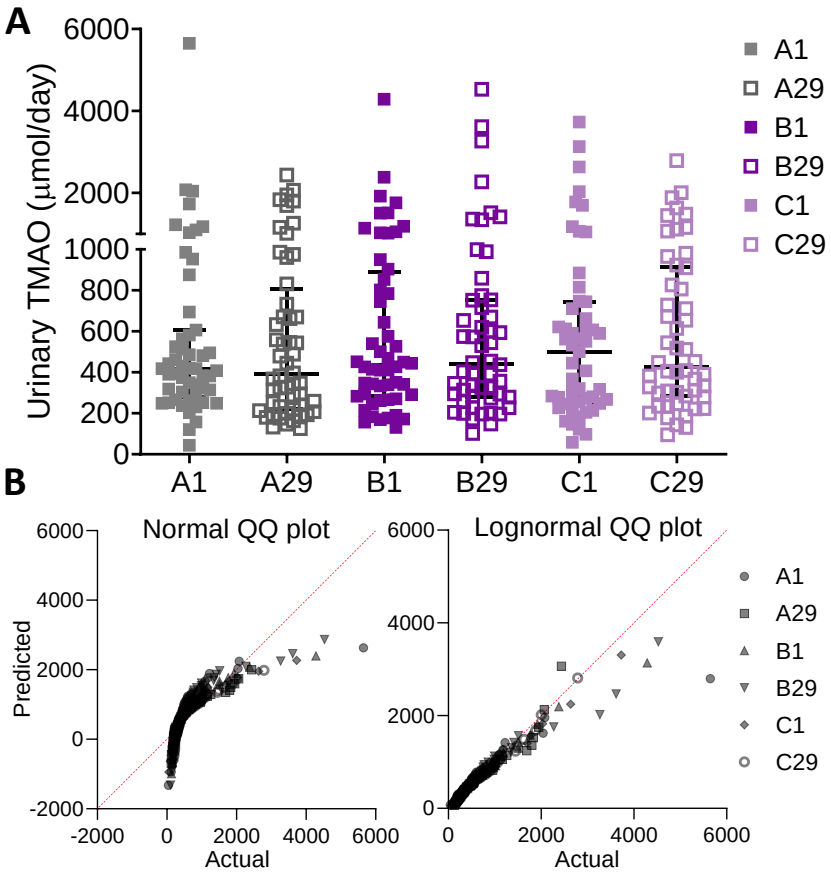

Figure 3-4 Urinary TMAO levels in the BERI study cohort (n=52)

A) Median and IQ range (all values displayed as symbols) urinary TMAO of 52 participants undertaking a randomised placebo-controlled 3-way crossover 28-day intervention study, values were measured before and after each 28-day treatment in a total urinary output per 24 h (Day 1 and Day 29 for treatment A, B and C).

B) QQ plot of Gaussian (normal) distribution and lognormal distribution of actual vs predicted values measured by D'Agostino & Pearson test.

Due to some missing values of participants that did not provide urine on some days, Kruskal-Wallis test with Dunn's multiple comparison test was performed instead of Two-way ANOVA, showing no significant differences between the medians of each timepoint ($p = 0.98$), therefore, the six measurements per participant can be considered independent values.

The variation in plasma TMAO concentration prompted calculation of CV of urinary TMAO levels for each participant. The data in **Figure 3-5** shows the CV for each individual participant and the median and range of urinary TMAO (µmol/24h) for the six independent samples (n=52). Interestingly, only 16 participants showed < 50% CV in their urinary TMAO over the 6 timepoints, with 22 participants showing CV between 50 and 100%, whilst 14 individuals had CV > 100%. Moreover, some participants that showed stable plasma TMAO concentrations were highly variable in their urinary TMAO levels. Performing the same stratification according to their CV in urinary TMAO, with a CV cutoff of 50% categorised as stable (n=16) and the remaining participant classified as changeable (> 50% CV; n=36). To illustrate the differences between these two measures of participant TMAO status, a matrix of variability in plasma and urinary TMAO levels is plotted in **Figure 3-6.** The highest number of participants (n=23) had changeable plasma and urinary TMAO levels. Thirteen participants showed changeable TMAO values in urine but stable values in plasma. The opposite was the case for participants changeable in plasma but stable in urinary TMAO levels (n=10). The smallest group of participants had low variation in TMAO measured in plasma and in urine (n=6).

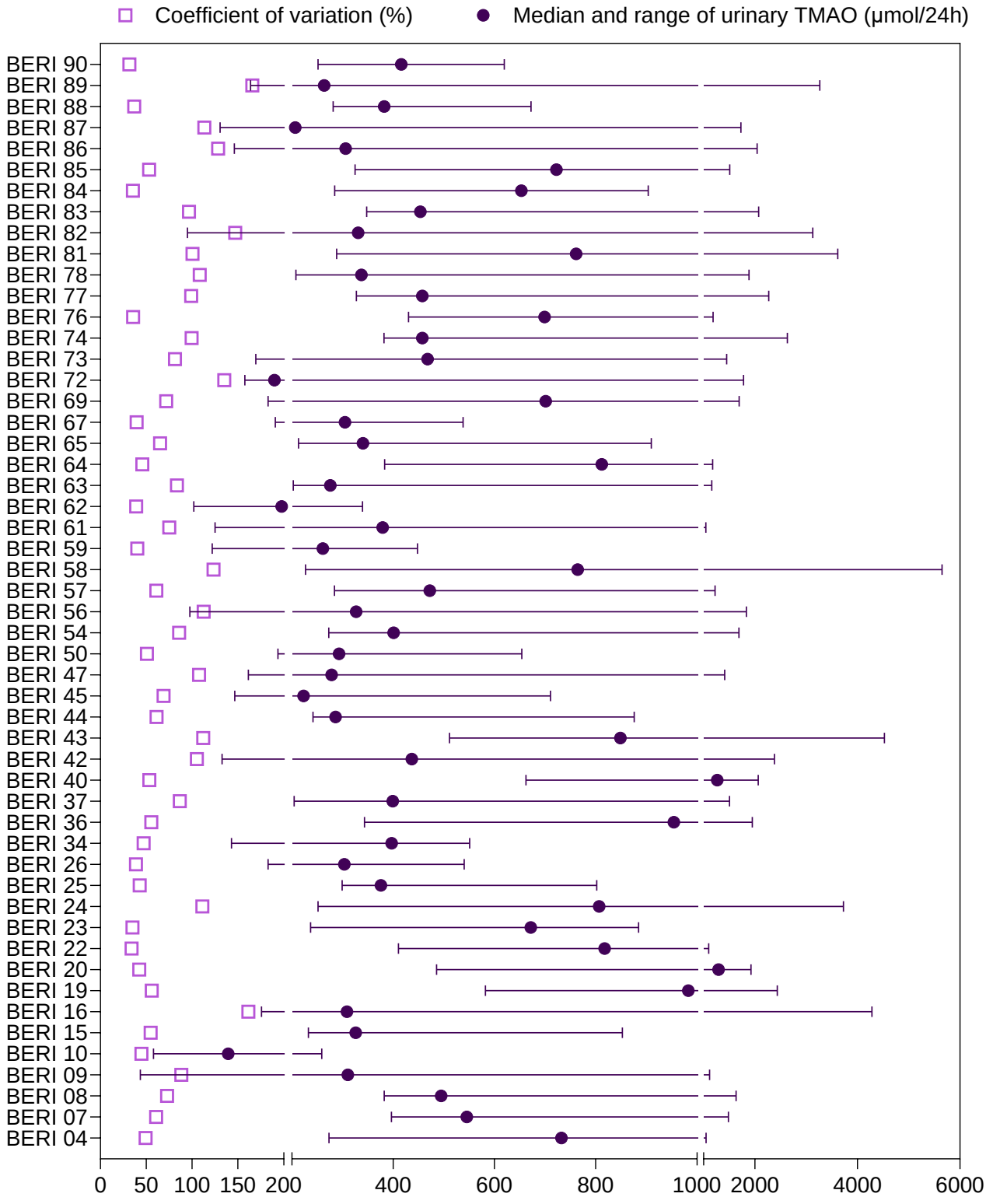

Figure 3-5 Variation in urinary TMAO levels of 52 participants

Median value and range of values of urinary TMAO in each participant measured at 6 separate timepoints in 52 participants (µmol/24h) and the coefficient of variation from these 6 timepoints (%).The X axis is split into three segments with left side ranging from 0 – 200 to display the coefficient of variation (%), middle part ranging from 200 – 1000 µmol/24h to show the median urinary TMAO values for majority of participants and right side ranging from 1000 – 6000 to display the range and high urinary TMAO producers.

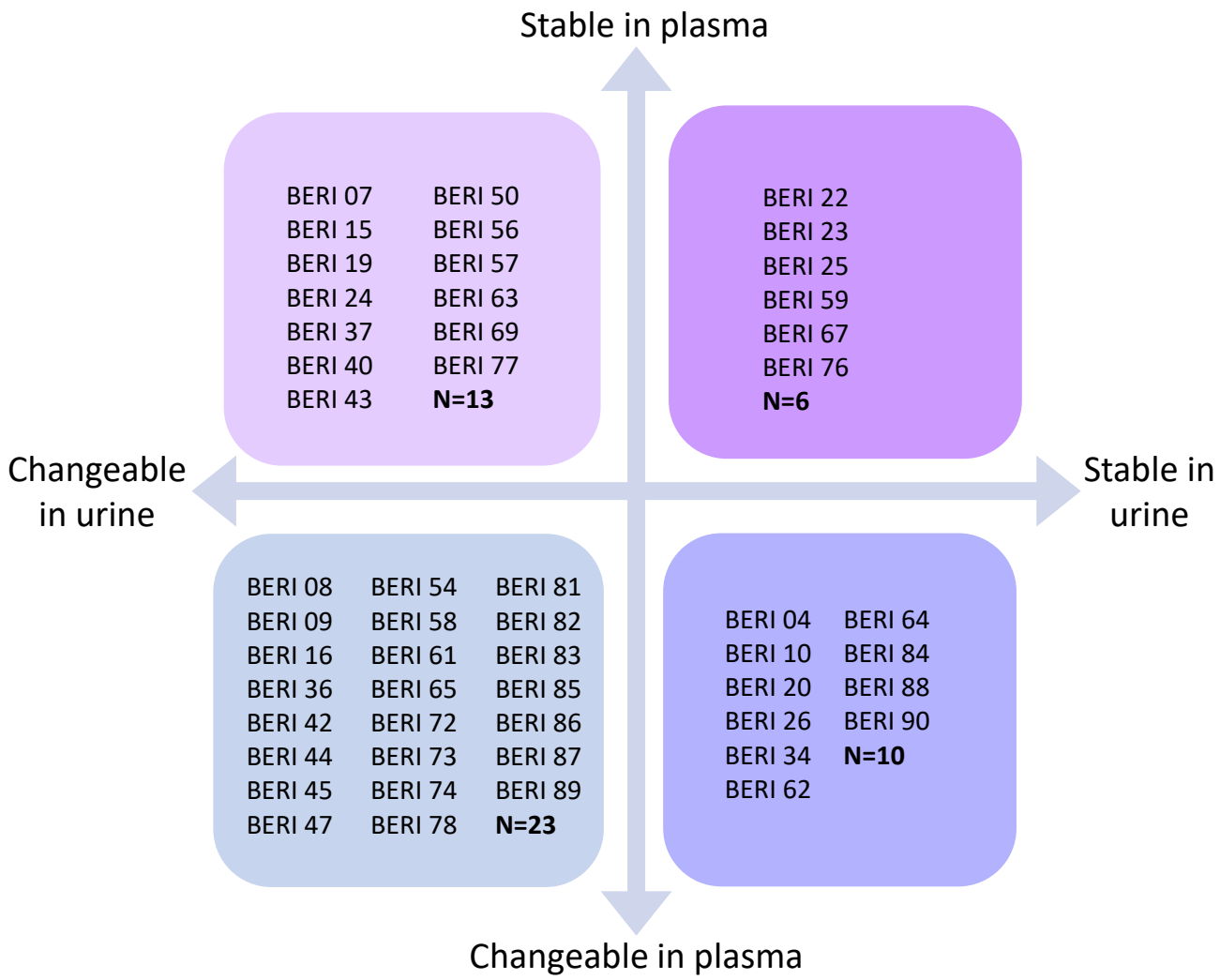

Figure 3-6 Matrix of TMAO status variability

3.5 Are there correlations between plasma and urinary TMAO levels?

The relationship between the plasma and urinary TMAO levels in each participant was investigated. Pairs of matched plasma and urinary values for each timepoint for each participant are displayed in a scatterplot with linear regression curve and its resulting equation, shown in **Figure 3-7**, panel **A**. The line of best fit had a low R^2 value as points were not distributed along the line and instead clustered together around the median values of plasma and urinary TMAO levels indicating weak linearity. The equation for linear regression curve is annotated on the scatter plot and 95% confidence bands are displayed. The 95% confidence intervals for the linear regression curve are reported in bottom part of panel B. Despite weak linearity, there was a significant correlation between plasma and urinary TMAO levels measured by Spearman correlation (Spearman r= 0.58, $p < 0.0001$). The confidence intervals of the Spearman r together with p value of Spearman correlation to demonstrate the significance of this relationship are reported in top half of panel B. The interactions of individual timepoints for both plasma and urine TMAO levels are displayed in the correlation matrix in panel C, where values for pA1 and pA29, pB1 and pB29, pC1 and pC29 represent plasma TMAO levels of 52 participants. Urinary levels of TMAO at each timepoint are denoted by uA1 and uA29, uB1 and uB29, and uC1 and uC29. Each timepoint for plasma TMAO was compared to all other plasma TMAO timepoints as well as all urinary TMAO levels to assess the correlation between these values. Additionally, the CV for each timepoint for 52 participants in plasma and urinary TMAO were compared and correlation between the variation was assessed.

There were multiple significant correlations between plasma TMAO levels at different timepoints. Namely, A1 was correlated with A29 ($r = 0.31$, $p = 0.03$) and B1 ($r = 0.34$, $p = 0.01$), whilst B1 was also correlated with A29 ($r = 0.34$, $p = 0.01$). A29 was correlated with C29 ($r = 0.34$, $p = 0.01$) but C29 was otherwise only correlated to C1 ($r = 0.33$, $p = 0.02$). There were significant correlations between all the urine and plasma TMAO values in individual timepoints (such as pA1 and uA1, significance annotated with stars to indicate $p < 0.0001$), but not between other unpaired timepoints (e.g. pA1 and uA29). The only other plasma and urinary timepoints that were significantly correlated were plasma at A29 with urine at B1 ($r = 0.34$, $p = 0.01$) and C1 ($r = 0.28$, $p = 0.05$), plasma TMAO at B1 with urine at A1 ($r = 0.31$, $p = 0.03$), and plasma TMAO at C29 with urine at A29 ($r = 0.30$, $p = 0.03$) and C1 ($r = 0.38$, $p = 0.006$). The coefficient of variation in plasma TMAO was significantly correlated with the variation in urinary TMAO ($r = 0.39$, $p = 0.004$). Plasma and urinary TMAO levels were correlated for individual datapoints, although there was a weaker relationship between different timepoints for participants.

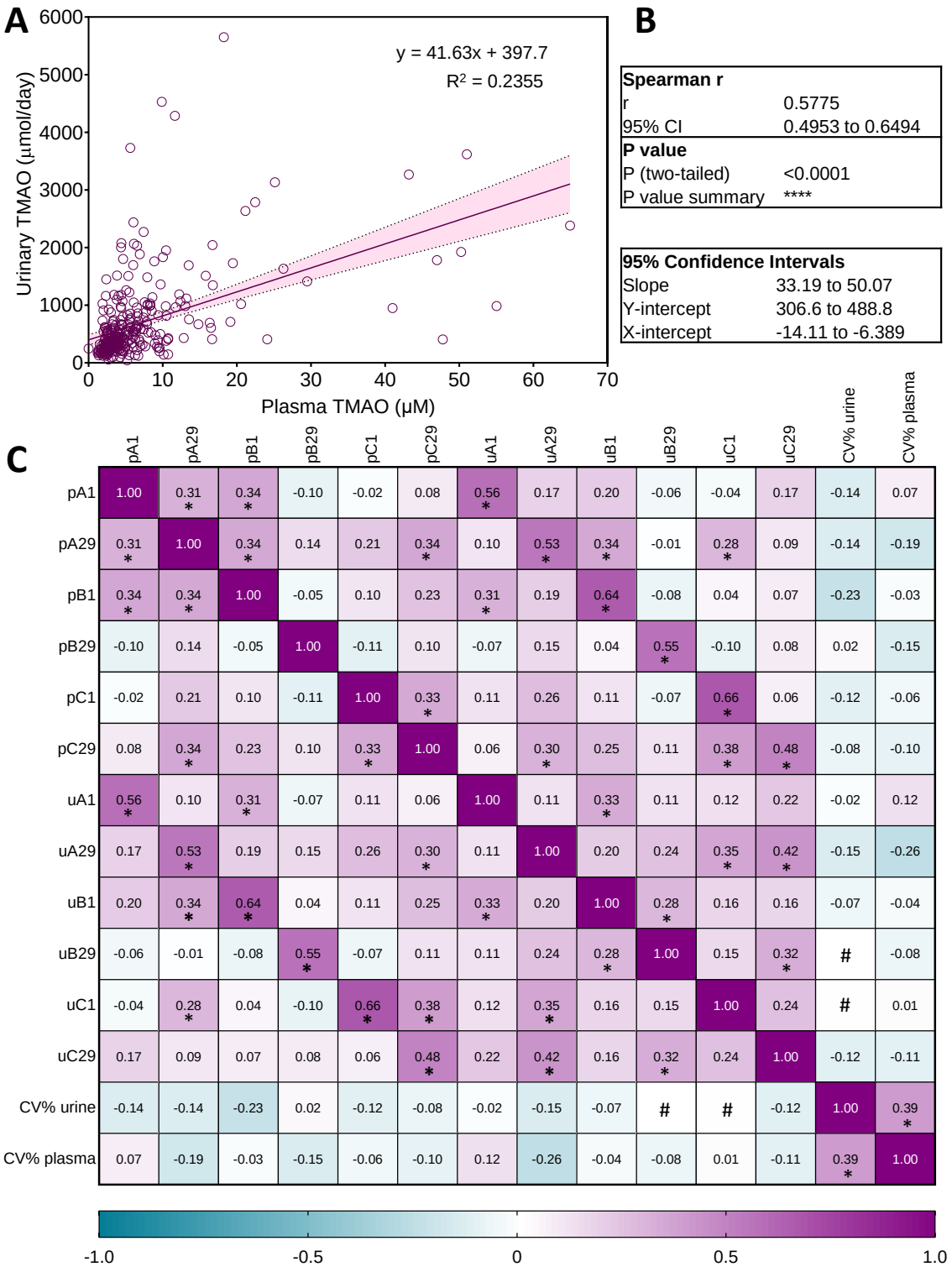

Figure 3-7 Correlation between plasma and urinary TMAO levels.

***A**) Pairs of matched plasma and urinary values for each timepoint for each participant are displayed in a scatterplot with linear regression curve and the 95% confidence bands of the regression curve. The curve equation is displayed with the R^2 linearity value.*

***B**) Results of Spearman correlation of all plasma and urinary matched values showing Spearman r and 95% confidence interval of r, with two-tailed p value. The confidence intervals for slope and intercept of the linear regression curve are reported.*

***C**) Correlation matrix of plasma TMAO values (p) of 52 participants and urinary levels of TMAO (u) at each timepoint are compared to all other plasma and urinary TMAO timepoints. The coefficient of variation (CV%) for each timepoint for 52 participants in plasma and urinary TMAO was correlated. Significant values ($p < 0.05$) are marked with *. Values with # represent r <0.001.*

3.6 Are there correlations between TMAO status and dietary precursors of TMAO?

To establish the relationships of plasma and urinary TMAO levels with dietary precursors, the levels of choline, betaine, and L-carnitine were measured in plasma and urine, with choline and TMA also quantified in faecal slurries. The distribution of values is shown in **Figure 3-8** with plasma and urine measures, and faecal concentrations from a subset of 23 participants who were also faecal donors. The dataset of correlations and distribution of TMAO precursors for all 52 participants is available as supplementary data.

The median choline values were 5.3 µM (12.1 µM range, n = 138 values) in plasma, 9.5 µmol/24h (56.3 µmol/24h range, n = 138 values) in urine, and 15.0 µM (41.6 µM range, n = 53 values) in faecal samples. Several participants had no detectable choline in urine and the values indicated a high CV of 97.6%. The concentration of TMA in faecal samples was low with median 7.5 µM (16.2 µM range, n = 53 values). Plasma had similar concentrations of betaine and carnitine, that were higher than the choline concentration (**Figure 3-8**). Likewise, urine also had similar (but highly variable) concentrations of betaine and carnitine, that were higher than the choline concentration. The distribution and high variability again pointed towards a lognormal distribution of these variables, and the non-parametric Spearman correlation was used to investigate the relationships between plasma and urinary precursor levels and TMAO status.

The Spearman correlation matrix of r values from interactions of precursors with plasma and urinary TMAO levels is displayed in **Figure 3-9** panel **A** with p-values shown in panel **B**. There were multiple significant correlations between the different precursors in plasma, for example, betaine was positively correlated with TMAO ($r = 0.24$, $p = 0.004$) and choline ($r = 0.27$, $p = 0.001$) but not with carnitine. Carnitine was correlated with choline ($r = 0.20$, $p = 0.016$), but not with betaine or TMAO. Plasma choline was not correlated with plasma or urinary TMAO, nor with choline measured in urine or faeces. Interestingly, there was a negative correlation between plasma choline and urinary carnitine ($r = -0.28$, $p = 0.001$) but no other correlation with urinary precursors was detected. Plasma choline was negatively correlated with faecal TMA ($r = -0.53$, $p < 0.0001$). This relationship was similar for the other precursors in plasma, with betaine showing a weaker corelation of $r = -0.22$ but this correlation was not significant ($p > 0.05$) possibly due to the reduced samples size (53 values of faecal TMA compared to other pairwise correlations performed with 138 values). Plasma TMAO but not urinary TMAO showed a significant negative correlation with TMA in faeces ($r = -0.30$, $p = 0.028$). Urinary betaine and carnitine were correlated with both plasma TMAO ($r = 0.21$, $p = 0.012$ and $r = 0.24$, $p = 0.005$, respectively) and urinary TMAO ($r = 0.37$, $p < 0.0001$ and $r = 0.28$, $p = 0.001$,

respectively). Urinary betaine was also correlated with urinary carnitine (r = 0.58, p < 0.0001), and interestingly with faecal choline (r = 0.45, p = 0.001).

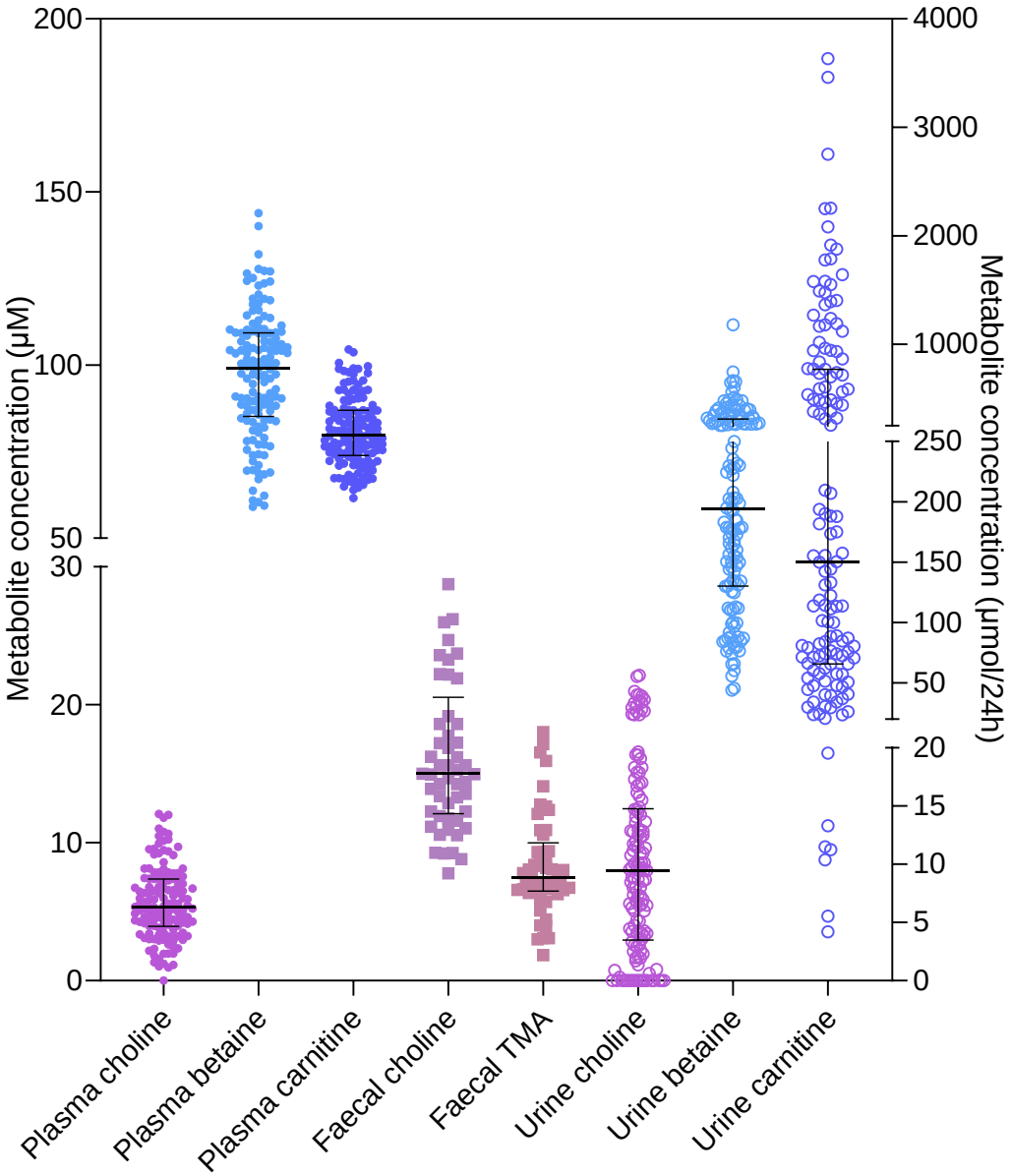

Figure 3-8 TMAO precursors levels in plasma, urine, and faecal samples of participants in the BERI study

Abundance of TMAO precursors in plasma and in urine at Day 1 and Day 29 of all treatments, with choline and TMA concentration measured in faecal samples of 23 participants at Day 1 and 10 participants at D29. Left y axis indicates metabolite concentration of TMA and choline in faecal samples, and choline, betaine, and carnitine concentration in plasma. Right y axis is split into 3 sections and depicts the metabolite and precursor levels for choline, betaine, and carnitine in urine (µmol/24h). Values are median and interquartile range.

Figure 3-9 Correlation matrix of levels of plasma and urinary precursors with TMAO status

The correlation compared the abundance of TMAO precursors in plasma and in urine at Day 1 and Day 29 of all treatments, with choline and TMA concentration measured in faecal samples of 24 participants at Day 1 and 10 participants at D29. These were correlated with all other abundances of precursors and metabolites with the heatmap showing Spearman correlation r values in panel A. Panel B shows the p-values indicating the significance of the correlation relationships between variables. Cells with # indicate p < 0.0001. Other p values are reported to three decimal places and p > 0.05 is shown in white.


A
Plasma TMAO (µM)
Urine TMAO (µmol/24h)
Plasma betaine (µM)
Plasma carnitine (µM)
Plasma choline (µM)
Urine betaine (µmol/24h)
Urine carnitine (µmol/24h)
Urine choline (µmol/24h)
Faecal choline (µM)
Faecal TMA (µM)
1.00 0.56 0.24 0.10 0.13 0.21 0.24 0.05 0.01 -0.30
0.56 1.00 0.17 0.17 0.16 0.37 0.28 -0.13 0.21 -0.25
0.24 0.17 1.00 -0.04 0.27 0.26 0.21 0.07 -0.06 -0.22
0.10 0.17 -0.04 1.00 0.20 0.11 -0.08 -0.05 -0.03 -0.06
0.13 0.16 0.27 0.20 1.00 0.12 -0.28 0.02 -0.06 -0.53
0.21 0.37 0.26 0.11 0.12 1.00 0.58 -0.15 0.45 -0.13
0.24 0.28 0.21 -0.08 -0.28 0.58 1.00 0.03 0.04 0.14
0.05 -0.13 0.07 -0.05 0.02 -0.15 0.03 1.00 -0.14 -0.13
0.01 0.21 -0.06 -0.03 -0.06 0.45 0.04 -0.14 1.00 0.06
-0.30 -0.25 -0.22 -0.06 -0.53 -0.13 0.14 -0.13 0.06 1.00
1.0
0.5
0
-0.5
B
Plasma TMAO (µM)
Urine TMAO (µmol/24h)
Plasma betaine (µM)
Plasma carnitine (µM)
Plasma choline (µM)
Urine betaine (µmol/24h)
Urine carnitine (µmol/24h)
Urine choline (µmol/24h)
Faecal choline (µM)
Faecal TMA (µM)
0.004 0.228 0.120 0.012 0.005 0.600 0.956 0.028
0.041 0.046 0.061 # 0.001 0.132 0.138 0.076
0.004 0.041 0.642 0.001 0.002 0.013 0.420 0.646 0.112
0.228 0.046 0.642 0.016 0.182 0.374 0.528 0.857 0.693
0.120 0.061 0.001 0.016 0.146 0.001 0.796 0.673 #
0.012 # 0.002 0.182 0.146 # 0.071 0.001 0.357
0.005 0.001 0.013 0.374 0.001 # 0.739 0.799 0.307
0.600 0.132 0.420 0.528 0.796 0.071 0.739 0.325 0.336
0.956 0.138 0.646 0.857 0.673 0.001 0.799 0.325 0.665
0.028 0.076 0.112 0.693 # 0.357 0.307 0.336 0.665
0.05
0.04
0.03
0.02
0.01

RELATIONSHIP BETWEEN THE TMA PRODUCTION CAPACITY OF FAECAL MICROBIOTA AND HOST TMAO STATUS

The hypothesis that an individual's TMAO status correlates with the capacity of their faecal microbiome to generate TMA from the precursor choline was tested. In the BERI intervention study, faecal samples were collected from a subset of 23 participants on Day 1 and Day 29 of each treatment (138 samples collected). In addition, faecal glycerol stocks were prepared from one Day 1 faecal sample for each of the 23 participants who donated stool samples, as a representative baseline (23 glycerol stocks). Finally, for 10 participants, glycerol stocks were also prepared from faecal samples collected on Day 29 of each treatment (30 glycerol stocks). Glycerol stocks were used to inoculate *in-vitro* colon models and their capacity to generate TMA from supplemented choline was measured. Additionally, DNA was extracted from the 138 unprocessed faecal samples for shotgun metagenomics sequencing. These data were used to determine the relative abundance of TMA producing bacteria, genes encoding enzymes involved in TMA production and utilisation, and the overall structure and function of the microbiome.

3.7 What is the capacity of an individual's faecal microbiota to produce TMA from choline?

An example of a choline fermentation kinetic curve involving the production of TMA from supplemented choline is shown in **Figure 3-10**. Choline abundance (mol%) is depicted as a solid green line, where 100% is the maximum choline concentration measured in the colon model vessel (~ 2 mM). In this example, most of the supplemented choline was metabolised between 12 h and 24 h, and this information was used to calculate the choline metabolism rate. Specifically, the choline concentration at 24 h (c2) was subtracted from the choline concentration at 12 h (c1), and the value divided by the difference in time between these two timepoints (Δt) to calculate the rate of choline metabolism per hour of fermentation – for choline, this value was derived from the actual concentration and is expressed as µM/h. TMA produced from choline (mol%) is shown as a solid red line (**Figure 3-10**), and represents the TMA concentration as a percentage of the maximum choline concentration in each of the vessels (with the initial background TMA subtracted from all timepoints). In the example (**Figure 3-10**), the TMA concentration (mol%) was low for the first 12 h of choline fermentation, after 24 h reached around 80 mol% (which was the Maximum TMA concentration (mol%) for this vessel) and then slightly declined over the next 24 h of fermentation to ~ 64 mol% (Final TMA). The rate of TMA production from choline calculated as

the difference between mol%$_2$ at 24 h and mol%$_1$ at 12 h (same Δt as for choline metabolism) is expressed as mol%/h.

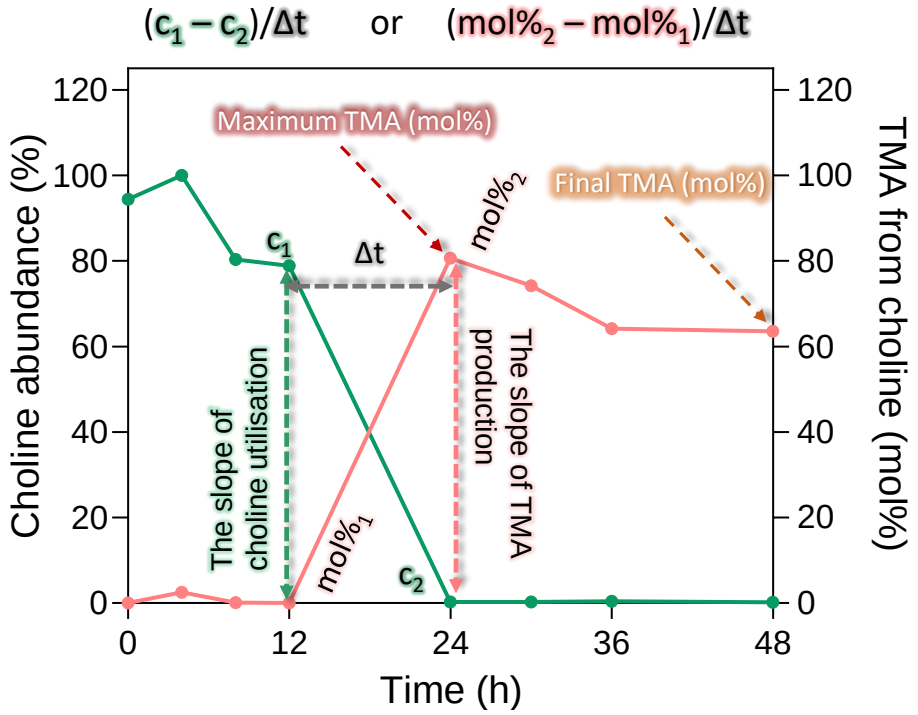

Figure 3-10 Example of choline metabolism and TMA production in the colon model

The figure depicts the kinetics of choline degradation plotted on the left Y axis against time on the X axis. The abundance of choline is expressed as percentage of maximum choline measured in the colon model vessel. In this example, maximum choline concentration was measured at T4. TMA from choline is measured as the proportion of inoculated choline converted into TMA (Concentration at timepoint - initial TMA at T0/Maximum choline concentration) and expressed as mol%.

Choline metabolism rate (μM/h), TMA production rate (mol%/h) together with the Maximum TMA concentration (mol%) and Final TMA concentration (mol%) are used to describe the capacity to produce TMA from choline of individual participants. For the 13 participants that only had one Day 1 glycerol stock prepared and no Day 29s, these samples were used as a representative of their TMA-producing capacity. For participants that also had their three Day 29 samples prepared as glycerol stocks, a mean response from these four faecal glycerol stock samples (1 x Day 1 and 3 x D 29 for 10 participants = 40 samples) was used to characterise each participant when used for further analysis of correlations between the *in-vitro* capacity to produce TMA and their plasma TMAO status. More in-depth analysis of intra-individual variation in these variables is available in Supplementary data.

The individual kinetic curves of choline degradation and TMA production are displayed in **Figure 3-11**, showing all participants at Day 1 (one colon model vessel per participant, n = 23). Choline abundance (mol%) and TMA from choline (mol%) are displayed on the Y axis, while the X axis shows the incubation time with samples collected at 0, 4, 8, 12, 24, 30, 36 and 48 h.

Most participants showed complete choline utilisation within 24 h (BERI 22, 23, 25, 26, 43, 47, 56, 62, 63, 64 and 67) or 30 h (BERI 07, 09, 34, 44, 57 and 72), however, some participants showed incomplete choline metabolism with limited TMA production (BERI 08, 20 and 58). For some participants, a stochiometric production of TMA from choline was detected (BERI 34, 44 and 67), but most participants showed partial conversion of choline to TMA, despite all choline being utilised. This was apparent in BERI 09, 16, 22, 47, 61, 64 and 72, where the Maximum TMA concentration (mol%) reached around 50 % of the supplemented choline concentration. This could indicate metabolism of some choline by a route that did not involve TMA formation, TMA utilisation by other microbial species present in the faecal samples, and/or non-biological TMA disappearance during the *in-vitro* fermentation or sample processing. Some participants (e.g. BERI 07, 23, 25, 26, 34, 36, 44 and 67) showed relatively stable TMA levels with around 80 mol% Maximum TMA produced from choline. It is important to note that most participants showed similar choline metabolism rates between 12 h and 24 h or 24 h and 30 h, however, BERI 16 and BERI 36 showed extremely rapid choline metabolism with total choline utilisation within 12 h and 8 h, respectively. Interestingly, in BERI 16, this rapid choline metabolism did not result in the Maximum TMA concentration being produced in 12 h. After an initial increase to ~50%, the TMA concentration gradually rose to ~68% in 30 h. The biggest change in metabolite concentration between two closest timepoints was used for determination of the metabolic rates. For more details on the calculations and individual concentrations, rates, and percentages for each fermenter, together with replicate vessels for the same Day 1 samples and Day 29s for the 10 participants, all data are available published in an online repository.

The overall characterising variables of TMA production capacity by the gut microbiota of all faecal donors (n = 23, total of 53 glycerol stock faecal samples) are displayed in **Figure 3-12**. On the left Y axis, metabolite abundance is displayed in terms of mol% of TMA produced from choline to show the distribution of Maximum TMA and Final TMA concentration (mol%) measured in each sample. The axis is split into two sections with the bottom section ranging from 0 – 40 mol% to display the samples with low TMA production capacity and the top section ranges from 40 – 140 mol% with the majority of values clustering around ~60 – 80 mol%. However, the range for Maximum TMA concentration was 126.0 mol% with some participants reaching more than 100 mol% production of TMA, indicating that some TMA might have been produced from other sources present in the faecal matrix. For 13 samples, the Maximum TMA concentration was below 30 mol%. The CV for the Maximum TMA concentration was 58.4 % with a median value of 63.0 mol% (n = 53). Similar values were detected for Final TMA where the median reached 57.1 mol% (range of 124.5 mol%, n = 53) and CV of 62.1 %.

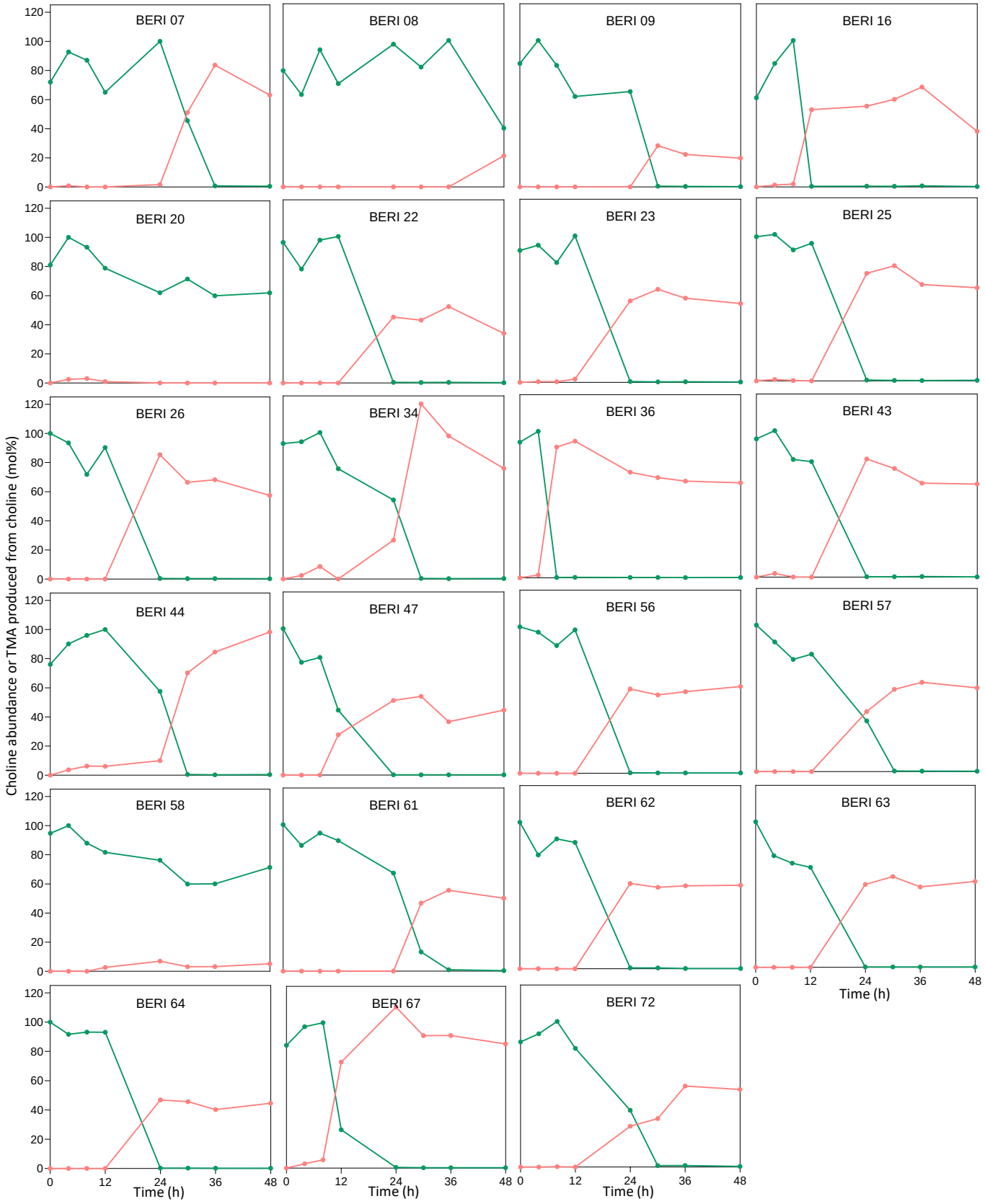

Figure 3-11 Choline metabolism and TMA production for Day 1 glycerol stock faecal samples of 23 BERI participants

Choline abundance (%) and TMA from choline (mol%) are displayed on the Y axis. The X axis shows the incubation time of experiments with sample collection at 0, 4, 8, 12, 24, 30, 36 and 48 h. Vessels were incubated at 37ºC under constant mechanical stirring, at pH 6.6-7.2 and continuously sparged with N_2 to maintain anaerobic conditions. Each participant Day 1 sample was fermented in a separate vessel due to low availability. Values are expressed as percentages where choline abundance (%) indicates the percentage of highest molar concentration of choline measured in the vessel. TMA from choline (mol%) is a molar equivalent of supplemented choline that was converted into TMA – for more information, see Calculations in Methods section.

Most rates of TMA production clustered around the median (4.8 mol%/h with range of 31.1 mol%/h). However, there were also samples that showed very slow or no TMA production, that corresponded to the cluster of participants with the lowest Maximum and Final TMA mol% values. Choline metabolism rate is predominantly displayed on the top range of the right Y axis (40 – 600 µM/h) but some also showed rates below 30 µM/h (10 samples). The median choline metabolism rate was 149.9 µM/h with range 516.1 µM/h (the maximum rate being a capacity to fully utilise supplemented choline within 4 hours, as seen in BERI 16 and 36 in **Figure 3-11**).

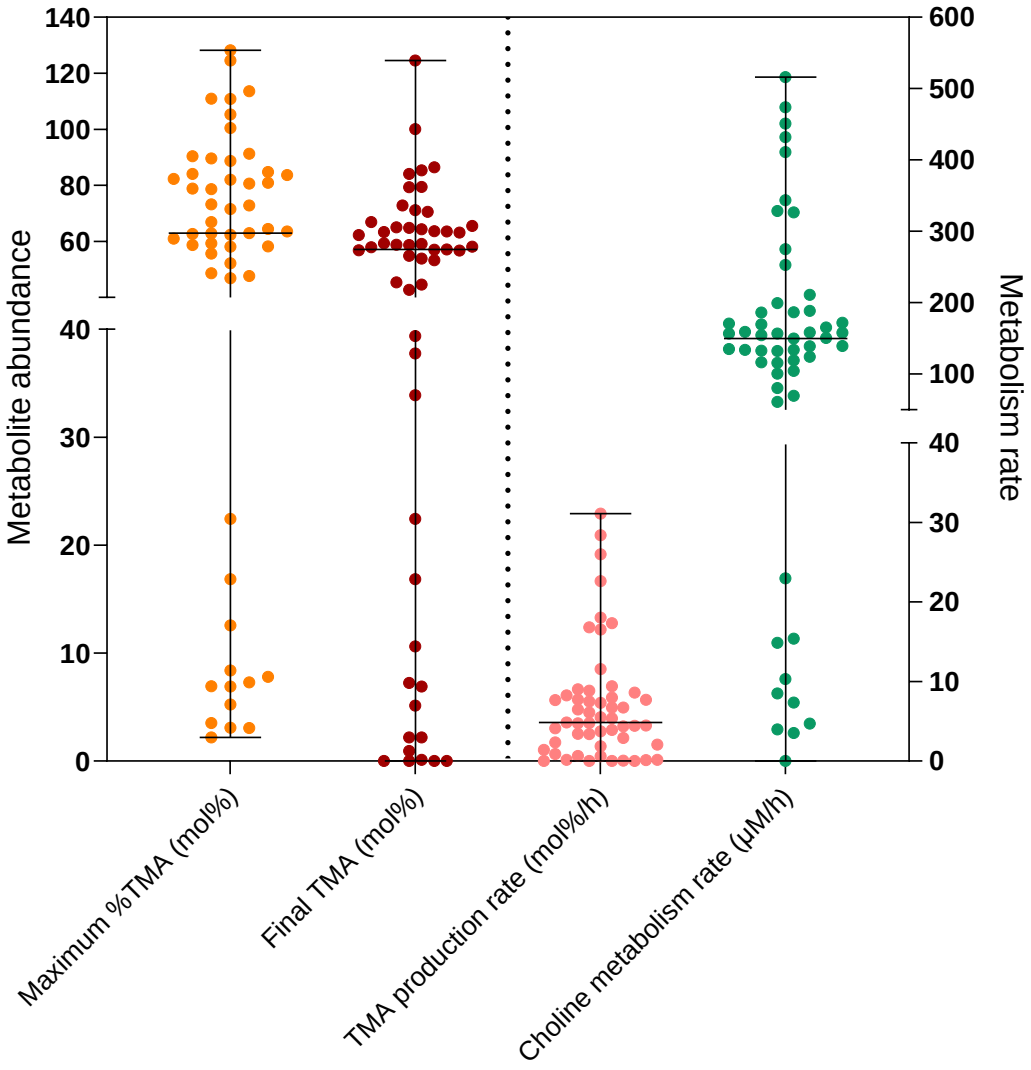

Figure 3-12 Characteristic variables of individual's capacity to produce TMA measured using in-vitro colon model

Maximum mol% of TMA from choline for all faecal glycerol stocks from the BERI study (n = 53) shown with median and range. Final TMA mol% from each faecal glycerol stock measured at the 48 h timepoint of the experiment. Rate of TMA production (mol%/h) displayed for each faecal glycerol stock with median and range, displayed on the right Y axis. Choline metabolism rate (µM/h) presented as individual values for all BERI faecal glycerol stocks and their median and range, split spanning across both sections of the right Y axis.

3.8 Does *in-vitro* ability to metabolise choline to TMA correlate with *in-vivo* markers of TMAO status?

The relationship between *in-vivo* markers of TMAO status from BERI study participants and the metabolic capacity of the corresponding participant faecal samples to metabolise choline to TMA *in-vitro* was investigated. Only the subset of 23 faecal donors included in *in-vitro* fermentations tests of choline to TMA by their faecal microbiome (53 faecal samples) were examined for correlations with *in-vivo* markers of their TMAO status.

For each of the 53 faecal samples, the main outcomes of the investigation of *in-vitro* TMA production capacity (Choline metabolism rate, TMA production rate and Maximum TMA produced from choline) were plotted against plasma TMAO concentration. These data are presented in scatterplots in **Figure 3-13** where plasma TMAO concentration (μM) is plotted on the X axis and the corresponding variables are plotted on the Y axis, negative part of the Y axis is displayed to plot 95% confidence bands of linear regression curves. A linear regression was calculated to produce a curve of best fit with 95% confidence bands drawn as dashed lines. In panel **A**, the rate of TMA production (mol%/h) and plasma TMAO concentration showed a slight negative correlation, with Spearman r of -0.10 that was not statistically significant. There was no correlation between plasma TMAO concentration and choline metabolism rate (μM/h) as displayed in panel **B**. Interestingly, samples that showed very fast *in-vitro* choline metabolism to TMA had average TMAO detected in the plasma (between 2 – 10 μM), with higher plasma TMAO concentrations associated with typical and lower *in-vitro* choline metabolism rates. There was a stronger, but not significant negative correlation of plasma TMAO concentration and Maximum TMA produced from choline (mol%) where Spearman r was -0.19 ($p > 0.05$), shown in panel **C**. There was a cluster of samples that produced a small quantity of TMA from choline *in-vitro* yet showed typical plasma TMAO concentrations.

In a similar way to markers of plasma TMAO status, there were also no significant correlations between urinary TMAO status and any of the *in-vitro* fermentation variables, with Spearman correlation values for all interactions reported in **Figure 3-14**.

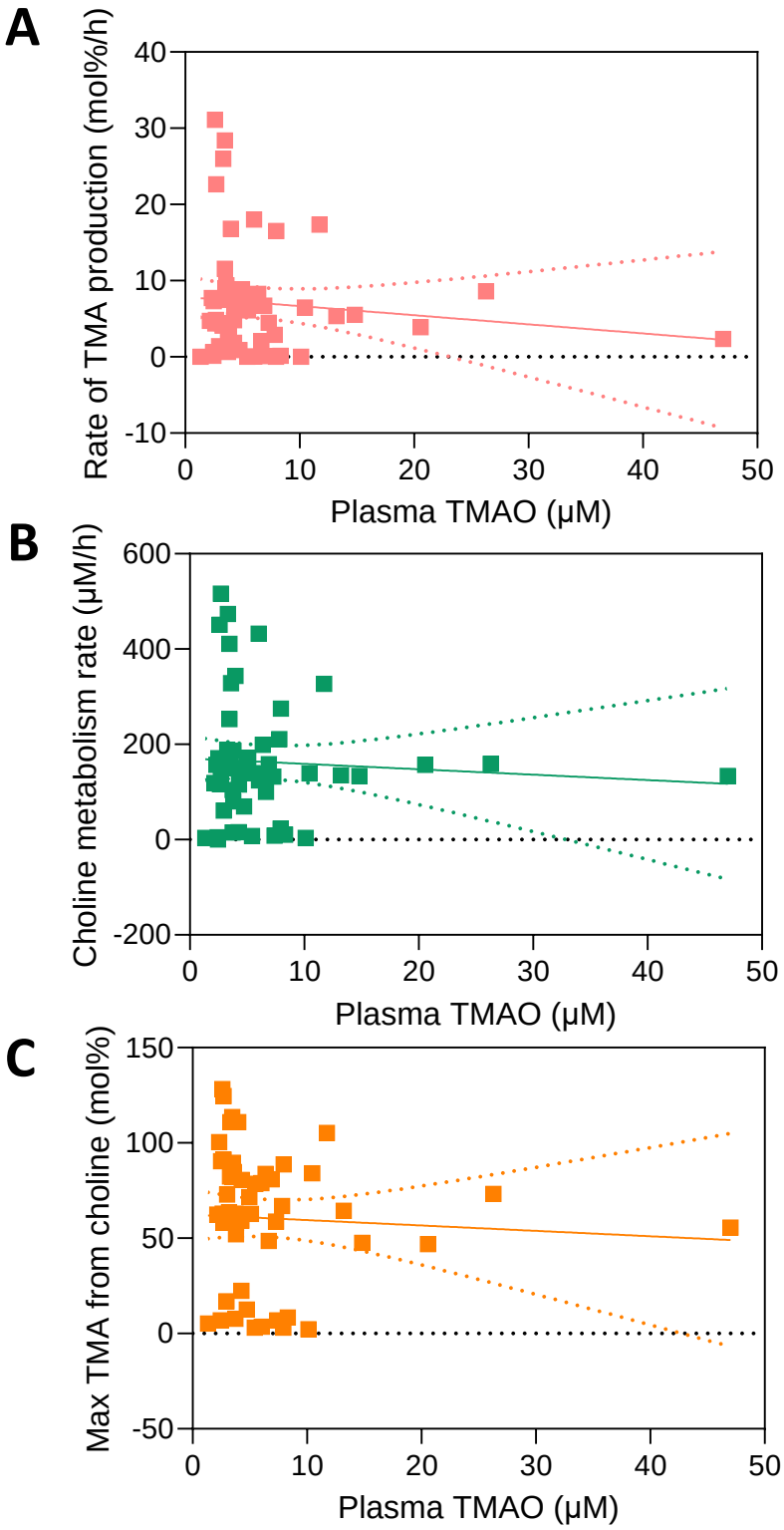

Figure 3-13 Correlations of plasma TMAO concentration with characterising variables of capacity to produce TMA in-vitro

***A**) Scatterplot of plasma TMAO concentration (µM) against TMA production rate (mol%/h). Linear regression curve of best fit with 95% confidence bands displayed as dashed lines. Spearman r = -0.10, p > 0.05.*

***B**) Scatterplot of plasma TMAO concentration (µM) and choline metabolism rate (µM/h) with linear regression curve of best fit with 95% confidence bands displayed as dashed lines. Spearman r = -0.02, p > 0.05.*

***C**) Scatterplot of plasma TMAO concentration (µM) and Maximum TMA produced from choline (mol%) with linear regression curve of best fit with 95% confidence bands displayed as dashed lines. Spearman r = -0.192 (p > 0.05).*

Plasma and urinary TMAO levels can be affected by the dietary patterns of individuals and as demonstrated in the previous section, they are correlated with the presence of some of the precursors in plasma and urine. The next question was whether these abundances of precursors in plasma and urine (which could possibly mean their absence in the lower GI tract) have any relationship with the characteristic variables determined from choline metabolism into TMA measured using the *in-vitro* colon models. Despite only choline being tested for the capacity to produce TMA, correlations with the other precursors were also explored.

In **Figure 3-14**, top area of the matrix depicts the correlation values between the *in-vitro* variables and plasma precursors, which showed no significant relationship between rate of TMA production or choline metabolism rate. There was a weak negative correlation of final choline abundance (mol%) at 48h and plasma betaine (r = -0.20), but this was not significant. Slightly higher correlations were detected with precursors in urine, for example, urinary betaine showed positive correlation with both choline metabolism rate (r = 0.22) and TMA production rate (r = 0.25), but neither of those were significant. Interestingly, very little association was found between the faecal concentrations of choline and TMA and the rates of metabolism *in-vitro*, together with maximum and final measures of TMA produced from choline and final measure of choline abundance at 48 h.

All variables calculated from *in-vitro* fermentation of faecal glycerol stocks from BERI study participants were significantly correlated with each other ($p < 0.0001$). Choline metabolism rate showed strong correlation with rate of TMA production (r = 0.86) but slightly weaker relationship with maximum concentration of TMA produced from choline (r = 0.79) and final TMA concentration (mol%) at 48 h (r = 0.62). There was a negative correlation between the rate of choline metabolism and the final abundance (mol%) of choline at 48 h (r = -0.55). Negative correlation was even higher between the TMA production rate and final choline levels at 48 h (r = -0.60). Notably, rate of TMA production was less correlated with final TMA levels (r = 0.77) compared to maximum TMA levels (r = 0.89). These findings elucidate the stronger associations between variables arising from *in-vitro* metabolism of choline and the weak relationships these have with the *in-vivo* measures of TMAO status and the abundance of other precursors in plasma and urine.

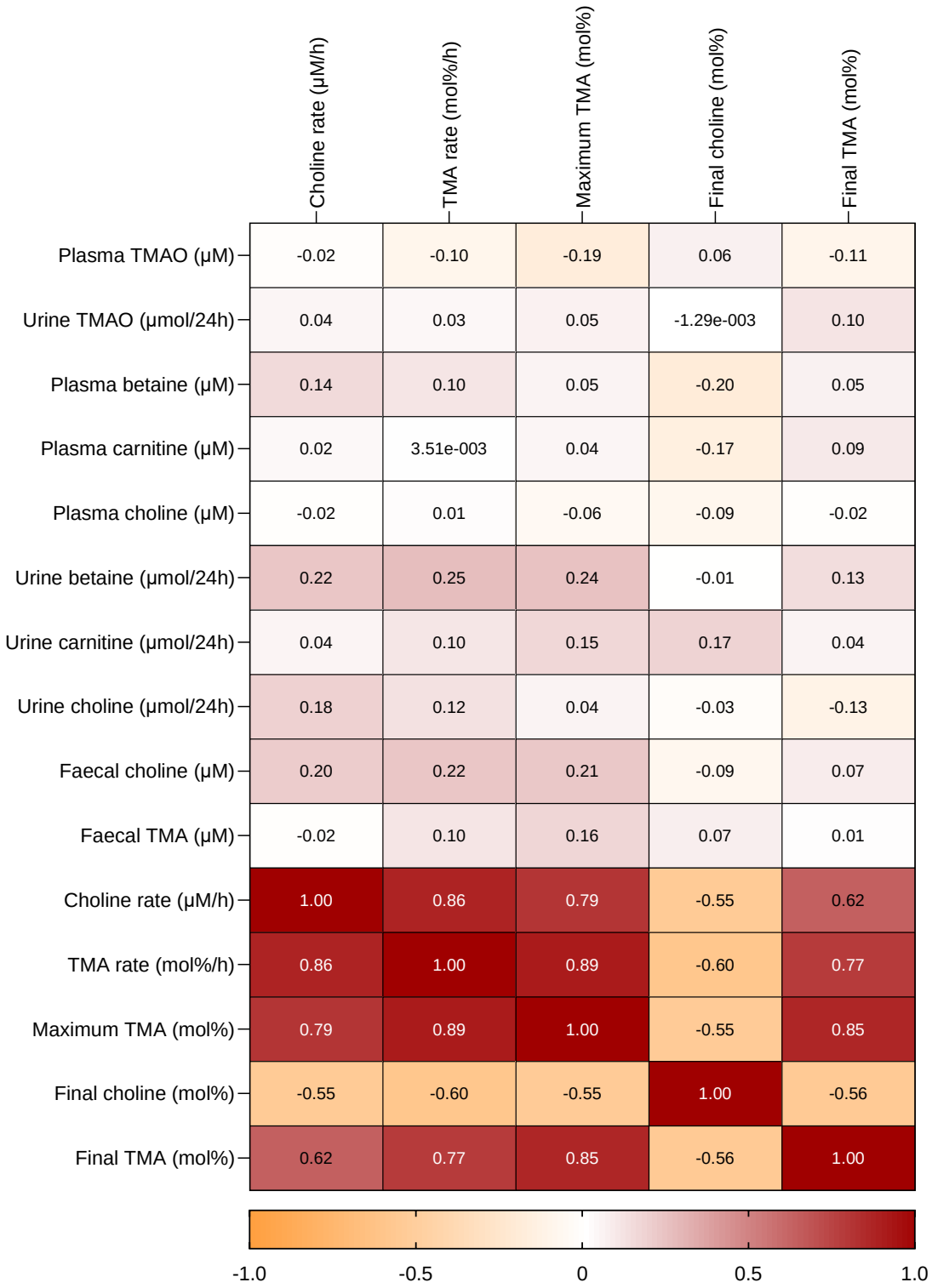

Figure 3-14 Spearman correlation matrix of metabolomics-derived measures from in-vivo samples and their relationship with in-vitro-determined capacity to produce TMA from choline.

Spearman correlations r between all variables from the human study metabolomics for a subset of 23 participants that donated faecal samples (137 samples) and the characteristic variables derived from the in-vitro investigation of TMA production capacity of individual's faecal samples (53 samples). The correlations for plasma and urine metabolites were previously reported in Figure 3-9 and are not displayed here.

To further determine the differences between the characterised groups introduced earlier in this chapter based on the variability of *in-vivo* TMAO status, two sets of categories were assigned to BERI participants based on their *in-vitro* capacity to produce TMA from choline. The respective category assignments per participant are displayed in **Figure 3-15**, plotted in a variability matrix of *in-vivo* TMAO status. The *in-vitro* capacity to produce TMA from choline was categorised based on the ability of participant microbiota to fully metabolise choline within the 48-h incubation. This category was based on their Day 1 choline fermentation test or an average value from all available choline fermentation tests per participant. Participants who fully metabolised choline were categorised as **completers** and are annotated in **bold** font in the *in-vivo* TMAO variability matrix, whereas non-completers are shown in regular font. Additionally, the time it took to metabolise supplemented choline was used for assigning a category based on rate of choline metabolism. Participants who showed <20 mol% choline abundance at 24 h were considered fast, whereas those that showed >20 mol% of choline abundance at that timepoint were categorised as slow. This category is shown in *underlined italics* for *fast* participants, and if they are fast and completers, it is shown in ***bold underlined italics.*** Those who were non completers and slow metabolisers are shown in regular font.

In the upper right quadrant, BERI 22, 23, 25 and 67 showed stable plasma and urinary levels of TMAO and 3 out of 4 participants in this group did not fully metabolise choline. Despite incomplete metabolism, BERI 22 was a fast metaboliser, same as BERI 67 who also fully metabolised choline within 48 h. The opposite category with changeable plasma and urinary TMAO status had representation of all *in-vitro* categories, where BERI 08 and 58 showed slow incomplete metabolism of choline and BERI 44, 61 and 72 were slow metabolisers but fully metabolised supplemented choline. BERI 09, 16, 36 and 47 were all fast metabolisers but BERI 09 was the only one that did not manage to fully utilise choline within the 48-h period. Participants that had stable TMAO levels in urine but changeable in plasma were mostly slow non-completers (BERI 20, 26, 34) with BERI 62 and 64 showing fast complete metabolism of choline.

Overall, there did not seem to be a relationship between the *in-vitro* capacity to metabolise choline to TMA and the TMAO status measured *in-vivo*. However, to determine the potential capacity of the faecal microbiota to produce TMA from other precursors that were not tested *in-vitro*, the gut microbial structure and function was explored in the next section.

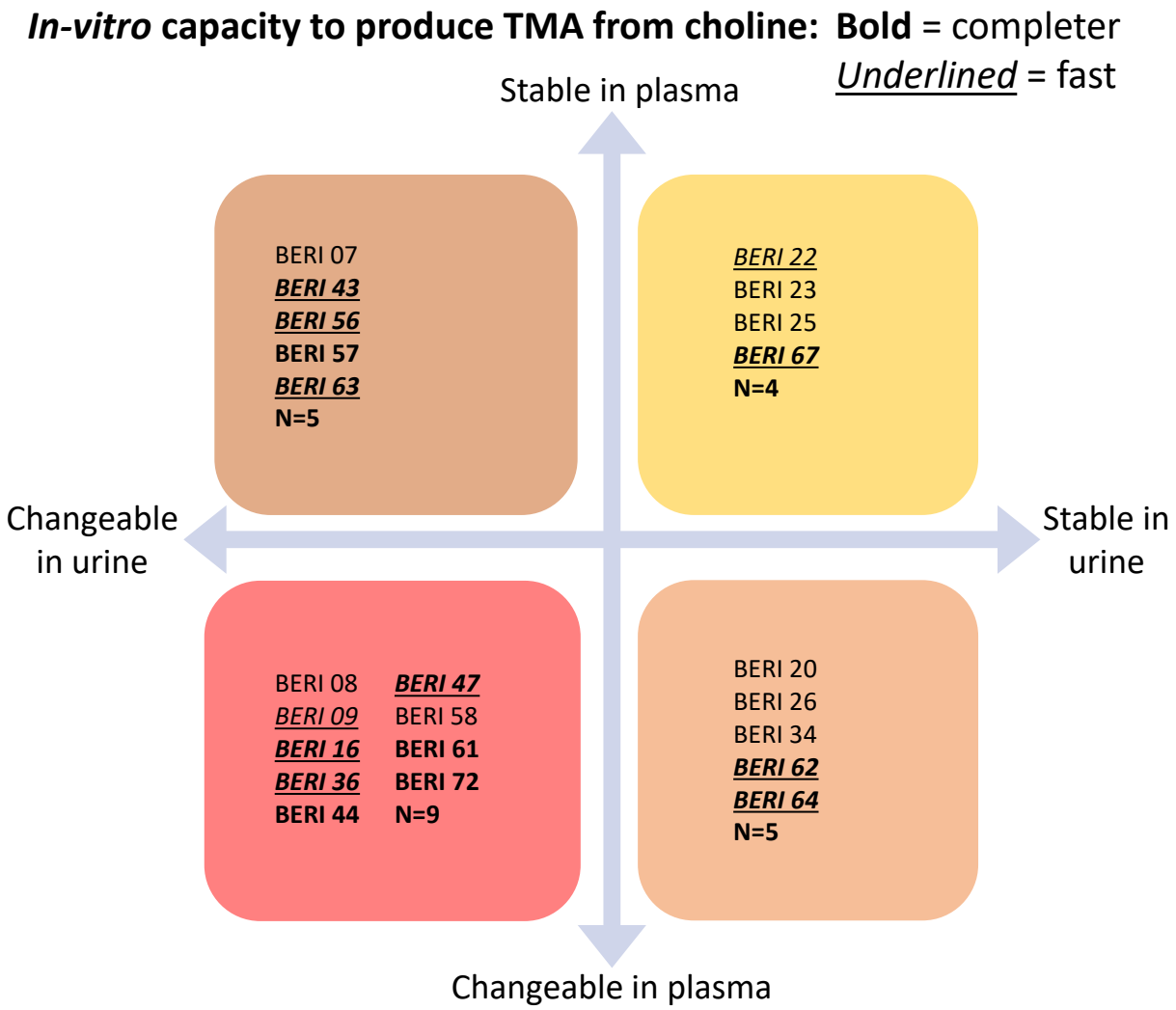

Figure 3-15 Categories of in-vivo TMAO status and in-vitro capacity to metabolise choline to TMA

Plasma TMAO category was established by calculating the CV of 6 independent plasma TMAO measures and stable participants fell below the cut-off point of 50% CV. This cut off was also used to determine Urine TMAO category as described in Sections 3.3 and 3.4. The 'completer' category was characterised based on the Day 1 or the average response from all available in-vitro choline fermentation tests where participant faecal samples that managed to fully utilise choline over the 48-h incubation time were classified as completers. Rate of metabolism category was derived from the abundance of choline at 24 h where <20 mol% choline abundance was categorised as fast rate and >20% at 24 h was a slow rate.

IS THERE A RELATIONSHIP BETWEEN THE STRUCTURE OF THE FAECAL MICROBIOTA, *IN-VIVO* TMAO STATUS AND THE CAPACITY TO METABOLISE CHOLINE TO TMA MEASURED *IN-VITRO*?

The BERI study faecal samples that were collected from a subset of 23 participants at the six independent timepoints of the human intervention were submitted for sequencing using shotgun metagenomics. The microbiota structure of 23 BERI participants was then correlated with plasma TMAO concentration, urinary TMAO levels and the *in-vitro*-determined characterising variables of TMA production capacity (choline metabolism rate, TMA production rate and maximum TMA produced from choline).

3.9 The faecal microbiota structure of the BERI study participants

To investigate the stability of the microbiota structure, the presence of the 10 most abundant genera were determined with MetaPhlAn v3.0.2. This tool was used with high quality trimmed reads that were mapped to the ChocoPhlAn database of specific marker genes that are unique to different clades to identify microbes and their abundance in metagenomic samples. The histograms in **Figure 3-16** represent the relative abundance of the top 10 genera in the BERI cohort over the six independent timepoints (Day 1 and Day 29 in rows, and each participant represented as a bar in columns A, B and C). Some variation in the relative abundance of the genera was observed between participants, most notably BERI 26 consistently showed a much lower overall relative abundance of the 10 major genera compared to other participants. There was also some variation in the abundance of the 10 major genera between samples from each participant, with genus *Bifidobacterium*, *Ruminococcus* and *Prevotella* being some of the most variable. BERI 20, for instance, showed over 50% relative abundance of *Prevotella* genus at B29, C1 and C29 but was less abundant at B1, A1 and A29. BERI 56 showed consistently high abundance of *Bifidobacterium* across the timepoints (A1 was not collected for this participant) with low abundance of *Bacteroides*. To investigate the structure of the microbiota in more depth, the relative abundance of the top 20 species was plotted in a heatmap displayed in **Figure 3-17.**

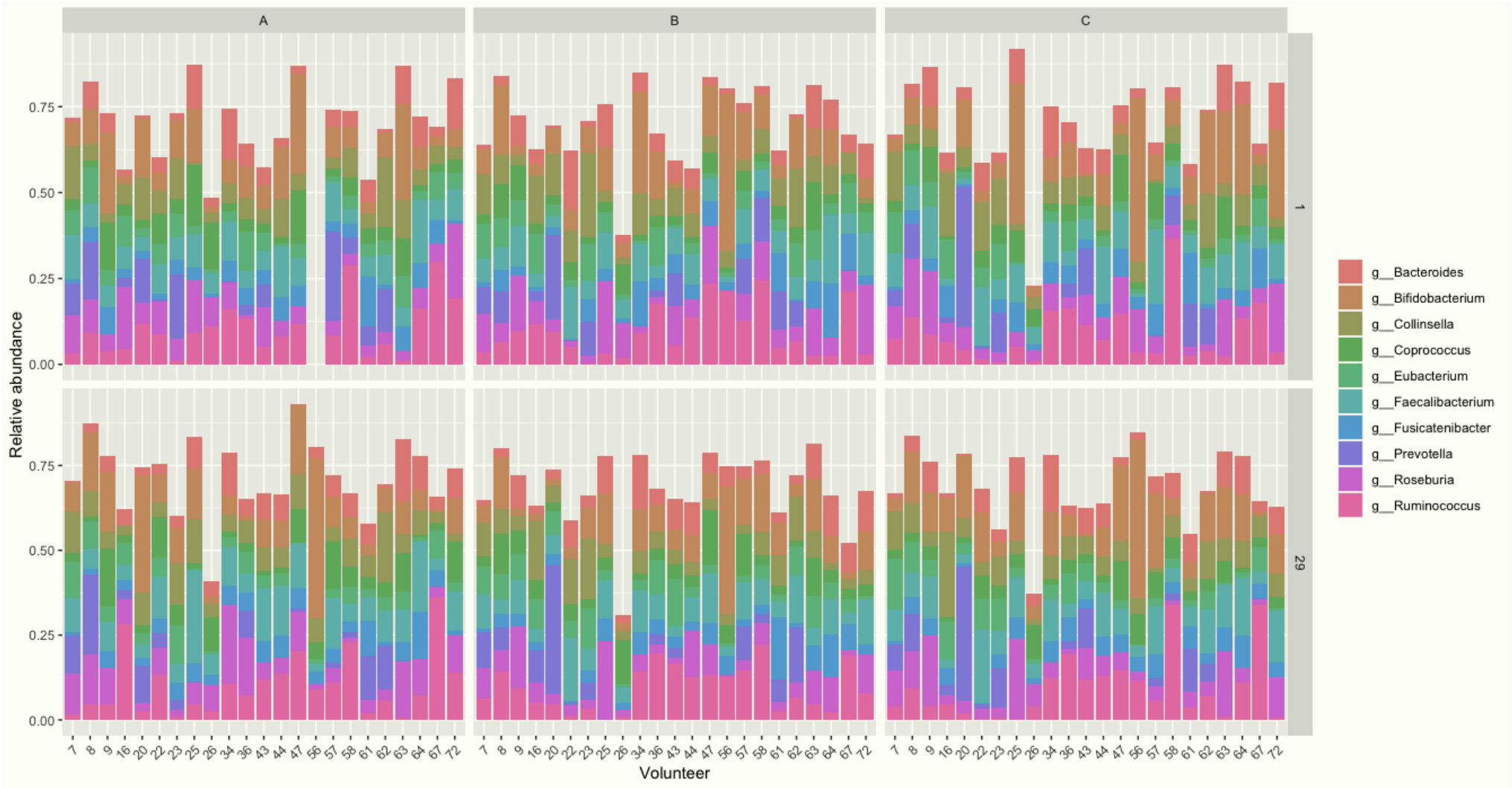

Figure 3-16 The relative abundance of the top 10 genera per participant at each independent timepoint.

The abundance of species in **Figure 3-17** revealed differences between participants, for instance, *Prevotella copri* was very abundant in BERI 07, 16, 20, 23 and 62, but not in BERI 09, 25, 26, 34, 36, 43, 44 and 47. BERI 22, 56, 57 and 58 showed more variability in the abundance of *Prevotella copri* across the different timepoints. The genus *Bifidobacterium* was represented predominantly by *B. adolescentis* which was abundant in a majority of participants with only BERI 36 and 61 showing a distinct absence of this species across all timepoints. *B. angulatum* was only present in BERI 07 and 20. A similar variation in abundance across different participants and timepoints was observed with *Roseburia* genus, where *Roseburia faecis* was the dominant species across the cohort with *R. inulinivorans* and *R. hominis* showing a lower abundance but similar variation across participants. Other species from the family Lachnospiraceae (*Coprococcus comes*, *Dorea longicatena* and *Fusicatenibacter saccharivorans*) were present throughout the whole cohort to different degrees. The family Lachnospiraceae belongs to the order Eubacteriales that was also represented by the families Eubacteriaceae and Oscillospiraceae that contain the genus *Eubacterium*, and *Faecalibacterium* with *Ruminococcus*, respectively. The three most abundant species from the *Eubacterium* genus - *E. rectale*, *Eubacterium sp. CAG. 180* and *E. eligens* were highly transient across participants and timepoints. Two different species of the genus *Ruminococcus* were abundant in a high number of participants, with *R. bromii* showing high abundance in BERI 34, 36, 43, 44, 47, 56, 57, 58, 64 and 67 but it was not detected in BERI 23, 61 and 63. Interestingly, BERI 26 showed a very high abundance of *Escherichia coli*, which was not detected in a majority of the cohort apart from some timepoints in BERI 07, 36, 43 and 62.

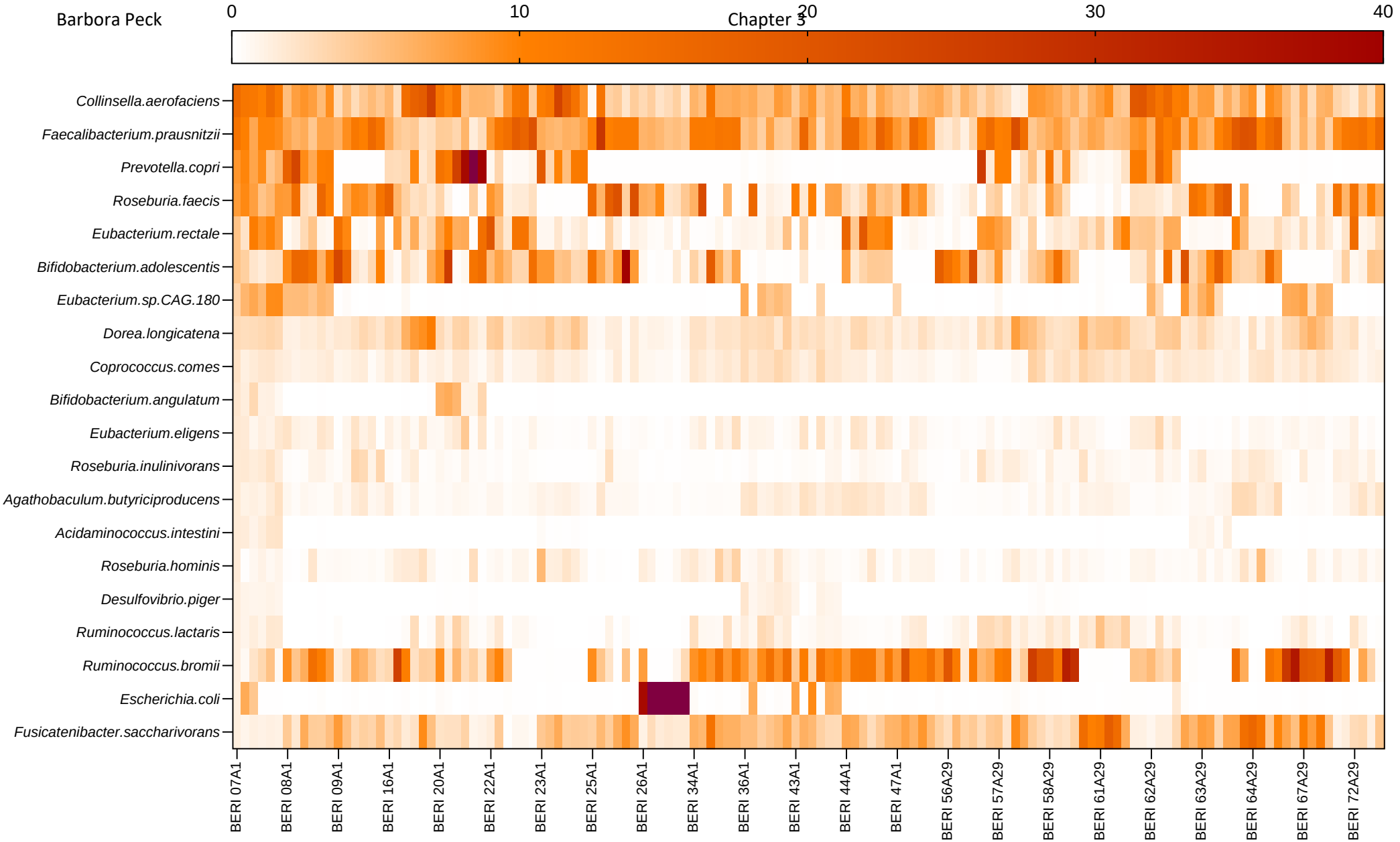

Figure 3-17 The relative abundances of the top 20 bacterial species per participant at each independent timepoint.

When comparing individual participants, there were notable differences between the abundances of the top 10 genera and the top 20 species reported in **Figure 3-16** and **Figure 3-17**, respectively. To determine the extent of these differences, the Shannon Diversity Index (SDI) was used to reflect the complexity and balance of the gut microbiota by calculating the richness and evenness of microbes in each faecal sample. There were few differences in SDI per participant over the 6 independent measures, therefore, an average value was used per participant to compare the differences between individuals whilst considering their *in-vivo* and *in-vitro* classification of TMAO status and capacity to produce TMA from choline, respectively.

To determine if these categories are related to the diversity of the gut microbiota, the SDI was plotted in **Figure 3-18** based on the *in-vivo* TMAO status of participants (either stable or changeable in plasma (TMAOp) and urinary (TMAOu) TMAO levels) with participants annotated based on their *in-vitro* capacity to metabolise choline (completers in **bold** and fast metabolisers in *underlined italics*) as described in **Figure 3-15**. The SDI in **Figure 3-18** panel **A** revealed no notable differences between the different categories (using an unpaired t-test for each category), except urinary TMAO status. Most participants showed SDI between 3.0 and 3.5 with BERI 26 displaying the lowest average diversity of 2.2 and BERI 36 showing the highest average diversity of 3.6, with both participants showing changeable plasma TMAO status. The SDI for participants grouped based on their urinary TMAO status showed slight differences with most changeable participants showing higher SDI than the stable groups. Therefore, an unpaired t-test was used to compare SDI between changeable and stable urinary TMAO, showing significantly higher SDI in the changeable group compared to stable ($p = 0.0365$, 95% CI = [0.02 to 0.46] of difference between means). The mean values between the groups are displayed in panel **B** of **Figure 3-18**. The SDI also significantly correlated with urinary TMAO levels according to the Spearman test ($r = 0.31$, $p = 0.026$). The SDI did not significantly correlate with plasma TMAO levels according to the Spearman test.

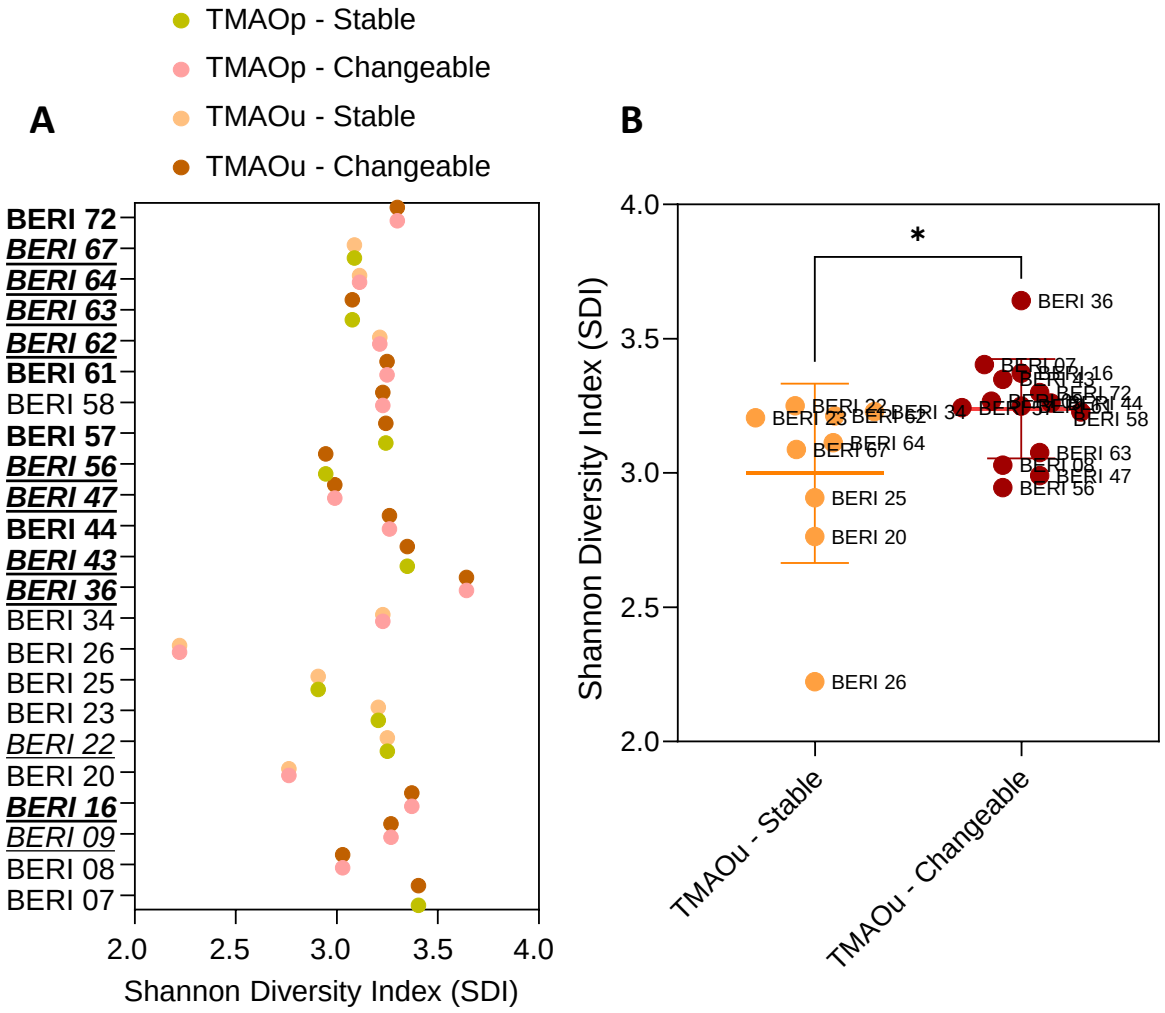

Figure 3-18 Shannon Diversity Index (SDI)

***A**) SDI plotted for each participant based on their in-vivo TMAO status (either stable or changeable in plasma (TMAOp) and urinary (TMAOu) TMAO levels) with participants annotated on the Y-axis according to their in-vitro capacity to metabolise choline (completers in **bold** and fast metabolisers in underlined italics).*

***B**) SDI in BERI participants based on their urinary TMAO status with individual values labelled with participant number and the mean ± SD of each group. * indicates significant difference between the means of the two groups measured by an unpaired t-test (p = 0.0365, 95% CI = [0.02 to 0.46] of difference between means).*

3.10 Are there correlations between the faecal microbiota species and the TMAO status of BERI study participants?

To establish relationships between the most abundant bacterial species and the *in-vivo* and *in-vitro* markers of TMAO status for BERI study participants, Spearman correlations were calculated and the r values are depicted in a heatmap with significant p values annotated in the relevant cells in **Figure 3-19**. Multiple species showed positive correlations with markers of *in-vivo* TMAO status. Urinary TMAO levels were more strongly associated with abundance of different species than plasma TMAO concentration, where *Prevotella copri* was the only significant relationship detected (Spearman r = 0.23, p = 0.007). Urinary TMAO levels were correlated with the abundance of *P. copri* (r = 0.24, p = 0.005), *B. angulatum* (r = 0.21, p = 0.015), *Agathobaculum butyriciproducens* (r = 0.22, p = 0.010) and *Desulfovibrio piger* (r = 0.34, p < 0.0001). *B. angulatum* was also associated with plasma and urinary betaine and carnitine levels, and *A. butyriciproducens* showed a negative correlation with plasma betaine (r = -0.24, p = 0.004). *Roseburia faecis* showed a negative correlation with plasma betaine concentration and a positive correlation with urinary carnitine levels. *D. piger* was positively correlated with plasma and urinary carnitine, urinary betaine, and faecal choline. Plasma carnitine was also positively correlated with *R. inulinivoran* and *Ruminococcus lactaris*, while urinary carnitine showed a negative correlation with *R. inulinivorans* (r = -0.17, p = 0.045). Urinary carnitine was also positively associated with *E. coli* (r = 0.37, p < 0.0001) and negatively associated with *Fusicatenibacter saccharivorans* (r = -0.34, p < 0.0001). This species was also negatively correlated with urinary betaine, faecal choline and abundance of archaea determined by 16S qPCR but positively correlated with plasma choline. Faecal choline was positively associated with *A. butyriciproducens* and *Dorea longicatena* but negatively correlated with *B. adolescentis*. Faecal TMA was correlated with *Coprococcus comes* and negatively associated with *Eubacterium* sp. CAG. 180.

From the *in-vitro* markers of TMA producing capacity, choline metabolism rate was significantly positively correlated with *D. longicatena* which was also correlated with maximum TMA produced from choline. TMA production rate showed no significantly positive correlations with any of the top 20 species but was negatively correlated with *A. butyriciproducens*. Another negative correlation was detected for *B. adolescentis* with maximum TMA produced from choline and final TMA concentration. Final TMA concentration but not maximum TMA concentration was also associated with *Faecalibacterium prausnitzi* and *B. angulatum*. The abundance of archaea in the samples was also negatively associated with multiple species, namely, *Eubacterium sp.* CAG. 180, *B. angulatum, R. inulinivorans, A. butyriciproducens* and *Acidaminococcus intestini.*

Cohort participant BERI 26 showed a distinctive microbial structure (on genera and species levels) compared to other study participants. But the distinctive microbiota did not seem to impact on the *in-vivo* measures of TMAO or the *in-vitro* metabolism of choline to TMA, which were similar.

A metagenomics approach was now used to determine gut microbiota function with respect to TMA production and utilisation pathways, and to investigate their relationship with the species described above, and to explore correlations with *in-vivo* and *in-vitro* TMAO status.

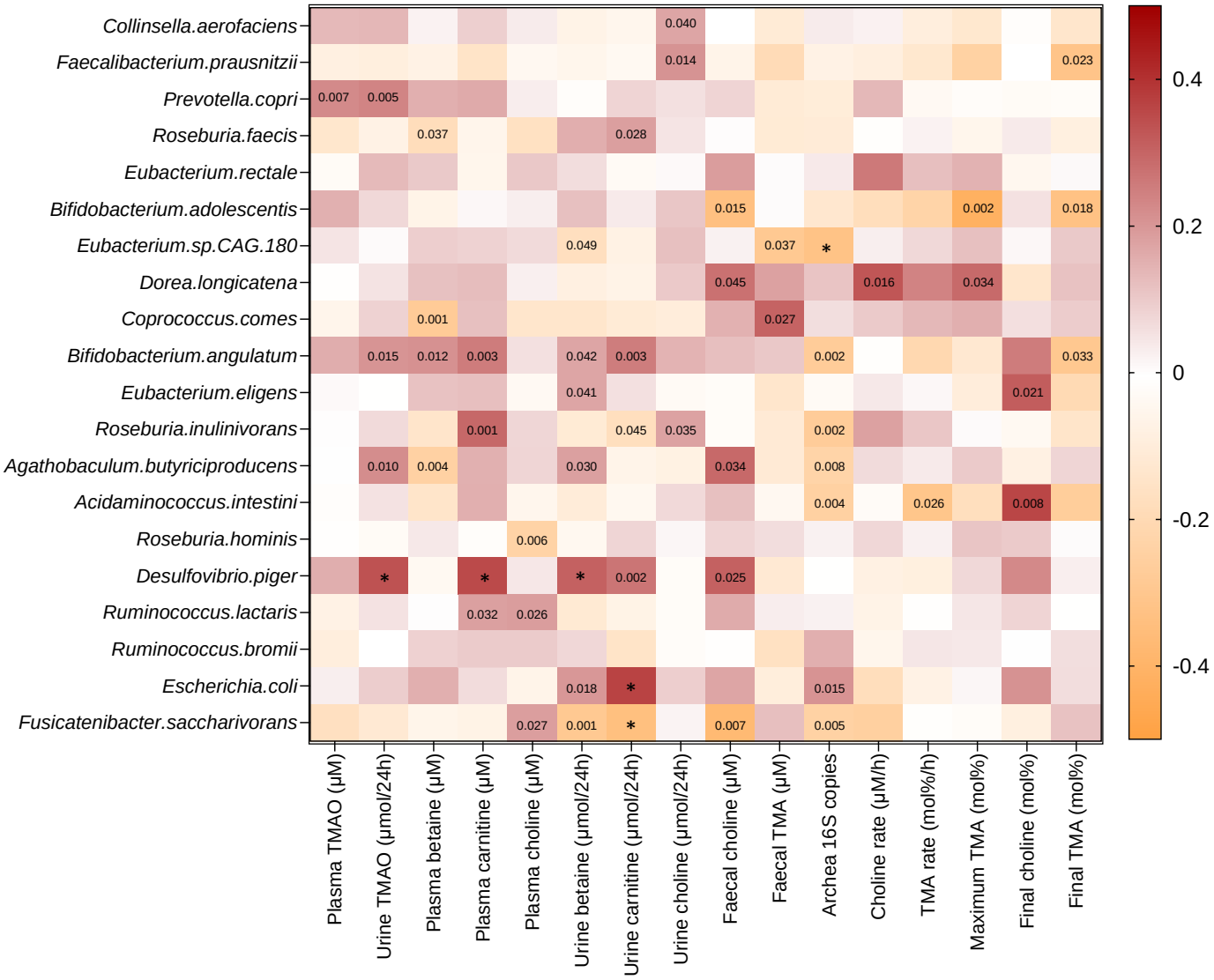

Figure 3-19 Spearman correlation matrix of in-vitro and in-vivo TMAO status with abundances of the top 20 bacterial species

*Correlation of 20 most abundant species with markers of TMAO status from in-vivo human study (plasma and urinary) and measures of in-vitro metabolism of choline to TMA. Heatmap depicts values between r = -0.5 to 0.5 and significant correlations are denoted with p value number in the respective cells or by asterisk * if p < 0.0001.*

IS THERE A RELATIONSHIP BETWEEN THE FUNCTION OF THE FAECAL MICROBIOTA, *IN-VIVO* TMAO STATUS AND THE CAPACITY TO PRODUCE TMA MEASURED *IN-VITRO*?

3.11 The association between the abundance of genes encoding choline-TMA lyase and the most abundant species detected in the faecal microbiota

The relative abundance of the top 20 species detected in the faecal microbiota of BERI participants showed both positive and negative correlations with markers of TMAO status. The hypothesis that these top 20 species were associated with the abundance of genes encoding TMA production via choline-TMA lyase was now tested. The abundances of reactions, pathways, enzymes, proteins, gene families and other variables in the BERI study metagenomics dataset were generated using the functional profiling tool HUMAnN 3.0. The abundances were normalised to copies per million (CPM) and the abundance of the genes encoding choline-TMA lyase reaction (RXN-13946) was correlated to the top 20 bacterial species, displayed in **Table 3-1**. *Collinsella aerofaciens* was positively correlated with genes encoding choline-TMA lyase (Pearson r = 0.45, p < 0.0001) together with *D. piger*, *C. comes* and *D. longicatena*, while *R. faecis* and *E. coli* showed a negative correlation with choline-TMA lyase associated reaction RXN-13946.

Table 3-1 The Pearson correlation of genes encoding choline-TMA lyase associated reaction RXN-13946 and most abundant bacterial species

Only significantly correlated species are reported. Species abundance was determined using MetaPhlAn v3.0.2 and abundance of RXN-13946 associated with choline-TMA lyase was determined using HUMAnN 3.0 with values normalised to copies per million (CPM)

choline-TMA lyase (CPM) *vs.*	*Collinsella aerofaciens*	*Roseburia faecis*	*Dorea longicatena*	*Coprococcus comes*	*Desulfovibrio piger*	*Escherichia coli*
Pearson r	0.45	-0.38	0.36	0.39	0.30	-0.19
95% CI	0.31, 0.58	-0.51, -0.22	0.20, 0.50	0.24, 0.52	0.14, 0.45	-0.35, -0.02
R^2	0.20	0.14	0.13	0.16	0.09	0.04
P (two-tailed)	<0.0001	<0.0001	<0.0001	<0.0001	0.0004	0.0278
P value	****	****	****	****	***	*

The distribution of the abundance of genes encoding choline-TMA lyase is displayed in **Figure 3-20** panel **A.** The abundance showed a large distribution, but most values clustered around ~0.5 CPM with the gene not detected in some samples. To investigate how the abundance of genes encoding choline-TMA lyase related to markers of TMAO status, the Spearman correlation was calculated with plasma and urinary TMAO levels shown in **Figure 3-20** panels **B**

and **C**. There was a weak correlation between the abundance of the choline-TMA lyase reaction and plasma TMAO concentration (Spearman r = 0.17, p = 0.042) but this relationship was not linear. Urinary TMAO levels were not significantly correlated with the abundance of the genes encoding choline-TMA lyase (Spearman r = 0.16, p = 0.067).

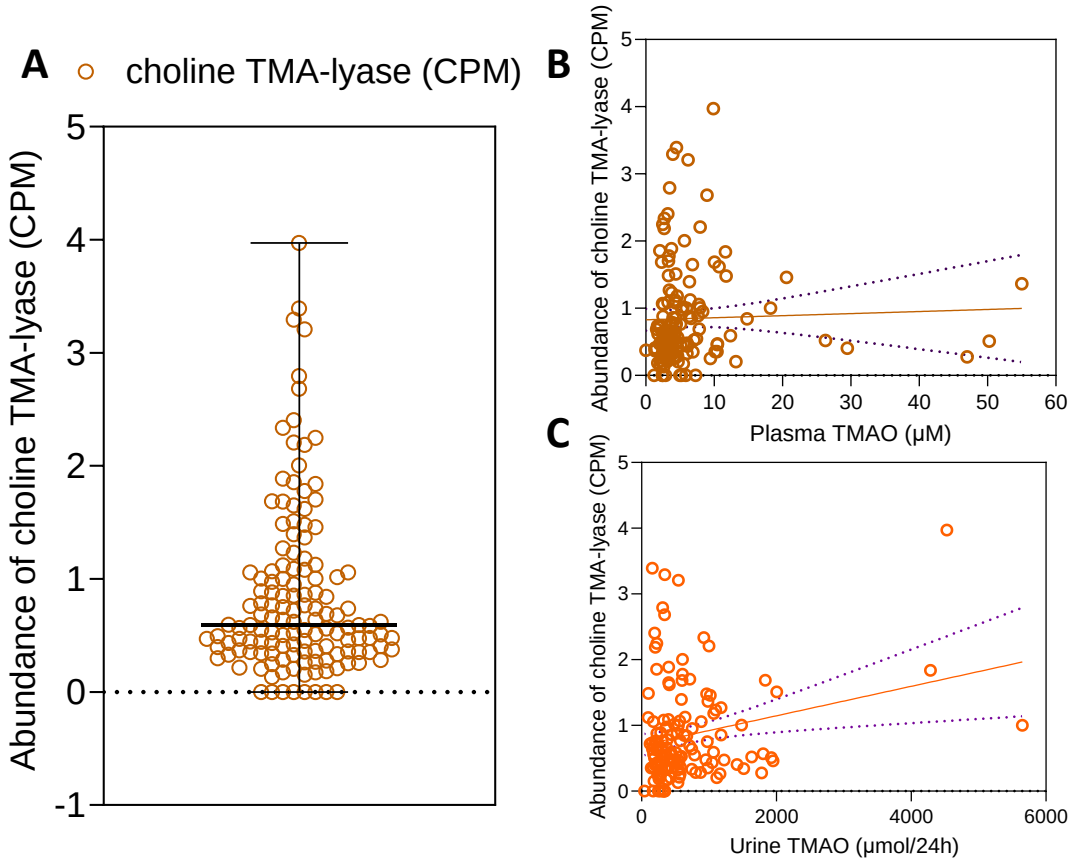

Figure 3-20 Distribution and correlation of genes encoding TMA producing reaction RXN-13946 associated with choline-TMA lyases in the BERI metagenomic dataset and its correlation with plasma and urinary TMAO.

***A**) Distribution of genes encoding TMA producing reaction RXN-13946 associated with choline-TMA lyases in the BERI metagenomic dataset. The abundance of choline-TMA lyase (CPM) is plotted for all faecal samples with median and range.*

***B**) Scatterplot of plasma TMAO concentration and the abundance of genes encoding choline-TMA lyase (CPM) with linear regression line of best fit and 95% confidence bands.*

***C**) Scatterplot of urinary TMAO levels and the abundance of genes encoding choline-TMA lyase (CPM) with linear regression line of best fit and 95% confidence bands.*

3.12 The abundance of genes encoding TMA-producing and utilising pathways in the BERI study cohort

Figure 3-21 panel **A** shows the EC (Enzyme Classification) numbers of enzymes that are involved in the production of TMA from multiple precursors and the pathways involved in TMA breakdown. This figure depicts the distribution of genes encoding these EC numbers in the BERI cohort. Glycine dehydrogenase was very abundant across the entire cohort, particularly in

BERI 26. Another highly abundant encoded enzyme was glycine/sarcosine *N*-methyltransferase (EC 2.1.1.156). Choline sulfatase, betaine reductase and sarcosine/dimethylglycine N-methyltransferase (EC 2.1.1.157) were transiently abundant throughout the cohort. BERI 26 included a high frequency of genes encoding EC numbers that were not particularly abundant amongst other cohort participants (exceptions included some timepoints for BERI 07, 36, 43 and 44). The encoded enzymes included trimethylamine-N-oxide reductase (cytochrome c), dimethylsulfoxide reductase, betaine aldehyde dehydrogenase, catalase peroxidase TMA dehydrogenase and choline dehydrogenase. Choline-TMA lyase has been previously described in more detail in **Figure 3-20**, and was not particularly abundant (E.C. 4.3.99.4) (**Figure 3-21** panel **A**). Pathways associated with carnitine breakdown were not very abundant, based on EC classification. However, other tools and databases can be used to further classify or further characterise these pathways such as Pfam domains displayed in panel **B** of **Figure 3-21**.

The Pfam domains are protein families represented by multiple sequence alignments and hidden Markov models (HMMs). Top five rows show the *in-vitro* TMA producing capacity from choline which indicates TMAO status. A group of methyltransferases associated with genes *mauE*, *mttB*, *mtmB* together with dimethylamine methyltransferase showed very low abundance compared to other Pfam domains in this cohort, with the *mttA* family of proteins being much more abundant in BERI 07, 23, 25, 26, 47 and 61. Genes encoding the betaine/carnitine/choline transporter family were transiently abundant across the cohort but very highly abundant in BERI 26, 58 and 61. A gene encoding the glycine/sarcosine/betaine reductase component B subunit was abundant in this cohort, as well as the reductase selenoprotein B (GRDB). The glycine reductase complex selenoprotein A was also similarly represented in this cohort. The *caiF/grlA* transcriptional regulator was only abundant in BERI 26. Genes encoding ethanolamine utilisation proteins were transiently abundant within the cohort, but very highly abundant in BERI 26.

KEGG orthology (KO) database entries related to TMA production and utilisation are displayed in **Figure 3-21** panel **C**. Some of these KEGG orthology entries are associated with EC numbers that were described in panel **A**, however, one KO can have multiple EC numbers assigned to it and *vice-versa*, but KO entries are always linked to sequence information. The SAM dependent methyltransferase and formate C-acetyltransferase were highly abundant in the BERI cohort. The betaine/carnitine/choline transporter BCCT family (mentioned in the Pfam domain analysis) seemed to be less abundant according to this analysis and only highly abundant in participants BERI 09, 23, 26, 43, 58 and 61. BERI 26 again showed a different pattern to other members of the cohort, including a distinct high abundance of KO entries associated with carnitine breakdown into crotonobetaine and crotonobetainyl-CoA, together with TMAO

reductase. There was a very low abundance of the different methyltransferases whereas the lipopolysaccharide cholinephosphotransferase was highly abundant across the entire cohort apart from low levels in BERI 16. There were also high abundances of reactions associated with breakdown of intermediate products of carnitine metabolism such as crotonyl CoA reductase. There was a very high abundance of the TMAO reductase associated with menaquinones which is linked to the *torA* and *torC* genes in BERI 07, 08, 09, 23, 25, 26, 34 and 36 (but only in some timepoints).

Figure 3-21 Abundance of genes encoding pathways with TMA producing and utilising enzymes in the BERI study cohort with their in-vitro markers of capacity to produce TMA from choline

A) EC (Enzyme Classification) numbers of enzymes that are involved in the production of TMA from multiple precursors and the pathways involved in TMA breakdown into smaller compounds

B) Pfam domains associated with proteins families involved in TMA production and utilisation.

C) KEGG orthology (KO) database numbers related to TMA production and utilisation


A
Choline rate (μM/h)
TMA rate (mol%/h)
Maximum TMA (mol%)
Final choline (mol%)
Final TMA (mol%)
1.4.4.2: Glycine dehydrogenase (aminomethyl-transferring)
2.1.1.156: Glycine/sarcosine N-methyltransferase
1.7.2.3: Trimethylamine-N-oxide reductase (cytochrome c)
1.8.5.3: Dimethylsulfoxide reductase
3.1.6.6: Choline-sulfatase
1.21.4.4: Betaine reductase
1.2.1.8: Betaine-aldehyde dehydrogenase
1.11.1.21: Catalase peroxidase - trimethylamine dehydrogenase
1.1.99.1: Choline dehydrogenase
2.1.1.157: Sarcosine/dimethylglycine N-methyltransferase
1.6.6.9: Trimethylamine-N-oxide reductase
4.3.99.4: Choline trimethylamine-lyase
6.2.1.48: carnitine-coA ligase
4.2.1.149: Crotonobetainyl-CoA hydratase
2.8.3.21: L-carnitine CoA-transferase
2.7.7.15: Choline-phosphate cytidylyltransferase
2.7.8.24: Phosphatidylcholine synthase
1.3.8.13: crotonobetainyl-coA reductase
2.7.1.32: Choline kinase
3.1.4.4: Phospholipase D
1.5.8.2: Trimethylamine dehydrogenase
BERI 07A1
BERI 08A1
BERI 09A1
BERI 16A1
BERI 20A1
BERI 22A1
BERI 23A1
BERI 25A1
BERI 26A1
BERI 34A1
BERI 36A1
BERI 43A1
BERI 44A1
BERI 47A1
BERI 56A29
BERI 57A29
BERI 58A29
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BERI 62A29
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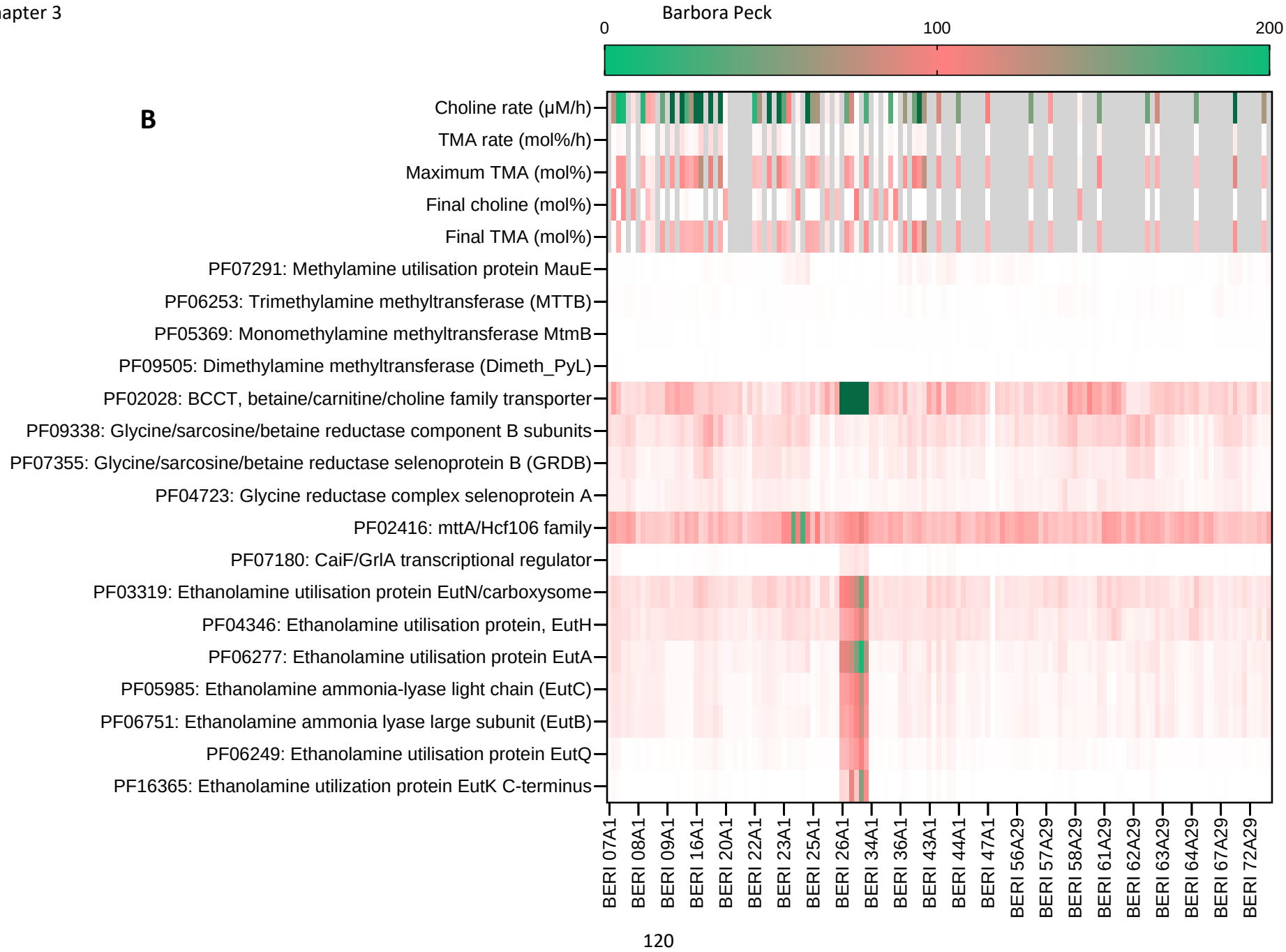
BERI 67A29
BERI 72A29
300
200
100
0

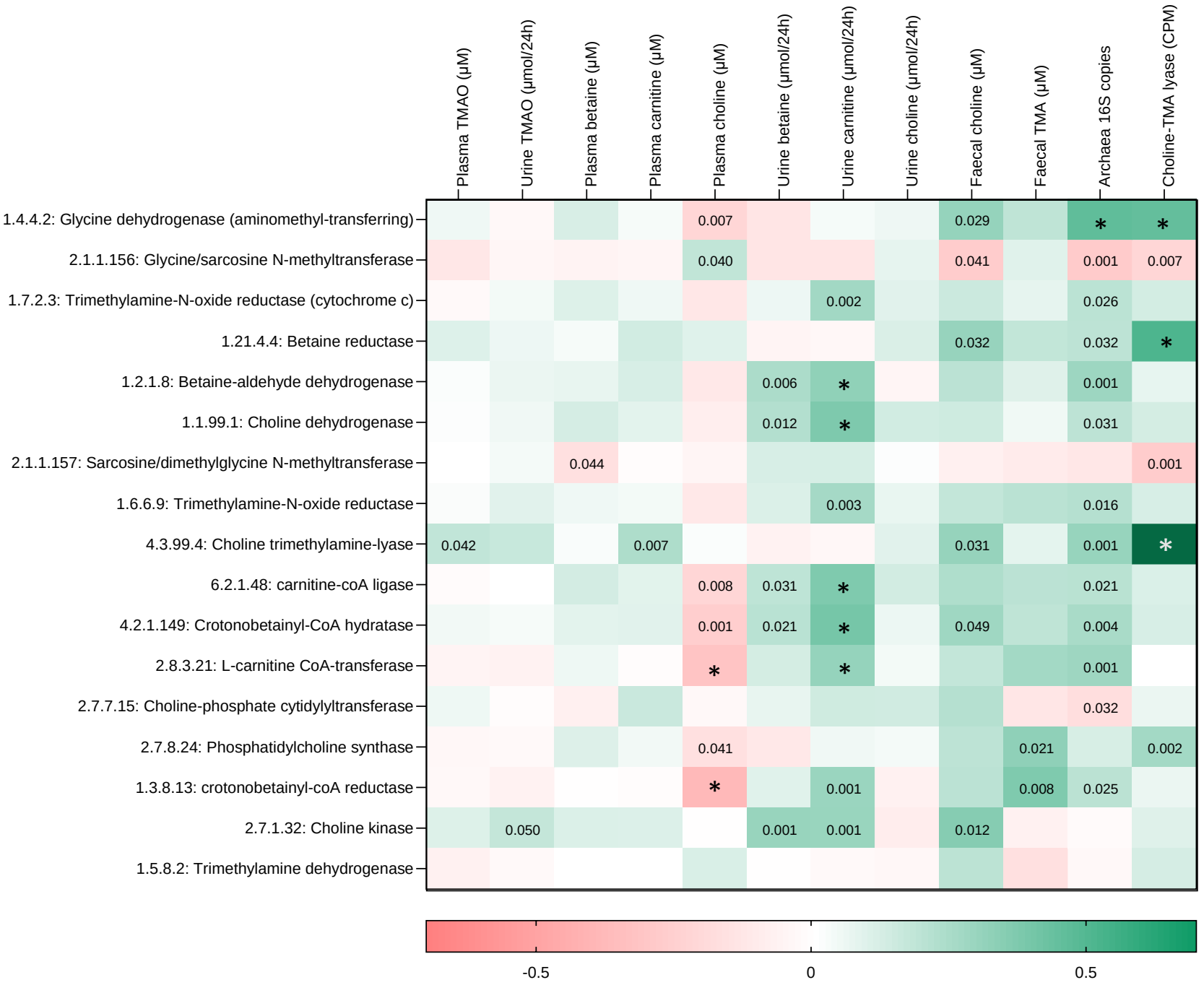

B
Barbora Peck
0
100
200
Choline rate (µM/h)
TMA rate (mol%/h)
Maximum TMA (mol%)
Final choline (mol%)
Final TMA (mol%)
PF07291: Methylamine utilisation protein MauE
PF06253: Trimethylamine methyltransferase (MTTB)
PF05369: Monomethylamine methyltransferase MtmB
PF09505: Dimethylamine methyltransferase (Dimeth_PyL)
PF02028: BCCT, betaine/carnitine/choline family transporter
PF09338: Glycine/sarcosine/betaine reductase component B subunits
PF07355: Glycine/sarcosine/betaine reductase selenoprotein B (GRDB)
PF04723: Glycine reductase complex selenoprotein A
PF02416: mttA/Hcf106 family
PF07180: CaiF/GrlA transcriptional regulator
PF03319: Ethanolamine utilisation protein EutN/carboxysome
PF04346: Ethanolamine utilisation protein, EutH
PF06277: Ethanolamine utilisation protein EutA
PF05985: Ethanolamine ammonia-lyase light chain (EutC)
PF06751: Ethanolamine ammonia lyase large subunit (EutB)
PF06249: Ethanolamine utilisation protein EutQ
PF16365: Ethanolamine utilization protein EutK C-terminus
BERI 07A1
BERI 08A1
BERI 09A1
BERI 16A1
BERI 20A1
BERI 22A1
BERI 23A1
BERI 25A1
BERI 26A1
BERI 34A1
BERI 36A1
BERI 43A1
BERI 44A1
BERI 47A1
BERI 56A29
BERI 57A29
BERI 58A29
BERI 61A29
BERI 62A29
BERI 63A29
BERI 64A29
BERI 67A29
BERI 72A29


C
0
50
100
150
200
Choline rate (μM/h)
TMA rate (mol%/h)
Maximum TMA (mol%)
Final choline (mol%)
Final TMA (mol%)
RXN-13946: choline trimethylamine-lyase (acetaldehyde-forming)
RXN-9515: crotonyl-[acyl-carrier protein] reductase (NADP)
CHOLINE-SULFATASE-RXN: NO_NAME
RXN0-5264: Trimethylamine-N-oxide reductase (menaquinone)
RXN-12558: crotonyl-CoA reductase (NAD+)
RXN-9657: crotonyl-[acp] reductase
K06969: putative SAM-dependent methyltransferase
K00656: formate C-acetyltransferase [EC:2.3.1.54]
K03451: betaine/carnitine transporter, BCCT family
K02002: glycine betaine/proline transport system substrate-binding protein
K07307: anaerobic dimethyl sulfoxide reductase subunit B (DMSO reductase iron- sulfur subunit)
K07308: anaerobic dimethyl sulfoxide reductase subunit C (DMSO reductase anchor subunit)
K02182: crotonobetaine/carnitine-CoA ligase
K07306: anaerobic dimethyl sulfoxide reductase subunit A [EC:1.8.5.3]
K07812: trimethylamine-N-oxide reductase (cytochrome c) 2
K07811: trimethylamine-N-oxide reductase (cytochrome c) [EC:1.7.2.3]
K02000: glycine betaine/proline transport system ATP-binding protein [EC:3.6.3.32]
K08279: carnitine operon protein CaiE
K00108: choline dehydrogenase [EC:1.1.99.1]
K03532: trimethylamine-N-oxide reductase (cytochrome c), cytochrome c-type subunit TorC
K08298: crotonobetainyl-CoA:carnitine CoA-transferase
K02168: high-affinity choline transport protein
K05245: L-carnitine/gamma-butyrobetaine antiporter
K00130: betaine-aldehyde dehydrogenase [EC:1.2.1.8]
K05020: glycine betaine transporter
K07821: trimethylamine-N-oxide reductase (cytochrome c) 2, cytochrome c-type
K10672: betaine reductase
K16176: methylamine---corrinoid protein Co-methyltransferase [EC:2.1.1.248]
K14083: trimethylamine---corrinoid protein Co-methyltransferase [EC:2.1.1.250]
K01004: phosphatidylcholine synthase [EC:2.7.8.24]
K00968: choline-phosphate cytidylyltransferase [EC:2.7.7.15]
K07271: lipopolysaccharide cholinephosphotransferase
BERI 07A1
BERI 08A1
BERI 09A1
BERI 16A1
BERI 20A1
BERI 22A1
BERI 23A1
BERI 25A1
BERI 26A1
BERI 34A1
BERI 36A1
BERI 43A1
BERI 44A1
BERI 47A1
BERI 56A29
BERI 57A29
BERI 58A29
BERI 61A29
BERI 62A29
BERI 63A29
BERI 64A29
BERI 67A29
BERI 72A29

The correlations of the interactions between the genes encoding enzymes (EC numbers) that were abundant in this cohort, *in-vivo* markers of TMAO status and the archaea 16S gene copy number and choline-TMA lyase abundance are displayed in **Figure 3-22** (heatmap of Spearman r where positive correlations are shown in green and negative correlations in pink with p values annotated in cells with significantly correlated relationships). Glycine dehydrogenase was significantly negatively associated with plasma choline concentration and positively correlated with faecal choline concentration. The opposite was the case for the glycine/sarcosine *N*-methyl transferase which showed positive correlation with plasma choline concentration but was negatively correlated with faecal choline concentration, archaea 16S copy number and choline-TMA lyase abundance. The abundance of TMAO reductase was positively correlated with urinary carnitine level and with archaea 16S copy number. Faecal choline concentration was correlated with betaine reductase which was also correlated with archaea 16S copy number and choline-TMA lyase abundance. Betaine aldehyde dehydrogenase, which is another step in the betaine breakdown pathway, was positively associated with urinary betaine and carnitine levels. Choline dehydrogenase was positively associated with urinary betaine and carnitine levels. Choline-TMA lyase was positively correlated with plasma TMAO levels, plasma carnitine and faecal choline concentrations. This EC number was also very strongly correlated with the abundance of Choline-TMA lyase associated reaction (RXN 13946) identified on MetaCyc. Most of the enzymes associated with carnitine utilisation pathway were negatively correlated with plasma choline concentration but positively correlated with urinary carnitine and betaine levels. Phosphatidylcholine synthase was negatively correlated with plasma choline level but positively correlated with faecal TMA concentration and with the abundance of choline-TMA lyase reaction. Choline kinase was also associated with urinary TMAO, urinary betaine, urinary carnitine, and faecal choline levels.

The correlations of genes encoding enzymes (EC numbers) with *in-vitro* capacity to produce TMA from choline are presented in **Figure 3-23**. Choline-TMA lyase abundance was positively correlated with rate of choline metabolism and TMA production, but negatively associated with final choline concentration. Betaine reductase was also correlated with choline metabolism rate and TMA production rate, as well as maximum TMA produced from choline. Maximum TMA concentration was also correlated with TMAO reductase, trimethylamine dehydrogenase and phosphatidylcholine synthase, however, not all these enzymes correlated with final TMA produced from choline.

Figure 3-22 Abundances of genes encoding enzymes (EC numbers) associated with TMA production and utilisation and their correlation with in-vivo TMAO status and the abundance of choline-TMA lyase associated reaction RXN-13946


Plasma TMAO (µM)
Urine TMAO (µmol/24h)
Plasma betaine (µM)
Plasma carnitine (µM)
Plasma choline (µM)
Urine betaine (µmol/24h)
Urine carnitine (µmol/24h)
Urine choline (µmol/24h)
Faecal choline (µM)
Faecal TMA (µM)
Archaea 16S copies
Choline-TMA lyase (CPM)
1.4.4.2: Glycine dehydrogenase (aminomethyl-transferring)
2.1.1.156: Glycine/sarcosine N-methyltransferase
1.7.2.3: Trimethylamine-N-oxide reductase (cytochrome c)
1.21.4.4: Betaine reductase
1.2.1.8: Betaine-aldehyde dehydrogenase
1.1.99.1: Choline dehydrogenase
2.1.1.157: Sarcosine/dimethylglycine N-methyltransferase
1.6.6.9: Trimethylamine-N-oxide reductase
4.3.99.4: Choline trimethylamine-lyase
6.2.1.48: carnitine-coA ligase
4.2.1.149: Crotonobetainyl-CoA hydratase
2.8.3.21: L-carnitine CoA-transferase
2.7.7.15: Choline-phosphate cytidylyltransferase
2.7.8.24: Phosphatidylcholine synthase
1.3.8.13: crotonobetainyl-coA reductase
2.7.1.32: Choline kinase
1.5.8.2: Trimethylamine dehydrogenase
-0.5
0
0.5

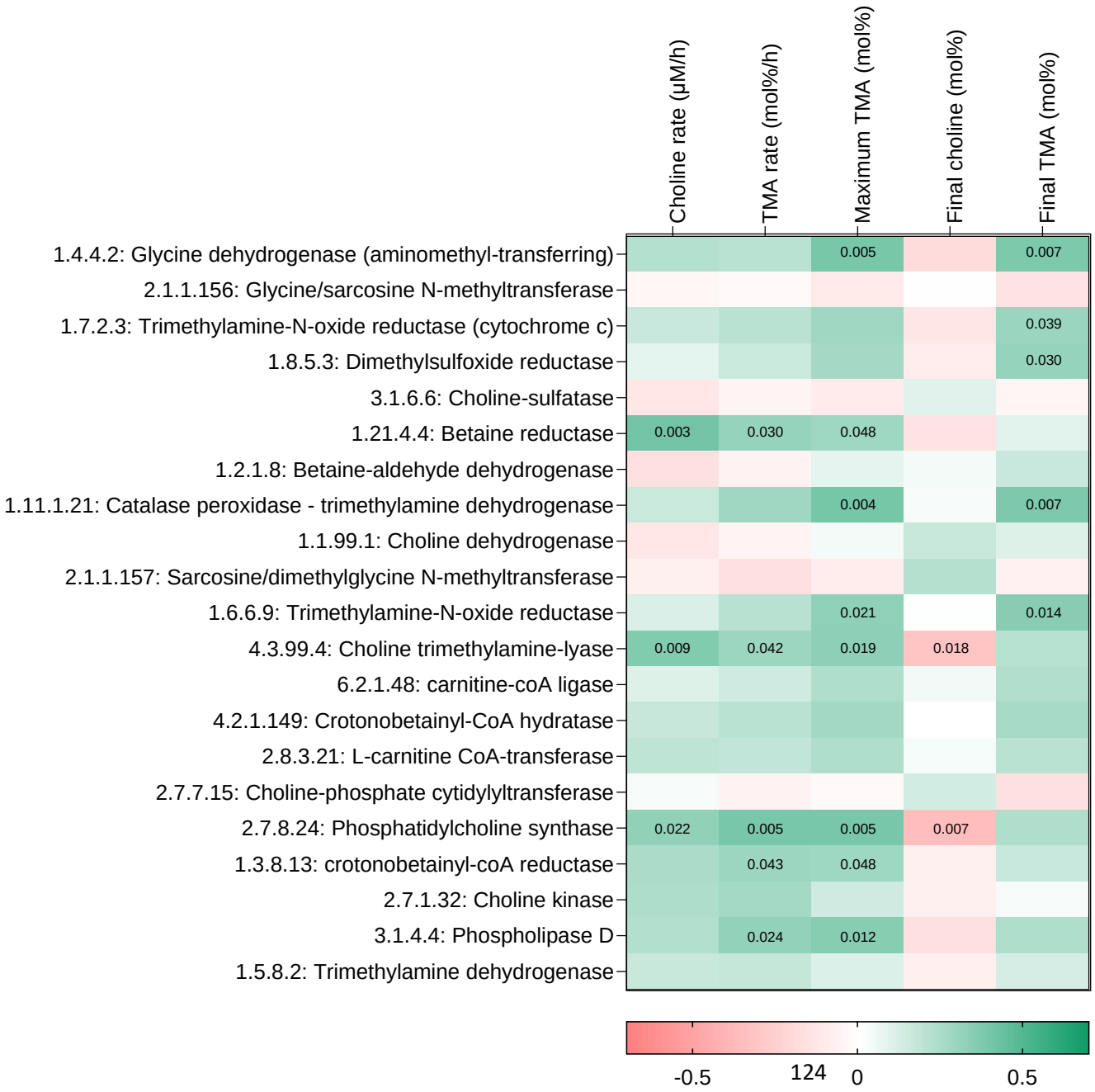


Figure 3-23 Abundance of genes encoding enzymes (EC numbers) associated with TMA production and utilisation and their correlation with in-vitro TMA production capacity

To determine if any of these genes were associated with the top 20 abundant species, Spearman correlation was used to identify relationships between these variables. A heatmap of Spearman r values is displayed in **Figure 3-24** (significant correlations are denoted by *, p values are available in supplementary information). There was a strong positive correlation between TMAO reductase associated reaction (RXN-19619) and related enzymes (EC numbers) and the abundance of *E. coli*. A weaker positive correlation of TMAO reductase was observed with *D. piger* and the abundance of this enzyme had a negative correlation with *F. prausnitzii*, *F. saccharivorans* and *B. adolescentis*. *D. piger* was positively correlated with many TMA producing enzymes (EC numbers) but was negatively correlated with methyltransferase pathways. Interestingly, choline sulfatase and betaine reductase were strongly correlated with multiple species but were most often mutually exclusive (i.e. *Prevotella copri* was negatively correlated with choline sulfatase but positively correlated with betaine reductase). Betaine reductase was positively correlated with *C. aerofaciens*, *E. rectale*, *R. lactaris, D. piger*, and most strongly associated with *D. longicatena*. This enzyme was negatively correlated with *F. prausnitzii* and *R. faecis*. Most of the dehydrogenases connected with betaine and choline metabolism were positively correlated in *E. coli* and *D. piger* but negatively associated with *B. adolescencentis, F. saccharivorans* and *F. prausnitzii*. Some of the methyltransferases were positively associated with *F. prausnitzii*, *R. inulinivorans*, *E. rectale, F. saccharivorans* and *A. intestini* but most of the species involved in TMA production were negatively correlated with these enzymes. EC number 4.3.99.4: Choline-TMA lyase was positively correlated with *C. aerofaciens*, *D. piger*, *C. comes*, and *D. longicatena* as previously established for the reaction associated with this enzyme, but *E. rectale, P. copri, R. hominis* and *R. lactaris* also showed a significant positive correlation with this enzyme. The only negatively correlated species that was significantly associated with choline-TMA lyase was *R. faecis*. Choline-TMA lyase, choline-phosphate cytidylyltransferase and betaine reductase were all positively correlated with Shannon diversity index.

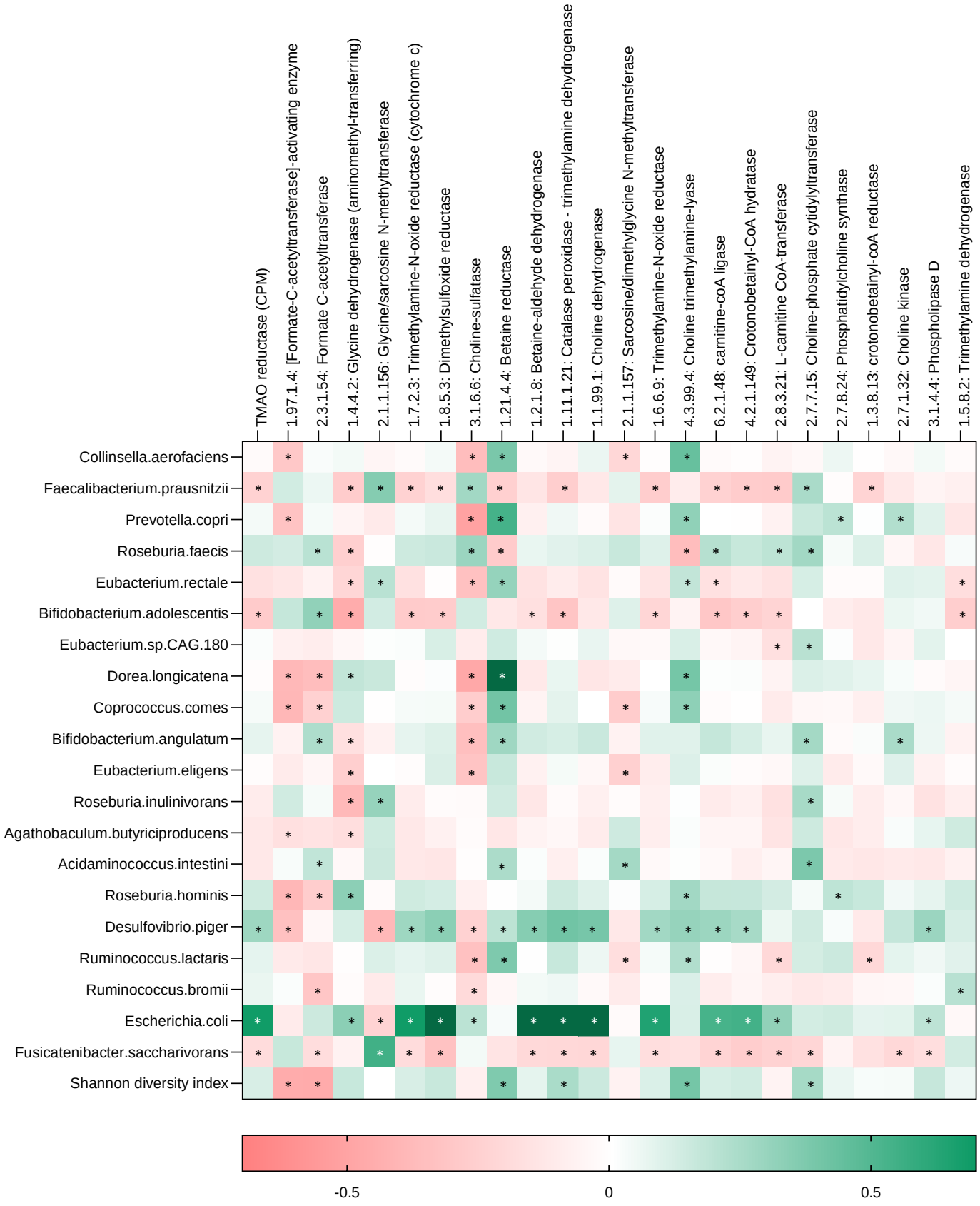


Figure 3-24 Abundance of genes encoding enzymes (EC numbers) associated with TMA production and utilisation and their Spearman correlation with the top 20 most abundant bacterial species in the BERI study cohort.

DISCUSSION

The aim of this chapter was to test the hypothesis that the capacity of individuals to produce TMA (measured using *in-vitro* fermentation and metagenomic analysis of faecal samples) correlates with their plasma and urinary TMAO levels.

3.13 Plasma and urinary TMAO levels show a positive correlation

To test this hypothesis, one of the objectives was to establish *in-vivo* TMAO status of participants from the BERI human study cohort by analysing their plasma and urinary TMAO levels on six independent occasions. Both plasma and urinary TMAO levels were highly variable in some participants across the different timepoints which resulted in stratification of participants based on their TMAO status variability into stable and changeable groups. Regardless of this variation, plasma and urinary TMAO levels for individual samples were significantly correlated in this cohort, although there was a weaker relationship between different timepoints for participants. Variation in plasma TMAO levels was previously reported by Kuehn *et al.* (244) who measured fasted plasma TMAO concentrations from two samples of 100 participants collected one year apart and reported Spearman correlation $r = 0.29$ ($p = 0.003$) between the two plasma TMAO measures. McEntyre and colleagues (245) also reported variability in TMAO levels and some of its precursors measured at 4 timepoints over 2 years, corroborating the notion that single measurements of TMAO may be of limited clinical use as a disease marker.

Wu *et al.* (47) attempted to identify a TMAO-producer phenotype by measuring fasted plasma and urinary TMAO levels in vegetarians and omnivores prior to undergoing an oral carnitine challenge test (OCCT) which was proposed as an alternative measure of capacity to produce TMAO. They showed very high correlation ($r = 0.92$, $p < 0.0001$) between plasma and urinary TMAO levels, but their observations were based on single matched measurement of these variables. Tang *et al.* (246) reported positive correlation between plasma and total urinary TMAO levels (Spearman's $r=0.58$, $P<0.001$) in a cohort of 40 participants undergoing a phosphatidylcholine challenge. Other reports argue that the variability in plasma and urinary TMAO levels could stem from consumption of TMAO precursors prior to measurements of plasma and urinary TMAO, despite baseline concentrations measured in fasted state (152, 154, 247, 248). This could have been a factor influencing the variation in plasma and urinary TMAO levels at the independent occasions in the BERI cohort since dietary intake was not controlled beyond exclusion of anthocyanin-rich foods. Therefore, the contribution of foods containing TMAO precursors to plasma and urinary TMAO levels in this study was highly likely. A number

of studies reported increase in both plasma and urinary TMAO levels as well as changes in dietary precursors in these biological samples with increased consumption of foods rich in TMAO substrates such as red meat, fish and eggs (54, 76, 245, 249-253). The relationships between TMAO status and the abundance of dietary precursors measured in plasma and urine of the BERI study participants was established by calculating Spearman correlation of these variables. Overall, the abundance of carnitine, betaine and choline measured in urine and plasma were quite variable for the urinary substrates and quite low for choline in plasma. The measured concentrations of carnitine and betaine were higher than those reported in other studies (31, 96, 137, 243, 254, 255), but this could have been influenced by dietary intake or differences in absorption of these TMAO precursors in the BERI cohort. This data suggests that consumption and absorption of carnitine and betaine was higher than the consumption and absorption of choline based on its plasma levels.

Choline was also measured in faecal samples and showed higher concentration than when measured in plasma and urine. However, the relationships between urinary, plasma and faecal choline concentrations are not well understood with most studies focusing on the impact of the precursors on TMAO rather than investigating the associations of these substrates in different biological matrices (50, 256-261). Furthermore, it is not clear if faecal choline can be representative of the amount of choline that was not absorbed in the upper GI tract, since a proportion of the unabsorbed dietary choline could have been converted to TMA by the gut microbiota, resulting in lower faecal choline concentrations. To determine the physiological significance of the abundance of TMAO precursors in different biological samples, dietary intake would have to be considered. The measured TMA concentration in faeces was very low, possibly due to re-absorption of TMA into circulation prior to bowel movements or further breakdown of TMA into smaller compounds that were not quantified.

Regardless of the abundance of these precursors in the different biological samples in the BERI study cohort, both plasma and urinary TMAO levels were correlated with the concentrations of carnitine and betaine in urine, but these correlations were much weaker between plasma betaine and carnitine and urinary TMAO levels. Urinary choline also revealed a negative correlation with urinary TMAO and showed no correlation with plasma TMAO. Urinary betaine was also correlated with urinary carnitine, and interestingly, with faecal choline. However, the physiological processes behind plasma and urinary levels of TMAO precursors are not well explored and the capacity of faecal microbiota to produce TMA from these precursors needs to be investigated. The ability of the gut microbiota to metabolise choline into TMA was investigated in faecal glycerol stocks from BERI study participants fermented with supplemented choline using an *in-vitro* colon model.

3.14 The *in-vitro* faecal microbial capacity to produce TMA from choline is not correlated with *in-vivo* TMAO status

Another objective of this chapter was to establish the capacity of the faecal microbiota to produce TMA from choline using outcome measures from the *in-vitro* colon model tests with faecal microbiota of BERI study participants, and to test the hypothesis that the capacity of an individual's gut microbiota to produce TMA from choline correlates with their *in-vivo* TMAO status. Choline metabolism rate, TMA production rate, maximum TMA produced from choline, and final concentration of choline and TMA at the end of incubation were used as markers of *in-vitro* TMAO status and capacity to produce TMA from choline. Additionally, based on the ability of participants to fully utilise supplemented choline within a given timeframe, participants were further categorised into fast or slow metabolisers who either completely or partially utilised all choline over 48 h of incubation. These markers were used for establishing the relationship between *in-vivo* TMAO status and *in-vitro* TMA production capacity. There were no significant correlations between the *in-vivo* and *in-vitro* markers of TMAO status, thus the variation in *in-vivo* TMAO levels was not associated with the capacity of the gut microbiota to metabolise choline to TMA (as measured in *in-vitro* tests). High *in-vivo* TMAO levels at individual timepoints may have been diet-related, and did not reflect an increased capacity of the gut microbiota to metabolise choline to TMA. Alternative approaches used by some workers have included feeding dietary precursors of TMAO and measuring the effect on TMAO concentration in plasma (22, 47, 262, 263).

However, *in-vitro* fermentation markers of TMAO status showed high correlations with each other. The rate of choline metabolism and TMA production were highly correlated, with the rate of TMA production showing weaker correlations with final TMA levels compared to maximum TMA levels. This suggests that the differences between maximum and final TMA levels might arise from the abundance of TMA-utilising microbes. Moreover, the TMA production capacity variables are only reflecting the metabolism of choline, and the potential of TMA production from other precursors still needs to be explored. Fermentation of L-carnitine or betaine using BERI faecal samples could have yielded different associations with *in-vivo* TMAO status, therefore, the potential ability of the gut microbiota to metabolise other substrates to TMA was investigated *in-silico*, using a taxonomic and function profiling of the faecal gut microbiota of BERI study participants.

3.15 Some of the most abundant species of the faecal microbiota showed associations with TMAO status

To determine the structure of the gut microbiota and its relationship with *in-vivo* TMAO status and *in-vitro* capacity to produce TMA from choline, the abundance of prevalent genera and

species were measured. The abundance of the top 10 bacterial genera and top 20 bacterial species in the faecal microbiota of each participant across 6 independent occasions was relatively stable, but there were considerable differences between participants. The Shannon Diversity Index was considered when comparing the different categories of participants based on the stability of their TMAO status *in-vivo* and their capacity to produce TMA from choline measured *in-vitro*. Participants with stable urinary TMAO status showed a significantly lower Shannon Diversity Index than those with changeable urinary TMAO status. The Shannon Diversity Index was previously used to correlate microbial metabolites measured in plasma where Wilmanski, Rappaport (264) reported Pearson's r = 0.68 in a cohort of 399 volunteers.

Several species were associated with the *in-vitro* capacity to produce TMA from choline and the *in-vivo* TMAO status. *Desulfovibrio piger* was positively correlated with urinary TMAO, and plasma and urinary levels of TMAO precursors carnitine and betaine, respectively. Other members of the *Desulfovibrio* genus have been previously associated with choline metabolism as described by Craciun and Balskus (57) who identified a gene cluster responsible for anaerobic choline degradation within the genome of a sulfate-reducing bacterium *D. desulfuricans* ATCC 27774. *D. alaskensis* G20 was also later confirmed to contain *cutC/D* genes encoding choline-TMA lyase with 91% and 76% similarity for *cutC* and *cutD* with *D. desulfuricans* ATCC 27774, respectively. This genus was identified as positively correlated with plasma TMAO levels by Manor and colleagues (130) in a cohort of 648 participants during their multi-omics association study.

Dorea longicatena was positively associated with choline metabolism rate and maximum TMA produced from choline as well as faecal choline concentration. This could be interpreted as a stimulatory relationship where abundance of choline in faeces promotes growth of *D. longicatena* which in turn translates to high choline metabolism rate and maximum TMA produced from choline. The abundance of *Dorea* species has been previously associated with TMA producing capacity in a metagenomics assembled genome (MAG) analysis by Borton and colleagues (265) in their Methylated Amine Gene Inventory of Catabolism database (MAGICdb). This database was developed to identify gut microbial gene content modulating human cardiovascular disease and contains 6,341 microbial genomes involved in production and utilisation of TMA. *Dorea* sp. 5-2 was also investigated by others for its involvement in choline metabolism and for its phylogenetic similarity to other representative TMA producers (2, 140, 231).

Bifidobacterium adolescentis was negatively correlated with maximum and final TMA concentrations measured *in-vitro*. Another strain from the *Bifidobacterium* genus, *B. angulatum* was negatively correlated with final TMA but not with maximum TMA produced

from choline, indicating a possible involvement of this genus in TMA utilisation. This genus was previously reported for its negative association with TMAO levels (130), specifically with *Bifidobacterium longum*, but the mechanisms behind the involvement of this genus in TMAO status remain unexplored. Other species negatively associated with the *in-vitro* capacity to produce TMA from choline were *Acidaminococcus intestini* and *Faecalibacterium prausnitzii* which were negatively correlated with TMA production rate and final TMA produced from choline, respectively. *A. intestini* was positively associated with final choline concentration, suggesting that abundance of this species could lead to lower TMA production. *Eubacterium eligens* was associated with low choline utilisation due to its positive correlation with final concentration of choline at 48 h of *in-vitro* fermentation. Some of these genera were identified as both TMA-producing and TMA-utilising in the MAGICdb taxonomic assignment of TMA classification developed by Borton *et al.* (265). In their investigation of the expression of MTTB superfamily of methytransferases within published metatranscriptome data sets, they identified *Eubacterium* as one of the genera detected in the transcriptome as well as in their methylated amine supplemented fermentation experiments.

3.16 Functional pathways involved in TMA production and utilisation are correlated with some of the most abundant species in the BERI cohort

Functional pathways involved in TMA production and utilisation by the gut microbiota were investigated in the BERI cohort to establish correlations between the functional profile of the gut microbiota and TMAO status. EC number correlations showed several enzymes that were associated with plasma choline, urinary betaine, and carnitine. Some of the EC numbers were also associated with faecal choline, showing negative correlation with carnitine-CoA ligase, crotonobetainyl-CoA hydratase, and L-carnitine CoA-transferase which are all microbial enzymes involved in carnitine breakdown. Interestingly, these pathways were positively correlated with urinary carnitine, but the mechanisms of this correlation have not been previously explored. Choline TMA lyase was the only EC number significantly associated with plasma TMAO concentration and was also positively correlated with faecal choline. This correlation could indicate that choline abundance in the gut (and measured in the faeces) stimulates growth of bacteria containing choline-TMA lyases and possibly also genes encoding betaine reductase. These two enzymes were also significantly associated with choline metabolism rate, TMA production rate and maximum TMA produced from choline, suggesting their involvement in choline metabolism measured *in-vitro*.

The *in-vitro* capacity to metabolise choline also revealed a negative relationship between phosphatidylcholine synthase and final choline concentration, but a positive correlation with

TMA production. The positive correlation of maximum TMA produced from choline and TMAO reductase associated pathways also hints towards TMA production from alternative substrates such as TMAO. Another pathway that has been proposed in TMAO metabolism is dimethylsulfoxide reductase (*dmsABC*) which was also positively correlated with final TMA concentration. This enzyme was examined for its involvement in TMAO metabolism together with methionine sulfoxide reductase (*msrP*) and biotin sulfoxide reductase (bisC) in *E. coli* and *Klebsiella* spp. genomes (266, 267). In the BERI study dataset, top 20 species were correlated with genes encoding key enzymes (EC numbers) for TMA formation and degradation, and this revealed that the abundance of *E. coli* was indeed correlated with TMAO reductase as well as dimethylsulfoxide reductase, and enzymes involved in metabolism of choline into betaine (betaine-aldehyde dehydrogenase and choline dehydrogenase). However, *E. coli* showed no association with betaine reductase, suggesting that it may not be able to produce TMA via this pathway. *D. piger* showed a positive correlation with most of the TMA producing pathways, both from choline and betaine, and was also associated with some of the carnitine breakdown enzymes. Several species were correlated with choline-phosphate cytidylyltransferase encoded by the gene *licC* which is involved in choline conversion into phosphatidylcholine. The strong correlation of *D. longicatena* with *in-vitro* markers of TMA producing capacity is reflected in its association with choline TMA-lyase and betaine reductase as was also the case for *P. copri*, *Collinsela aerofaciens*, *E. rectale* and *C. comes*. Interestingly, *R. hominis* and *R. lactaris* also showed a positive association with choline TMA lyase EC number which was not the case for the associated reaction 13946. This observation highlights the importance of using multiple databases to determine interactions between TMAO status and microbial and gene abundances. Mapping metagenome assembled genomes to a database of TMA producing and utilising genomes like MAGICdb would help identify other species and genera associated with metabolism of substrates and further understanding of the TMA production capacity. That way, individual participants could be tested for their *in-vitro* metabolism of substrate which was most represented in terms of bacterial metabolism pathways. Regardless, other studies have previously reported associations of different microbial taxa and TMAO levels (130, 268-270), but this chapter showed an in-depth analysis of associated EC numbers with *in-vivo* and *in-vitro* markers of TMAO status and abundances of dominant species.

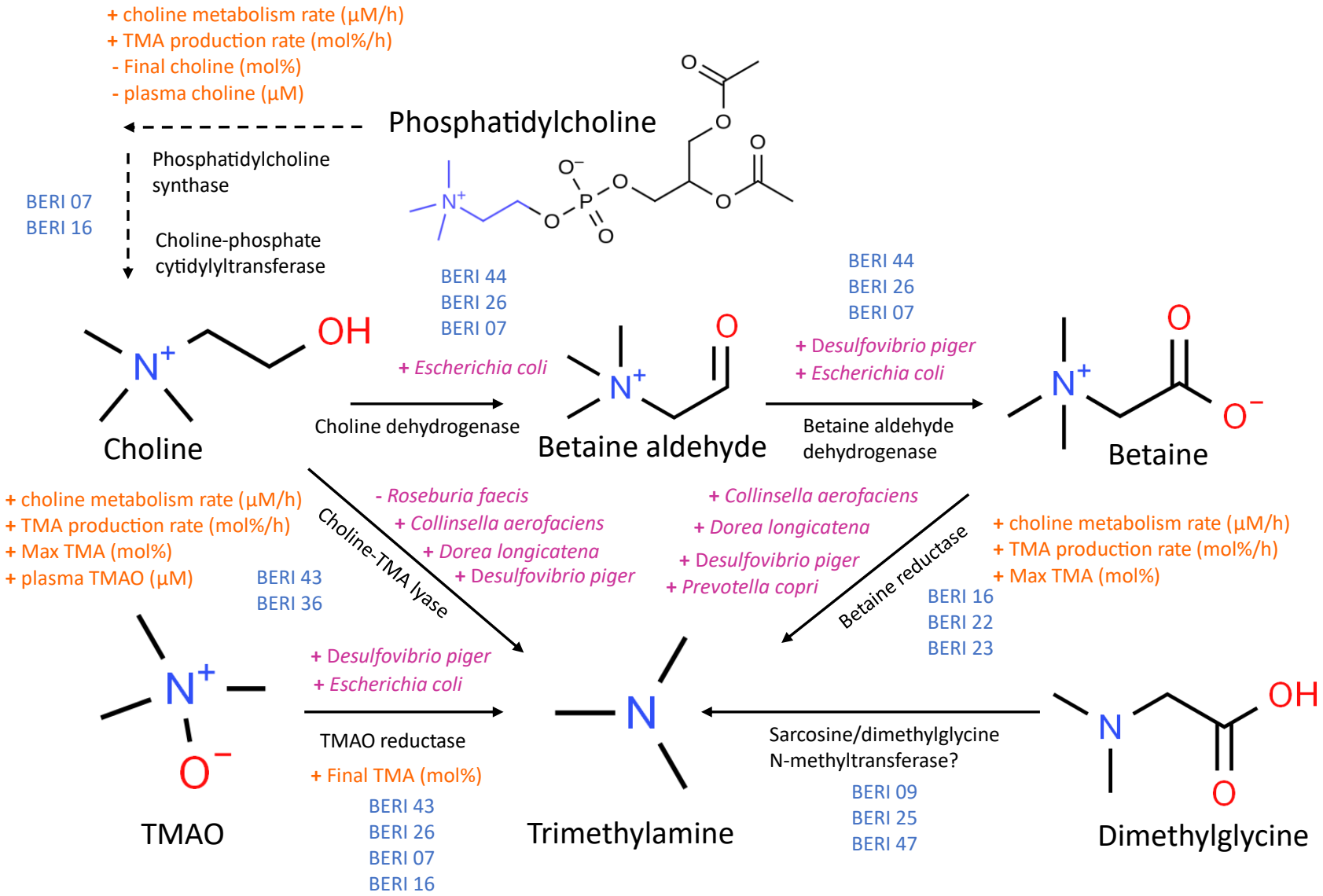


Figure 3-25 TMA producing pathways that have been associated with in-vitro capacity to produce TMA from choline and with the abundance of the top 20 species in the BERI cohort

3.17 Limitations and future research

There were some limitations in the observations made in this chapter connected to the BERI human trial, notably the BERI study was not designed to include individuals with a consistently high or low TMAO status that responded to dietary supplementation. The initial selection of individuals with a known consistently high or low TMAO status would be beneficial. To minimise the impact of factors influencing TMAO levels, some additional measures and study design adjustments would have to be carried out. The consumption of foods rich in TMAO precursors, such as red meat, fish, and eggs, can significantly influence TMAO levels. Therefore, dietary habits must be carefully controlled or adjusted for accurate assessment, which was not done in this study. Otherwise, collecting a 3-day food diary prior to each study day would help establish both habitual intake of TMAO substrates and account for increased fasting TMAO levels due to consumption of choline, carnitine, or betaine rich foods. Additionally, instructing participants to avoid these foods prior to study days would give insight into variation of fasted plasma TMAO levels without the influence of dietary factors.

There were some technical aspects that limited the observations of *in-vitro* capacity to produce TMA from methylated amines. Firstly, due to the complexity of the *in-vitro* fermentation experiments, only choline metabolism into TMA was analysed. The *in-vitro* capacity to produce TMA from carnitine, betaine and other more complex precursors was not investigated and these outcomes could have elicited different associations with *in-vivo* TMAO status and revealed relationships with gut microbial taxonomic and functional profiling. Secondly, repeating the *in-vitro* fermentation studies on multiple occasions or inoculating glycerol stocks in more replicate fermenters would improve the reliability of the observations and better determine the *in-vitro* capacity to metabolise choline into TMA. Additionally, having glycerol stocks made from all collected faecal samples would allow for direct comparison between the *in-vivo* and *in-vitro* measures at different occasions, together with giving an insight into the relationships between changes to the microbial structure and function and the capacity to produce TMA from methylated amines. Utilising high-throughput models like CMBB or CMHT with flash-frozen faecal samples and frozen glycerol stocks would improve the reproducibility of observations and allow for testing of multiple different supplemented substrates. Alternatively, other microbiota-rich samples representative of different parts of the human colon could give insight into the site-specific differences in the metabolism of these substrates. And finally, measuring additional outcomes of *in-vitro* fermentation studies such as changes to microbial composition after supplementation with methylated amines or investigating the expression of individual pathways using metatranscriptomics would facilitate validation of involvement of certain enzymes and genes that encode them in TMA production.

Investigating other microbial metabolites produced during *in-vitro* fermentations and determining their relationship with *in-vivo* TMAO markers and the host metabolome would elucidate the multi-pathway interactions between the gut microbiota, diet and the host.

The exploration of the structural and functional relationships between gut microbiota and *in-vivo* and *in-vitro* markers of TMAO status also had its limitations. Using available databases for taxonomic profiling of metagenomic datasets is dependent on the quality of the data set as well as the database annotation. For bacteria present in low abundance, some profilers might not be suitable due to default cut off point settings and limited diversity in annotated organisms. In this study, MetaPhlAn and HUMAnN 3.0 were used for investigating the structure and function of the gut microbiota but their sensitivity for non-bacterial microorganisms may be lower compared to other profilers and targeted approaches such as PCR-based assays or metatranscriptomics that reveal not just the gene contents of these samples but also the involvement of individual pathways based on transcription levels. Utilisation of tools specific for identifying microbial genomes involved in production and utilisation of TMA such as MAGICdb could highlight the importance of certain microbes and establish a TMA-producing and utilising gut microbial signatures.

CONCLUSION

In-vivo markers of TMAO status from the human BERI study cohort were generally stable with occasional samples having an elevated TMAO concentration. There was no relationship between *in-vitro* ability to metabolise choline to TMA and *in-vivo* TMAO status. Occasional high *in-vivo* TMAO samples were not associated with a change in capacity of the gut microbiota to convert choline to TMA, and were associated with another factor, possibly diet. Metagenomic analysis revealed that the structure and function of the gut microbiota were similar for most members of the human cohort, indicating that similar genera/species and enzymic pathways were likely involved in TMA formation and degradation. The structure and function of the gut microbiota was different for one cohort participant (BERI 26), but this had no detectable impact on *in-vivo* TMAO status or *in-vitro* metabolism of choline to TMA.

The hypothesis that the capacity of individuals to convert choline to TMA (measured using *in-vitro* fermentation and metagenomic analysis of faecal samples) correlates with their *in-vivo* plasma and urinary TMAO levels was not supported. Most individuals had a similar *in-vitro* ability to metabolise choline to TMA, structure/function of the gut microbiota, and *in-vivo* TMAO status. Occasional high *in-vivo* TMAO samples were not associated with a change in the capacity of gut microbiota to convert choline to TMA nor in structure/function of the gut

microbiota but were probably diet-related. It would be interesting to retest the hypothesis with a cohort of individuals with a consistently low or high *in-vivo* TMAO concentration.

Chapter 4

The effect of probiotics on microbial production of TMA from choline

ABSTRACT

Background

There is a need to develop effective sustainable approaches to reduce TMAO production from dietary precursors. Probiotic bacteria have been proposed as a new therapeutic target due to their ability to contribute towards re-instating gut microbial homeostasis and combatting gut dysbiosis. There are some published reports of the effects of supplementing single probiotic strains on plasma TMAO in human participants and animal models, however, the impacts of mixed probiotics in a simulated human colon model remain unexplored. The overall aim was to test the hypothesis that supplementing human faecal fermentations of choline with a 14-strain mixed probiotic product (Bio-Kult) and/or the individual strains within it would reduce the conversion of choline to TMA.

Approaches and Methods

The effects of the probiotics were investigated using (i) *in-vitro* colon models inoculated with human faecal samples which were incubated at 37 ^{o}C in anaerobic conditions in media designed to simulate the micronutrient and pH environment of the human large intestine and (ii) a TMA-producing strain (*Proteus mirabilis* DSM 4479) cultured in similar conditions. Choline was supplemented into the media at 2 mM final concentration. Treated vessels were supplemented with probiotic strains from Bio-Kult supplement. Samples were collected at multiple time points over 24/48 hours and the kinetics of choline disappearance and TMA production were quantified. Samples were analysed using LC-MS/MS method with isotopically labelled methylated amine internal standards to quantify choline and TMA concentrations. Microbial growth was measured using viable counts and optical density at 600 nm wavelength.

Results

There was no effect of the Bio-Kult mixed probiotic supplement ($1.7x10^7$ CFU/mL) on TMA production from choline in the human colon model inoculated with 1% faecal slurries from 7 individual donors. Neither the rate of choline metabolism (mean ± SEM; Bio-Kult = 149.0 ± 30.7 µM/h vs control = 160.4 ± 17.6 µM/h; p = 0.56) nor the area under the curve (AUC) of TMA produced from choline (Bio-Kult = 1813 ± 429 mol% vs control = 2158 ± 331 mol%; p = 0.29) nor the maximum TMA concentration generated from choline (Bio-Kult = 59.9 ± 13.0 mol% vs control = 71.3 ± 6.4 mol%; p = 0.39) were significantly different. In a further test, there was a significant decrease in AUC of TMA produced from choline (mol%) in three tested doses ($1.7x10^6$ CFU/mL, $8.4x10^6$ CFU/mL and $1.7x10^7$ CFU/mL final concentration of added Bio-Kult supplement) measured in pooled faecal sample from two donors ($p < 0.0001$). However, two higher doses did not have a significant effect. *Lactobacillus acidophilus* PXN 35 and *Streptococcus thermophilus* PXN 66 from Bio-Kult supplement incubated with a pooled faecal sample significantly decreased the AUC of choline (p = 0.0049 and p = 0.0128, respectively) compared to the control. The AUC of TMA produced from choline was significantly lower with strain *L. acidophilus* PXN 35 (p = 0.0028) and significantly higher with *Lactobacillus rhamnosus* PXN 54 (p = 0.0455) based on Two-way ANOVA. None of the single strains from the Bio-Kult supplement significantly altered the kinetics of choline disappearance when co-cultured with *Proteus mirabilis* DSM 4479, but there was a significantly lower percentage of TMA produced from choline by all single strains (TMA at 4 h = 17.2 ± 8.0 mol% for all Bio-Kult strains compared to 53.1 ± 1.5 mol% in control (p = 0.0007)).

Conclusion

These data show that some individual probiotic strains (and their culture supernatants) from the Bio-Kult supplement were effective in reducing TMA production by faecal microbiota and the TMA-producing strain *Proteus mirabilis* DSM 4479. However, the findings do not support the hypothesis that the Bio-Kult mixed-strain probiotic supplement can be an effective approach for reducing TMA production from choline in a complex faecal matrix.

INTRODUCTION

The modulation of gut microbiota can be achieved through probiotic supplementation (189, 196, 201, 212, 271, 272). This modulation can result in shifting of the microbial composition, increasing the number and diversity of beneficial microbes, and inhibition of pathogenic microorganisms. Some of these processes can have an impact on production of gut microbial metabolites such as short-chain fatty acids, bile acids or methylamines from dietary sources. To explore the effects of probiotic strains on TMA production, probiotic supplement Bio-Kult® Advanced Multi-Strain Formulation manufactured by ADM Protexin was used due to the extensive evidence of beneficial effects exhibited by the supplement strains in a vast array of clinical outcomes (273-275). The mixed probiotic supplement, containing proprietary strains that are members of the genus *Lactobacillus*, *Bifidobacterium* and other genera commonly used as probiotics, has been the target of studies in pre-clinical settings and human clinical trials. The antimicrobial activity of these bacterial strains has been tested against *Clostridioides difficile*, *Salmonella typhimurium* and *Helicobacter pylori* where they demonstrated significant inhibition through different mechanisms of action (276, 277). Most of the previous research has focused on the alleviation of symptoms of disease and improvement of clinical outcomes, such as the clinical efficacy in the management of infantile colic (278, 279), paediatric acute gastroenteritis (280), childhood constipation (281) and necrotising enterocolitis (NEC) of preterm infants (282). Research into the use of the Bio-Kult® strains has also expanded into the investigation of the gut-brain axis in a study of the clinical efficacy of probiotics in chronic and episodic migraines or their effects on major depressive disorder (273, 283). Other systemic effects of probiotics manufactured by Protexin have been explored in the context of metabolic syndrome, NASH, NAFLD and other metabolic diseases (284, 285), with additional evidence available on the Protexin website (286). The use of Bio-Kult probiotics for modulation of the gut microbiota in metabolic diseases has ignited interest in their potential effect on TMA production from dietary precursors.

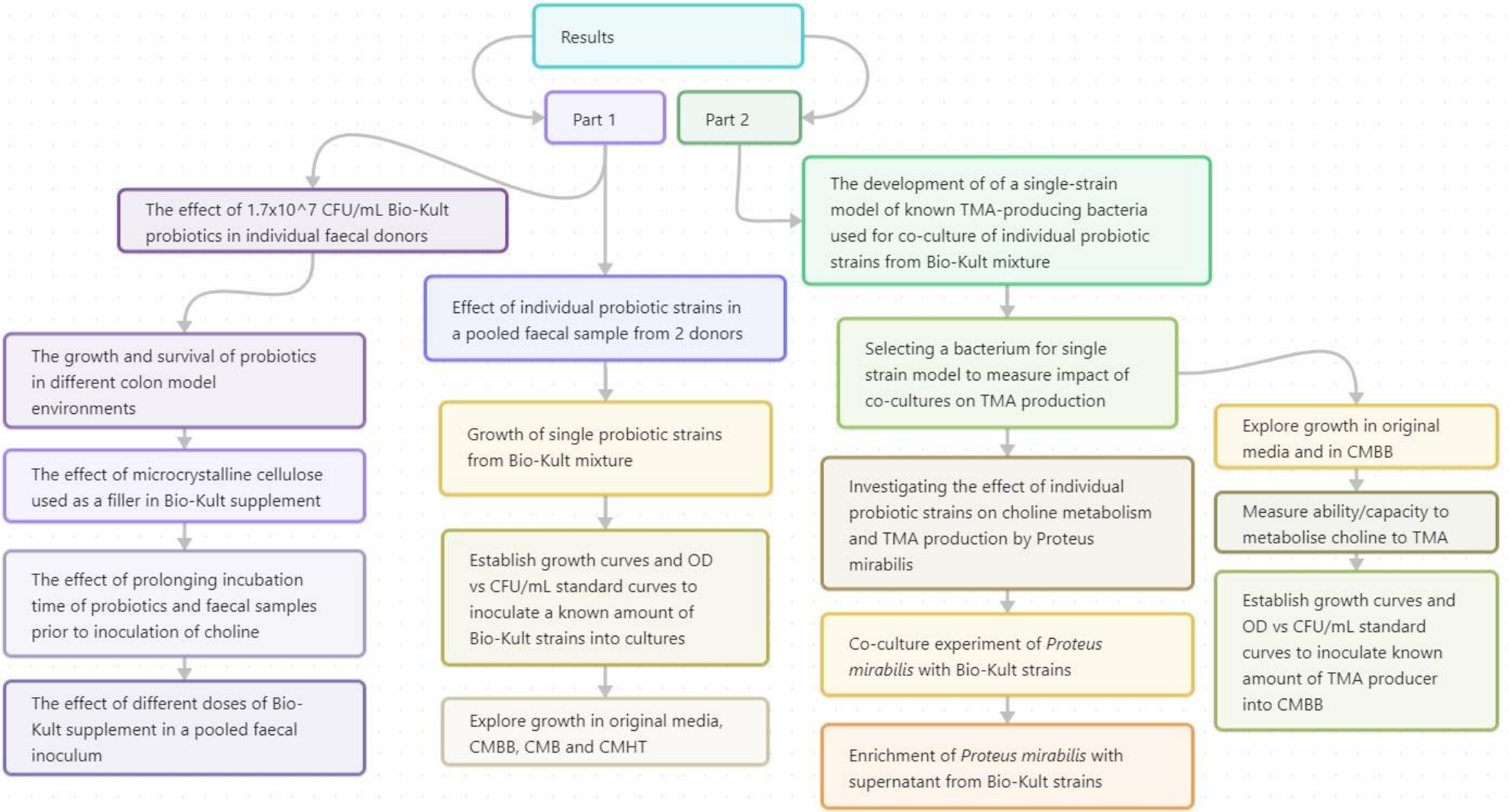
4.1 Hypothesis and aims

The overall aim was to investigate the effect of probiotics on choline metabolism and TMA production by the human gut microbiota. Specifically, testing the hypothesis that supplementing human faecal fermentations of choline with a 14-strain mixed probiotic product Bio-Kult and a subset of its individual strains would reduce the conversion of choline to TMA.

4.2 Objectives and approaches

Objective	Approach
Characterise the Bio-Kult supplement and establish a dose to be tested in the *in-vitro* colon model of choline fermentation	Using *in-vitro* colon model fermentations enriched with probiotics inoculated with human faecal samples, incubated at 37 ^{o}C in anaerobic conditions in media designed to simulate the micronutrient and pH environment of the human large intestine
Test a selected dose of Bio-Kult supplement in multiple faecal donors to investigate the effect on choline conversion to TMA	
Establish probiotic survival and growth in colon model media with and without enrichment with faecal microbiota	
Explore the effect of different doses of probiotic supplement on choline metabolism to TMA in a pooled faecal sample	
Characterise a subset of individual probiotic strains from Bio-Kult supplement to be used in probiotic enrichments of choline fermentations in a pooled faecal sample	
Test established *cutC*-containing bacterium for its ability to metabolise choline when supplemented with individual probiotic strains from the Bio-Kult supplement	Using a single-strain model of known TMA-producing bacterium (*Proteus mirabilis* DSM 4479) used for co-culture of individual probiotic strains to ferment choline in similar conditions as the *in-vitro* colon model

The methodology and study design of the experiments is further detailed in Chapter 2 and the development of the models is described in Appendix I. The results section is separated into two parts mirroring the two approaches for fulfilling the objectives of this chapter, some results are contained in Appendix I.


Results
Part 1
Part 2
The effect of 1.7x10^7 CFU/mL Bio-Kult probiotics in individual faecal donors
The growth and survival of probiotics in different colon model environments
The effect of microcrystalline cellulose used as a filler in Bio-Kult supplement
The effect of prolonging incubation time of probiotics and faecal samples prior to inoculation of choline
The effect of different doses of Bio-Kult supplement in a pooled faecal inoculum
Effect of individual probiotic strains in a pooled faecal sample from 2 donors
Growth of single probiotic strains from Bio-Kult mixture
Establish growth curves and OD vs CFU/mL standard curves to inoculate a known amount of Bio-Kult strains into cultures
Explore growth in original media, CMBB, CMB and CMHT
The development of of a single-strain model of known TMA-producing bacteria used for co-culture of individual probiotic strains from Bio-Kult mixture
Selecting a bacterium for single strain model to measure impact of co-cultures on TMA production
Investigating the effect of individual probiotic strains on choline metabolism and TMA production by Proteus mirabilis
Co-culture experiment of Proteus mirabilis with Bio-Kult strains
Enrichment of Proteus mirabilis with supernatant from Bio-Kult strains
Explore growth in original media and in CMBB
Measure ability/capacity to metabolise choline to TMA
Establish growth curves and OD vs CFU/mL standard curves to inoculate known amount of TMA producer into CMBB

RESULTS

4.3 Investigation of the effect of Bio-Kult mixed-strain probiotic supplement on choline metabolism and TMA production in a complex microbial environment using an *in-vitro* colon model

4.3.1 The Bio-Kult probiotic supplement

The Bio-Kult supplement was selected for investigation of the effects of probiotics on TMA production due to the extensive literature supporting its effectiveness in improving clinical outcomes across multiple diseases and its ability to act within the gastrointestinal tract and beyond. For this investigation, the supplement was used in its original form without the protective shell, undigested by enzymes of the stomach, assuming that at least half of the supplement contents were delivered to the colon, based on the evidence of previously reported bacterial survival of Bio-Kult in different pH (287). According to the product information, one Bio-Kult capsule contained $2x10^9$ CFU, equivalent to 10^{10} CFU/g of the supplement. The packaging recommended a dose of up to 4 capsules a day taken with food for children aged 12+ and adults. The purpose of the batch colon model used for this investigation was to simulate a single bolus of probiotic supplement in the colonic environment enriched with added choline, therefore only a single dose of 1.7 mg/mL ($1.7x10^7$ CFU/mL) of supplement in the fermentation culture was considered, amounting to around half of one capsule. **Table 4-1** lists the proprietary Protexin® strains in the Bio-Kult supplement and the source from which they were isolated.

Table 4-1 Probiotic strains in Bio-Kult supplement and their origin.

Probiotic strain (with updated nomenclature acc. 2020)	**Origin**
Bacillus subtilis PXN® 21™	Soil
Bifidobacterium bifidum PXN® 23™	Infant faeces
Bifidobacterium breve PXN® 25™	Intestine of infant
Bifidobacterium infantis PXN® 27™	Intestine of infant
Bifidobacterium longum PXN® 30™	Intestine of adult
Lactobacillus acidophilus PXN® 35™	Human
Lactobacillus delbrueckii ssp. *bulgaricus* PXN® 39™	Bulgarian yoghurt
Lactobacillus casei PXN® 37™ (*Lacticaseibacillus casei)*	Cheese
Lactobacillus plantarum PXN® 47™ *(Lactiplantibacillus plantarum)*	Pickled cabbage
Lactobacillus rhamnosus PXN® 54™ (*Lacticaseibacillus rhamnosus*)	Human
Lactobacillus helveticus PXN® 45™	Dairy
Lactobacillus salivarius PXN® 57™ (*Ligilactobacillus salivarius*)	Human
Lactococcus lactis ssp*. lactis* PXN® 63™	Dairy product
Streptococcus thermophilus PXN® 66™	Pasteurised milk

The supplement also contained additional ingredients that acted as a filler or as a protective shell to deliver viable bacteria to the intended site of action. Microcrystalline cellulose (MCC)

was used as a bulking agent and the supplement was encased in a vegetable capsule made from hydroxypropyl methylcellulose, packaged in air-tight blister packs. The proportions of the strains or the overall weight of the live bacteria content was not stated, therefore the biomass of live bacteria was estimated from reported nutritional fact sheets of other commercially available probiotic supplements and the results of calculations of the different components are reported in **Table 4-2**.

Table 4-2 Information about the contents of the Bio-Kult supplement

Parameter	Value	Information source
Average bacterial loading (CFU/mg of different supplements)	1.45×10^9	Reported in the supplement fact sheet of 8 randomly selected commercially available products, some that reported weight and bacterial load of individual components of the blend were entered as individual values contributing to the average bacterial loading (CFU/mg)
SD (CFU/mg)	0.8×10^9	SD from 21 bacterial loading and weight reports (from 8 supplement fact sheets)
Bio-Kult capsule bacterial loading (CFU)	2×10^9	Factory packaging information
Estimated weight of probiotic bacteria in Bio-Kult capsule (mg)	14	Bacterial load per mg based on the supplement fact sheet of 8 randomly selected commercially available products.
The weight of each capsule based on CFU/g (mg)	200	The average CFU/g of the Bio-Kult supplement is 10^{10}, therefore $2x10^9$ CFU per capsule means $2x10^9$CFU in capsule / 10^{10} per g = weight of capsule contents
Filler weight (mg)	186	Therefore 100 mg (for the dose of 1.7 mg/mL) of supplement contains about 93.1 mg of microcrystalline cellulose (MCC)

4.3.2 The effect of Bio-Kult probiotics in individual faecal donors

The kinetics of choline metabolism to TMA were previously explored in Chapter 3 and this chapter investigated the ability of probiotics to modify choline utilisation and the production of TMA from choline. Therefore, the probiotic supplement was compared against a controlled condition (no added probiotic), where a 2 mM final concentration of choline supplemented to a 1% faecal culture was incubated in the batch colon model and monitored over 48 h of incubation through frequent sampling. Due to the variation in the patterns of choline metabolism reported in Chapter 3, multiple individual faecal samples were used to determine the potential of probiotic supplement to decrease TMA production.

Faecal samples from 7 individual donors (5 from BERI study and 2 others) were independently enriched with a $1.7x10^7$ CFU/mL dose of commercially available mixed-strain probiotic

supplement Bio-Kult (PRX, equivalent to 1.7 mg/mL of supplement in faecal fermentation cultures) showed no effect on TMA production from choline compared to the control (no added Bio-Kult). The mean response (and SEM) is shown in Error! Reference source not found..

The choline abundance gradually decreased over 48 h of incubation, with the final abundance of choline in control was 2.6 ± 1.6 mol% (mean ± SEM, n=7) compared to 14.3 ± 11.2 mol% in Bio-Kult (PRX) supplemented condition. TMA produced from choline gradually increased over 20 h, reaching a plateau of 47.8 ± 9.9 mol% at 48 h with Bio-Kult (PRX) compared to 63.1 ± 4.4 mol% in the control, however, this difference was not statistically significant ($p > 0.05$).

To quantify the differences in individual donors, the mean Area Under Curve (AUC) of each participant was calculated for choline abundance and TMA produced from choline. This is shown in **Figure 4-2**.

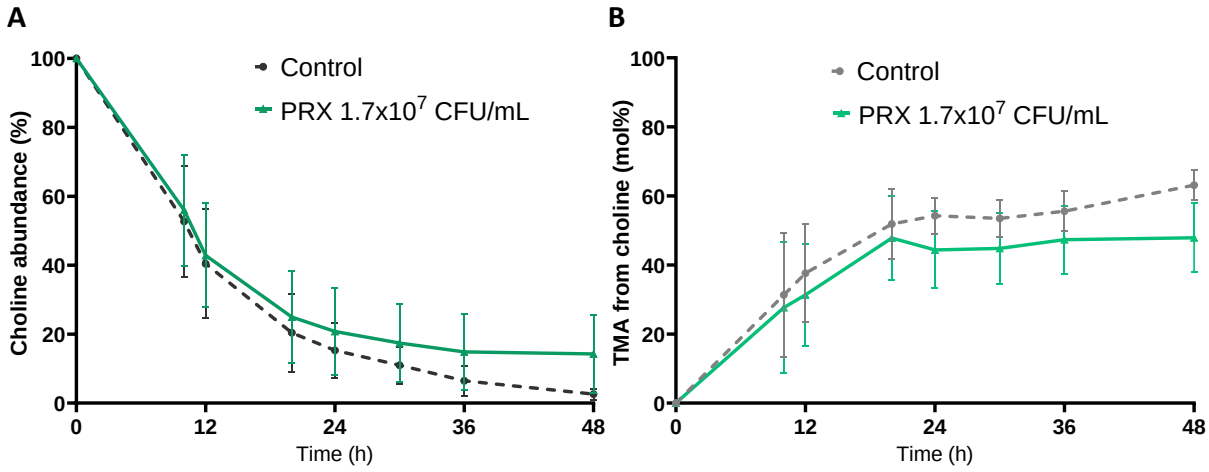


Figure 4-1 The mean effect of addition of Bio-Kult probiotics on choline to TMA metabolism kinetics

***A**) Choline abundance (mol%) and **B**) TMA from choline (mol%) with 1.7x10^7 CFU/mL of Bio-Kult mixed strain probiotic (equivalent to 1.7mg/mL of supplement) compared to untreated (absence of Bio-Kult) control. Samples were collected at 0, 10, 12, 20, 24, 30, 36 and 48 h. Vessels were incubated at 37ºC under constant mechanical stirring, at pH 6.6-7.2 and continuously sparged with N_2 to maintain anaerobic conditions. The experiment was repeated with 7 different donors where each donor had 2-5 technical replicates of each condition, depending on availability of vessels.*

Values are expressed as percentages where choline abundance (mol%) indicates the percentage of highest molar concentration of choline measured in each technical replicate of each donor. TMA from choline (mol%) is a molar equivalent of supplemented choline that was converted into TMA – for more information, see Calculations in Methods section. There was no significant difference between control and Bio-Kult (PRX) 1.7x10^7 CFU/mL at any of the measured timepoints using 2-way ANOVA (all p>0.05). Values are mean ± SEM (n=7).

There was no statistically significant difference in AUC of choline abundance between the Bio-Kult (PRX) addition and the control ($p = 0.0709$), or in AUC of TMA produced from choline ($p = 0.0565$). When using the Tukey's multiple comparison test, donor CM059 showed a significantly higher AUC of choline abundance ($p = 0.0018$, 95% CI = [2637, 649]) and a

significantly lower AUC of TMA produced from choline (p = 0.0076, 95% CI = [472, 2880]) in Bio-Kult (PRX) supplemented condition. However, for donor CM059 there was an exceptionally large variation between independent tests leading to the conclusion that this effect was not reproducible, and for this reason it was not studied further. Overall, the difference in AUC of participants accounted for 79 % and 58 % of total variance for choline abundance and TMA produced from choline, respectively, when comparing PRX treated condition and control.

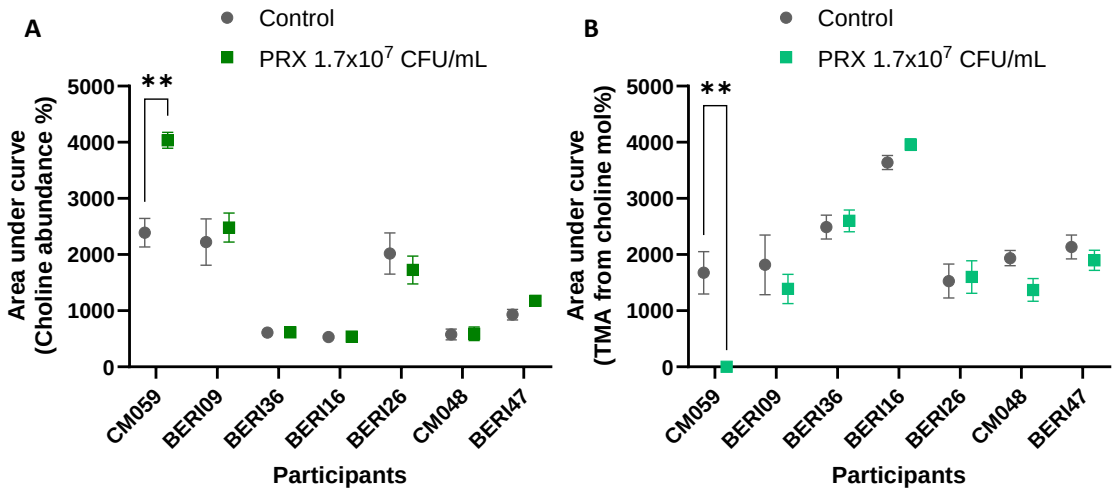


Figure 4-2 The effect of addition of Bio-Kult probiotics on choline to TMA metabolism for 7 individual faecal donors.

*The AUC of **A**) choline abundance (mol%) and **B**) TMA from choline (mol%) with 1.7x10^7 CFU/mL of Bio-Kult mixed strain probiotic (equivalent to 1.7mg/mL of supplement) compared to untreated (absence of Bio-Kult) control. Values are mean ± SEM, each donor had 2-5 technical replicates of each treatment. ** indicates significant difference ($p < 0.05$) between supplemented condition and control using a 2-way ANOVA with Tukey's multiple comparison test.*

Other measures defining the changes in the capacity to metabolise choline and produce TMA between the control (no Bio-Kult) and Bio-Kult (PRX) supplemented condition are displayed in **Figure 4-3**. The mean of difference in the maximum concentration of TMA produced from choline was 11.4 ± 12.5 mol% lower (mean ± SEM) with Bio-Kult (PRX) addition compared to the control. The choline metabolism rate was 11.5 ± 23.6 µM/h higher in the control compared to Bio-Kult (PRX) addition, and the TMA production rate was 2.1 ± 1.5 mol%/h lower with Bio-Kult (PRX) addition. However, these differences were not statistically significant using a paired t-test.

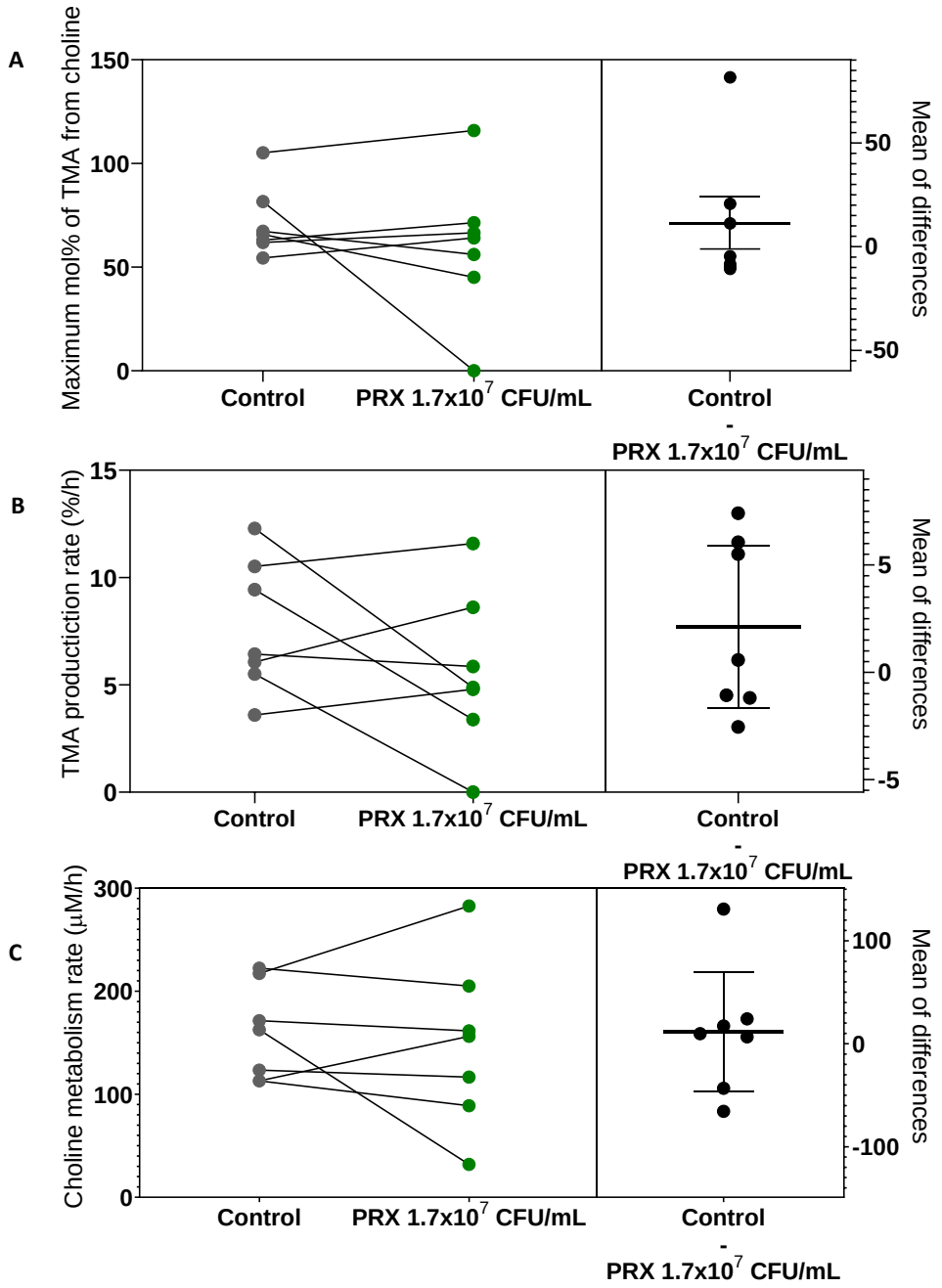


Figure 4-3 Differences in choline metabolism characteristic variables between addition of Bio-Kult (PRX) and control (no PRX) for all participants (n=7).

***A**) Maximum concentration of TMA from choline (left) shown as a mean value per participant calculated from technical replicates of each donor in control (grey circles) and in Bio-Kult (PRX) supplemented condition (green circles), matched donor values are connected by a line; mean + SEM of differences in maximum concentration (mol%) of TMA between the two conditions (right)*

***B**) Rate of TMA production (mol%/h) displayed and calculated as in A (left) and the mean + 95% CI of differences between the rate of TMA production displayed and calculated as in A (right).*

***C**) Choline metabolism rate (µM/h) presented as mean value of each participant's replicates calculated same as in A (left); the mean + 95% CI of differences between the rate of choline metabolism per participant (right).*

Bio-Kult (PRX) supplemented condition had an estimated 1.7x10^7 CFU/mL of probiotic strains added to colon model vessels, equivalent to 100 mg dose of Bio-Kult mixed strain probiotic supplement in 60 mL of total culture volume (1.7 mg/mL). Untreated control contained no Bio-Kult (PRX). The experiment was undertaken with 7 different donors where each donor had 2-5 technical replicates of each condition. There was no significant difference between control and Bio-Kult (PRX) supplemented condition in any of these characterising variables (paired t-test).

4.4 Determining factors contributing to the null effect of probiotic supplement Bio-Kult on TMA production from choline

To investigate factors contributing to the overall null effect of the probiotic supplement on choline metabolism to TMA in the colon model with multiple faecal donors, several hypotheses were tested, as described in the upcoming subsections:

a) The null effect was attributed to poor growth and survival of the added probiotics. The growth and survival of probiotics was explored using the batch fermentation model and the CMB medium in non-pH-controlled conditions to investigate the effect of pH and dilution on their ability to thrive with and without the faecal microbiota (**Section 4.4.1**)

b) The null effect (and occasional variable response) was due to low homogeneity in the added probiotics. The homogeneity of weighed supplement that was inoculated into the treatment vessels was explored to understand the variation in delivered probiotics (**Section 4.4.2**).

c) The null effect was due to interference by the supplement filler. The effect of the supplement filler, microcrystalline cellulose (MCC), was tested to determine if probiotic bacteria had been entrapped in the filler matrix or if any other interactions occurred (**Section 4.4.3**).

d) The null effect was because of a short incubation period. The fermentation period was extended to enable probiotics from the supplement to integrate within the faecal microbiota in fermentation vessels prior to addition of choline into the culture to investigate the impact of probiotics on TMA production (**Section 4.4.4**).

e) The null effect was due to the probiotic concentration. The dose of probiotics added to the fermentation vessels was altered to determine if lower or higher additions affected TMA production from choline (**Section 4.4.5**).

4.4.1 Testing the growth and survival of probiotics in different colon model environments

Since there was no effect of Bio-Kult (PRX) supplementation in faecal samples, probiotic supplement was grown in pH-controlled (using batch colon model) and non-pH-controlled (using cultures grown in anaerobic cabinet) environment without the presence of faecal sample in colon model medium (CMB). Different probiotic doses were tested in the batch colon model and the anaerobic cabinet was used for comparison between the use of the whole capsule and the capsule contents.

The initial viable count for each concentration of added Bio-Kult (PRX) was ~2-3 orders of magnitude lower than that estimated by the manufacturer. All probiotic doses in both CMB environments multiplied exponentially and plateaued between 20-24 h of incubation, apart from 1.7x10^6 CFU/mL dose in pH-controlled environment, **Figure 4-4** panel **A,** where the viable count decreased in the first 16 h and only later began exponential phase. In the non-pH-controlled environment of the anaerobic cabinet, the initial concentration in the vial with whole capsule was substantially lower at 0 h but reached the same final concentration as the capsule contents at 12 h.

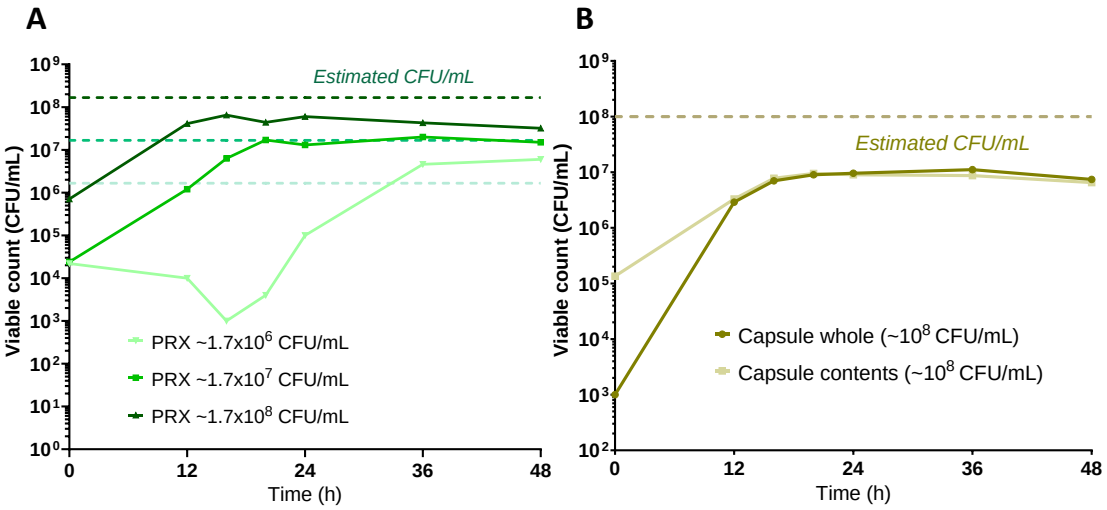


Figure 4-4 The growth of Bio-Kult probiotic supplement in different colon model environments.

A) *Viable count (CFU/mL) of three probiotic doses (10 mg, 100 mg and 1000 mg per 60 mL) measured over 48 h in a pH-controlled environment (pH 6.5-7.2) of batch colon model are displayed in solid lines compared to the estimated supplemented concentration (CFU/mL) from Bio-Kult (PRX) doses in dashed lines.*

B) *Viable count (CFU/mL) of a single capsule of Bio-Kult mixed strain probiotic inoculated whole or as capsule contents only into anaerobic batch media, incubated in the anaerobic cabinet (no external pH control). Dashed line represents estimated concentration (CFU/mL) of supplemented Bio-Kult (PRX) probiotics based on information on the product packaging. Viable counts are single measurements counted on Wilkins-Chalgren agar plates.*

To determine bacterial growth when supplementing Bio-Kult (PRX) into faecal samples, two doses were tested compared to faecal only control in a pH-controlled environment of the colon model and in the anaerobic cabinet without pH control. **Figure 4-5** displays the total viable count of anaerobic bacteria in these conditions over 48 h.

There were negligeable differences between total viable count in the vessels despite the different doses of Bio-Kult (PRX) supplemented into 1% faecal sample in both tests. In the anaerobic cabinet where pH control was not available, the total viable microbial count in the control vial with 1% faecal decreased from 24 h compared to the Bio-Kult (PRX) supplemented conditions. In these tests, it was not possible to distinguish the growth/survival of Bio-Kult (PRX) probiotics from the bacteria in the faecal matrix.

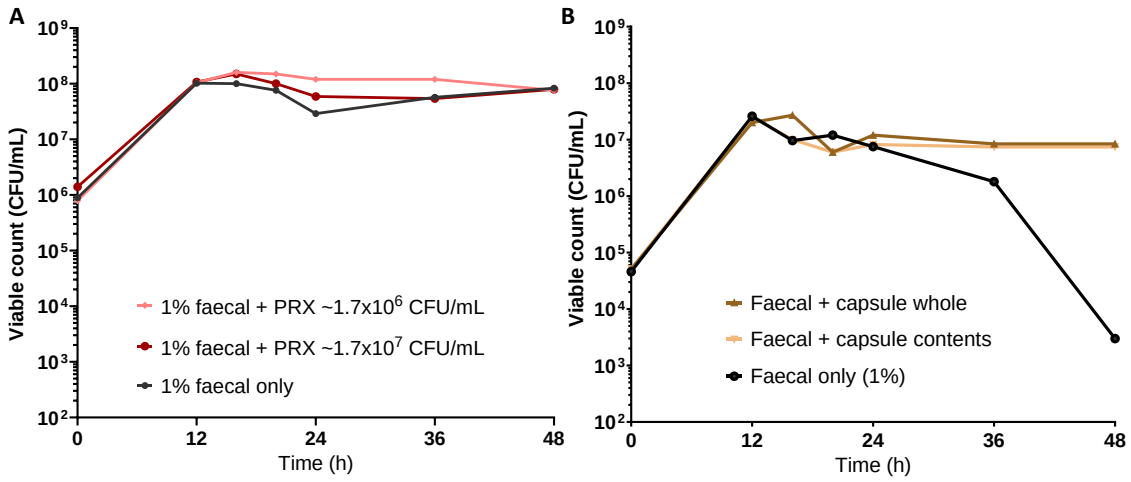


Figure 4-5 The growth of Bio-Kult probiotic supplement in different colon model environments enriched with faecal inoculum.

***A**) Total viable count (CFU/mL) of two probiotic doses (10 mg and 100 mg per 60 mL) added to 1% faecal inoculum compared to 1% faecal sample only, measured over 48 hours in a pH-controlled environment (pH 6.5-7.2) of batch colon model.*

***B**) Total viable count (CFU/mL) of a single capsule of Bio-Kult mixed strain probiotic inoculated whole or as capsule contents only into anaerobic batch media enriched with 1% faecal compared to 1% faecal only control, incubated in the anaerobic cabinet (no external pH control).*

Values are single measurements counted on Wilkins-Chalgren agar plates.

To determine the concentration of probiotic strains in both pH environments, these cultures (**Figure 4-5**) were now also enumerated on Lactobacilli selective MRS agar plates. It was assumed that all Bio-Kult (PRX) strains were able to grow on MRS. **Figure 4-6** displays the viable count on MRS agar at 0 h, 12 h, 24 h and 48 h of incubation. It was confirmed that Bio-Kult (PRX) strains grew in all tests, and that the faecal material contained bacteria able to form colonies on MRS. In the colon model, both faecal enriched conditions showed no colonies at 0 h, indicating that sample was too dilute. At 12 h, Bio-Kult (PRX) supplemented 1% faecal culture had 10^7 CFU/mL compared to 10^6 CFU/mL in Bio-Kult (PRX) only condition. Faecal only

control decreased in viable count at 48 h compared to Bio-Kult (PRX) enriched conditions, regardless of pH control.

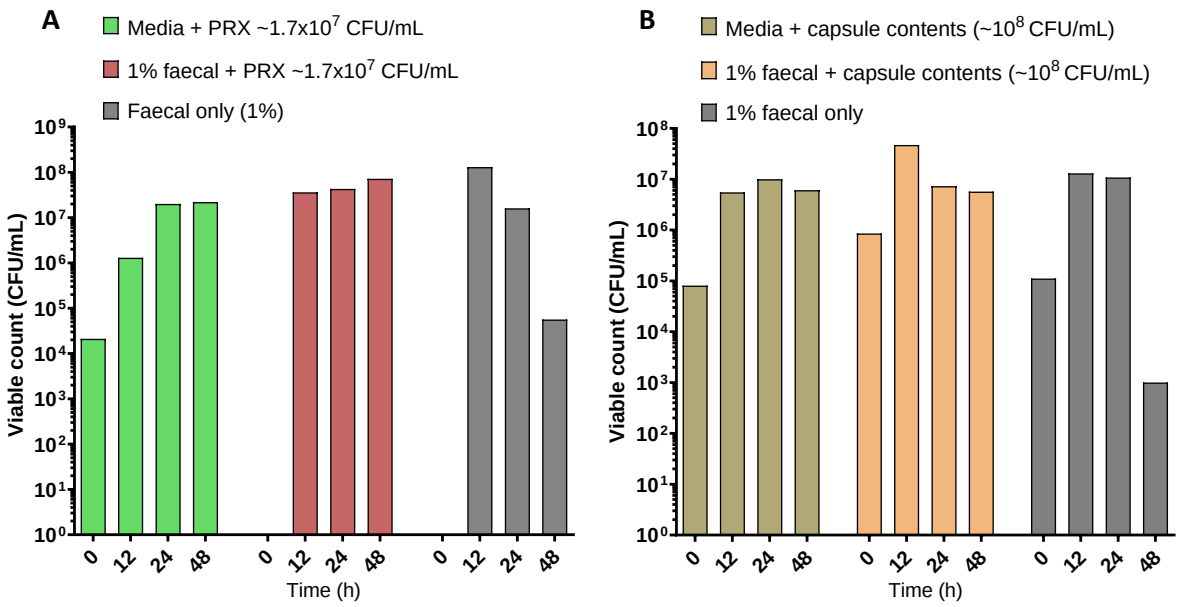


Figure 4-6 The growth of Bio-Kult probiotic supplemented cultures enumerated on MRS agar.

***A**) Viable count (CFU/mL) of probiotic enriched cultures with or without added faecal sample compared to faecal sample only control grown on MRS agar, measured at 0, 12, 24 and 48 h. Cultures were incubated in batch colon model with pH control.*

***B**) Viable count (CFU/mL) of contents from a single capsule of Bio-Kult mixed strain probiotic incubated with or without added faecal sample compared to faecal sample only control grown on MRS agar, measured at 0, 12, 24 and 48 h. Cultures were grown in the anaerobic cabinet using CMB media without pH control. Values are single measurements.*

To establish the reason for lower viability of bacteria in faecal samples without Bio-Kult (PRX) supplementation, the pH of the non-controlled cultures was measured (using litmus paper) over the period of incubation in the anaerobic cabinet, displayed in **Figure 4-7**. The pH of colon model fermentation cultures was kept between 6.5 - 7.2 and not measured in this way.

All cultures in the anaerobic cabinet started at pH 7, decreased down to pH 4 in all faecal enriched samples by 12 h. The decrease in Bio-Kult (PRX)-only inoculated vials was slower, reaching pH 4 within 20 h and further decreased to pH 3 by 48 h. The changes in pH were most likely reflective of substrate fermentation resulting from bacterial growth, indicating initial growth of fast-growing bacteria in the first 12 h of the fermentation and then further decrease in pH suggesting a slower increase in growth between 36-48 h. In faecal only vials, the pH decreased to ~4 and remained constant between 10 h and 48 h, indicating a fast initial growth and metabolism of substrate before 12 h and stationary phase onwards.

The confirmation of the viability of probiotic strains from the Bio-Kult (PRX) supplement indicated that the hypothesis that lack of probiotic effect was attributed to poor

growth/survival of the added probiotics was not supported, and that another factor was responsible for the null effect of probiotics on TMA production.

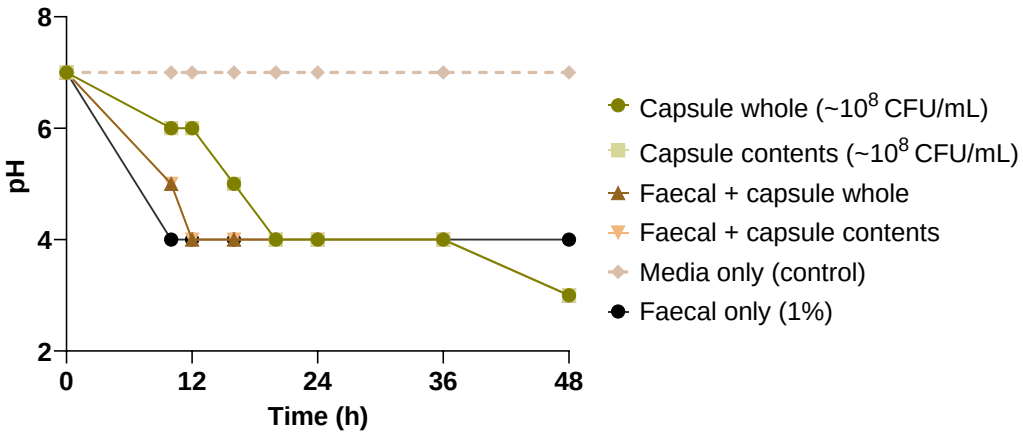


Figure 4-7 Estimated pH of anaerobic cultures grown in the anaerobic cabinet without pH control.

Probiotic-supplemented cultures (either with whole capsule or capsule contents) incubated with or without faecal sample compared to a medium only control (MFC) and a faecal only control that contained 1% faecal inoculum. All conditions contain CMB. The pH are single measurements made with litmus paper of individual vials at 0, 10, 12, 16, 20, 24, 36 and 48 h.

4.4.2 Testing the homogeneity of weighed Bio-Kult supplement

The initial low concentration of bacteria in tests inoculated with whole probiotic capsule, as shown in **Figure 4-4**, led to the decision to use the contents of the Bio-Kult supplement capsule for inoculation of colon model vessels. To ensure that the weighed amount of supplement added to each fermentation vessel was consistent, the homogeneity (total number of bacteria per inoculated culture) of the probiotic supplement after decanting and weighing of a 100 mg dose (to make a final concentration of 1.7×10^7 CFU/mL in colon model) was demonstrated by enumeration (on Wilkins-Chalgren agar) of CMB cultures grown in the anaerobic cabinet. Additionally, the survival of the strains in the CMB medium was measured after 24 h of incubation, compared to the initial bacterial concentration at 0 h.

Figure 4-8 shows that the inoculated concentration of probiotics was $4.0 \pm 0.1 \times 10^7$ CFU/mL (mean ± SEM) at 0 h. After 24 h incubation, the viable count was significantly lower with mean difference ± SEM of $1.6 \pm 0.1 \times 10^7$ ($p < 0.0001$, 95% CI = [1.2×10^7, 2.2×10^7]). Despite this decrease, the survival of Bio-Kult (PRX) in CMB was adequate as it was still in the same order of magnitude. The confirmation of the viability of probiotic strains from the Bio-Kult (PRX) supplement indicated that the hypothesis that lack of probiotic effect was attributed to low homogeneity of the added probiotic supplement was not supported, and that another factor was responsible for the null effect of probiotics on TMA production.

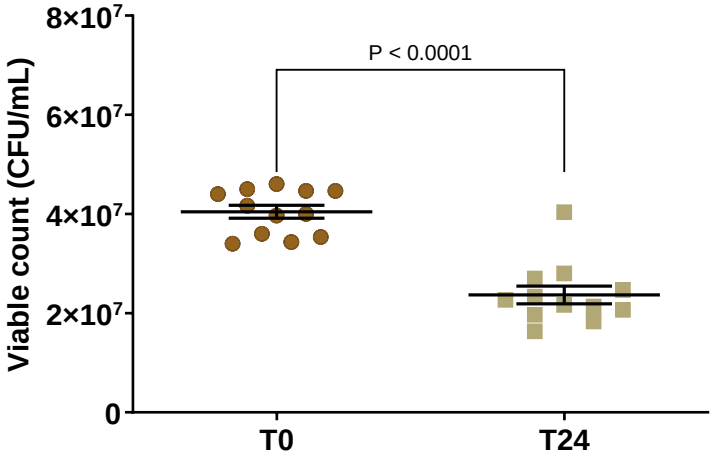


Figure 4-8 The survival of Bio-Kult probiotic supplement in CMB over 24 h without pH control.

Viable count (CFU/mL) of 10 mg/mL dose of Bio-Kult in non-pH-controlled environment of the anaerobic cabinet, incubated in CMB over 24 h (n=12), and counted on Wilkins-Chalgren agar. Values are mean ± SEM of 3 replicates of each vial at 0 h and at 24 h with individual means shown in scatter dot plot. Paired t-test indicated p < 0.0001.

4.4.3 Testing the effect of microcrystalline cellulose used as a filler in Bio-Kult supplement

A test was carried out to determine whether microcrystalline cellulose (MCC) restricted the ability of Bio-Kult probiotics to inhibit choline metabolism and TMA production. The original intention was to compare MCC component of the Bio-Kult supplement with or without the probiotic strains, however, the probiotic mixture without the MCC filler was not available. Therefore, an equivalent dose of MCC that was present in 100 mg of probiotic supplement was tested. This dose of the probiotic supplement was previously used to investigate the effectiveness of Bio-Kult (PRX) in multiple faecal donors (Error! Reference source not found.**)**. The actual MCC used in the manufacturing of the Bio-Kult capsule was not available, therefore, an autoclaved MCC from a different manufacturer was used in individual and pooled faecal samples, using CMB and CMBB models, respectively.

There was little effect of MCC on TMA production, as reported in **Figure 4-9**, therefore, any effect of the Bio-Kult probiotic supplement was attributable to the probiotic strains. In CMB, the final TMA produced from choline (mol%) was lower in MCC than in control (no MCC), but this effect was not observed in CMBB, likely being faecal donor specific. In CMBB, choline abundance was 21.9 ± 0.5 mol% lower (mean ± SD) in control (no MCC) compared to MCC at 12 h, resulting in 19.3 ± 3.0 mol% difference in TMA produced from choline at 12 h. However, this difference diminished in the later timepoints of the experiment. No effect of MCC on choline metabolism to TMA confirmed that the null effect of the Bio-Kult (PRX) supplement was not due to interference by the supplement filler.

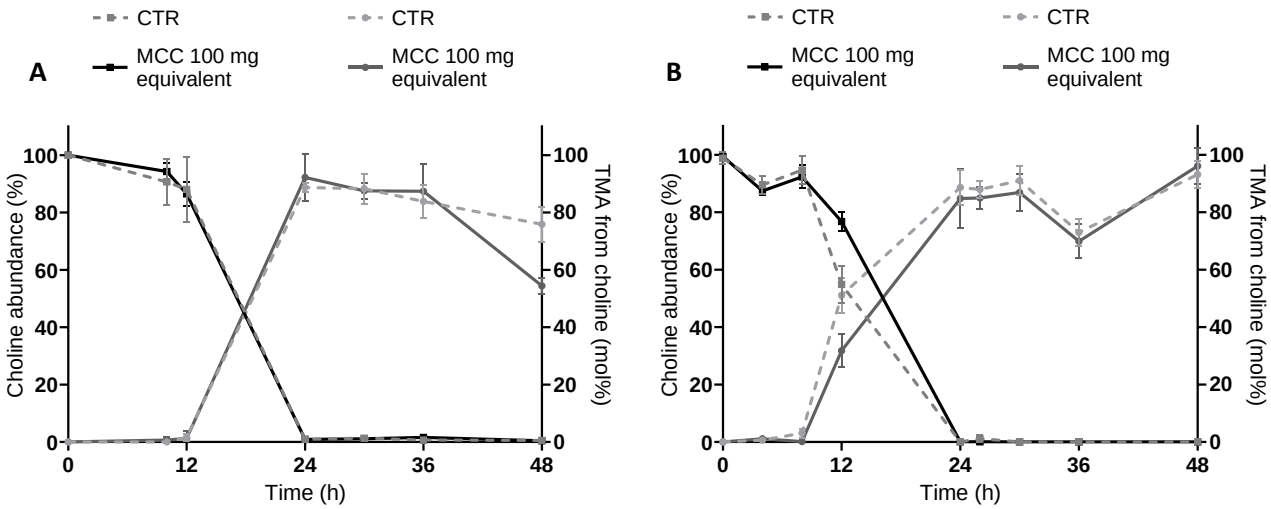


Figure 4-9 The effect of microcrystalline cellulose on choline metabolism and TMA production in two different colon models.

***A**) Choline abundance (mol%) and TMA from choline (mol%) in 100 mg equivalent dose of microcrystalline cellulose (MCC) that is used as a filler in probiotic supplement compared to untreated control in the batch model (CMB). Values are mean + SD (n=3 for CTR and n=4 for MCC of replicates per condition) using a single donor inoculum.*

***B**) Choline abundance (mol%) and TMA from choline (mol%) in 100 mg equivalent dose of microcrystalline cellulose (MCC) that is used as a filler in probiotic supplement compared to untreated control in the batch-batch model (CMBB). Values are mean ± SD (n=3 replicates per condition) using a pooled donor inoculum.*

4.4.4 The effect of prolonging incubation time of probiotics and faecal samples prior to inoculation of choline

It was previously shown (Section 4.4.1) that probiotic strains in the Bio-Kult (PRX) supplement grew within 12 h, and during their growth possibly produced antimicrobial compounds that could inhibit proliferation of TMA producing species in faecal samples. In the previous work in Section 4.3.2, the Bio-Kult supplement was added directly to the faecal samples enriched with choline, and it was now tested whether pre-incubation of the probiotic mixture with faecal samples prior to choline addition impacted subsequent TMA production. Faecal samples and Bio-Kult (PRX) probiotic supplement ($1.7x10^7$ CFU/mL) were pre-incubated for 12 h after which a choline dose of 2 mM was delivered in fresh medium that formed 1/5 of the 0 h culture (for more details, see methods). Pre-incubation of probiotic strains and faecal sample in media prior to choline supplementation had no effect on TMA production compared to control with no probiotics (n=3). The effect of Bio-Kult (PRX) probiotics was investigated in three individual faecal donors with different choline metabolism kinetics, resulting in high variation in participant responses, reported in **Figure 4-10**. The initial decrease in choline abundance between inoculation and 4 h was 40.8 ± 28.0 mol% (Mean ± SEM) in control (no Bio-Kult

added) with 46.4 ± 27.5 mol% (Mean ± SEM) decrease in Bio-Kult (PRX). In both the control and Bio-Kult (PRX), choline was not fully depleted at 24 h, with 11.4 ± 8.6 mol% and 10.6 ± 9.8 mol% choline abundance, respectively. TMA production slowly increased after the initial rise between inoculation and 4 h, reaching 46.6 ± 12.5 mol% in the control and 42.8 ± 2.4 mol% in Bio-Kult (PRX). However, the AUC of both choline and TMA concentration displayed in **Figure 4-10** panel **C,** showed no difference between the control and pre-incubated samples, and between pre-incubated and previous non-pre-incubated tests (section 4.3.2). Therefore, increasing the time of incubation to allow growth of probiotics within Bio-Kult (PRX) had no effect on TMA production.

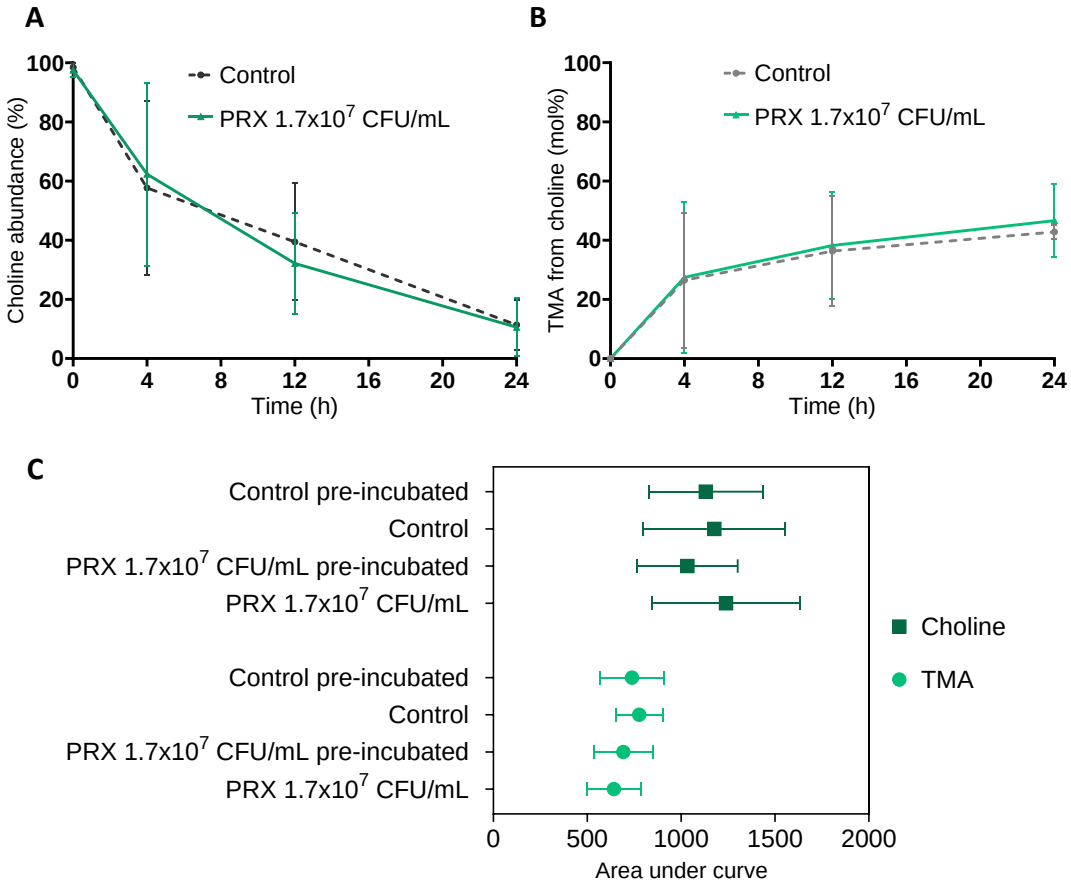


Figure 4-10 The effect of pre-incubation of probiotic (Bio-Kult) supplement with faecal samples prior to choline addition.

***A**) Choline abundance (mol%) and **B**) TMA produced from choline (mol%) in 100 mg dose of Bio-Kult mixed strain probiotic (PRX 100 mg) equivalent to 1.7x10^7 CFU/mL compared to untreated control. Samples were collected at 0, 4, 12 and 24 h after inoculation of choline into faecal cultures. Probiotics (Bio-Kult) were pre-incubated with faecal samples for 12 hours prior to addition of choline when sample collection then started. The experiment was repeated with 3 different donors where each donor had 3-4 technical replicates of each treatment, depending on availability of vessels. There was no significant difference between the control (no Bio-Kult added) and Bio-Kult (PRX) at any of the measured timepoints using 2way ANOVA. Values are Mean ± SEM (n=3).*

***C)** Area under curve for choline abundance (mol%) and TMA produced from choline (mol%) in pre-incubated condition (n=3) and regular fermentation condition (section 4.3.2, n=7), only timepoints 0, 12 and 24 h were used for AUC comparison as these were the only timepoints in common. Values are Mean + SEM of AUC per condition.*

4.4.5 The effect of different doses of Bio-Kult supplement in a pooled faecal inoculum

To determine whether probiotic concentration impacted on the effectiveness of Bio-Kult supplement, five doses of Bio-Kult (PRX, $1.7x10^6$, $8.4x10^6$, $1.7x10^7$, $3.3x10^7$ and $8.4x10^7$ CFU/mL) were tested ($1.7x10^7$ CFU/mL tested previously in CMB) in a complex microbial community of pooled faecal sample from two donors. Bio-Kult (PRX) probiotics were pre-incubated for 12 hours in CMBB medium without faecal microbiota to allow the rehydration of the supplement and growth of strains at pH 6.6-7.2 and 37 ºC. Sampling commenced after faecal inoculation (0 h). Choline metabolism and TMA production were measured in Bio-Kult (PRX) supplemented bottles and compared to control (no Bio-Kult (PRX)), sterile microbiota-free control (MFC), and microbiota-free control with Bio-Kult (MFC+PRX) that had $1.7x10^7$ CFU/mL dose of probiotic supplement without addition of faecal sample.

There were significant differences between Bio-Kult (PRX) addition and controls without Bio-Kult (PRX) addition, although Bio-Kult (PRX) concentration had little consistent effect on choline concentration. Bio-Kult (PRX) $8.4x10^6$ showed 24.8 mol% higher choline concentration compared to the control at 12 h ($p = 0.003$, 95% CI = [6.6, 43.0]), noted in **Figure 4-11** panel **B** with hash (###). More substantial changes were observed in TMA produced from choline at 12 h, 24 h, 30 h and 48 h across the different doses, with the mean differences, 95% confidence intervals and p values reported in

Table 4-3 and marked with asterisks (*) in **Figure 4-11**. In the control condition (dashed line), choline abundance remained relatively unchanged in the first 8 h, rapidly decreasing by 12 h to 54.9 ± 6.5 mol% (mean ± SD) and was fully depleted at 24 h. This was reflected in TMA produced from choline that increased to 51.1 ± 8.7 mol% in 12 h. The rate of TMA production decreased between 12 h and the end of incubation, with TMA produced from choline decreasing briefly to 73.0 ± 8.2 mol% at 36 h, after which it increased to 93.2 ± 8.5 mol% by the end of the experiment. This dip in TMA produced from choline was observed across many of the Bio-Kult (PRX) supplemented conditions, notably in the $8.4x10^6$ and $1.7x10^7$ CFU/mL doses where the TMA decrease occurred earlier compared to control, at 30 h. This phenomenon was not observed in the lowest probiotic dose, where TMA concentration at 48 h was significantly lower than control, at 74.2 ± 6.6 mol%. While significant differences between addition and absence of Bio-Kult (PRX) were identified at 4 timepoints (12, 24, 30, 48 h) (

Table 4-3), at 4 other timepoints (4, 8, 26 and 36h) the differences were not significant ($p > 0.05$).

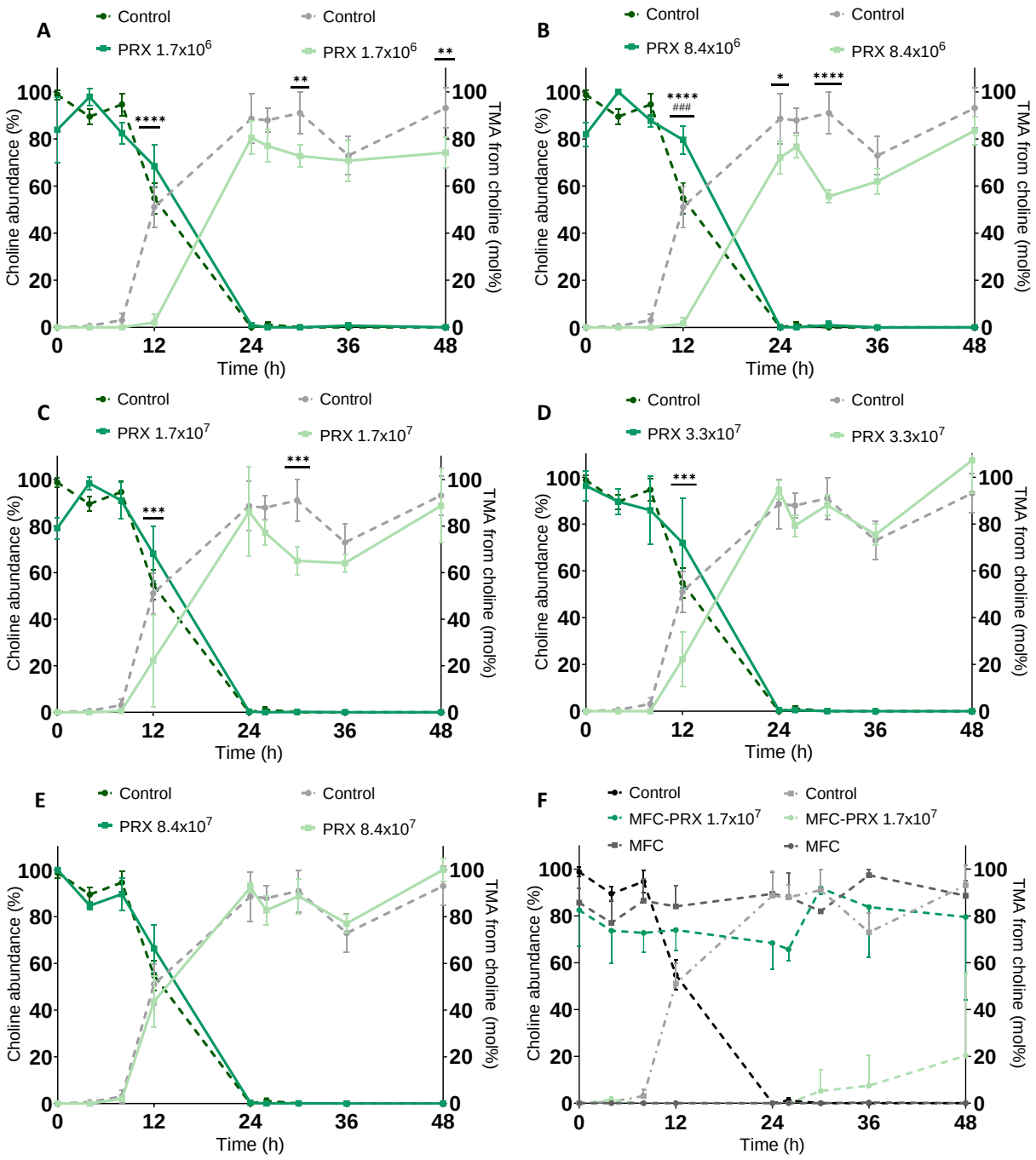


Figure 4-11 Choline abundance (mol%) and TMA produced from choline (mol%) with different doses of Bio-Kult (PRX) supplement in a pooled faecal sample from two donors.

***A**) 10 mg dose equivalent to 1.7x10^6, **B**) 50 mg dose equivalent to 8.4x10^6, **C**)100 mg dose equivalent to 1.7x10^7, **D**)200 mg dose equivalent to 3.3x10^7 and **E**) 500 mg equivalent to 8.4x10^7 CFU/mL of Bio-Kult (PRX) mixed strain probiotic compared to Bio-Kult-free control. **F**) Microbiota free controls without (MFC) or wit 1.7x10^7 CFU/mL of Bio-Kult supplement (MFC-PRX) compared to faecal enriched control.*

Samples were collected at 0, 4, 8, 12, 24, 26, 30, 36 and 48 h after inoculation of choline into faecal cultures. Serum bottles were sparged with CO_2 prior to inoculation and were incubated static at 37ºC at pH 6.6-7.5. Each condition had three technical replicates. Values are expressed as percentages where choline abundance (mol%) indicates the percentage of highest molar concentration of choline measured in each technical replicate. TMA produced from choline (mol%) is a molar equivalent of highest measurement of supplemented choline that was converted into TMA. Significant differences are indicated by asterisks calculated using 2way ANOVA with Dunnett's multiple comparisons test. Values are Mean ± SD (n=3). Left Y-axis legend of each graph corresponds to choline abundance and the right Y-axis legend to TMA concentration. For convenience of comparison, control (no added Bio-Kult (PRX)) is included in each graph.

Table 4-3 Mean differences in TMA produced from choline (mol%) at 12, 24, 30 and 48 h after inoculation with different concentrations of Bio-Kult (PRX).

The individual doses are shown in each row and their mean difference with 95% confidence intervals and Dunnett's multiple comparison test adjusted P values are shown in different columns of each time-point. Differences are calculated as control – PRX. Differences in other timepoints (0, 4, 8, 26 and 36 h) were not significant (p > 0.05) and are not reported.

Dose	12 h			24 h		
	Mean	**95% CI**	**p value**	**Mean**	**95% CI**	**p value**
PRX 1.7x10^6	49.0	[31.3, 66.7]	<0.0001	8.1	[-7.9, 24.2]	ns
PRX 8.4x10^6	49.6	[31.9, 67.2]	<0.0001	16.5	[0.4, 32.5]	0.0412
PRX 1.7x10^7	29.0	[11.3, 46.6]	0.0002	2.5	[-13.6, 18.6]	ns
PRX 3.3x10^7	28.9	[11.2, 46.5]	0.0002	-6.0	[-22.1, 10.0]	ns
PRX 8.4x10^7	7.3	[-10.3, 25.0]	ns	-3.9	[-19.9, 12.2]	ns
Dose	**30 h**			**48 h**		
	Mean	**95% CI**	**p value**	**Mean**	**95% CI**	**p value**
PRX 1.7x10^6	18.2	[2.2, 34.3]	0.018	18.9	[2.9, 35.0]	0.0128
PRX 8.4x10^6	35.3	[19.3, 51.4]	<0.0001	9.6	[-6.4, 25.7]	ns
PRX 1.7x10^7	25.9	[9.9, 42.0]	0.0002	4.4	[-11.6, 20.5]	ns
PRX 3.3x10^7	2.8	[-13.2, 18.9]	ns	-14.1	[-30.2, 1.9]	ns
PRX 8.4x10^7	2.2	[-13.8, 18.3]	ns	-6.9	[-22.3, 9.1]	ns

The AUC of the mean response from each dose is displayed in **Figure 4-12** with the choline abundance (mol%) not significantly different in the absence or presence of Bio-Kult (PRX). However, for the 3 lowest concentrations of Bio-Kult (PRX), the AUC of TMA produced from choline (mol%) was significantly lower than the control (**Figure 4-12**), while for the 2 highest Bio-Kult (PRX) doses, the AUC of TMA produced from choline (mol%) was not significantly different. Dose 1.7x10^6 CFU/mL of Bio-Kult resulted in 721 lower AUC (95% CI = [-944, -498]) of TMA produced from choline (mol%) than in no Bio-Kult control. Similar difference in AUC of TMA produced from choline (mol%) was observed in 8.4x10^6 CFU/mL Bio-Kult dose with mean decrease of 894 AUC (95% CI = [-1117, -671]) compared to Bio-Kult free-control. Slightly smaller decrease but still significant was shown in 1.7x10^7 CFU/mL dose (mean = -530, 95% CI= [-753.3, -306.7]) with all significant differences having adjusted $p < 0.0001$. Other doses and AUC of choline abundance (%) were not significantly different to Bio-Kult-free control. Therefore, although some differences were noted, there was no consistent dose-dependent effect of Bio-Kult probiotic concentration (PRX, 1.7x10^6, 8.4x10^6, 1.7x10^7, 3.3x10^7 and 8.4x10^7 CFU/mL) on the conversion of choline to TMA. It may be, however, that with other faecal samples and/or with optimisation, the Bio-Kult supplement can be used to inhibit choline metabolism to TMA.

Panel **B** in **Figure 4-12** displays three variables introduced in Chapter 3 (choline metabolism rate, TMA production rate, and maximum TMA produced from choline) characterising the microbial capacity to metabolise choline to TMA. Interestingly, none of the variables were

significantly different between the control and the 5 different Bio-Kult (PRX) doses, possibly due to the variation in replicates. However, this suggested that TMA AUC can be significantly different in the presence/absence of Bio-Kult (PRX) even if the rates of metabolism and maximum percentage of TMA produced from choline do not differ, possibly pointing to probiotic involvement in gut modulation towards a lower TMA concentration phenotype via either decreased TMA formation or increased TMA utilisation. However more robust evidence is needed to test this theory. Overall, while some differences were noted with lower doses, there was no consistent effect of Bio-Kult probiotic concentration on the metabolism of choline to TMA. The testing of other doses of Bio-Kult should be considered in further work.

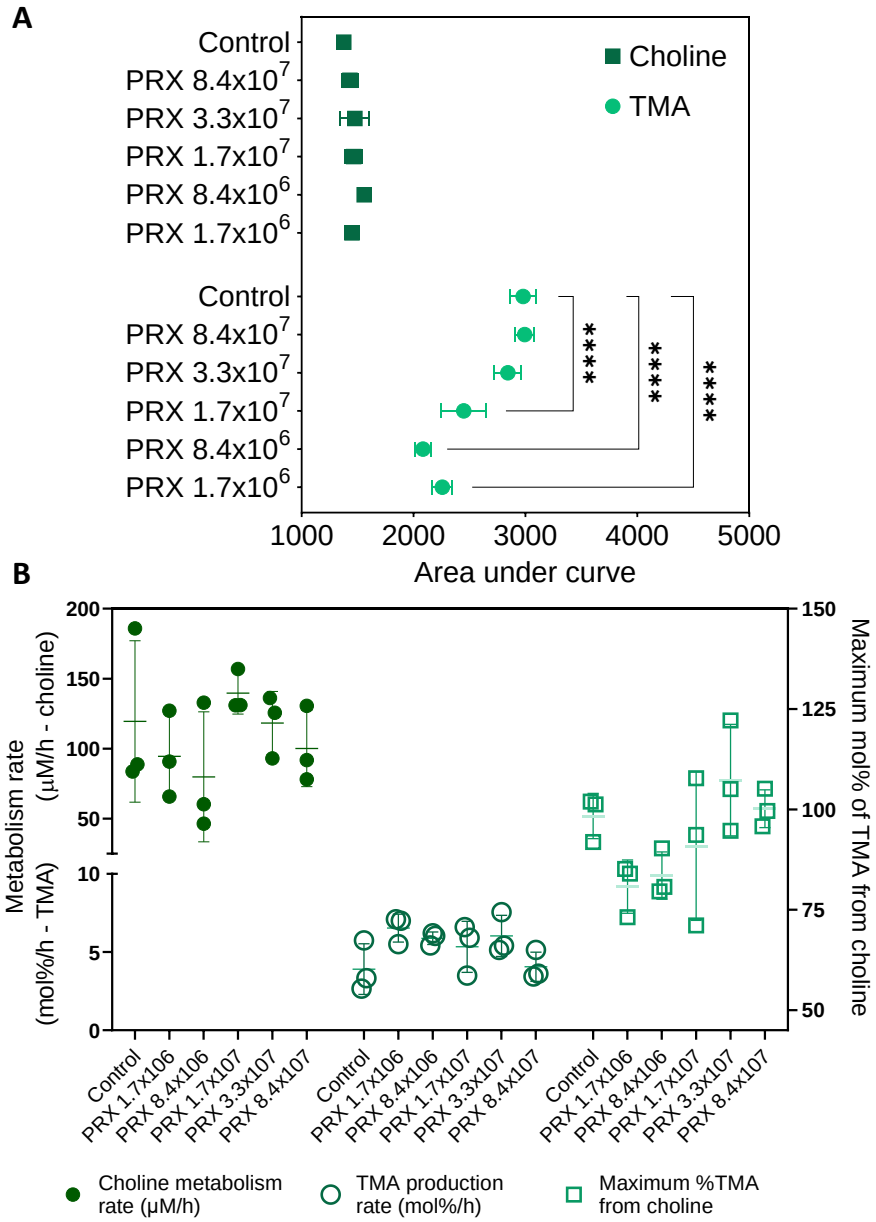


Figure 4-12 The dose response of PRX supplement on choline metabolism and TMA production.

***A**) Area under curve for choline abundance (mol%) and TMA produced from choline (mol%) in each probiotic dose compared to non-supplemented control. Values are Mean + SD of AUC per condition. ****Two-way ANOVA indicated p < 0.0001.*

***B**) Choline metabolism rate (left), TMA production rate (middle) and Maximum %TMA from choline (right) in the five different doses of PRX supplement compared to control. Mean + SD calculated from rates and percentages of each replicate per condition are displayed.*

4.5 The effect of individual Bio-Kult strains on TMA production from choline in faecal microbiota

The previous section of this chapter demonstrated that there was no consistent effect of the 14-strain Bio-Kult supplement on TMA production in the context of multiple faecal fermentations of choline. However, evidence from other studies hinted that the effects of probiotics on TMA production are most likely strain specific, with multiple strains influencing TMAO formation in animal and human studies. Therefore, individual strains from the Bio-Kult supplement were investigated for their effect on choline metabolism and TMA production in a complex matrix of human faecal microbiota with pooled samples from two donors. The proportions of the individual strains in the Bio-Kult supplement were not known, therefore, a final concentration of 10^6 cells/mL of each individual Bio-Kult strain was tested.

After establishing the methodology for inoculating a known number of probiotic cells into faecal cultures detailed in Appendix I, a trial was conducted to establish the effect of a probiotic reported to show an effect on TMA production - *L. rhamnosus* GG (LRGG). This was previously attempted in CMB but high acid production by *L. rhamnosus* GG during fermentation and the need for extensive pH buffering, resulted in high dilution of the probiotic dose. A different fermentation model, CMBB, was chosen to explore the role of single probiotic strains in modulating TMA production in faecal samples supplemented with choline. This model was optimised for inoculation with fresh faecal samples with a final concentration of 0.35% from 10% faecal slurry made with PBS (w/v). Where fresh samples were not available, snap frozen faecal samples slowly defrosted at 4°C in anaerobic atmosphere were used.

Figure 4-13 shows that there was no effect of the single probiotic strain *L. rhamnosus* GG on choline abundance or TMA produced from choline (mol%). The curve of TMA production from choline was slightly lower from 16 h onwards, however, these differences were not significant. Alongside this single strain, the 8 individual Bio-Kult (PRX) strains were tested for their response compared to the control (no probiotic). The kinetics of choline utilisation and TMA production are displayed in **Figure 4-14**.

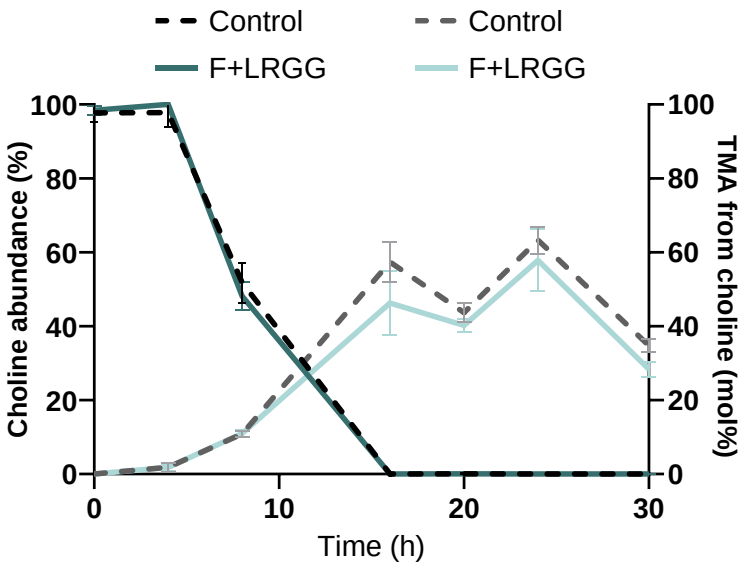


Figure 4-13 The effect of L. rhamnosus GG on TMA production from choline.

*Choline abundance (mol%) and TMA from choline (mol%) in a pooled faecal sample supplemented with 10^6 CFU/mL final concentration of L. rhamnosus GG (**F+LRGG**) compared to untreated **Control** (no LRGG). Values are Mean ± SD (n=3 replicates per condition). Samples were collected at 0, 4, 8, 16, 20, 24 and 30 h of incubation in CMBB medium with 0.35% faecal inoculum from two donors, with or without addition of L. rhamnosus GG. Cultures were incubated in an anaerobic cabinet at 37ºC, static in a deep 96-well plate covered with adhesive plate seal between sampling to prevent evaporation and contamination.*

Figure 4-14 displays the individual responses of 8 individual strains from Bio-Kult supplement, (details previously reported in **Table**). In the control condition, 2 mM choline was inoculated in a batch vial supplemented with faecal sample, thoroughly mixed by inverting inside an anaerobic cabinet and the homogenised culture was aliquoted into wells of a 96-well plate in triplicate. The control (no strain added) is plotted as a dashed line in black for choline abundance (mol%) and TMA produced from choline (mol%) in grey. Choline abundance decreased between 4 and 8 h and was fully depleted by 20 h. Despite the rapid decrease in choline abundance by 8 h, only 10.8 ± 0.7 mol% of TMA from choline was produced at that timepoint. By 20 h, metabolised choline was converted to 57.4 ± 5.4 mol% of TMA and remained relatively stable with slight dips at 24 h and 30 h, where final TMA from choline dropped to 43.7 ± 1.9 mol%.

All cultures supplemented with individual probiotic strains showed a higher decrease in choline abundance 4 h after inoculation compared to the control, but this effect only persisted until 8 h in strain 1 (*L. acidophilus*) and strain 8 (*S. thermophilus*), with strain 4 (*B. longum*) showing a significantly higher choline abundance than the control. The ANOVA of TMA produced from choline (mol%) revealed significant differences at 16 h for strains 1, 6, 7 and 8, showing lower values compared to control. The opposite was the case for strains 6, 7 and 8 at 20 h, where TMA produced from choline (mol%) was significantly higher than the control. There was a

rapid decrease in abundance of TMA produced from choline between 20 h and 24 h in strains 5, 6, 7 and 8, but other strains did not show this pattern. By 30 h, most of the strains reached similar final TMA produced from choline (mol%) apart from strain 3 that showed significantly higher TMA levels compared to control. The differences in choline abundance between individual strains and the control are reported in **Table 4-4**. Differences in TMA from choline between individual strains and the control are reported in **Table 4-5**.

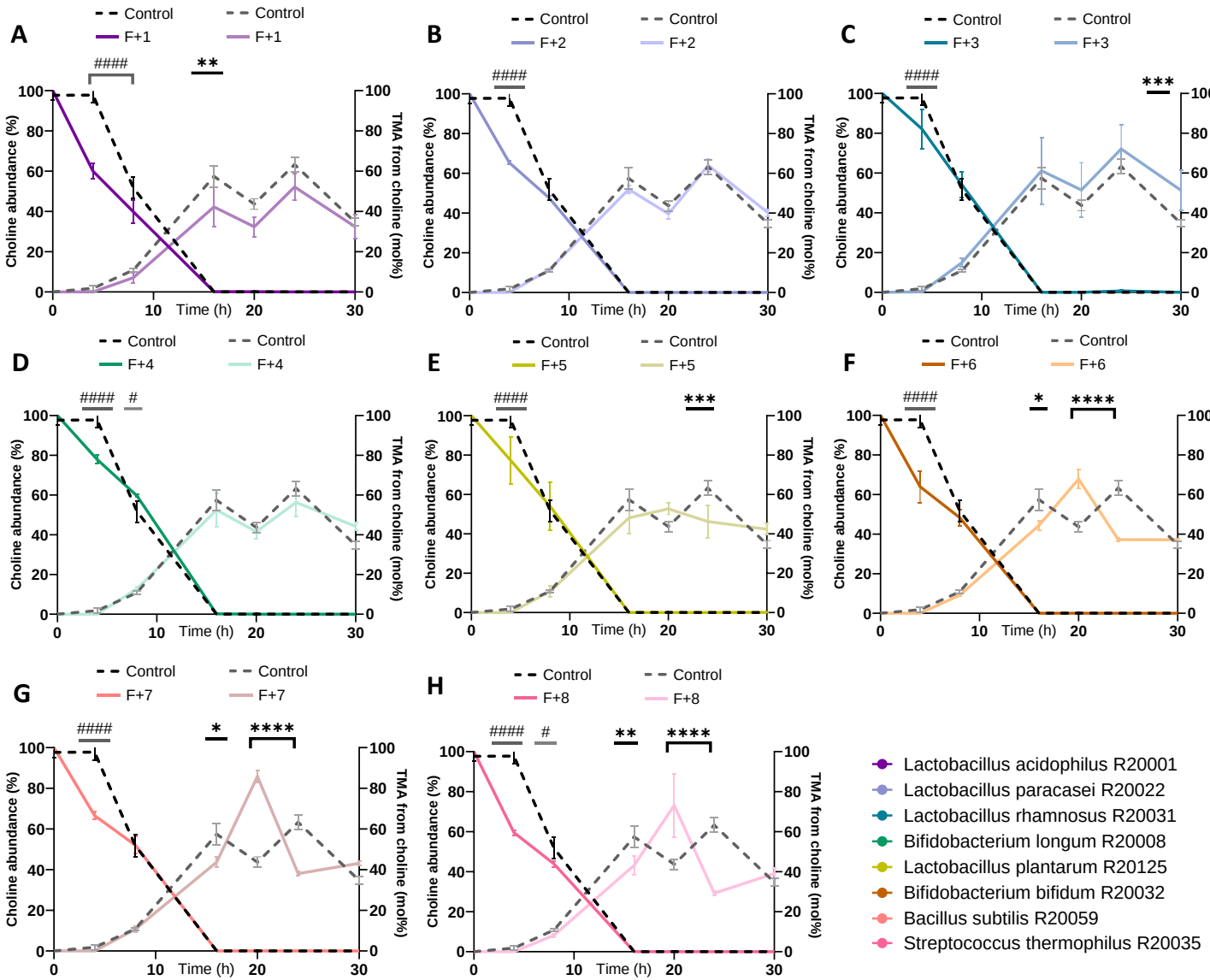


Figure 4-14 The effect of 8 individual probiotic strains from Bio-Kult supplement enriched with faecal samples on choline metabolism and TMA production.

A – H**) Choline abundance (mol%) and TMA from choline (mol%) in pooled faecal sample supplemented with 10^6 CFU/mL final concentrations of 8 individual probiotic strains (**F+strain #**) compared to Bio-Kult strain-free **Control**. Values are mean ± SD (n=3). Legend for choline abundance is on left of each graph and TMA from choline is on the right of each graph. Colour codes for each strain are displayed at bottom right of the plot. For ease of comparison, the control response is plotted in each graph. Significant differences in choline abundance (mol%) between control and individual Bio-Kult (PRX) strains are annotated with # ($p < 0.05$) and #### ($p < 0.0001$); significant differences in TMA from choline (mol%) are marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and * ($p < 0.0001$). Significance was calculated by Two-way ANOVA with Dunnett's multiple comparison test. Full details of p values and confidence intervals are reported in Table 4-4 and Table 4-5.*

Table 4-4 ANOVA of choline abundance (mol%) in individual Bio-Kult (PRX) strains enriched with faecal samples.

ANOVA results	4 h			8 h		
	Mean	**95% confidence interval**	**Adjusted p value**	**Mean**	**95% confidence interval**	**Adjusted p value**
Control vs. F+1	37.8	[31.0, 44.6]	<0.0001	12.0	[5.1, 18.8]	<0.0001
Control vs. F+2	32.5	[25.7, 39.3]	<0.0001	4.2	[-2.6, 11.0]	ns
Control vs. F+3	15.7	[8.9, 22.5]	<0.0001	-2.4	[-9.2, 4.5]	ns
Control vs. F+4	19.9	[13.1, 26.7]	<0.0001	-7.8	[-14.7, -1.0]	0.0165
Control vs. F+5	20.5	[13.7, 27.3]	<0.0001	-2.2	[-9.1, 4.6]	ns
Control vs. F+6	33.8	[27.0, 40.6]	<0.0001	3.5	[-3.4, 10.3]	ns
Control vs. F+7	31.2	[24.4, 38.0]	<0.0001	-0.3	[-7.1, 6.6]	ns
Control vs. F+8	38.2	[31.4, 45.0]	<0.0001	7.9	[1.1, 14.7]	0.0152

Table 4-5 ANOVA of TMA produced from choline (mol%) in individual PRX strains enriched with faecal samples.

	16 h			20 h		
	Mean	**95% confidence interval**	**Adjusted p value**	**Mean**	**95% confidence interval**	**Adjusted p value**
Control vs. F+1	15.1	[3.8, 26.5]	0.0032	11.3	[-0.1, 22.7]	ns
Control vs. F+6	13.0	[1.6, 24.3]	0.0170	-24.2	[-35.5, -12.8]	<0.0001
Control vs. F+7	13.7	[2.3, 25.0]	0.0103	-42.2	[-53.5, -30.8]	<0.0001
Control vs. F+8	14.2	[2.9, 25.6]	0.0068	-29.5	[-40.9, -18.1]	<0.0001
	24 h			**30 h**		
	Mean	**95% confidence interval**	**Adjusted p value**	**Mean**	**95% confidence interval**	**Adjusted p value**
Control vs. F+3	-8.8	[-20.2, 2.6]	ns	18.9	[2.9, 35.0]	0.0128
Control vs. F+5	17.1	[5.7, 28.4]	0.0006	-7.6	[-19.0, 3.7]	ns
Control vs. F+6	26.1	[14.7, 37.5]	<0.0001	-2.4	[-13.8, 9.0]	ns
Control vs. F+7	25.2	[13.9, 36.6]	<0.0001	-8.4	[-19.8, 4.0]	ns
Control vs. F+8	34.0	[21.3, 46.7]	<0.0001	-4.4	[-17.1, 8.4]	ns

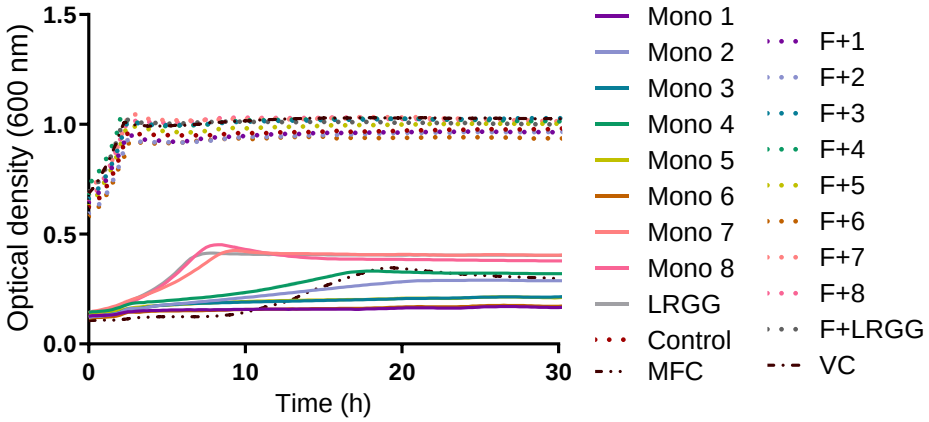


Figure 4-15 Optical density of Bio-Kult (PRX) monocultures and faecal enriched cultures in CMBB.

Optical density was measured over 30 h during in-vitro fermentation of choline in anaerobic environment, using a plate-reader programmed to shake a sealed plate for 5 second before measurements taken every 15 minutes. The plate was inoculated using a multichannel pipette from the experimental 96-well plate used for metabolomics analysis, therefore 3 replicates were measured per condition (error bars not displayed).

Throughout this work, growth of faecal enriched cultures and also Bio-Kult (PRX) monocultures was measured as optical density by spectrophotometry at 600 nm in an anaerobic environment, shown in **Figure 4-15.** There were some differences in the lag phase of monoculture growth with strains 7, 8 and *L. rhamnosus* GG reaching stationary phase within 10 h of incubation whereas strains 2 and 4 showed slower growth pattern. Strains 1, 3 and 5 showed a very small increase in optical density throughout the experiment, yet some of them affected the kinetics of choline metabolism and TMA production (**Figure 4-14**). The differences between the growth of strains could be due to lower inoculation load reported in Error! Reference source not found., where strains 1, 2 and 3 showed lower mean concentration of stock.

The mean responses of all individual Bio-Kult (PRX) strains in pooled faecal sample and Bio-Kult (PRX) monocultures (no faecal microbiota) compared to the control (no Bio-Kult (PRX) strains, same control curves as in **Figure 4-14**) are shown in **Figure 4-16**, panel **A**. The mean response for individual monocultures (no faecal microbiota) showed a 20.5 mol% decrease (79.7 ± 5.6 mol% vs 59.2 ± 2.6 mol%, mean ± SEM) in choline abundance between the initial measurement and the abundance at 8 h. The choline concentration in the Bio-Kult (PRX) monocultures (no faecal microbiota) then remained relatively unchanged over the period of incubation but was not associated with TMA production as the average maximum TMA produced from choline (mol%) only reached 4.3 ± 1.0 mol% (mean ± SEM) at 24 h after inoculation. The effect of individual strains on choline abundance in samples enriched with faecal microbiota remained significantly different in the mean response from all strains, showing a lower choline abundance at 4 h (mean = 28.7 mol%, 95% CI =[15.7, 41.8], $p < 0.0001$) as indicated with the hashes in **Figure 4-16**, panel **A**. Some differences persisted in the mean response of individual strains on TMA produced from choline (mol%), namely at 20 h and 24 h (mean = -11.9, 95% CI = [-21.5, -2.3], $p = 0.0128$ and mean = 13.9, 95% CI = [4.3, 23.4], $p = 0.0034$, respectively). The AUC was now calculated (**Figure 4-16**) for individual probiotic strains enriched with faecal samples, and compared to the control (see **Figure 4-14** for original data).

When considering the individual strain responses in terms of AUC of choline metabolism and TMA production (mol%), the only significantly lower AUC for choline abundance was in strain 1 ($p = 0.0049$) and strain 8 ($p = 0.0128$) compared to control, other strains had no significant effect. The AUC of TMA produced from choline was significantly lower in strain 1 ($p = 0.0028$) and significantly higher in strain 3 ($p = 0.0455$) based on Two-way ANOVA with Dunnett's multiple comparisons test. In summary, strains specific effects were identified, with only one of the Bio-Kult strains, strain 1, showing a significant decrease in TMA production.

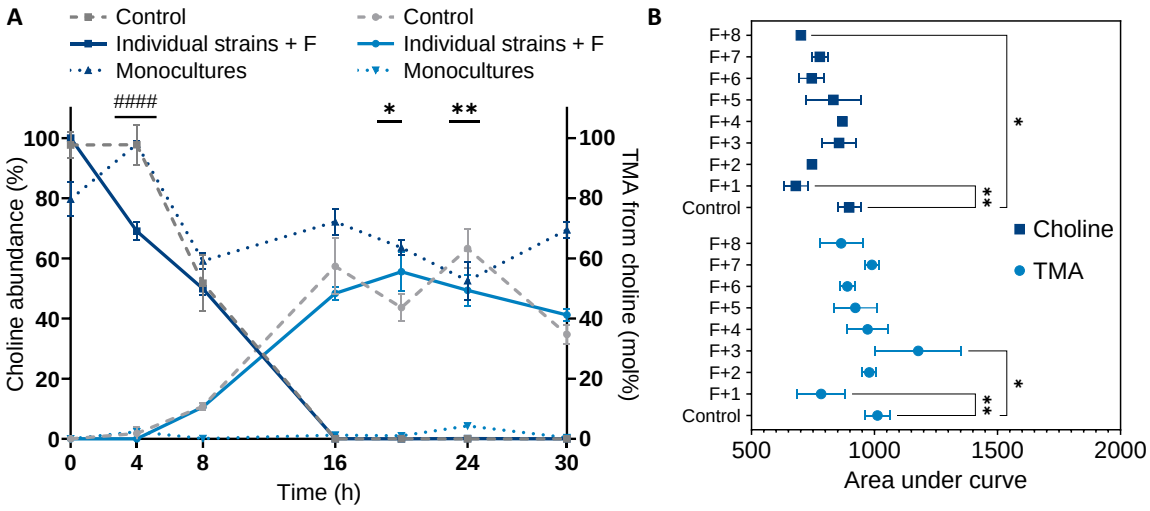


Figure 4-16 The mean response of all individual probiotic strains in choline metabolism and TMA production, and the individual strain differences in their AUC when enriched with faecal samples.

***A**) Choline abundance (mol%) and TMA from choline (mol%) in pooled faecal sample supplemented with 10^6 CFU/mL final concentrations of 8 individual probiotic strains (**Individual strains + F**) compared to no probiotic **Control** and **Monocultures** that have no faecal microbiota added. Values are mean ± SEM (n=8) for Individual strains + F and Monocultures; mean ± SD for control (n=3). Significant differences in choline abundance are marked with # and TMA produced from choline by *, calculated by Two-way ANOVA with Dunnett's multiple comparisons test.*

***B**) Area under curve of individual probiotic strains enriched with faecal samples compared to control – top part of figure shows AUC ± SD of choline abundance (mol%) and bottom part shows TMA from choline (mol%). Significant values are marked with an asterisk calculated with Two-way ANOVA with Dunnett's multiple comparisons test.*

To explore the effects of the probiotic strains outside of the complex ecological environment of the faecal matrix, a model of a single TMA-producing strain was developed to investigate the interactions between a known TMA producer and the Bio-Kult strains in a co-culture model of choline fermentation to TMA.

4.6 Investigation of the effect of individual Bio-Kult probiotic strains on choline metabolism and TMA production by a known *cutC*-containing bacterium

A more simplified approach was now taken to test the ability of single strain Bio-Kult cultures to act directly on a TMA-producing species that contained *cutC/D* genes encoding choline-TMA lyase. The aims were to test the hypothesis that Bio-Kult strains inhibit growth or modify the function of a TMA producing strain with *cutC/D* genes in a co-culture model, and then to investigate the mechanism of action. For this purpose, the development of new model system was undertaken, and this method with the results is described in Appendix I.

4.6.1 The effect of individual Bio-Kult strains in co-culture with *Proteus mirabilis* DSM 4479 on choline metabolism and TMA production

The aim of the co-culture experiments with *P. mirabilis* and Bio-Kult strains was to investigate the direct effect of probiotics on TMA production by a single *cutC*-containing strain in a simplified model. Prior investigation of the characteristics of both *P. mirabilis* and the Bio-Kult strains revealed that all grew in CMBB, and this was selected for *in-vitro* fermentations of supplemented 2 mM choline and enriched with 0.1% glucose to boost bacterial growth. An estimated inoculation load of 10^6 cells/mL was added to monocultures and co-cultures, calculated using equations in previous sections of this chapter. The number of viable bacteria was confirmed by plating of 10^8 CFU/mL diluted stock on appropriate agar (MRS, BHI or Wilkins-Chalgren agar for probiotics and BHI, Nutrient agar or Wilkins-Chalgren agar for *P. mirabilis*).

Cultures were prepared by inoculating stock into pre-reduced choline enriched CMBB dispensed into plastic Falcon tubes and then transferring 1 mL of culture in triplicate to a sterile 96-deepwell plate. A portion of the cultures in deep well plate was used for inoculation of the 96-well plate, for optical density readings and for sampling of cultures for metabolomics analysis. Original batch cultures in Falcon tubes were then used for enumeration of viable bacteria (CFU/mL). Over the incubation period of 24 h, samples were taken at 0 h, 2 h, 4 h, 6 h, 8 h, 10 h and 24 h after inoculation of strains into CMBB.

Multiple measures were investigated:

- choline and TMA concentration in probiotic monocultures, co-cultures and *P. mirabilis* supplemented with choline (control) at 0 h, 2 h, 4 h and 10 h (**Figure 4-17**)
- bacterial growth in monocultures (**Figure 4-18 A**) and co-cultures (**Figure 4-18 B**) using optical density at 600 nm measured over 24 h

- confirmation of inoculation load by counting colonies (CFU/mL) of diluted stocks of probiotic strains and *P. mirabilis* (**Figure 4-18 C**)
- viable count (CFU/mL) of Bio-Kult strains in monocultures on MRS and BHI at 8 h (**Figure 4-18 D**)
- viable count (CFU/mL) of probiotic strains in co-culture with *P. mirabilis* plated on BHI agar at 8h (**Figure 4-18 E** – left)
- viable count (CFU/mL) of *P. mirabilis* in co-culture with probiotic strains plated on BHI agar at 8 h (**Figure 4-18 E** – middle)
- viable count (CFU/mL) of all bacteria in co-culture plated on WCH agar at 8h (**Figure 4-18 E** – right)

4.6.2 Choline metabolism and TMA production

Figure 4-17 shows choline abundance (mol%) in panel **A**, where choline was rapidly depleted between 0 – 2 h in control condition (*P. mirabilis* + choline, black circle) and when *P. mirabilis* was in co-culture with probiotic strains (the mean response for all 8 probiotic strains tested individually with *P. mirabilis* is shown, green triangle). There was a slight decrease in choline detected in probiotic monocultures (grey square) that do not contain *cutC* genes, suggesting another mechanism of choline degradation or potential contamination of some monocultures. TMA production from choline (mol%) displayed in panel **B** reflects apparent low choline degradation in probiotic monocultures with 9.4 ± 7.2 mol% TMA (mean ± SEM, n=8 strains) at 2 h with a lower TMA concentration at later time points. There was no difference between the co-cultures and control (*P. mirabilis* only) in the quantity of TMA produced at 2 h, however, TMA concentration significantly decreased in co-cultures at 4 h to 17.2 ± 8.0 mol% ($p = 0.0007$) whilst the control (*P. mirabilis* only) remained unchanged at this timepoint. This difference was not maintained at 10 h with all conditions showing a very low TMA concentration, indicating that *P. mirabilis* itself had degraded TMA obscuring the effect of the probiotic strains. It is unclear what caused the decrease of TMA in both co-culture and control conditions, as there are presently no reports of *P. mirabilis* converting TMA into dimethylamine or monomethylamine or via another mechanism. The more rapid fall in TMA concentration with the probiotic strains at 4 h is notable. Further investigations should be undertaken with additional time points to determine the reproducibility and full significance of the observations.

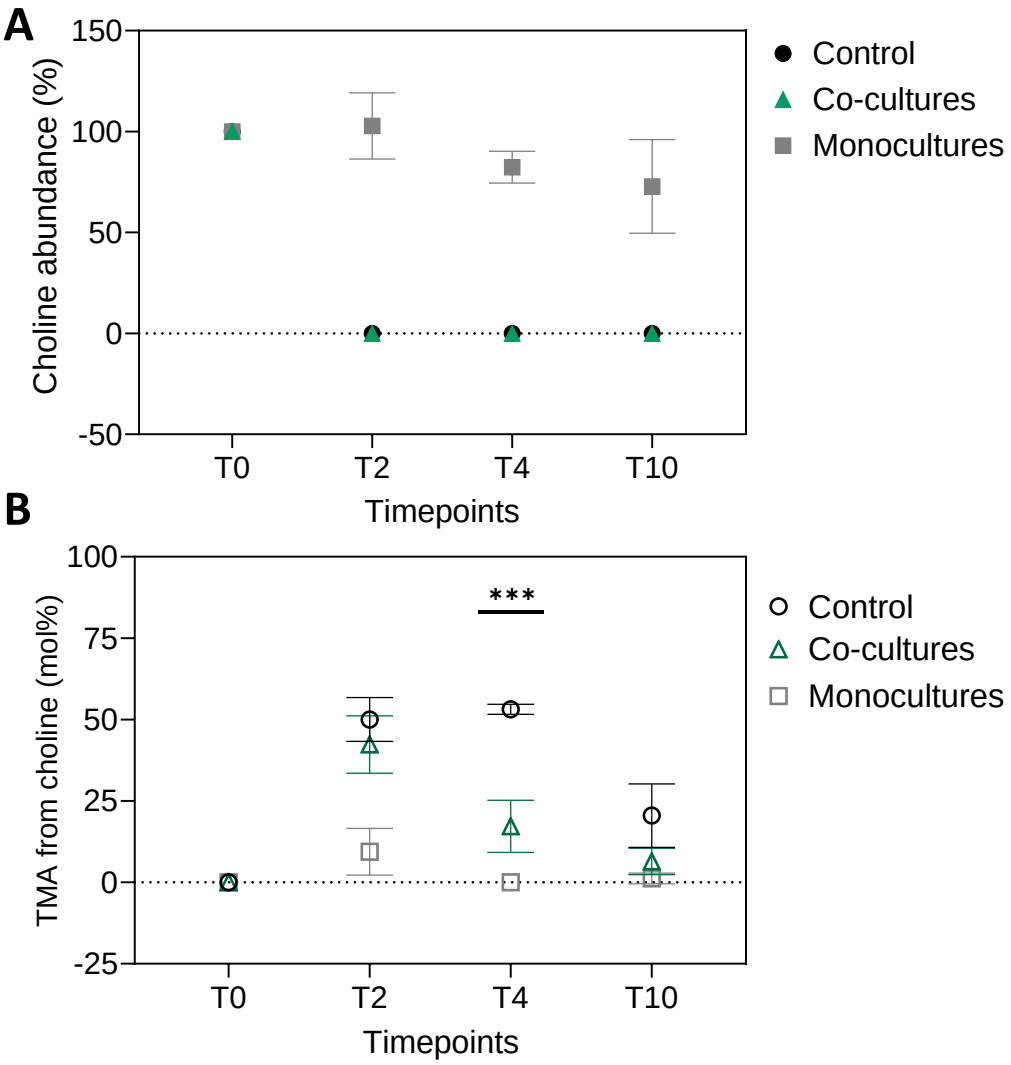


Figure 4-17 The mean effect of individual probiotic strains on choline metabolism to TMA by P. mirabilis in anaerobic co-culture using CMBB

***A**) Choline abundance (mol%) and **B**) TMA from choline (mol%) in co-culture of P. mirabilis and individual Bio-Kult strains (triangles) compared to P. mirabilis as a positive control (circles) and individual Bio-Kult monocultures (not containing cutC gene) supplemented with choline as a negative control (squares). Metabolite concentration was measured at 0, 2, 4 and 10 h of incubation. Values are mean ± SEM of 8 probiotic co-cultures and monocultures (n=3 replicates each) and mean ± SD for control (n=-3). Asterisks denote significant difference between co-cultures and control (p = 0.0007).*

4.6.3 Growth of monocultures and co-cultures measured using optical density

To investigate the interactions of individual Bio-Kult strains with *P. mirabilis* further, growth of monocultures and co-cultures was measured using a 96-well plate reader in anaerobic conditions over 24 h. **Figure 4-18** panel **A** illustrates the growth of single strains, probiotic monocultures, *P. mirabilis* and microbiota-free controls (optical density measured at 600 nm). *P. mirabilis* control and *P. mirabilis* with no choline showed a rapid increase in OD from 2 h to 6 h, with Bio-Kult monocultures showing a more gradual increase from around 8 h for strain 6, 12 h for strain 5, and after 16 h for strains 4 and 7. Strains 1, 2, 3 and 8 showed limited increase in optical density over the duration of the experiment.

Panel **B** reflects the changes in optical density of *P. mirabilis* cultures enriched with individual Bio-Kult strains. Growth of co-cultures with strains 7 and 8 showed more rapid growth than *P. mirabilis* or the Bio-Kult strain alone. This could be due to the additive effect of both bacteria multiplying, and/or stimulation. Growth of the other co-cultures (panel **B**) was either slightly more rapid than or similar to that of *P. mirabilis* alone (panel **A**). However, these small differences did not influence choline utilisation as both control and co-cultures showed full depletion of choline by 2 h. Optical density measurement alone cannot distinguish the response of Bio-Kult strains and *P. mirabilis* in co-cultures, therefore, enumeration of viable count of individual strains was investigated using different types of agar.

4.6.4 Viable bacterial counts of Bio-Kult strains and *P. mirabilis* in co-cultures and monocultures

Firstly, to confirm that enough bacteria were inoculated into the cultures, **Figure 4-18** panel **C** displays the diluted bacterial stock concentration that was plated on BHI agar in triplicates. This stock was diluted to an intended 10^8 CFU/mL (based on optical density measurement and OD vs viable count (CFU/mL) equations for Bio-Kult strains and *P. mirabilis*). Strains 1, 2 and 3 failed to reach the desired inoculation concentration, which could explain the low optical density increase in these monocultures in panel **A** of **Figure 4-18**. Strain 4 showed limited growth in 2nd passage therefore all harvested pellet was used for inoculation of the experiment and no stock remained for confirmation of the inoculated concentration. Strains 5, 6, 7, 8 and *P. mirabilis* showed good growth and exceeded the 10^8 CFU/mL requirement.

Secondly, similar viable counts (CFU/mL) were obtained for Bio-Kult monocultures plated on MRS and BHI agar after 8 h of incubation in CMBB medium (panel D). Lower viable counts were obtained for strains 1, 2, 3 and 4 than for strains 5, 6, 7 and 8. The differences reflect a combination of the initial inoculum concentration and growth in CMBB. *P. mirabilis* had a

viable count of 4.7 ± 0.6 x 10^8 CFU/mL at 8 h in choline supplemented control condition, and a similar viable count with no choline when plated on BHI (it does not grow on MRS).

When grown in co-culture and plated on BHI, it was possible to readily distinguish colonies formed by the Bio-Kult strains and those formed by *P. mirabilis* (morphology showing swarming pattern around *P. mirabilis* colonies). Bio-Kult strains grew better in the presence of *P. mirabilis* in CMBB co-cultures plated on BHI agar at 8 h, as displayed in panel **E** (left), compared to their monoculture growth shown in panel **D**. The concentration of *P. mirabilis* in monoculture was unaffected by the presence of a Bio-Kult strain in co-culture, displayed in the middle part of panel **E** with colours corresponding to the Bio-Kult strains *P. mirabilis* was co-cultured with. Similar viable counts were reached on WCH agar that was intended to represent total anaerobic bacteria in co-cultures, however, it was uncertain if all Bio-Kult strains were able to grow on this type of agar as this was not tested prior to this experiment.

Overall, in hindsight, *P. mirabilis* may not have been an ideal candidate for Bio-Kult co-culture experiments due to its fast growth in CMBB and rapid utilisation of choline. Despite the fast growth of Bio-Kult strains in their recommended medium, CMBB was less favourable, and this could have limited their effect on choline metabolism. Nevertheless, Bio-Kult strain supplementation did influence TMA concentration during some later time points, pointing towards a further investigation needed to understand the loss of TMA in Bio-Kult co-cultures with *P. mirabilis*.

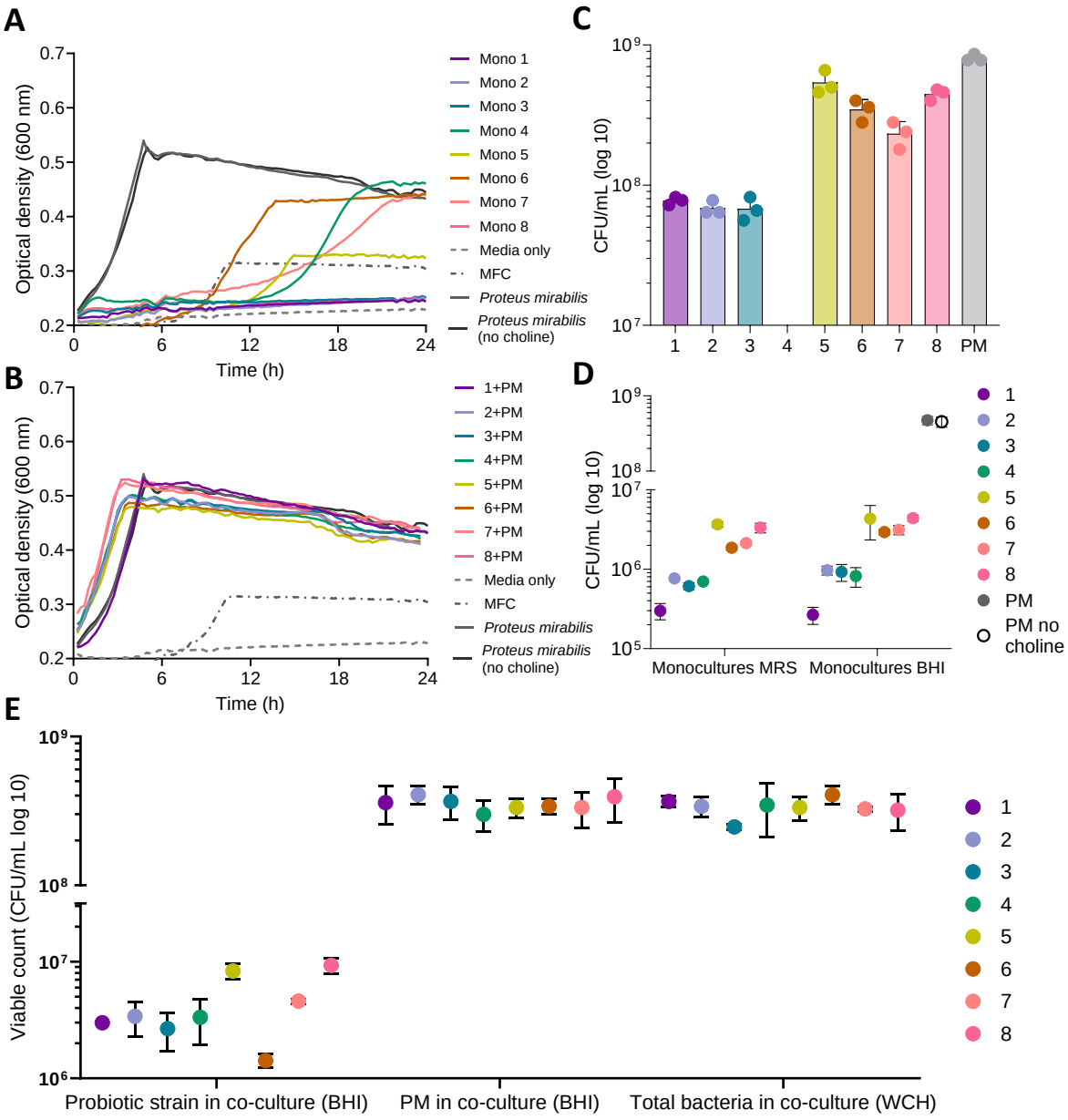


Figure 4-18 Growth of individual Bio-Kult monocultures and co-cultures with P. mirabilis during investigation of choline metabolism into TMA in CMBB

***A**) Growth curve of individual Bio-Kult monocultures and P. mirabilis controls (with or without choline) compared to media only control and choline supplemented microbiota free control (MFC). Optical density was measured at 600 nm every 15 minutes in an anaerobic plate reader sealed with breathable film over 24 h of incubation.*

***B**) Growth curve of individual Bio-Kult co-cultures with P. mirabilis compared to P. mirabilis monoculture controls (with or without choline) and microbiota free controls – same values displayed for controls as in A) for ease of comparison. Optical density was measured as in A).*

***C**) Viable count (CFU/mL) of bacterial stock diluted to 10^8 CFU/mL (estimated from OD) in PBS used for inoculation of co-cultures and monocultures to final concentration of 10^6 CFU/mL. All dilutions were inoculated in triplicate on BHI plates and incubated for 24 hours. Values are Mean ± SD (n = 3). Strain 4 had low growth prior to harvesting, therefore all stock was used for inoculation, and none remained for confirmation of inoculation load.*

***D**) Viable count (CFU/mL) of Bio-Kult monocultures and P. mirabilis controls (incubated with or without choline) at 8 h after inoculation. Bio-Kult monocultures from CMBB experiment were plated on MRS and BHI and P. mirabilis was plated on BHI only. CFUs were counted after 24 h of incubation in an anaerobic cabinet at 37 °C.*

***E**) Viable count (CFU/mL) of Bio-Kult strains and P. mirabilis co-culture after 8 h incubation in CMBB. Bio-Kult strains counted on BHI (left), P. mirabilis counted on BHI (middle), and total anaerobic bacteria count in co-culture plated on WCH (right). All cultures were sampled at 8 h after inoculation, plated in triplicate. Values are mean ± SD (n = 3).*

4.7 The effect of Bio-Kult cell-free supernatants on growth of *Proteus mirabilis* DSM 4479 and its ability to metabolise choline to TMA

The aim of this investigation was to determine if Bio-Kult strains exerted an effect on *P. mirabilis* growth and choline utilisation through the metabolites that they produced during growth in their recommended media. This could be an important mechanism by which Bio-Kult strains influence TMA production *in-vivo.* When Bio-Kult strains were inoculated in MRS or BHI, the pH of these cultures radically decreased throughout the growth as metabolites of fermentation were produced. *In vivo*, the pH buffering capacity is likely to be greater; however, it was important to determine whether lowering the pH affected growth and choline use by *P. mirabilis*.

Bio-Kult strains were grown as described previously for harvesting pelleted cells used for inoculation of co-culture experiments. The supernatant was harvested, and filter sterilised in an anaerobic environment. Two different conditions were investigated to establish the role of pH. In one condition, supernatant was filter sterilised and directly added to *P. mirabilis* specific medium (PMS) in 1:2 ratio of one part supernatant and two parts PMS supplemented with ~2 mM choline – this condition had non-adjusted pH. A final concentration of 10^6 cells/mL of *P. mirabilis* grown for 12 h in Nutrient broth, harvested by centrifugation and diluted to 10^8 CFU/mL stock concentration was inoculated into the culture. The second condition had the supernatant adjusted to a neutral pH (6.5-7.0) with NaOH (3 M) prior to filter sterilisation and addition to PMS medium – this condition had adjusted pH.

The cultures were incubated in an anaerobic environment for 24 h at 37 °C. Choline enriched PMS medium with *P. mirabilis* inoculum and anaerobic PBS added instead of Bio-Kult supernatant was used as a control (*P. mirabilis* + choline). To simulate the conditions of the supernatant enrichment, sterile MRS medium at neutral pH (*P. mirabilis* + MRS control) and MRS adjusted to the pH that matched grown Bio-Kult cultures (pH ~3.7 at 12h growth) were used as additional controls. While the use of MRS medium was an important control, it was not a perfect match to the nutritional composition of the supernatant as the metabolite profile after Bio-Kult growth would be vastly different and most carbon sources in the supernatant would have been likely depleted. A further condition with a pooled supernatant from all Bio-Kult strains added was investigated to mimic the impact of supernatant from a mixed strain Bio-Kult enrichment (*P. mirabilis* + S-mixed).

4.7.1 Differences in growth of *P. mirabilis* based on the initial pH of the Bio-Kult supernatants

Growth of *P. mirabilis* was measured in choline-enriched PMS medium over 24 h using a 96-well plate reader in anaerobic conditions, and the growth curves are displayed in **Figure 4-19** panel **A**. The graph on the left depicts growth in non-adjusted pH supernatant and the right side shows the growth response in PMS medium supplemented with pH-adjusted Bio-Kult supernatant. Adding low pH supernatant (non-adjusted) caused a delay in *P. mirabilis* growth and flattening of the exponential curve. A higher optical density was reached in pH-adjusted conditions (separate controls were used) shown on the right side of panel **A**.

4.7.2 The effect of supernatant pH on choline abundance and production of TMA after 10 h of incubation

The choline concentration was measured at 0 h and 10 h, and the abundance (mol%) at 10 h is displayed in **Figure 4-19** panel **B**. There was total choline depletion in all cultures with pH-adjusted Bio-Kult supernatant, and in 6 out of 12 treatments with non-pH-adjusted Bio-Kult supernatant. However, for 6 other treatments with non-pH-adjusted supernatant (S1, S2, S5 and S7, MRS low pH control, pooled supernatant treatment) not all choline was degraded at 10 h. Although all choline was degraded with addition of pH-adjusted supernatant, there was less TMA produced from choline (mol%) at 4 h compared to MRS low pH and MRS control (panel **C**). These effects diminished by 10 h, matching TMA produced by *P. mirabilis* + choline control and MRS controls.

Figure 4-19 The effect of probiotic supernatant on growth of P. mirabilis and its ability to metabolise choline to TMA

P. mirabilis was inoculated into P. mirabilis specialised medium (PMS) supplemented with supernatant harvested from 12 h growth of probiotic strains in MRS or BHI. Probiotic supernatant was either adjusted to neutral pH with NaOH or remained at the final pH reached after 12 h of growth (~ 3.7). The supernatant was filter-sterilised and inoculated as 1/3 of final volume of PMS culture inoculated with 10^6 CFU/mL stock of P. mirabilis.

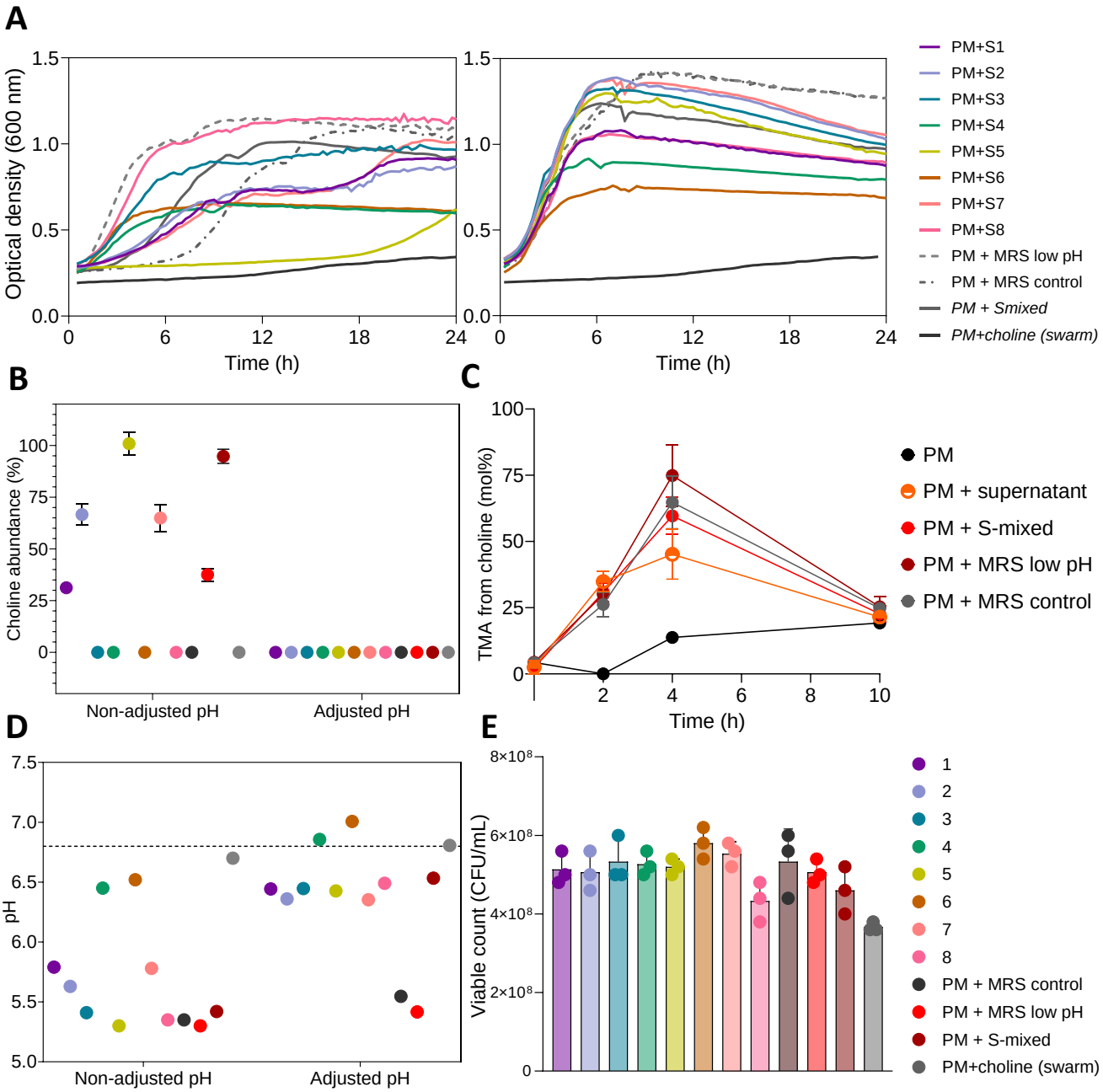
***A**) Growth of P. mirabilis in probiotic supernatant-supplemented PMS enriched with 2 mM choline. Optical density was measured at 600 nm every 15 minutes in an anaerobic plate reader sealed with breathable film over 24 h of incubation. Graph on the left represents cultures supplemented with pH-non-adjusted supernatant. Growth curve on the right represents the growth of P. mirabilis cultures with pH-adjusted supernatant.*

***B**) Choline abundance (mol%) at 10 h after addition of choline into P. mirabilis cultures supplemented with either pH-adjusted or non-adjusted probiotic supernatant. Values are mean ± SD of three replicates. Legend for this graph is available at bottom right corner of this plot next to figure E.*

***C**) TMA produced from choline (mol%) in P. mirabilis cultures supplemented with pH adjusted supernatant (values for P. mirabilis + supernatant are mean ± SEM of all supernatant enriched cultures) compared to P. mirabilis supplemented with choline as a positive control (P. mirabilis; mean ± SD, n=3) and mixed supernatant condition compared to MRS supplemented controls (either low or neutral pH). TMA concentration was measured at 0, 2, 4 and 10 h of incubation and 100% is molar equivalent of maximum choline concentration measured in each replicate vessel.*

***D**) pH measurements of 8 h P. mirabilis culture supplemented with either pH-adjusted or non-adjusted supernatant. pH was measured using a micro probe in aerobic environment at room temperature. Dashed line indicates initial pH value of PMS medium before supernatant inoculation. Values of individual vessels at inoculation were not measured. Legend for this graph is available at bottom right corner of this plot next to figure E.*

***E**) Viable count (CFU/mL) of P. mirabilis cultures enriched with pH-adjusted supernatant compared to P. mirabilis supplemented with choline as a positive control (P. mirabilis + choline (swarm)) and mixed supernatant condition compared to MRS supplemented controls (either low or neutral pH). Cultures were inoculated on WCH after 8 h incubation in PMS medium and were kept in the anaerobic cabinet for 24 h at 37 °C. All vessels were diluted, and all dilutions were plated in triplicates (mean ± SD, n=3).*


A
Optical density (600 nm)
Time (h)
PM+S1
PM+S2
PM+S3
PM+S4
PM+S5
PM+S6
PM+S7
PM+S8
PM + MRS low pH
PM + MRS control
PM + Smixed
PM+choline (swarm)
B
Choline abundance (%)
Non-adjusted pH
Adjusted pH
C
TMA from choline (mol%)
Time (h)
PM
PM + supernatant
PM + S-mixed
PM + MRS low pH
PM + MRS control
D
pH
Non-adjusted pH
Adjusted pH
E
Viable count (CFU/mL)
1
2
3
4
5
6
7
8
PM + MRS control
PM + MRS low pH
PM + S-mixed
PM+choline (swarm)

4.7.3 The effect of pH-adjustment of supernatants prior to inoculation on pH levels of *P. mirabilis* cultures at 8 h

The pH values of the *P. mirabilis* cultures were measured after 8 h (**Figure 4-19** panel **D**). When pH adjusted or non-pH-adjusted culture supernatant from S4 or S6 was added, the pH was around 6.5 and choline was fully degraded (**Figure 4-19** panel **B**). The addition of culture supernatants from S1, S2 or S7 led to a pH >6.0 and full metabolism of choline when the supernatant was pH adjusted, and a pH of 5.5-6.0 and incomplete metabolism of choline when non-pH adjusted. A similar pattern was observed for S5, except that the pH fell to <5.5 when non-pH-adjusted supernatant was added. Interestingly, the addition of culture supernatants from S3 or S8 led to a pH>6.0 and full metabolism of choline when the supernatant was pH adjusted, and a pH <5.5 and full metabolism of choline when the supernatant was non-pH adjusted. Thus, a lower pH alone was not sufficient to prevent *P. mirabilis* fully degrading the added choline. It is likely, therefore, that other factors present in the supernatants of S1, S2, S5 and S7 also have a role in preventing *P. mirabilis* fully degrading the added choline at a lower pH. Further work should be carried out to investigate this further, including identifying the factors, establishing their mode of action, and whether they could be used to prevent choline metabolism to TMA *in-vivo*.

4.7.4 Viability of *P. mirabilis* in pH adjusted supernatant-enriched cultures

To ensure that the differences in TMA production shown in panel **C** were not caused by different concentrations of *P. mirabilis*, viable counts (CFU/mL) at 8 h were measured by plating cultures on WCH agar and incubating for 24 h at 37 °C, with results displayed in panel **E**. Supernatant treatments showed a similar effect on growth of *P. mirabilis* in PMS medium to MRS controls and mixed supernatant, whereas *P. mirabilis* + choline control showed a significantly lower viable count to the average supernatant response ($p = 0.0002$; comparing *P. mirabilis* + choline (mean, SD, n=3) and Supernatant (mean, SEM, n=8) using One-way ANOVA with Dunnett's test of multiple comparisons). This could explain the lower TMA produced at 4 h compared to other conditions and the overall low conversion of choline to TMA despite complete choline depletion by 10 h (panel **C**).

4.7.5 Choline metabolism was not inhibited by a low *P. mirabilis cutC* gene copy number

Figure 4-19 panel **D** indicated the pH of the *P. mirabilis* culture at 8 h was strongly affected by the initial pH of supplemented supernatant. To investigate if the pH at 8 h correlated with the concentration of *P. mirabilis* bacteria capable of converting choline into TMA, the number of

copies of the *P. mirabilis cutC* gene was quantified in supernatant-enriched treatments compared to MRS controls. **Figure 4-20** panel **A** displays the *cutC* gene copy number measured at 8 h of incubation. The *cutC* gene copy number showed no difference for most pH-adjusted and non-adjusted supernatant treatments, apart from S5 that showed a significantly lower gene copy number in non-pH-adjusted compared to adjusted pH. The low degradation of choline in most tests where non pH-adjusted supernatant was added (**Figure 4-19** panel **B**) is therefore not due to a low concentration of *P. mirabilis*, an exception to this may be when non pH-adjusted supernatant from S5 was added. Interestingly, despite the low pH reached in other supernatant enriched conditions, such as in S3 and S8, there was no significant inhibition of *P. mirabilis* growth based on *cutC* gene copy number. A low concentration of *P. mirabilis* (as determined by *cutC* gene copy number) also does not appear to be an explanation for the observations on the formation of TMA from choline (**Figure 4-20** panel **B**).

Overall, pH influenced choline metabolism and TMA production by *P. mirabilis* treated with Bio-Kult supernatants, with a role of other unknown factors in the supernatants of S1, S2, S5 and S7 also identified. After adjusting the pH of supernatants to a neutral pH prior to supplementation into PMS medium enriched with *P. mirabilis*, there was no inhibition of choline metabolism. However, there were some effects of Bio-Kult pH-adjusted supernatant on TMA production compared to PMS medium supplemented with MRS controls. The responses of MRS-based controls were not consistent over the two separate tests; therefore, this experiment should be further replicated to make appropriate conclusions about the effects of Bio-Kult supernatants on TMA production by *P. mirabilis*. Further work is also needed to establish the mechanism of action of pH and the other factors, and their potential use to decrease choline metabolism to TMA *in-vivo*.

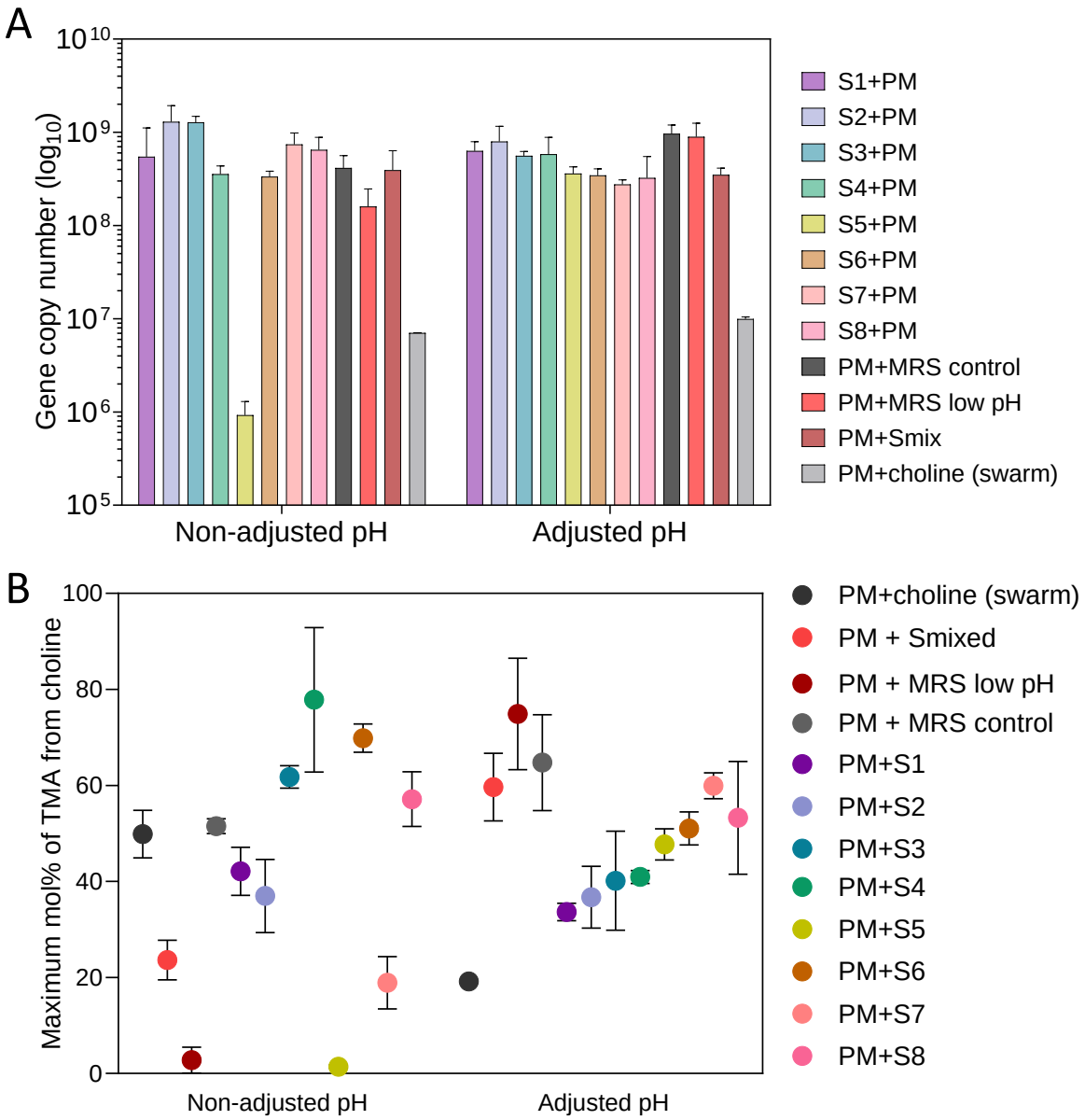


Figure 4-20 The effect of probiotic supernatant on the number of copies of P. mirabiliscutC gene in supernatant enriched cultures and the maximum concentration (mol%) of TMA produced from choline

***A**) Number of cutC gene copies per culture of P. mirabilis in cultures enriched with either pH adjusted or non-adjusted supernatant after 8 h of incubation. DNA was extracted from snap frozen cultures at 8 h (pooled culture from 3 technical replicates) as described in Methods. qPCR assay of cutC genes was performed using P. mirabilis -specific cutC primers binding to a serially diluted product of PCR assay used for amplification of gene fragment (495 bp) containing the fraction amplified by qPCR primers used in this assay to form a standard curve for absolute quantification of cutC-P. mirabilis gene copy number in each culture. Values are mean ± SD of triplicates of each condition.*

***B**) Maximum concentration (mol%) of TMA from choline in non-pH-adjusted supernatant-enriched cultures compared to cultures with pH adjusted supernatant. The same control conditions serve as a comparison (PM + choline(swarm), PM + Smixed, PM + MRS low pH and PM + control MRS). Maximum concentration (mol%) of TMA is a measure of the highest value of TMA measured at 0, 2, 4 and 10 h of incubation. Values are mean ± SD (n=3).*

DISCUSSION

The aim of this chapter was to test the impact of supplementing human faecal fermentations of choline with a 14-strain mixed probiotic product (Bio-Kult) and its individual strains on the conversion of choline to TMA by the gut microbiota. There was no effect of the mixed probiotic supplement when a dose of $1.7x10^7$ CFU/mL was inoculated into faecal samples from 7 individual donors. Currently, there are no published studies that investigated the impact of Bio-Kult strains on TMA production from choline using a complex faecal microbiota as inoculum in an *in-vitro* colon model. The application of probiotic strains was previously investigated in various disease conditions using different *in-vitro* models or to investigate the survival, engraftment, adhesion, and interactions of probiotics with pathogenic bacteria, other microorganisms, or mammalian cells (213, 214, 216, 217, 277, 288). These previous studies reported various degrees of success of probiotics impacting the investigated conditions, however, they rarely explored the possible reasons contributing to no effect of some probiotic strains. To understand the reasons behind the null effect of Bio-Kult mixed supplement demonstrated in this study, multiple factors were investigated, including the growth of probiotic bacteria in faecal samples, their survival in different batch fermentation media, the interference of other components of the supplement and the fermentation duration. None of these factors explained the null effect of that dose of the mixed Bio-Kult supplement, as good viability was established and prolonged fermentation of probiotics with faecal samples prior to testing of choline metabolism yielded similar results to overall TMA production in the 7 investigated faecal samples.

There was a significant impact of $1.7x10^7$ CFU/mL and two other lower doses of mixed Bio-Kult supplement on AUC of TMA produced from choline (mol%) when tested in a pooled faecal sample from two donors (but no effect at three higher doses). This raises the possibility that after some optimisation, the Bio-Kult supplement may be used to reduce choline metabolism to TMA both *in-vitro* and *in-vivo*. Exploring this in further work should be considered. In the published literature, there is currently no consensus on the appropriate dose of mixed probiotic supplement administered in human studies, animal models or *in-vitro* assays. Several recent reviews pointed out the challenges of unifying evidence of the beneficial effects of probiotics due to the variable doses and other factors such as duration of supplementation, delivery method and the nature of strain specificity of probiotic properties (196, 197, 234). In fact, when considering probiotic use for reduction of TMA and TMAO levels, reports from animal studies with mice fed choline and individual probiotic strains such as *Enterobacter aerogenes* ZDY01 (202), *Lactiplantibacillus plantarum subsp. plantarum* ZDY04 (201), *Lactobacillus amylovorus LAM1345 and Lactiplantibacillus plantarum LP1145* (289) contribute

to the evidence that probiotics can reduce TMAO and TMA levels through modification of the gut microbiota (at least in animal models). This was also reported when *Bifidobacterium animalis subsp. lactis* F1-3-2*, Bifidobacterium animalis subsp. lactis* F1-7 and *Lactiplantibacillus plantarum* F3-2 reduced the content of TMA in the intestine and downregulated the levels of TMA and TMAO in the serum of mice (290). The mechanism behind the decrease in TMA and TMAO levels reported by Liang and colleagues was the TMA-degrading capacity of supplemented strains that were specifically selected after *in-vitro* investigation of their utilisation of supplemented TMA. *Bifidobacterium breve* Bb4 and *Bifidobacterium longum* BL1 and BL7 also reduced plasma TMAO and plasma and caecal TMA concentrations (291) and many other strains have been associated with improved atherosclerosis outcomes, recently summarised in a review by El Hage and colleagues (109) and mentioned as one of the strategies to modulate the crosstalk between gut microbiota and the immune system response in atherosclerosis by Jing *et al.* (292).

The effect of individual strains from the Bio-Kult supplement was previously investigated for their ability to inhibit growth of pathogens, produce anti-microbial peptides and short-chain fatty acids. This chapter describes their capacity to alter TMA production by the faecal microbiota and by the TMA-producing strain *Proteus mirabilis* DSM 4479. Individual Bio-Kult strains performed better compared to the mixed Bio-Kult supplement in a complex faecal matrix, but only two out of the eight tested strains showed a significant decrease in choline abundance (measured as AUC) - *Lactobacillus acidophilus* PXN 35 and *Streptococcus thermophilus* PXN 66. Different strains of these species were previously investigated in a 12-week supplementation study with haemodialysis patients conducted by Borges and colleagues (203) who reported no effect of their probiotic strains on TMAO levels. Bio-Kult strain *L. acidophilus* PXN 35 also significantly decreased TMA produced from choline (measured as AUC), indicating either a lower production of TMA by the gut microbiota in the presence of this strain, or an increased proliferation or activity of TMA-degrading species (with Bio-Kult strains acting directly or indirectly). An opposite effect was observed in faecal cultures enriched with *Lactobacillus rhamnosus* PXN 54 where TMA produced from choline (measured as AUC) was significantly higher than in control. Similar findings were reported by Chen and colleagues who reported an increased abundance of *Faecalibacterium* at genus level that was associated with increased TMAO levels during a phosphatidylcholine challenge after supplementation of a mixed probiotic consisting of *L. rhamnosus* GG, *L. acidophilus*, *B. animalis* and *B. longum*. Their two-week probiotic supplementation did not have any effect on fasting serum TMAO levels or postprandial TMAO AUC measured in 40 healthy males (293).

In general, most previous studies report no effect of mixed or single strain probiotic supplementation on plasma or serum TMAO levels in both diseased and healthy human volunteers (204, 208-210, 294). However, the complex mechanisms contributing to the multi-pathway interactions of probiotics and TMAO production by the host are yet to be understood. To investigate the direct impact of probiotic strains from the Bio-Kult supplement on TMA production, a single strain model of TMA-producer *P. mirabilis* was developed in this study and used for investigation of this relationship in co-culture experiments. The mean response of single Bio-Kult strains was to bring about a more rapid and initially significant decrease in the concentration of TMA previously produced from choline by *P. mirabilis*. A greater impact of individual Bio-Kult strains may have been observed if tested with a lower concentration of *P. mirabilis*. It would be worthwhile to test whether a higher concentration of these single Bio-Kult strains has a greater impact on the metabolism of choline to TMA by *P. mirabilis*. Interestingly, Bio-Kult strains showed improved growth in presence of *P. mirabilis* compared to their growth in monocultures, despite good growth of individual strain established during previous investigation of growth of Bio-Kult strains in CMBB medium.

There are several published reports of co-culture experiments with probiotic strains, most often investigating the interactions of probiotics with pathogenic strains, their production of anti-microbial compounds and inhibition assays on solid media (213, 217, 233, 276, 277). The impact of probiotic strains on *cutC*-containing strains was explored in detail by Ramireddy and colleagues who investigated the effect of *Lactobacillus amylovorus* LAM1345 and *Lactiplantibacillus plantarum* LP1145 on their ability to inhibit growth of some known TMA-producing bacteria, such as *Providencia alcalifaciens, Escherichia fergusonii, Proteus mirabilis, Klebsiella pneumoniae, and Providencia rustigianii*. They used an agar diffusion method with spent supernatant supplemented into a well in solid media inoculated with TMA-producing strains; most probiotic strains that they tested created inhibition zones greater than or equal to 15 mm. There are currently no other reports investigating the effects of probiotic strains on TMA production by single *cutC*-containing strains using a co-culture method. The development of a single-strain model of *P. mirabilis* was established in this study, following methodology used in previous reports for investigating the production of TMA and gene expression of *cutC* and *cutD* in single strains and complex faecal matrix (232, 235, 295, 296). This model was used to investigate the effect of Bio-Kult strain supernatant on the growth of *P. mirabilis* which was inspired by the inhibition properties of probiotic supernatant on solid media by Ramireddy *et al.*(289), as discussed above.

In this chapter, the impact of Bio-Kult supernatant was investigated in the context of the pH lowering properties of Bio-Kult strains grown in nutrient rich media MRS and BHI. There were

notable differences in the pH change of *P. mirabilis* cultures based on initial pH of supplemented supernatant. When the supernatant pH was adjusted prior to supplementation into *P. mirabilis* cultures, pH changes were minimal and resulted in no effect of Bio-Kult supernatant on choline concentration at 8 h. However, addition of non-adjusted supernatant from strains *Lactobacillus acidophilus* PXN® 35™, *Lactobacillus casei* PXN® 37™, *Lactobacillus plantarum* PXN® 47™ and *Bacillus subtilis* PXN® 21™ demonstrated notable inhibition of choline metabolism. This was most likely due to the pH decrease that influences choline metabolism to TMA, however, other strains that elicited decrease in pH had no effect on choline metabolism and showed complete utilisation of choline at 8 h. This suggests that other factors present in the supernatants of some strains played an important role in preventing *P. mirabilis* fully degrading added choline at a lower pH. Future work should investigate the contribution of other factors such as availability of substrate sources or abundance of short chain fatty acids (SCFA) which is one of the proposed mechanisms of probiotic supernatant mode of action (63, 101, 214, 217, 233, 288, 297, 298). Regardless of the pH effect, probiotic cell-free supernatants, heat-killed strains, and purified metabolites have previously shown ability to alter the growth of pathogenic strains. This concept, referred to as postbiotics, has been extensively discussed in recent reviews (168, 299, 300) and the impact of postbiotics on TMA and TMAO production needs to be further investigated. Using co-culture experiments with single strains of TMA producing bacteria offers a platform for investigation of the interactions between candidate probiotic strains with TMA producers as well as investigation of the postbiotic effects on strain ability to produce TMA from methylated amines.

To determine whether the supernatant pH-dependent changes were associated with a reduction in *P. mirabilis* concentration, the abundance of *P. mirabilis cutC* gene copies was measured using qPCR. There was no difference in the number of copies of the *cutC* gene between the two pH conditions of supernatant. The exception was *P. mirabilis* supplemented with supernatant from *Lactobacillus plantarum* PXN® 47™ in non-adjusted pH, which showed a much lower number of copies of the *cutC* gene.

Limitations, gaps, and next steps

This chapter revealed that testing probiotic treatments in different batch colon models using glycerol stocks and fresh faecal samples is complex and multiple factors can affect the performance of probiotic strains in mixed formulation of a supplement or delivered as individual strains. Despite the effort to test a selected dose in multiple faecal donors and investigate the impact of different doses in a pooled faecal samples, this analysis only reflects the effect of the commercially available supplement in this limited population. Investigating the effects of the subset of strains from the supplement can elucidate the individual impact of

probiotics with varied properties, however, they should be further tested in multiple different faecal donors as the impact of probiotic strains is often dependent on the existing microbiota of the host (212). Some preliminary findings were reported on the impact of probiotic strains on *P. mirabilis* and its ability to produce TMA from choline, but the mechanisms behind this effect need to be further explored both in co-cultures and by incubating *P. mirabilis* with probiotic supernatant in liquid and solid media. In this present study of the impact of Bio-Kult supernatant on *P. mirabilis*, the maximum TMA produced from choline was highly variable across the different supernatant enrichments, with some non-adjusted cultures showing much higher maximum TMA than their pH-adjusted counterparts. Unfortunately, these two conditions were not a direct comparison of using the same supernatant only with changes in pH but rather two separate supernatants resulting from probiotic growth on different occasion (although using the same conditions). Therefore, this experiment should be reproduced using supernatant with the same metabolite composition only with adjusted pH to determine the effect of pH on *P. mirabilis* growth and metabolism of choline to TMA. In this study, samples were collected for further investigation of the effect of probiotics on several other outcomes, such as their persistence in cultures after multiple rounds of faecal inoculation, their effects on expression of *cutC* gene in *P. mirabilis*, the ability of supernatant to alter swarming differentiation of *P. mirabilis* cells and their impact on changing microbial signatures in colon model fermenters. Unfortunately, there was not enough time to investigate these further. Future work should further investigate how single probiotic strains and their supernatants reduce choline metabolism to TMA by *P. mirabilis in-vitro* (mode of action of pH and other factors), and then explore their potential use to prevent choline metabolism to TMA *in-vivo*.

CONCLUSION

The probiotic supplement Bio-Kult showed a very limited ability to reduce TMA production from choline by complex faecal microbiota, and there may be merit in trying to optimise this effect through further work. However, a subset of individual strains from the supplement tested in this environment showed decreased TMA production. Some of these strains and their culture supernatants were also successful in bringing about a more rapid decrease in the concentration of TMA that had been formed by *P. mirabilis* DSM 4479, and the mechanisms behind these effects warrant further investigation.

Chapter 5

The effect of polyphenols on microbial production of TMA from choline

ABSTRACT

Background

There is a need to develop an effective treatment or preventative strategy for elevated plasma TMAO levels in populations at risk of disease. Plant polyphenols and phytochemicals have shown promising effects on plasma TMAO levels both *in-vivo* and *in-vitro*. There are several food components and plant extracts that effectively inhibited choline metabolism to TMA, however, the mechanisms behind these effects are often unexplored or have not been reproduced in other studies.

Aims

The overall aim was to test the hypothesis that supplementing human faecal fermentations of choline with phenolic compounds identified in foods effective at reducing plasma TMAO would reduce the conversion of choline to TMA.

Approaches and Methods

Phenolic compounds were identified from the available evidence reporting an effect on TMAO levels with food components and plant extracts. Selected compounds were screened for their inhibitory properties towards choline conversion into TMA using a high-throughput batch colon model inoculated with pooled faecal samples. Faecal cultures were incubated at 37 ^{o}C in anaerobic conditions in well-buffered medium designed to simulate the micronutrient and pH environment of the human large intestine. Chlorogenic acid which was used as a positive control of choline metabolism inhibition was also assessed in a TMA-producing strain (*Proteus mirabilis* DSM 4479 and *Desulfovibrio desulfuricans* QI0028) cultured in their recommended medium. Samples were collected at multiple time points over 24/48 hours and the kinetics of choline disappearance and TMA production were quantified. Samples were analysed using LC-MS/MS method with isotopically labelled methylated amine internal standards to quantify choline and TMA concentrations. Microbial growth was measured using viable counts and optical density at 600 nm wavelength. Differences between the individual compounds were determined using Area Under Curve (AUC) of choline and TMA concentration over the incubation period and were tested for significance using One-way or Two-way ANOVA.

Results

The ability of chlorogenic acid to inhibit choline metabolism to TMA seemed to be dependent on the gut microbiota composition as seen in *in-vitro* colon models inoculated with faecal samples from different donors. Furthermore, by using the established single-strain model of TMA producing bacterium, chlorogenic acid caused non-lethal inhibition of choline metabolism in *P. mirabilis* but failed to maintain the inhibitory effects in *D. desulfuricans*. Luteolin, eriodictyol and resveratrol showed significantly higher AUC of choline metabolism compared to phenolic-free control, together with significant difference in AUC of TMA production (all $p < 0.0001$). Non-flavonoid compounds sinapic acid and 3-(4-hydroxyphenyl) propionic acid resulted in a significant reduction of AUC for TMA concentration, but similar effects were observed with all solvent-supplemented control conditions, posing difficulties in determining if effect was due to the presence of solvents or phenolic compounds. Mechanistic investigation showed that most compounds showed no inhibitory effect towards total viable count on anaerobic bacteria, with eriodictyol showing significantly higher viable count than polyphenol-free control ($p = 0.0376$).

Conclusion

The hypothesis that supplementing human faecal fermentations of choline with phenolic compounds identified in foods effective at reducing plasma TMAO would reduce the conversion of choline to TMA was supported. The findings identified that the structural properties of phenolic compounds used for reducing TMA production from choline play a significant role in their effectiveness with more complex compounds exhibiting better inhibitory activity.

INTRODUCTION

Plant polyphenols and their bioactive metabolites have been reported to affect disease outcomes through several mechanisms (100, 144, 166, 173), some of which include gut microbiota modulation and anti-inflammatory and antioxidant activity (143, 301-304). Their involvement in mitigation of cardiovascular disease and ability to reduce TMAO levels in plasma, serum and urine in animal models and human clinical trials has been reported (100, 177, 178, 205, 305, 306). Reviews of the published literature on the ability of phytochemicals to alter TMAO production revealed that most studies test whole foods, extracts or mixtures containing phenolic compounds of different types and classes (180, 181). Studies often focus on identifying novel strategies that inhibit TMA production from dietary precursors, but there is a lack of understanding as to what compounds from these complex structures show an effect on TMA production from choline.

Using *in-vitro* models of human faecal microbiota provides an opportunity to test the capacity of different components of phytochemical-rich foods to reduce choline metabolism to TMA and identify individual compounds that may be responsible for the TMA-reducing effects of more complex structures. Previous *in-vitro* studies of the effects of polyphenols on reducing TMA production investigated the impact of blonde grapefruit juice and pink grapefruit juice, which reduced TMA concentration by 91-94% compared to the non-supplemented control, as reported by Bresciani *et al.* (179). Additionally, they reported 79% and 91% reduction in TMA when pomegranate and blood orange juice were supplemented into faecal starters, respectively. They expanded their investigation by testing abundant phenolic compounds in orange juice such as hesperidin, narirutin and ferulic acid, as well as cyanidin-3-glucoside found in blood oranges. They reported no effect of any of the polyphenolic compounds tested on choline degradation into TMA by 24 hours of incubation, attributing the main effect of the fruit juices to their carbohydrate content.

The opposite approach was taken by Iglesias-Carres *et al.* who tested compounds found in tea, coffee, and cocoa, identifying catechin, epicatechin and chlorogenic acid in 2 mM concentration as effective inhibitors of choline conversion to TMA (228). They followed-up their findings with testing polyphenol-rich beverages from said foods (in either digested or undigested form), using their *ex vivo – in vitro* model that was also used in this thesis (307). Multiple different strategies for TMAO reduction have been identified in previous reports, but there is a lack of systematic approaches to establish an effective inhibitor. The available literature was analysed to determine the most effective phenolic-rich foods and extracts, and survey their phenolic compositions to identify compounds that could contribute to the

inhibitory effect. Therefore, the approach is to test pure compounds and investigate the impact of phenolic structure on their inhibitory properties and to reproduce and expand on findings of others, strengthening the available evidence for the effects of plant bioactive compounds on TMA production.

5.1 Aims and hypotheses

The aim was to test the hypothesis that phenolic compounds can reduce bacterial metabolism of choline into TMA.

5.2 Objectives and approaches

Objective	**Approach**
Test the suitability of chlorogenic acid for being used as a positive control for inhibiting choline metabolism to TMA	Reproduce findings reported by Iglesias-Carres *et al.* (228) and investigate the ability of chlorogenic acid to inhibit TMA production in higher doses of supplemented choline.
Select polyphenols for testing of their ability to reduce TMA production from choline	Exploring literature to select polyphenols to be used in screening for their ability to reduce TMA production from choline
Identify effective polyphenols that showed a reduction in TMA production	Investigate the effect of selected compounds on choline metabolism into TMA using a high-throughput *in-vitro* fermentation colon model inoculated with a pooled faecal sample and 2 mM concentration of phenolic compounds
Investigate the mechanisms of action in the most effective polyphenols	Testing the most effective polyphenols in a pooled faecal sample to establish their effect on bacterial growth, culture pH and capacity to produce TMA from choline.

RESULTS

5.3 The effect of chlorogenic acid on TMA production from choline

Based on previous reports utilising chlorogenic acid and DMB as non-lethal inhibitors of choline metabolism to TMA, these findings were now reproduced to investigate the suitability of chlorogenic acid as a positive control compared to DMB, a reported inhibitor of choline metabolism. The use of the 96-well high-throughput model optimised in the same publication by Iglesias-Carres *et al.* (228) was also tested to investigate if this model could be used for the screening of phenolic compounds as previously described, with some alterations to the methodology outlined in the Chapter 2 section 2.5.

5.3.1 Inhibition of choline metabolism by 5 mM chlorogenic acid in a fresh faecal inoculum

The fermentation of 100 µM final concentration of supplemented choline in a 2% fresh faecal slurry inoculated into CMHT nutritive media in a 96-well plate CMHT set-up served as a control condition in an experiment investigating the inhibition of choline metabolism by 5 mM chlorogenic acid and 10 mM DMB, displayed in panel **A** of **Figure 5-1**. Choline abundance (mol%) rapidly decreased between 4 h and 8 h in the control condition and DMB supplemented wells, compared to gradual decrease in choline abundance over 24 h in wells treated with 5 mM chlorogenic acid. This gradual depletion of choline resulted in steady increase in TMA produced from choline to 18.5 mol% at 10 h and final TMA concentration of 50.9 mol% which was comparable to TMA produced from choline in control and DMB conditions. Therefore, chlorogenic acid is an effective inhibitor of TMA production from choline, when compared to ineffective DMB and a treatment-free control.

5.3.2 Inhibition of choline metabolism by chlorogenic acid in a pooled faecal sample from two donors

A higher concentration of added choline was tested in multiple separate runs using the same pooled faecal sample on three separate occasions (at least 6 replicate wells per condition). **Figure 5-1** panel **B** shows the mean and standard error displayed for control condition with 500 µM choline concentration and polyphenol treated wells with 5 mM chlorogenic acid supplemented into 2% faecal culture in CMHT medium. The control response of choline metabolism in a pooled faecal sample showed rapid utilisation of choline within first 8 h, compared to chlorogenic acid treated condition that slower initial metabolism of choline in the first 6 h but then decreased to 14.1 ± 2.8 mol% abundance of choline at 8 h. There was no difference between the control and chlorogenic acid conditions at 12 h for both choline

abundance and TMA produced from choline. Furthermore, this method seemed reproducible when testing the same pooled faecal sample on different occasions. There was some variation in choline metabolism at certain time points at different runs, but in-plate variation in replicate wells was very low, as shown in panel **A** where multiple wells were inoculated across the 96-well plate (control = 30 wells, chlorogenic acid = 20 wells, DMB = 10 wells). This model was therefore used for further investigation of the ability of chlorogenic acid to inhibit choline metabolism and to determine how this ability was affected by the faecal microbiota of different donors. Furthermore, the CMBB medium and model were used to observe the differences in choline degradation patterns when supplemented with pooled faecal samples from different donors and in different final faecal concentrations.

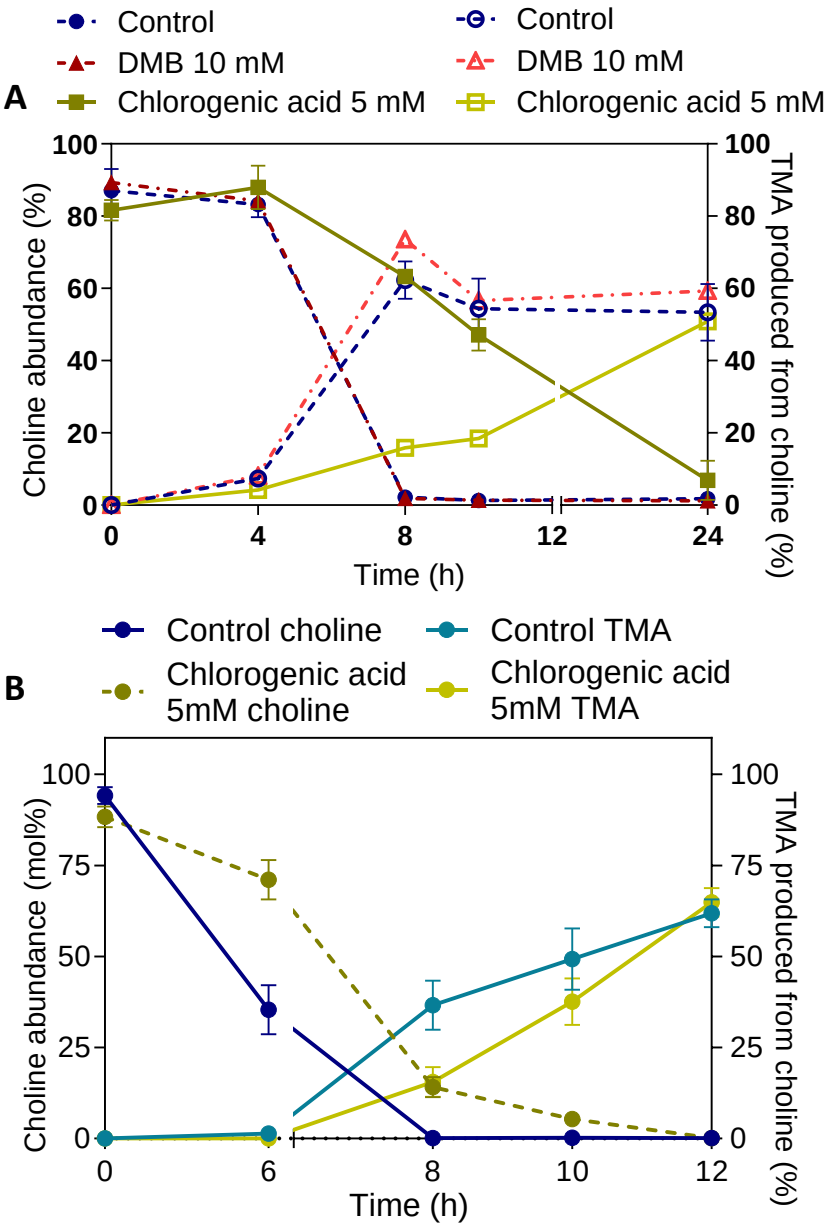


Figure 5-1 Chlorogenic acid and DMB inhibition of choline metabolism into TMA

A) Choline abundance (mol%) and TMA produced from choline (mol%) in 5 mM chlorogenic acid supplemented wells and 10 mM dimethyl-butan-1-ol (DMB) treated wells compared to untreated control inoculated in a 96-well plate (control = 30 wells, chlorogenic acid = 20 wells, DMB = 10 wells) with CMHT nutritive medium. Fermentation cultures had 100 µM final concentration of choline and 2% faecal concentration from a single fresh faecal sample. Fermentation samples were collected at 0 h, 4 h, 8 h, 10 h and 24 h. Values are mean ± SD.

B) Choline abundance (mol%) and TMA produced from choline (mol%) in 5 mM chlorogenic acid supplemented wells compared to untreated control. All wells were supplemented with 500 µM choline and 2% final concentration of faecal slurry pooled from frozen faecal samples of 2 donors. Values are mean ± SEM from three independent fermentations using the same sample (at least 6 replicate wells per condition per fermentation test). Concentrations of choline and TMA were analysed at 0 h, 6 h, 8 h, 10 h, and 12 h.

Values are expressed as percentages where choline abundance (mol%) indicates the percentage of highest molar concentration of choline measured in each technical replicate of each condition. TMA from choline (mol%) is a molar equivalent of supplemented choline that was converted into TMA – for more information, see Calculations in Chapter 2.

5.3.3 Investigating the effect of two doses of chlorogenic acid on TMA production from choline in pooled faecal samples using CMHT and CMBB fermentation systems

To understand if chlorogenic acid affected choline metabolism in a dose-dependent manner, two different doses were tested in a pooled faecal sample from 5 donors inoculated to 2% final faecal concentration in a CMHT medium supplemented with 2 mM choline. Chlorogenic acid in 2 mM final concentration and 5 mM concentration were compared to treatment-free control that had no chlorogenic acid added. **Figure 5-2** panel **A** shows the decrease in choline abundance in control within 20 h and further decrease to 23.1 ± 15.2 mol% choline concentration at 48 h. Both chlorogenic acid doses resulted in reduced choline metabolism compared to control until 38 h but resulted in complete utilisation of choline at 48 h and production of 55.6 ± 4.2 mol% and 67.2 ± 9.9 mol% of TMA from choline for wells supplemented with 5 mM and 2 mM chlorogenic acid, respectively.

Panel **B** in **Figure 5-2** shows a more apparent dose-response effect of 5 mM and 2 mM chlorogenic acid on choline metabolism. In this test, the CMBB was used with frozen pooled faecal sample from two donors in a final faecal concentration of 0.35% supplemented with the two doses of chlorogenic acid compared to control that had 2 mM final choline concentration and no treatment added. In the control condition, majority of supplemented choline was utilised in 20 h with complete metabolism at 24 h. TMA concentration gradually increased over the first 16 h with a more rapid production by 20 h, resulting in 84.5 ± 3.0 mol% of choline converted into TMA in the control condition at 24 h. Chlorogenic acid showed a delayed start of metabolism of choline in both doses, with 5 mM dose showing 53.7 ± 4.3 mol% abundance

of choline at 24 h, compared to 2 mM chlorogenic acid concentration that resulted in 16.1 ± 15.4 mol% of final choline concentration at 24 h and corresponding to 86.4 ± 8.2 mol% of TMA produced from choline, which showed no difference to the treatment-free control.

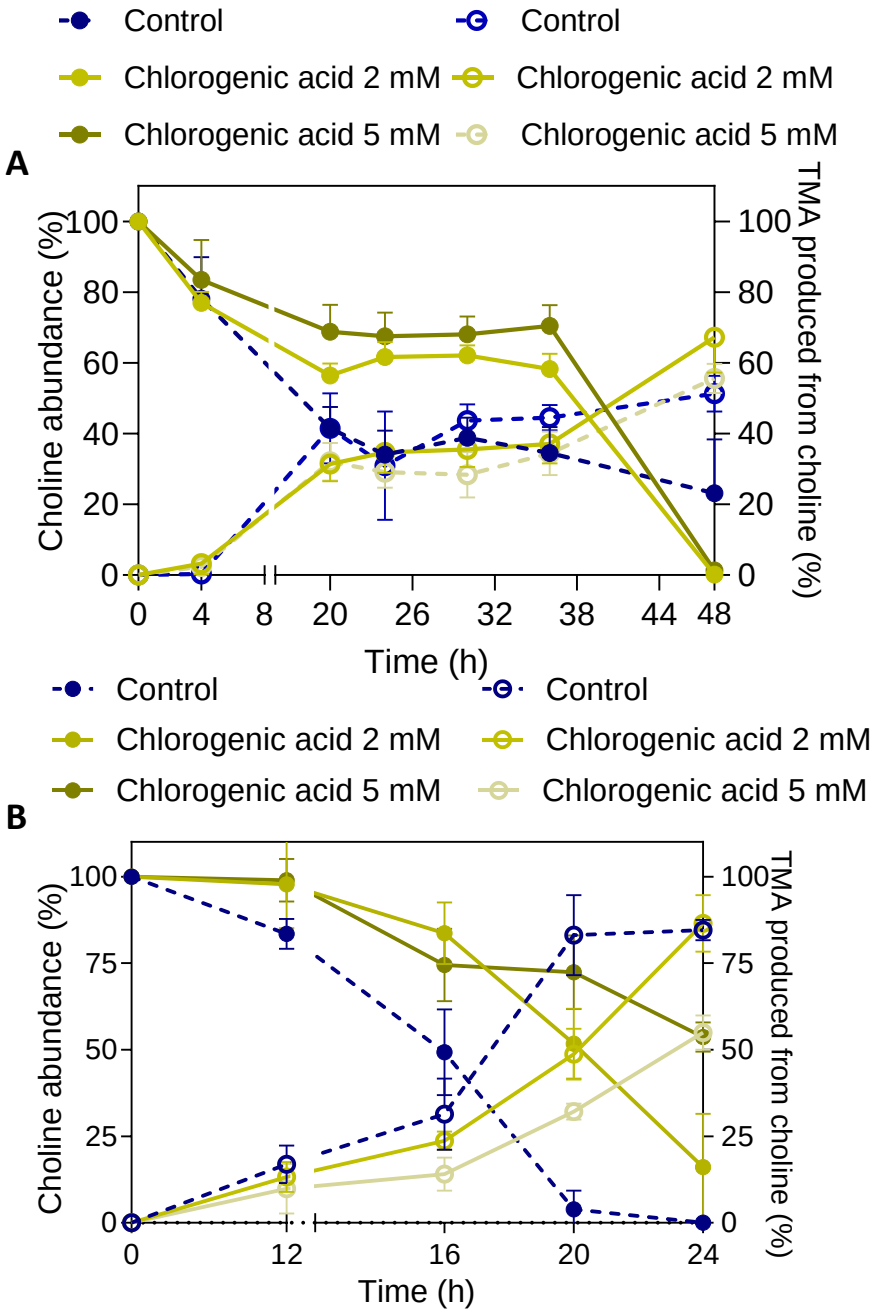


Figure 5-2 Dose response effect of chlorogenic acid on choline metabolism into TMA.

A) Choline abundance (mol%) and TMA produced from choline (mol%) in 2 mM and 5 mM chlorogenic acid supplemented wells compared to untreated control, using a CMHT 96-well system. All wells were supplemented with 2 mM choline and 2% final concentration of faecal slurry pooled from frozen glycerol stocks of 5 donors. Values are mean ± SD from 4 replicate wells per condition. Samples were collected and quantified for choline metabolism and TMA production at 0 h, 4 h, 20 h, 24 h, 30 h, 36 h and 48 h.

B) Choline abundance (mol%) and TMA produced from choline (mol%) in 2 mM and 5 mM chlorogenic acid supplemented vials compared to untreated control, using a CMBB fermentation system inoculated with 0.35% faecal concentration from a pooled faecal slurry of 2 donors and 2 mM final concentration of choline. Conditions were inoculated in 4 replicates and incubated anaerobically at 37 °C for 24 h. Samples were quantified at 0 h, 12 h, 16 h, 20 h and 24 h.

5.3.4 The microbiota-dependent response of choline metabolism to chlorogenic acid supplementation in three independent faecal samples

From these observations using individual and pooled faecal samples, the ability of chlorogenic acid to maintain inhibitory effect towards choline metabolism appears to be microbiota dependent. To investigate this further, three faecal samples from individual donors were assessed for their inhibitory response on choline metabolism by chlorogenic acid, alongside investigating the effects of chlorogenic acid on optical density of faecal samples as a proxy measure of bacterial growth. These results are reported in **Figure 5-3**.

The inhibition of choline metabolism by chlorogenic acid in Donor 1 is displayed in panel **A** of **Figure 5-3**, with growth curves depicting optical density changes over 24 h in untreated control, 5 mM chlorogenic acid (CA) and vehicle control (VC) that was inoculated with media and faecal sample only without any treatment or choline supplementation. Donor 1 showed limited choline metabolism and little TMA production over the 24 h incubation. The effect of chlorogenic acid was similar to control and there was a high variation in replicate wells (3 replicates per treatment). Despite limited metabolism of choline, the microbiota appeared active with increased growth in all conditions based on optical density increase between 12 and 24 h, however, the between-well variation was also very high for this measure. Panel **B** in **Figure 5-3** shows slow degradation of choline over 24 h in untreated control, where choline was only partially utilised (albeit with high variation) and resulted in 42.1 ± 30.9 mol% production of TMA from choline. Choline metabolism increased in CA supplemented wells compared to control at 24 h, fully degrading choline and resulting in 88.2 ± 20.6 mol% of TMA produced from choline. There were no notable differences in the microbial growth pattern between control and CA supplemented wells for Donor 2, but there was no apparent exponential growth of bacteria in either of the conditions over 24 h. Panel **C** displays the response of Donor 3 who rapidly metabolised majority of choline within 8 h in control condition and showed similar rate of choline metabolism in CA supplemented wells, but showing significantly higher abundance of choline at 4 h ($p = 0.0342$), 8 h ($p = 0.0327$), 10 h ($p = 0.0004$) and 24 h ($p = 0.0020$) compared to control. The effect of CA on TMA production was weaker, showing significantly lower TMA produced from choline at 8 h ($p = 0.0070$), but there was no difference in final TMA produced from choline between the two conditions. The growth curves of faecal microbiota were very comparable between the conditions, showing slightly higher OD in CA and VC conditions compared to choline supplemented control.

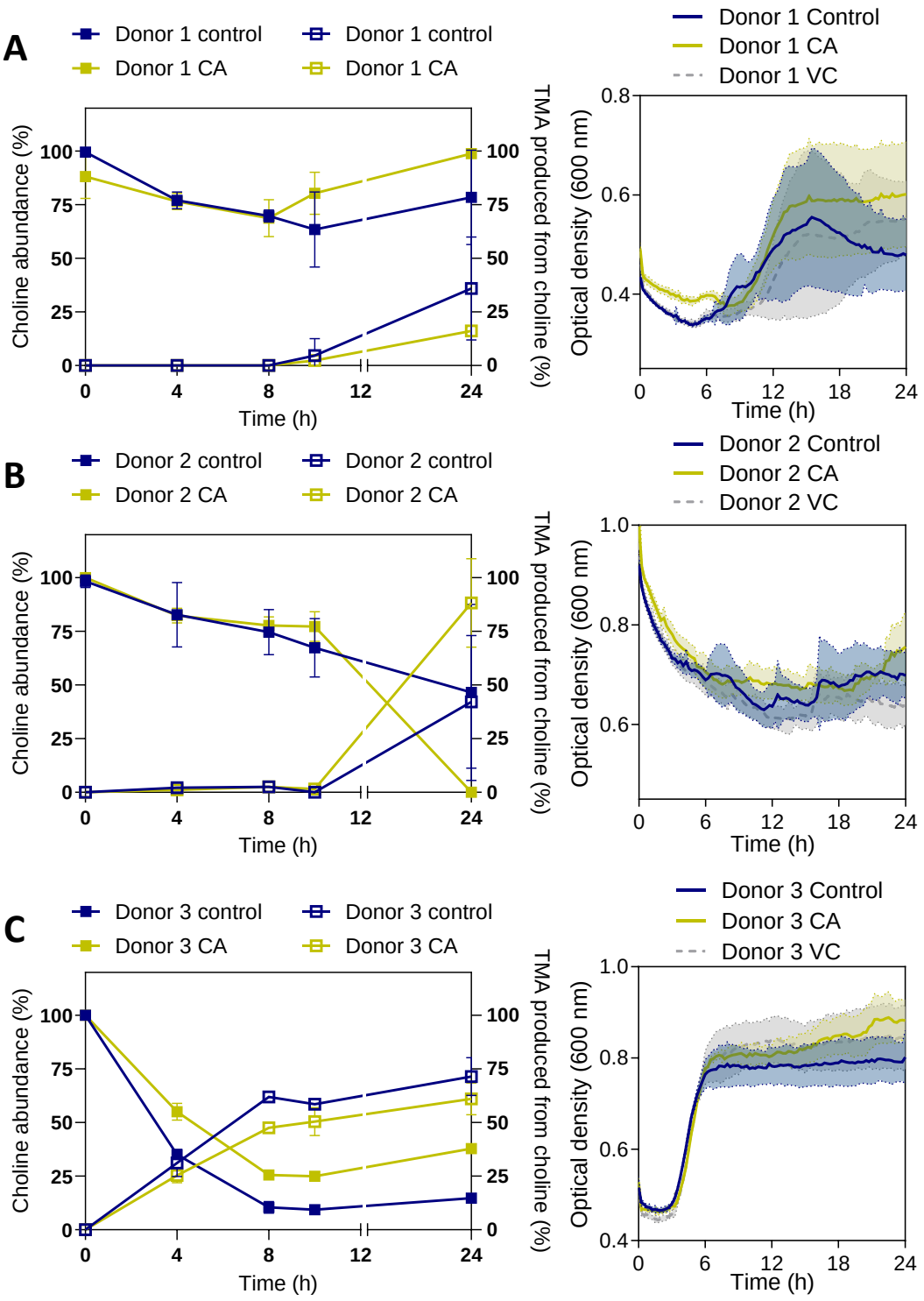


Figure 5-3 The effect of 5 mM chlorogenic acid on choline metabolism in 3 independent donors

*Choline abundance (mol%) and TMA produced from choline (mol%) in 5 mM chlorogenic acid supplemented wells compared to untreated control, using a CMHT 96-well system. All wells were supplemented with 2 mM choline and 2% final concentration of faecal slurry from frozen glycerol stocks of **A**) Donor 1, **B**) Donor 2 and **C**) Donor 3. Values are mean ± SD from 3 replicate wells per condition. Samples were collected and quantified for choline metabolism and TMA production at 0 h, 4 h, 8 h 10 h and 24 h.*

Optical density was measured over 24 h during in-vitro fermentation of choline in anaerobic environment, using a plate-reader programmed to shake sealed plate for 5 second before measurements taken every 15 minutes. Plate was inoculated using a multichannel pipette from the experimental 96-well plate used for metabolomics analysis, therefore 3 replicates were measured per condition. Figures show mean ± SD for each measurement (error bars displayed as filled area around solid line of each colour).

5.3.5 The inhibition of choline metabolism into TMA by chlorogenic acid and DMB in *P. mirabilis*

To investigate if the ability of chlorogenic acid to inhibit choline metabolism stemmed from its effect on the growth of TMA-producing bacteria, the inhibition of choline metabolism in a single strain model of TMA producing bacterium developed in Chapter 4 was now tested. Alongside the addition of chlorogenic acid, DMB was tested for its capacity to inhibit choline metabolism outside of the complex matrix of the faecal microbiota, investigating its capacity to inhibit choline metabolism by blocking the active site of the enzyme choline-TMA lyase. The metabolism was tested by incubating *P. mirabilis* in Nutrient broth for 24 h and measuring optical density and viable counts at different timepoints. **Figure 5-4** panel **A** shows choline metabolism in the three conditions and the production of TMA from choline over 24 h. The control condition was inoculated with *P. mirabilis* and 2 mM choline, with treatment conditions containing 5 mM chlorogenic acid and 10 mM DMB. Choline was rapidly degraded between 2 h and 4 h, remaining at ~20% for the rest of the incubation period. TMA produced from choline peaked at 66.8 ± 6.4 mol% at 6 h in control condition, gradually decreasing to 40.0 ± 3.4 mol% at 24 h. There was a near complete inhibition of choline metabolism in chlorogenic acid supplemented condition, showing maximum TMA produced from choline (mean + SD = 4.1 ± 1.5 mol%) at 2 h. The final choline abundance at 24 h was 86.7 ± 3.5 mol% in chlorogenic acid vials, compared to 16.2 ± 0.6 mol% in control. DMB showed similar ability to inhibit choline metabolism, resulting in final mean ± SD choline abundance of 66.7 ± 14.1 mol% at 24 h. For DMB, TMA production peaked at 6 h, showing 10.4 ± 0.6 mol% of TMA produced from choline, which then decreased to 2.8 ± 2.5 mol% of TMA from choline at 24 h.

Panel **B** shows the changes in optical density over 24 h, displaying rapid growth in control and DMB treated conditions, albeit with variation between vials. There was a notable inhibition of *P. mirabilis* growth with chlorogenic acid until 8 h, only increasing exponentially after 10 h. By 24 h, there was no difference in optical density between any of the treated conditions and control. The growth inhibition effect was more notable when enumerating viable counts, shown in panel **C** of **Figure 5-4,** where viable counts were significantly lower at 6 h and 24 h in chlorogenic acid treated compared to control, but not in DMB treated at 24 h. This suggests that despite optical density showing no difference between chlorogenic acid and control, viable counts show consistently lower CFU/mL in chlorogenic acid. However, *P. mirabilis* still managed to grow to ~10^7 in presence of chlorogenic acid. Therefore, inhibiting growth of TMA-producing bacteria might not be the only mechanism of action of chlorogenic acid. To investigate this further, another TMA-producing bacterium *D. desulfuricans* QI0028 was evaluated for the ability of chlorogenic acid and DMB to inhibit choline metabolism to TMA.

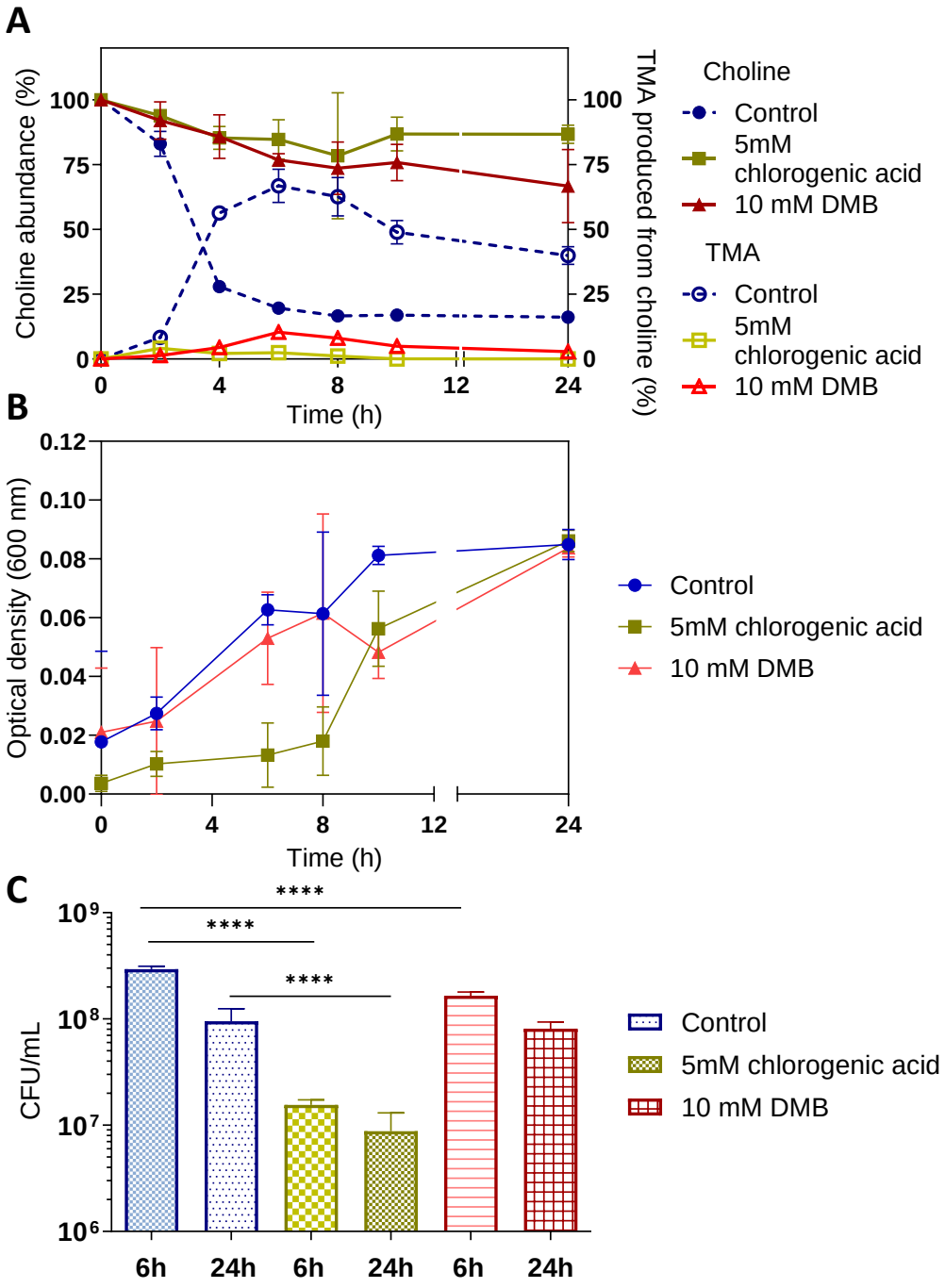


Figure 5-4 The effect of chlorogenic acid and DMB on growth and choline metabolism in P. mirabilis

A) Choline abundance (mol%) and TMA produced from choline (mol%) in 5 mM chlorogenic acid and 10 mM DMB supplemented vials compared to untreated control, inoculated into Nutrient broth. All vials were supplemented with 2 mM choline and 2% 2nd passage of P. mirabilis with measurements taken at 0 h,2 h, 4 h, 6 h, 8 h, 10 h and 24 h.

B) Optical density at 600 nm was measured over 24 h during in-vitro fermentation of choline by P. mirabilis in anaerobic environment, using cuvettes and a spectrophotometer. Figures show mean ± SD (3 replicates per condition).

C) Viable counts (CFU/mL) were enumerated at 6 h at 24 h of incubation in each condition grown on Nutrient agar, counted after 24 h of incubation in anaerobic environment.

5.3.6 The inhibition of choline metabolism into TMA by chlorogenic acid and DMB in *D. desulfuricans*

D. desulfuricans was incubated in ABB with 2 mM choline, without choline, with 2 mM choline and a dose of 50% methanol equivalent to what was used for dissolving 5 mM chlorogenic acid, and with the two tested treatments that had 2 mM and 5 mM chlorogenic acid (CA) or 10 mM DMB, respectively. Two measures of growth were considered to determine the effect of CA and DMB on *D. desulfuricans*, optical density measured in a microplate reader inside the anaerobic cabinet and turbidity of culture inside Hungate tubes measured by a turbidometer. Both measures had uninoculated blank ABB subtracted from the readings. Choline and TMA concentrations were measured at 0 h, 12 h, 16 h, 24 h and 38 h. *D. desulfuricans* was inoculated into ABB at 2% of 2nd passage inside the anaerobic cabinet and was incubated at 37 °C.

Panel **A** of **Figure 5-5** shows the increase in optical density of all conditions inoculated with *D. desulfuricans*. In CA supplemented wells, an increase in OD ~ 0.2 occurred in the first 2 h, which then gradually decreased to ~0.1 by 12 h, suggesting a possible change in absorbance measured at this wavelength rather than optical density change due to bacterial growth. This peak was not detected by turbidity measures displayed in panel **B** where CA supplemented vials showed a higher initial turbidity compared to controls and DMB treatment but showed lower turbidity levels compared to *D. desulfuricans* with and without choline and with 50% methanol after 18 h. DMB supplemented vessels showed lower OD than choline supplemented control in a plate-reader but still managed to increase ~0.1, but showed very limited increase in turbidity within the first 12 h in Hungate tubes and no further growth until 38 h. This inhibition of growth based on turbidity and optical density did not affect the ability of *D. desulfuricans* to metabolise choline into TMA, shown in panel **C** of **Figure 5-5**, where chlorogenic acid supplemented vials showed ~20 mol% choline abundance at 24 h and complete metabolism of choline by the end of the experiment. There were some inconsistencies in the initial choline concentration in the different conditions, but DMB showed very low metabolism of choline over 38 h which resulted in 4.4 ± 1.9 mol% maximum TMA produced from choline at 24 h, suggesting that some choline could have been converted into other metabolites or present in the bacterial cell pellet rather than the supernatant. In the control and CA treated conditions, TMA production peaked at 24 h, but the biggest difference was apparent at 16 h ($p = 0.0199$) showing lower concentration in CA supplemented vials. However, this effect slightly diminished at 24 h and was not significant due to high variation ($p = 0.2124$) and was much smaller at the end of the experiment. Both choline control and CA

conditions showed a decrease in TMA after 24 h, but this was not as notable in vials with choline and added methanol.

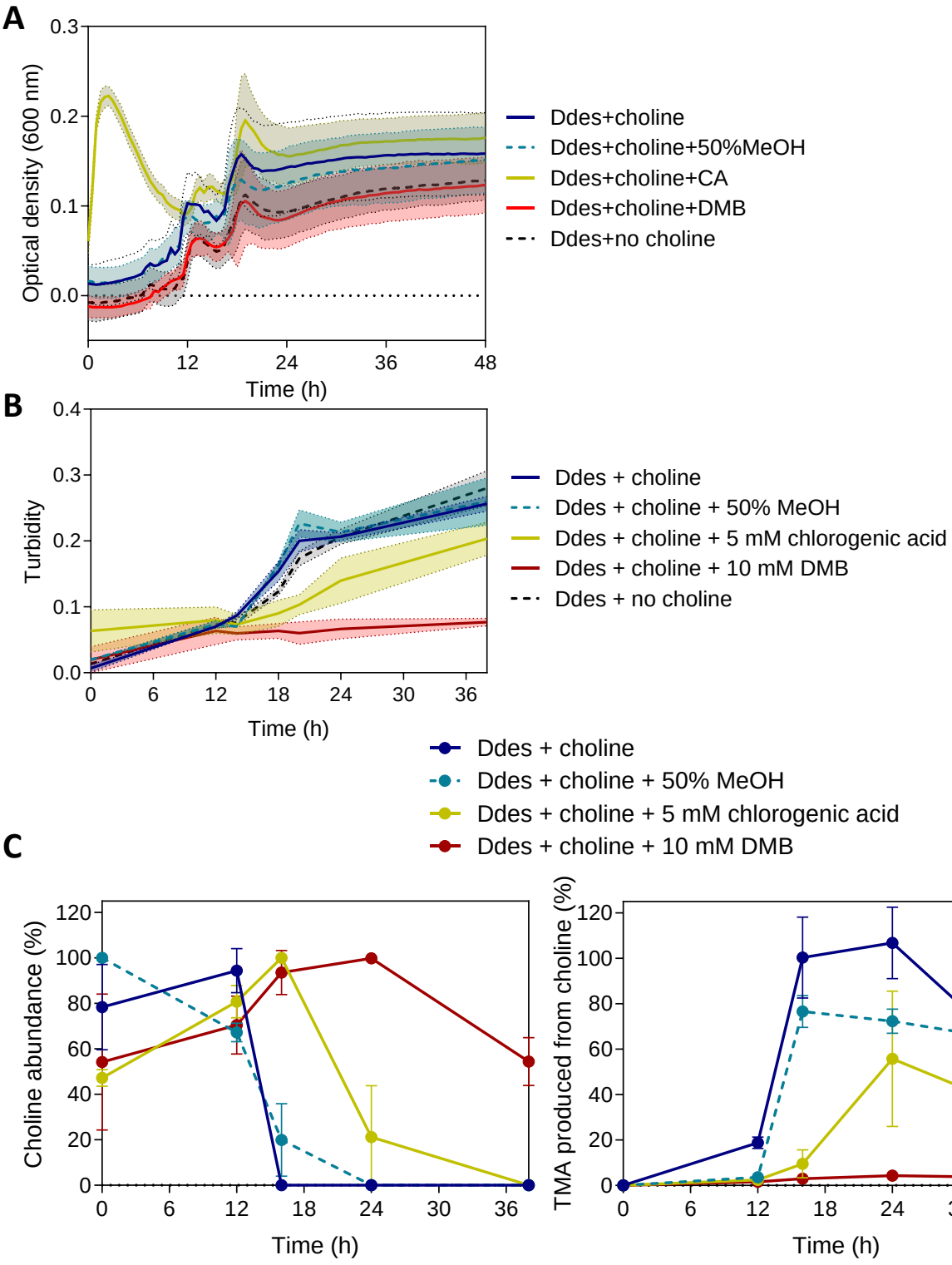
These findings further solidify the observation that the ability of chlorogenic acid to inhibit choline metabolism is dependent on the gut microbiota composition, as chlorogenic acid causes non-lethal inhibition of choline metabolism in some strains, but in other strains it only causes inhibition for a limited duration, possibly being dependent on the strains ability to break down chlorogenic acid into smaller metabolites that are no longer effective at choline metabolism inhibition. To investigate this, the presence of chlorogenic acid at different timepoints should be investigated and possible breakdown products could be quantified by LC-MS in future work.

Figure 5-5 The effect of chlorogenic acid and DMB on growth and choline metabolism in D. desulfuricans

A) Growth of D. desulfuricans (Ddes) in Anaerobic basal broth measured as optical density at 600 nm measured in anaerobic plate reader over 48 h (n = 6) Anaerobic basal broth was used as a blank and subtracted. The plate-reader was programmed to shake sealed plate for 5 second before measurements taken every 15 minutes. Figure shows mean ± SD for each measurement (error bars displayed as filled area around solid/dashed line of each colour.

B) Growth of D. desulfuricans (Ddes) in Anaerobic basal broth measured as turbidity in Hungate tubes at 0 h, 12 h, 14 h, 20 h and 38 h (n = 3). Anaerobic basal broth was used as a blank and subtracted.

C) Choline abundance (mol%) and TMA produced from choline (mol%) in 5 mM chlorogenic acid and 10 mM DMB supplemented vials compared to untreated control and a control condition with added methanol which was used as a solvent of chlorogenic acid, inoculated into Anaerobic basal broth. All vials were supplemented with 2 mM choline and 2% 2nd passage of D. desulfuricans (Ddes) with measurements taken at 0 h, 12 h, 16 h, 24 h and 38 h. Values are mean ± SD of 3 replicates per condition.


A
Optical density (600 nm)
0.3
0.2
0.1
0.0
0
12
24
36
48
Time (h)
Ddes+choline
Ddes+choline+50%MeOH
Ddes+choline+CA
Ddes+choline+DMB
Ddes+no choline
B
Turbidity
0.4
0.3
0.2
0.1
0.0
0
6
12
18
24
30
36
Time (h)
Ddes + choline
Ddes + choline + 50% MeOH
Ddes + choline + 5 mM chlorogenic acid
Ddes + choline + 10 mM DMB
Ddes + no choline
C
Ddes + choline
Ddes + choline + 50% MeOH
Ddes + choline + 5 mM chlorogenic acid
Ddes + choline + 10 mM DMB
Choline abundance (%)
120
100
80
60
40
20
0
0
6
12
18
24
30
36
Time (h)
TMA produced from choline (%)
120
100
80
60
40
20
0
0
6
12
18
24
30
36
Time (h)

5.4 Screening of polyphenol compounds for their ability to reduce choline conversion into TMA

Published literature was screened for reports focusing on modulation of TMAO levels, and two recent reviews (180, 308) were considered for deciding what type of polyphenol compounds were to be tested for their ability to reduce TMA production from choline *in-vitro*. The available evidence was collated, and word clouds were drawn based on the different food components, extracts, fermented products, and pure compounds that were used for the reviewed dietary interventions. Furthermore, the most abundant compounds and their metabolites identified in these foods by the authors of the publication, or the phenolic composition of these foods from available polyphenol database phenol-explorer were collated. A word map was derived from these to identify individual compounds associated with TMAO reduction. Additionally, the choice of phenolic compounds for screening was based on the tested structures reported by Iglesias-Carres *et al.* (228) and their follow up publication (229) where they tested compounds found in coffee and tea. From these structures, additional substitutions in OH or OCH_3 were tested to determine if the ability of phenolic compounds to inhibit TMA production was dependent on their structural properties. All compounds chosen for screening in the CMHT *in-vitro* fermentation system are listed in **Table 5-1**. The overview of the literature is available in **Figure 5-6**.

Table 5-1 Polyphenol compounds chosen for testing in the colon model.

Type/Subclass	Name	
Phenolic acids and non-flavonoid compounds	Gallic acid Quinic acid Caffeic acid Cinnamic acid Benzoic acid Phenylacetic acid Hydrocinnamic acid Phenylvaleric acid Vanillic acid Hippuric acid Catechol Ferulic acid Sinapic acid Ellagic acid	p-Coumaric acid Protocatechuic acid 4-Hydroxybenzoic acid 3-Hydroxybenzoic acid Syringic acid 3,4-Dihydroxyphenylacetic acid 4-Hydroxyphenylacetic acid 3-Hydroxyphenylacetic acid 3-(4-Hydroxyphenyl)propionic acid Dihydrocaffeic acid 3-(3-Hydroxyphenyl)propionic acid Homovanillic acid 3-Hydroxycinnamic acid 4-Methoxycinnamic acid
Flavan-3-ols	(+)-Catechin	
Flavanones	Eriodictyol	
Flavones	Luteolin	
Flavonols	Quercetin	
Isoflavonoids	3’-Hydroxydaidzein	
Anthocyanins	Cyanidin	
Stilbenes	Resveratrol	

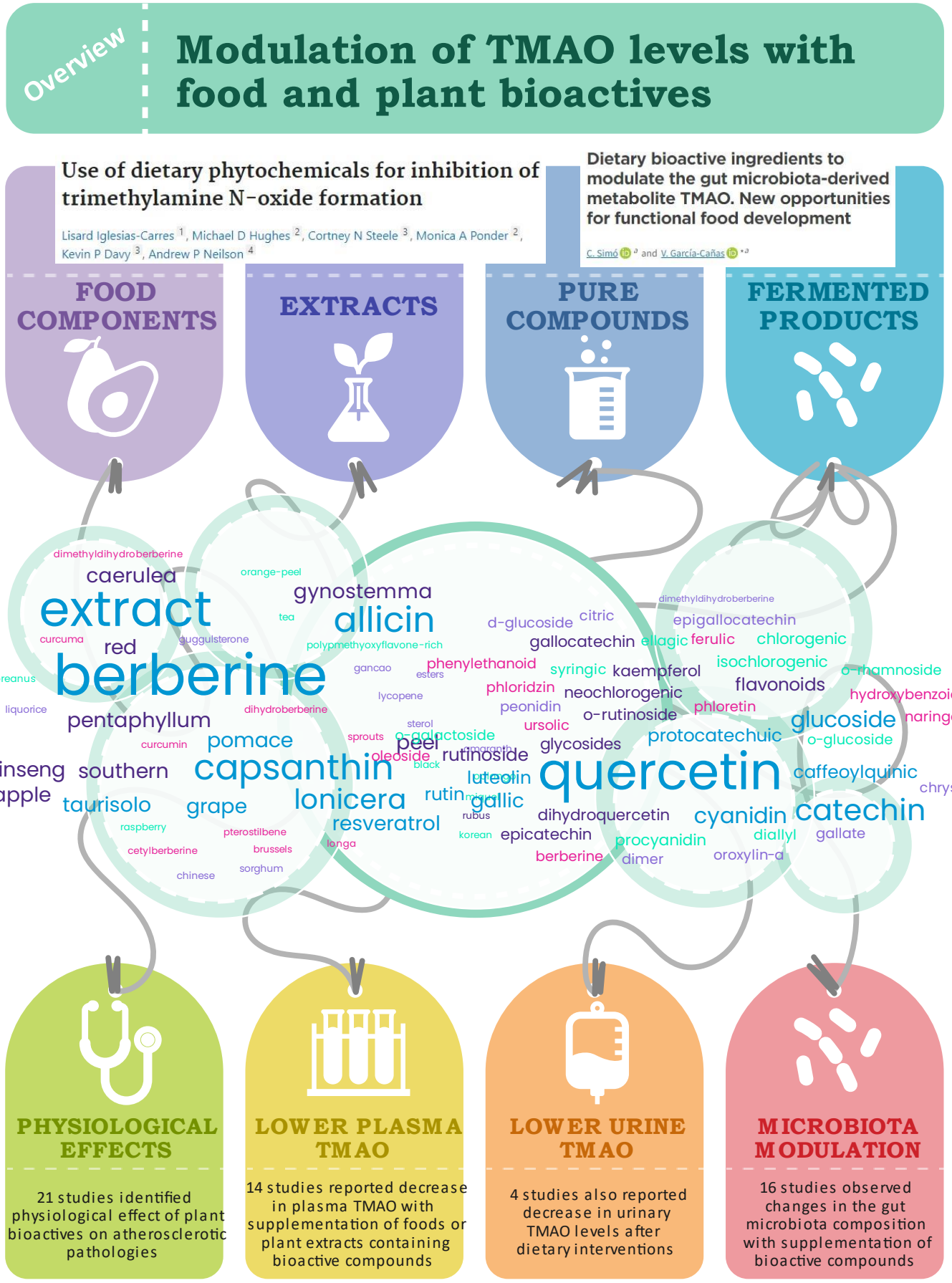


Figure 5-6 Overview of the evidence on the effects of plant bioactives on TMAO production

5.4.1 Testing bioactive compounds for their inhibitory effect on TMA production from choline using a pooled faecal sample in CMHT

The compounds listed in **Table 5-1** were tested in the CMHT 96-well *in-vitro* fermentation model for their effect on TMA production from choline. The aim of this work was to evaluate the impact of structural properties of phenolic compounds on their inhibitory effects. Representative compounds from each major class of flavonoid and non-flavonoid structures with different aliphatic chain length, hydroxylation and methylation level were assessed. Due to the high number of compounds, all non-flavonoid structures were evaluated during the same experiment, incubating each condition in 6 replicate wells, utilising two separate 96-well plates. Control condition that contained CMHT medium, 500 µM choline and 2% final faecal concentration was inoculated on each plate and 5 mM chlorogenic acid condition was used as a positive control. An additional control containing faecal sample with supplemented choline and 50% methanol mixture that was used for dissolving phenolic compounds was used to account for the effect of methanol (0.3% final concentration) on choline metabolism. Only two timepoints were evaluated for the effect of compounds on choline metabolism, with panel **C** in **Figure 5-7** showing choline abundance at 6 h and 10 h for each compound, and TMA produced from choline displayed as abundance at 6 h and 10 h. Two-way ANOVA was used to determine significant differences between the two timepoints for both choline and TMA abundance of treated conditions compared to control. AUC for 0 h, 6 h and 10 h was used to compare the effects of different compounds, with results displayed in panel **B**. The structures of the tested compound structurally related to benzoic acid are displayed in panel **A** of **Figure 5-7**.

Gallic acid, vanillic acid and CA showed significantly higher AUC compared to control for choline metabolism, indicating slower degradation of choline or incomplete utilisation. AUC of TMA production showed significantly lower values for CA and syringic acid, but significantly higher AUC in benzoic, protocatechuic and 4-hydroxybenzoic acid. Higher AUC for TMA suggests faster or higher production or sustained levels between 6 h and 10 h. In panel **C**, most tested compounds showed near complete utilisation of choline by 6 h, with vanillic acid being the only compound with significantly higher choline abundance but did not reach as high inhibition as CA. All compound showed full depletion of choline by 10 h. Some compounds accelerated TMA production compared to control at 6 h, but most compounds reached lower concentration of TMA at 10 h. However, there was a notable effect of methanol on TMA levels, showing similar final TMA to positive control CA, which was significantly lower than control.

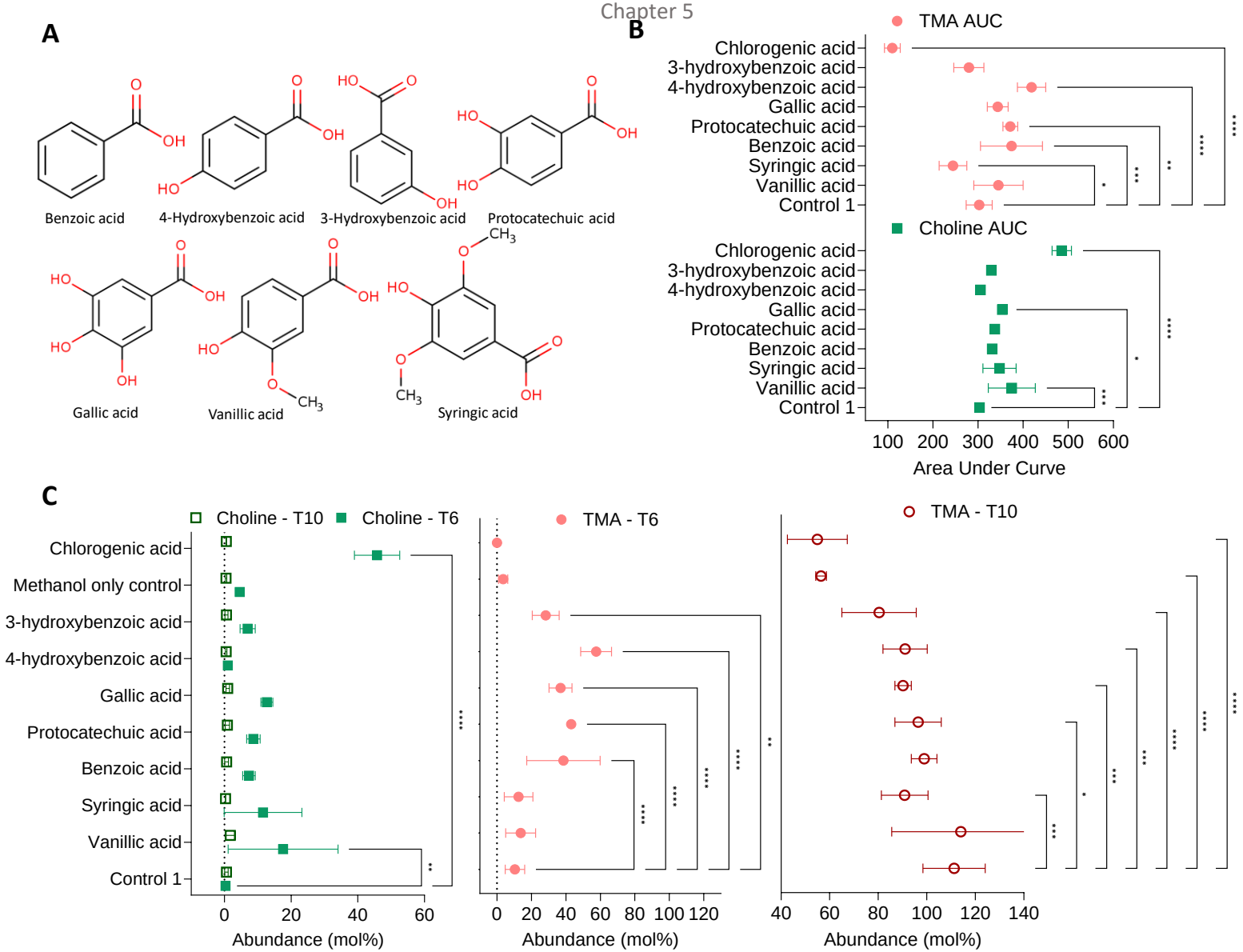


Figure 5-7 The effect of benzoic acid and its hydroxylated and methylated derivatives on choline metabolism to TMA

A**) Structures of benzoic acid and its hydroxylated and methylated derivatives **B**) Area under curve of choline metabolism and TMA production. **C**) as described in **Figure 5-8.

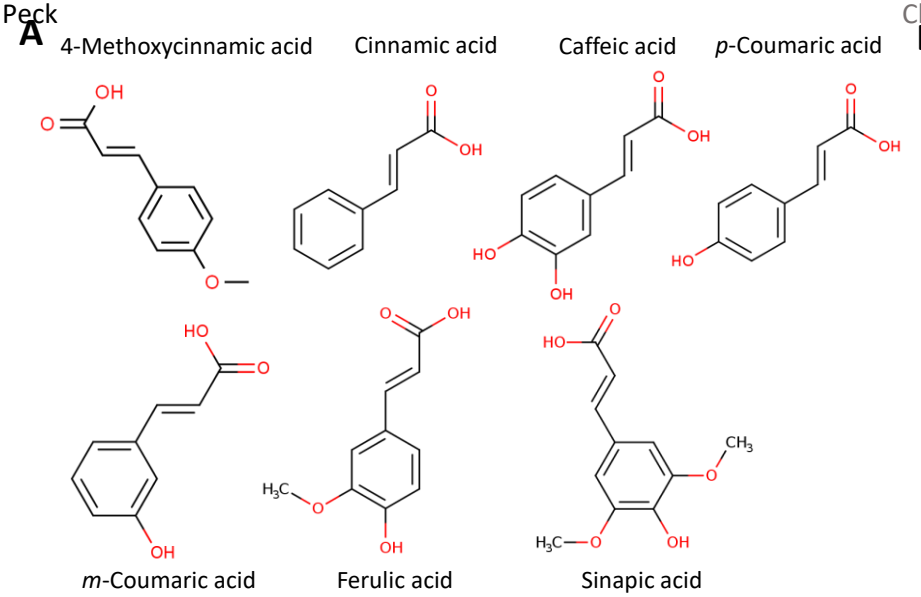
The structures of the tested compound structurally related to cinnamic acid are displayed in panel **A** of **Figure 5-8**. Panel **C** shows choline abundance at 6 h and 10 h for each compound, and TMA produced from choline displayed as abundance at 6 h and 10 h. Two-way ANOVA showed significantly higher abundance of choline at 6 h in sinapic acid, ferulic acid, 3-hydroxycinnamic acid and caffeic acid compared to control condition, but all of them showed lower choline abundance than the positive control CA. All compounds showed full depletion of choline at 10 h. These differences persisted and resulted in significantly higher AUC for choline metabolism when supplemented with sinapic acid, 3-hydroxycinnamic acid and caffeic acid, as well as CA, shown in panel **B**. Differences between the two timepoints for TMA abundance of treated conditions compared to control showed inhibition of TMA production by CA, 4-methoxycinnamic acid, sinapic acid and ferulic acid at 6 h, yet cinnamic acid and p-coumaric acid showed significantly higher TMA abundance at 6 h compared to control, indicating an acceleration of choline metabolism in presence of these compounds. All compounds showed significantly lower TMA compared to control at 10 h, however, this was the case also for methanol treated condition without any phenolics added. Nevertheless, AUC for TMA production was only significantly lower than control in 3-hydroxycinnamic, 4-methoxycinnamic, sinapic, ferulic and chlorogenic acid as displayed in panel **B**. This suggests that more complex compounds with higher methylation and hydroxylation show better potential for inhibitory properties towards choline metabolism. This was also the case for syringic acid which was the only compound that resulted in lower AUC of TMA production in **Figure 5-7** panel **B**.

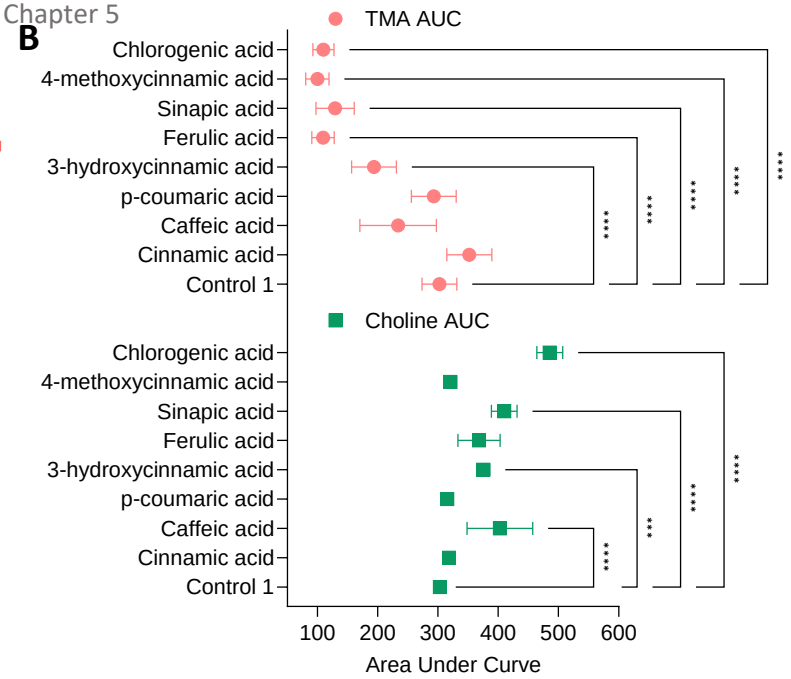
Figure 5-8 The effect of cinnamic acid and its hydroxylated and methylated derivatives on choline metabolism to TMA

***A**) Structures of cinnamic acid and its hydroxylated and methylated derivatives.*

***B**) Area under curve of choline metabolism and TMA production.*

***C**) Choline abundance (mol%) and TMA produced from choline (mol%) in 2 mM of non-flavonoid compounds and 5 mM chlorogenic acid compared to untreated control, using a CMHT 96-well system. All wells were supplemented with 500 µM choline and 2% final concentration of faecal slurry pooled from frozen faecal sample of 2 donors. Values are mean ± SD from 6 replicate wells per condition. Samples were collected and quantified for choline metabolism and TMA production at 0 h, 6 h, 10 h.*


A
4-Methoxycinnamic acid
Cinnamic acid
Caffeic acid
p-Coumaric acid
m-Coumaric acid
Ferulic acid
Sinapic acid


B
TMA AUC
Chlorogenic acid
4-methoxycinnamic acid
Sinapic acid
Ferulic acid
3-hydroxycinnamic acid
p-coumaric acid
Caffeic acid
Cinnamic acid
Control 1
Choline AUC
100 200 300 400 500 600
Area Under Curve

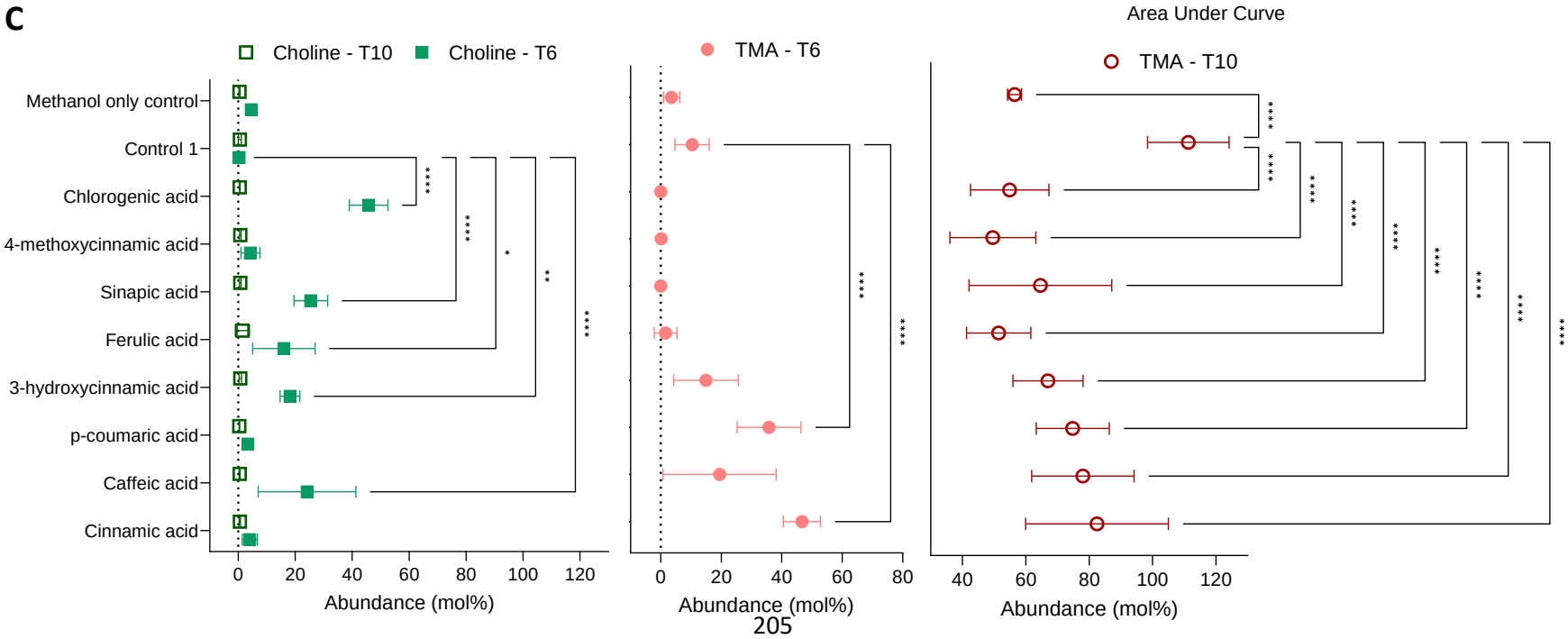
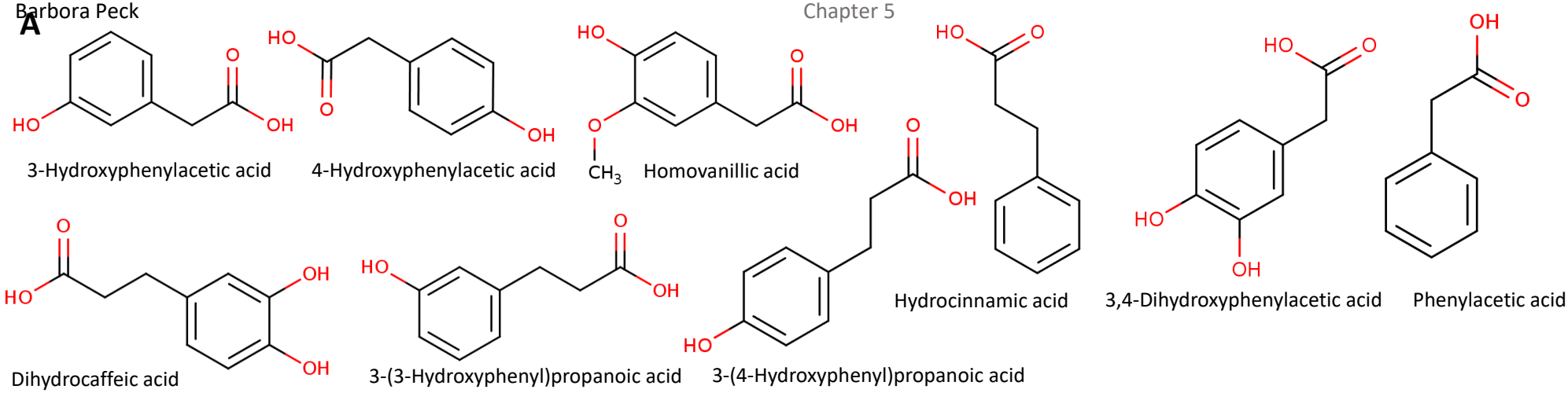

C
Choline - T10
Choline - T6
TMA - T6
TMA - T10
Methanol only control
Control 1
Chlorogenic acid
4-methoxycinnamic acid
Sinapic acid
Ferulic acid
3-hydroxycinnamic acid
p-coumaric acid
Caffeic acid
Cinnamic acid
Abundance (mol%)
Abundance (mol%)
Abundance (mol%)

Figure 5-9 panel **B** shows that there were some differences between the control in plate 2 and hydrocinnamic acid, homovanillic acid, 4-hydroxyphenylacetic acid and phenylacetic acid in choline abundance at 6 h, however by 10 h, all choline was metabolised in all supplemented conditions as well as control. There was very little TMA produced at 6 h in any of the conditions, and none of the phenolic compounds showed TMA reducing effects at 10 h compared to control. Some compounds also resulted in significantly higher TMA abundance than the control, however, the control wells showed high variation. The AUC reflected differences between control and choline metabolism in phenolic supplemented wells, displaying significantly higher AUC in phenylacetic acid, 4-hydroxyphenylacetic acid, homovanillic acid, hydrocinnamic acid and 3-(4-hydroxyphenyl) propionic acid. All phenolic-treated conditions showed significantly higher AUC for choline metabolism, suggesting that choline metabolism was slower or was not fully metabolised as fast as the control. There were some significant differences in AUC for TMA production, but these were significantly higher than the control, suggesting that phenolic acids with absence of double bonds on aliphatic chains might be contributing to an acceleration or increased production of TMA compared to control condition. In plate 2, there were also no significant effects of methanol control on TMA or choline production. However, it is worth noting that there was a difference between the two control conditions inoculated on each plate, therefore the statistical comparisons of compounds were only compared to the control condition inoculated on the same 96-well plate.

More complex compounds such as ellagic acid, hippuric acid and phenylvaleric acid were tested to determine their effect on TMA production, shown in **Figure 5-10.** Panel **A** shows the structural differences between compounds, including breakdown products of some polyphenols such as catechol with one benzene ring and two hydroxyl groups, and cyclohexane quinic acid which forms part of the effective inhibitor chlorogenic acid. Panel **C** shows significantly higher choline abundance at 6 h for positive control CA, catechol, hippuric acid and phenylvaleric acid. All phenolic compounds apart from CA increased TMA production at 6 h, but for quinic acid, catechol, hippuric acid and ellagic acid, TMA abundance at 10 h was significantly lower than in control, indicating decrease in TMA abundance or inhibition of choline conversion into TMA, favouring other intermediate or terminal metabolite. These differences were reflected in the AUC of TMA production shown in panel **B**, where all phenolic compounds showed significantly higher AUC compared to untreated control, most likely due to the acceleration of TMA production at 6 h. Only catechol and phenylvaleric acid showed significantly higher AUC for choline metabolism, arising as the most effective in this group of compounds based on their ability to delay choline metabolism to TMA.



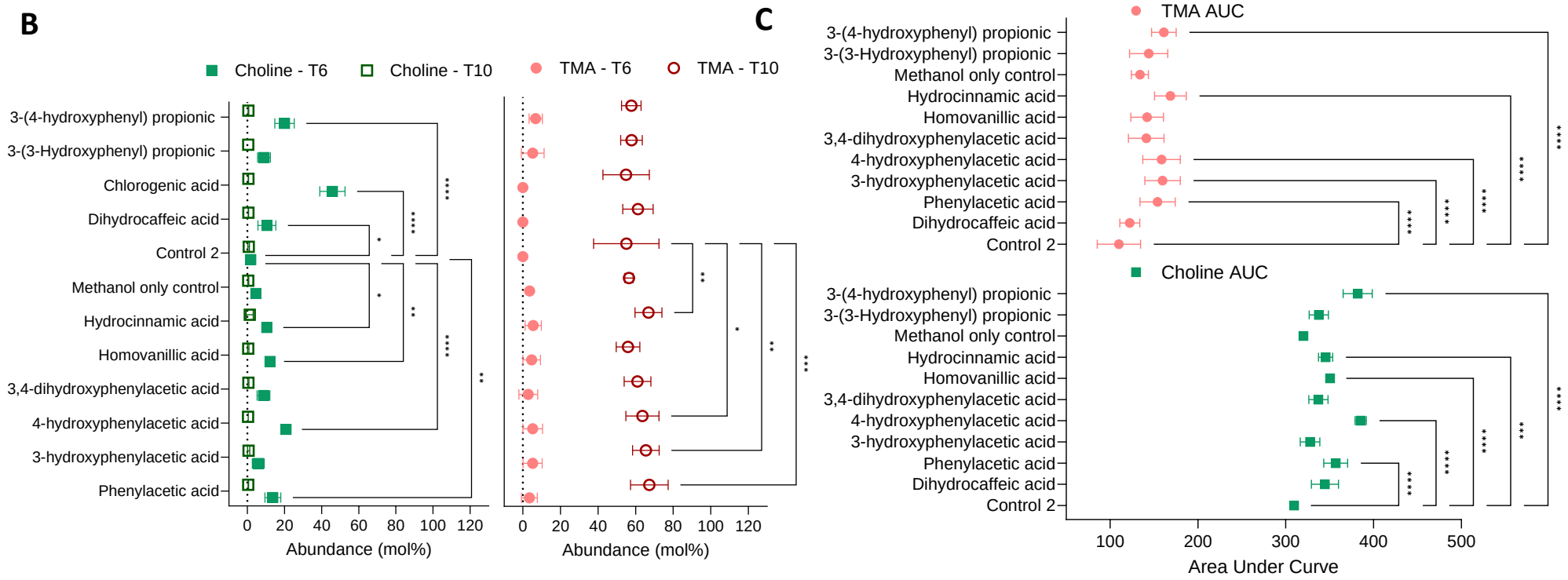


Figure 5-9 The effect of phenylacetic acid, (hydroxyphenyl)propanoic acid and their hydroxylated and methylated derivatives on TMA production.

__A__) Structures related to phenylacetic acid and (hydroxyphenyl)propanoic acid, __B__) Area under curve of choline metabolism and TMA production, __C__) as described in __Figure 5-8__

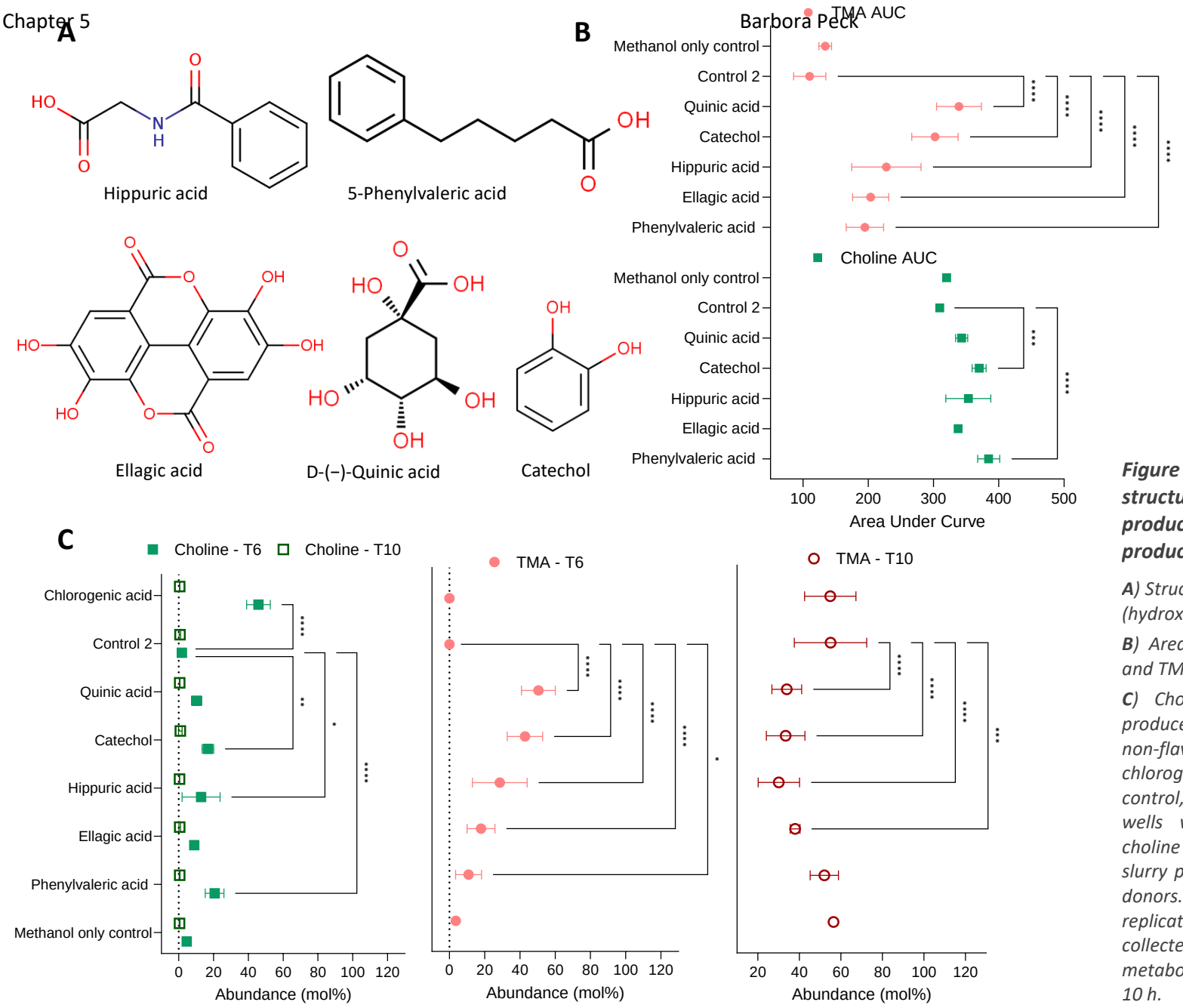


Figure 5-10 The effect of more complex structures and some breakdown products of phenolic acids on TMA production from choline.

***A**) Structures related to phenylacetic acid and (hydroxyphenyl)propanoic acid*

***B**) Area under curve of choline metabolism and TMA production*

***C**) Choline abundance (mol%) and TMA produced from choline (mol%) in 2 mM of non-flavonoid compounds and 5 mM chlorogenic acid compared to untreated control, using a CMHT 96-well system. All wells were supplemented with 500 µM choline and 2% final concentration of faecal slurry pooled from frozen faecal sample of 2 donors. Values are mean ± SD from 6 replicate wells per condition. Samples were collected and quantified for choline metabolism and TMA production at 0 h, 6 h, 10 h.*

Overall, the structural properties of individual non-flavonoid phenolic compounds had some effect on the inhibition of choline metabolism or reduction of TMA production. An overview is displayed in **Figure 5-11** where the mean abundance of choline and TMA at 6 h and 10 is plotted for each group of compounds. Complex compounds were the most effective in reducing choline metabolism at 6 h together with phenylacetic/(hydroxyphenyl)propionic acids, but complex compounds, cinnamic and benzoic acid groups accelerated production of TMA compared to control at 6 h. Due to the high variation between the two control conditions in different 96-well plates, only complex compounds showed a notable reduction in TMA produced from choline compared to control. Other compound groups such as cinnamic and phenylacetic/(hydroxyphenyl)propionic acids showed a weaker reduction in TMA produced from choline. Compounds derived from benzoic acid increased TMA abundance at 10 h, suggesting that shorter aliphatic chains or the absence of double bonds might result in a limited ability to inhibit TMA production.

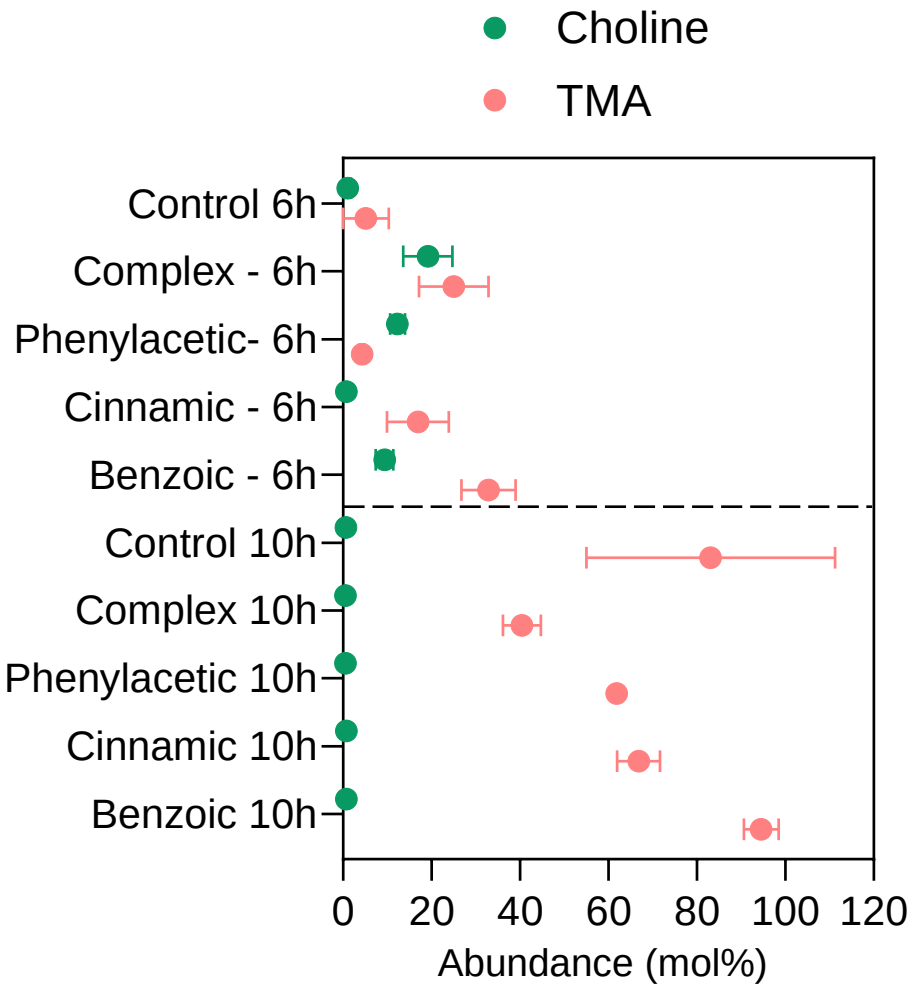


Figure 5-11 Summary of the effect of non-flavonoid compound groups on choline metabolism to TMA

Abundance for choline and TMA produced from choline (mol%) from benzoic acid related compounds (n = 7), cinnamic acid related compounds (n = 7), phenylacetic and (hydroxyphenyl)propionic acid (n = 9), complex compounds (n = 6) and control condition (n = 2). All values are Mean ± SEM (number of compounds in brackets) from 6 replicates per compound.

To confirm the hypothesis that more complex compounds are more effective at inhibiting TMA production from choline, flavonoid compounds were now tested. Representative compounds from each sub-class of flavonoids were selected and assessed in a pooled faecal sample from two donors using the CMHT 96-well fermentation system. Kinetic curves of choline metabolism and TMA production are displayed in **Figure 5-12** and **Figure 5-13**. The control condition had 500 µM choline with 2% final faecal concentration inoculated into CMHT nutritive media in 6 replicates. All phenolic compounds were dissolved in 50% methanol and final concentration of 2 mM of each compound was added to appropriate wells (6 replicates each). Chlorogenic acid was used as a positive control in a 5 mM final concentration inoculated in 6 replicates. Control and chlorogenic acid were plotted in all kinetics graphs for ease of comparison.

In the control condition (solid grey line), around ~60 % of supplemented choline was metabolised by 6 h, showing complete depletion of choline by 8 h timepoint. Quercetin, cyanidin and 3'-hydroxydaidzein, plotted in **Figure 5-12**, showed similar patterns of choline metabolism like chlorogenic acid (dashed grey line), delaying start of choline metabolism compared to control until after 6 h. Quercetin and cyanidin resulted in rapid production of TMA peaking at 10 h, reaching higher TMA produced from choline than control. Production of TMA in 3'-hydroxydaidzein supplemented condition was slower between 6 h and 8 h but increased rapidly by 10 h. Cyanidin and 3'-hydroxydaidzein also showed markedly lower final TMA concentration compared to control, indicating a mechanism of TMA conversion into other metabolites in presence of these compounds. This was not observed in control and CA conditions, where final TMA concentration was also the maximum TMA produced from choline.

Luteolin, resveratrol and eriodictyol were even more effective delaying the start of choline metabolism until after 6 h, 8 h and 6 h, respectively, but all these conditions showed full choline depletion by 10 h. This was not the case for (+)-catechin that showed ~40 mol% decrease in choline abundance at 6 h, comparable to the response seen in control which also resulted in ~30 mol% TMA produced from choline at 8 h and mean final TMA produced from choline of 67.1 ± 27.5 mol% (mean ± SD). Despite the ability of eriodictyol to delay choline metabolism and TMA production, final TMA concentration surpassed control (mean ± SD of 86.2 ± 13.7 mol% vs 60.6 ± 17.6 mol% for eriodictyol vs control, respectively). Resveratrol showed similar decrease in TMA concentration between 10 h and 12 h like cyanidin and 3'-hydroxydaidzein, but this effect was not observed in the other sub-classes of flavonoids displayed in **Figure 5-13.** To investigate the significance of these effects on choline metabolism and TMA production, AUC was calculated and is plotted in **Figure 5-14.**

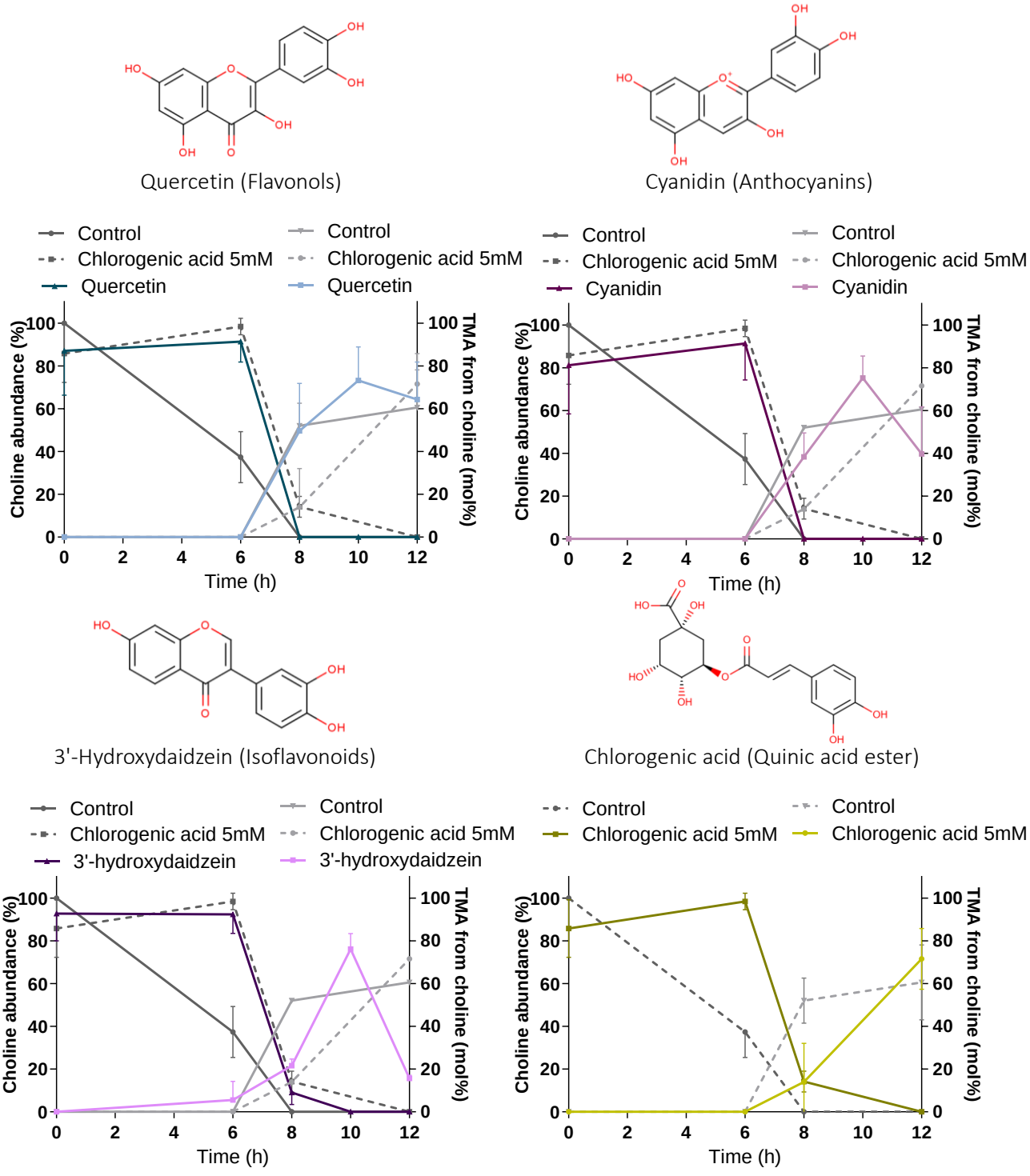


Figure 5-12 The effect of quercetin, cyanidin, 3'-hydroxydaidzein and chlorogenic acid on choline metabolism to TMA

Choline abundance (mol%) and TMA produced from choline (mol%) in 2 mM of flavonoid compounds and 5 mM chlorogenic acid compared to untreated control, using a CMHT 96-well system. All wells were supplemented with 500 µM choline and 2% final concentration of faecal slurry pooled from frozen faecal sample of 2 donors. Values are mean ± SD from 6 replicate wells per condition. Samples were collected and quantified for choline metabolism and TMA production at 0 h, 6 h, 8h, 10 h and 12 h (chlorogenic acid condition did not have 10 h samples included due to measurement error).

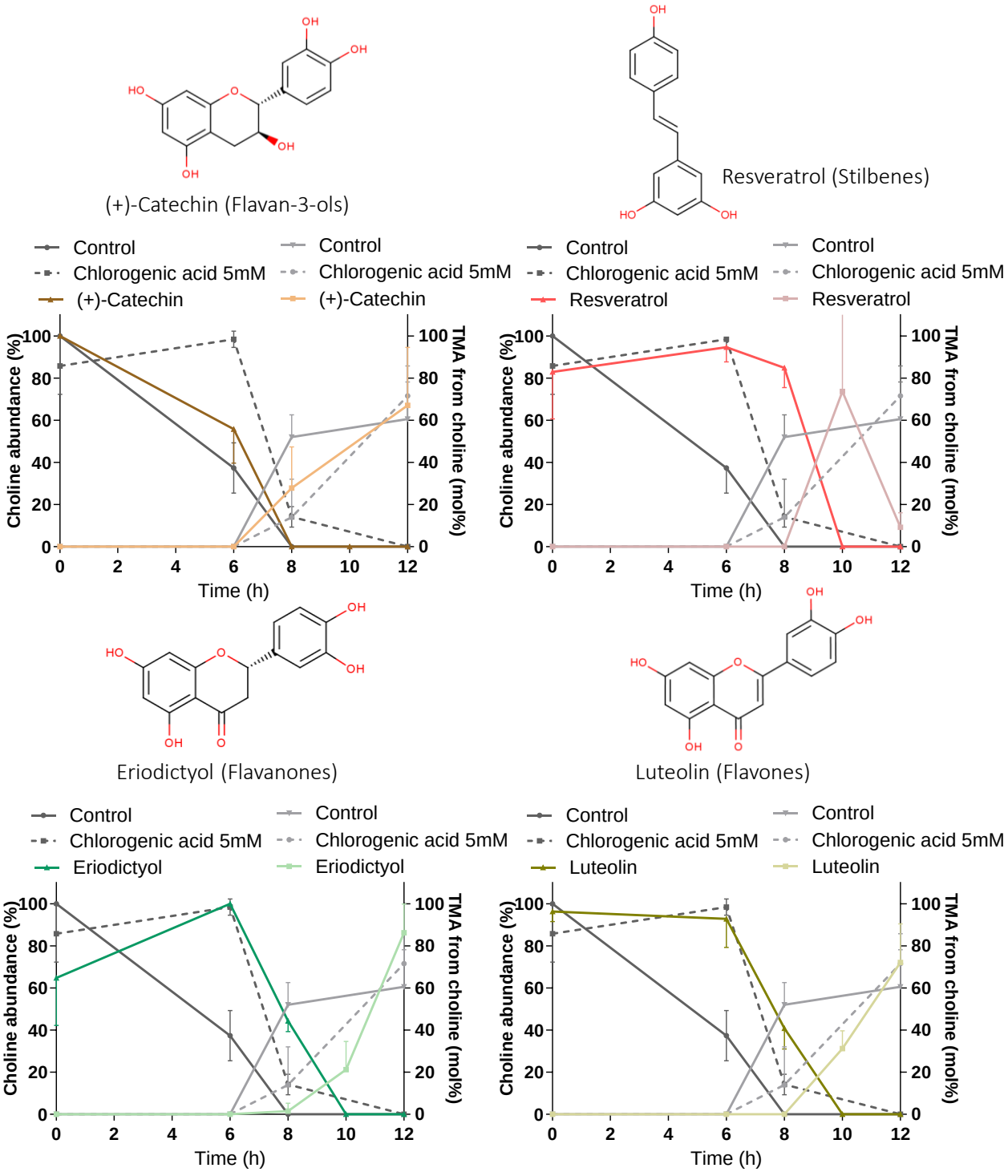


Figure 5-13 The effect of (+)-catechin, resveratrol, eriodictyol and luteolin on TMA production from choline

Choline abundance (mol%) and TMA produced from choline (mol%) were quantified from cultures as described in ***Figure 5-12****.*

The AUC of choline metabolism showed significant differences for all flavonoid compounds except for (+)-catechin, with all being significantly higher than control. No compounds showed significantly higher AUC of TMA production, maintaining the inhibitory effects observed in choline metabolism, however, only resveratrol, luteolin and eriodictyol were significantly lower compared to control condition, together with positive control CA.

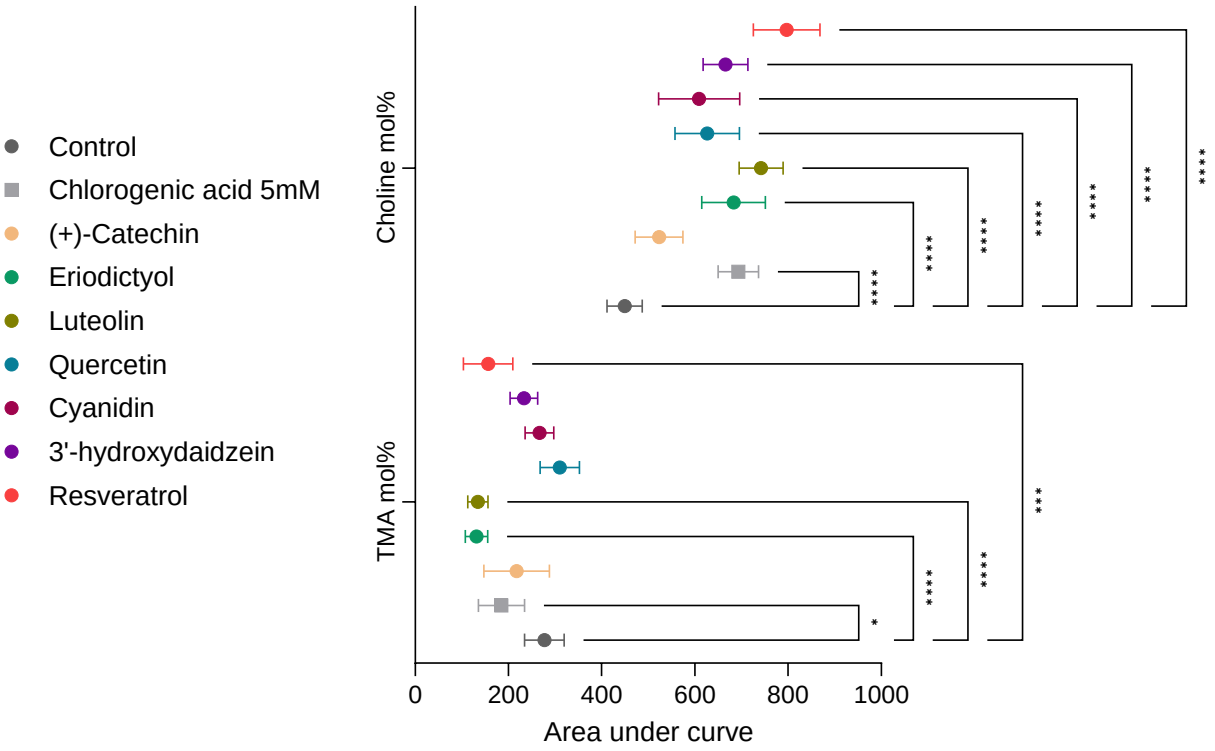


Figure 5-14 Area under curve for choline metabolism and TMA production measured in cultures supplemented with flavonoid compounds, resveratrol and chlorogenic acid.

*Areas were calculated from kinetic curves displayed and described in **Figure 5-12** and **Figure 5-13**.*

5.4.2 Investigating the mechanisms behind inhibitory properties of the most effective phenolic compounds

It is difficult to determine if these changes were due to structural differences of compounds or due to other factors, therefore, the most effective compounds from both non-flavonoid and flavonoid categories were selected for further analysis side by side. These compounds (catechol, 3-(4-hydroxyphenyl) propionic acid (3-4HPPA), sinapic acid, luteolin, resveratrol and eriodictyol) were inoculated in a 96-well plate using CMHT media with 2% final faecal concentration and 2 mM choline used as a substrate. Control condition was inoculated in 6 replicate wells and chlorogenic acid 5 mM was used as a positive control (6 replicate wells). All compounds were inoculated in 2 mM final concentration (4 replicate wells) and different

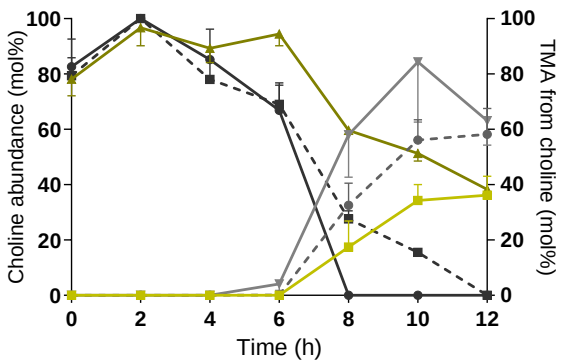
solvents were used due to previous resuspension of dissolved compounds upon inoculation into CMHT medium.

The kinetic curves of choline metabolism and TMA production are shown in **Figure 5-15**. Control and chlorogenic acid (CA) were plotted in all kinetics graphs for ease of comparison. In the control condition (solid black line), around ~30 mol% of supplemented choline was metabolised by 6 h, showing complete depletion of choline by 8 h. CA (dashed black line) showed slower metabolism of choline with ~30 mol% choline abundance at 8 h compared to fully depleted control and only showed complete metabolism of choline at 12 h. This resulted in maximum TMA production at 12 h with 58.2 ± 9.4 mol% for CA compared to 84.4 ± 21.8 mol% maximum TMA produced from choline in control at 10 h. For the selected flavonoids (luteolin and eriodictyol), both compounds showed substantial changes to the rate of choline metabolism, preventing complete utilisation of supplemented choline and inhibiting TMA production. The metabolism curves of choline looked remarkably similar for these compounds and less than ~40 mol% of TMA was produced from choline by 12 h. Even stronger inhibitory activity was seen in resveratrol supplemented condition where choline abundance decreased to 63.8 ± 11.5 mol% (mean ± SD) at 12 h and resulted in 22.4 ± 9.1 mol% (mean ± SD) of TMA produced from choline at that timepoint. Catechol showed no significant inhibition of choline metabolism, but maximum TMA produced from choline was lower compared to control for catechol-supplemented condition. The choline response to 3-4HPPA was slightly delayed with complete utilisation detected at 10 h, and maximum TMA produced from choline comparable to positive control CA (58.2 ± 9.4 mol% for CA vs 61.9 ± 1.5 mol% for 3-4HPPA; mean ± SD). Sinapic acid resulted in similar delay in choline metabolism to 3-4HPPA, but showed lower maximum TMA produced from choline than chlorogenic acid.

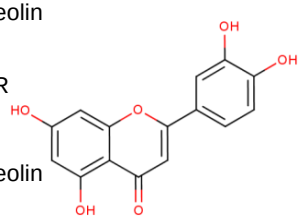
These results strengthen the hypothesis that the inhibitory capacity of phenolic compounds is dependent on the complexity of phenolic structures, with double bonds on aliphatic chains and methylation or hydroxylation on benzene rings associated with stronger inhibitory properties towards choline metabolism. To further investigate the effects of these compounds on the growth of microbes in the faecal samples, and the effect of solvent mixture controls on viability of bacteria was now investigated.

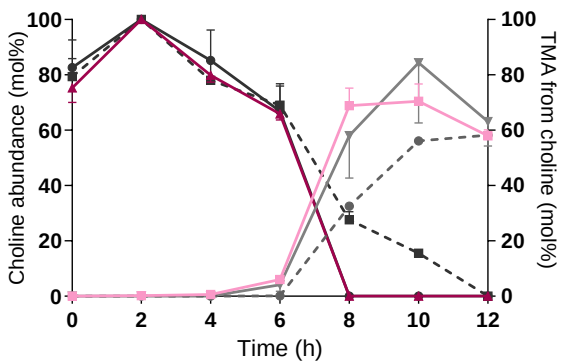
Figure 5-15 Investigating the inhibitory properties of 6 most effective compounds identified in screening experiments on choline metabolism to TMA.

Choline abundance (mol%) and TMA produced from choline (mol%) in 2 mM of flavonoid compounds and 5 mM chlorogenic acid (positive control) compared to untreated control, using a CMHT 96-well system. All wells were supplemented with 2 mM choline and 2% final concentration of faecal slurry pooled from frozen faecal sample of 2 donors. Values are mean ± SD from 4 replicate wells per condition. Samples were collected and quantified for choline metabolism and TMA production at 0 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h.

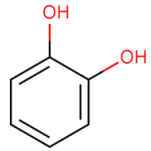


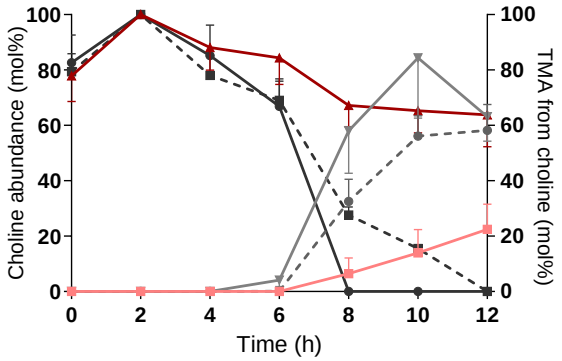
Choline
Luteolin
CA
CTR
TMA
Luteolin
CA
CTR



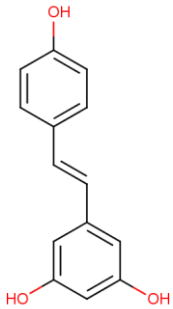


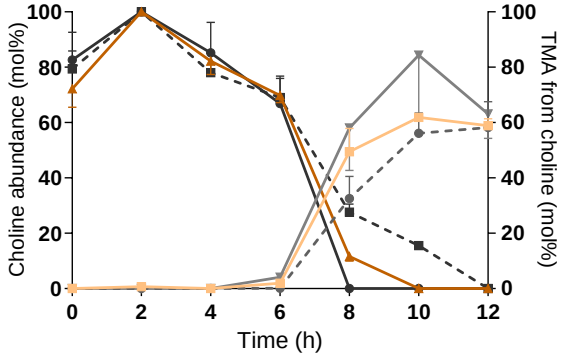
Choline
Catechol
CA
CTR
TMA
Catechol
CA
CTR



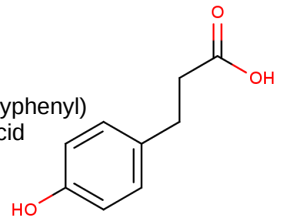


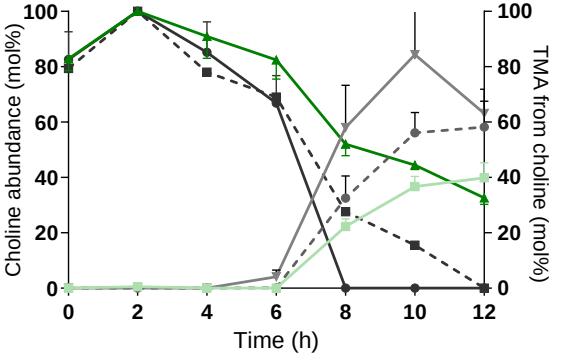
Choline
Resveratrol
CA
CTR
TMA
Resveratrol
CA
CTR



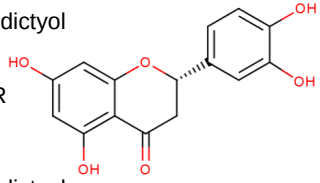


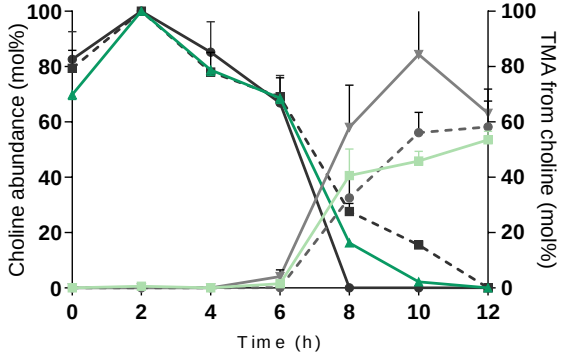
Choline
3-(4-hydroxyphenyl) propionic acid
CA
CTR
TMA
3-(4-hydroxyphenyl) propionic acid
CA
CTR



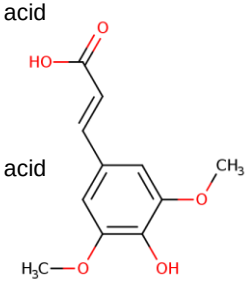


Choline
Eriodictyol
CA
CTR
TMA
Eriodictyol
CA
CTR





Choline
Sinapic acid
CA
CTR
TMA
Sinapic acid
CA
CTR



The phenolic compounds were dissolved in a mixture of solvents according to **Table 5-2**. Therefore, these mixtures were added to CMHT nutritive medium inoculated with 2 mM choline and 2% final faecal concentration to determine their effect on TMA production.

Table 5-2 Names of most effective compound for choline inhibition and the solvents used

Code	Name	Solvent and percentage
F1	Luteolin	100% DMSO
F2	Resveratrol	50% MeOH
F3	Eriodyctiol	25% MeOH/25% DMSO
NF1	Catechol	50% MeOH
NF2	3-(4-hydroxyphenyl) propionic acid	50% MeOH
NF3	Sinapic acid	50% MeOH/50% DMSO
CA	Chlorogenic acid (360 mM stock)	50% MeOH

The kinetic curves of choline metabolism and TMA production are shown in **Figure 5-16** panel **A**. All solvent mixtures had a slight inhibitory effect on choline metabolism and TMA production compared to untreated control that did not contain any solvent mixture. Solent-treated cultures reached a maximum TMA produced from choline between 48.4 – 56.9 mol% (lower and upper quartiles), with 50% MeOH/50% DMSO showing the highest TMA production and 25% MeOH/25% DMSO resulting in the lowest maximum TMA production from choline. This inhibition did not seem to influence the overall changes in optical density displayed in panel **B**, however there was a lack of exponential increase in OD for all conditions. To measure the changes in viability of bacteria in phenolic-treated conditions, panel **C** displays the total viable count (CFU/mL) of anaerobic microbes counted on Wilkins-Chalgren agar after 8 h of incubation of fermentation cultures. Most compounds showed no inhibitory effect towards total viable count, with eriodictyol showing significantly higher viable count to control ($p = 0.0376$). Only 3-4HPPA showed cytotoxic activity based on viable count, but also showed lower baseline OD measure (shown in panel **B**). None of the solvent mixture control showed inhibitory effects towards viable count, shown in panel **D**, with 100% DMSO resulting in significantly higher viable count than control ($p = 0.0003$). The inhibitory properties of flavonoids and resveratrol were not caused by changes to pH of cultures, with panel **E** showing a heatmap of pH values measured at 0 h, 8 h and 12 h, displaying similar pH between 6.54 and 7.02 across all cultures and timepoints.

Figure 5-16 Investigating the mechanisms behind the inhibitory properties of 6 most effective compounds identified in screening experiments

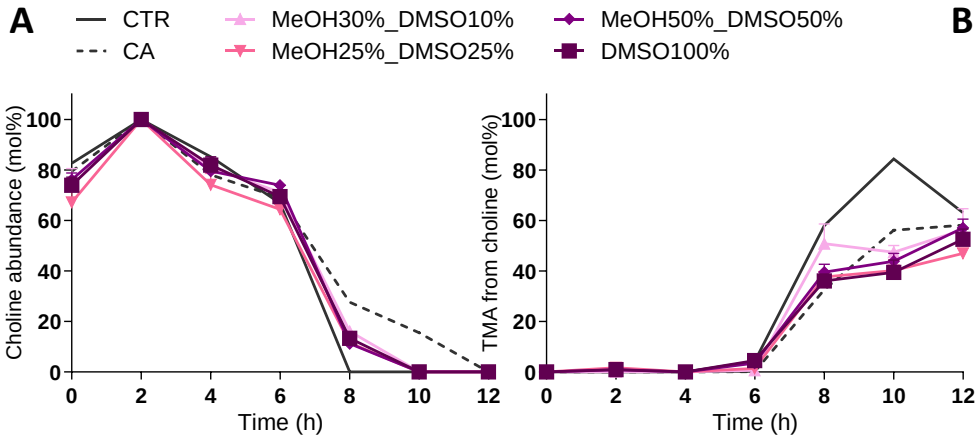
***A**) Choline abundance (mol%) and TMA produced from choline (mol%) in solvent controls (100% DMSO, 50% MeOH/50% DMSO, 25% MeOH/25% DMSO, 30% MeOH/10% DMSO) and 5 mM chlorogenic acid (positive control) compared to untreated control, using a CMHT 96-well system. All wells were supplemented with 2 mM choline and 2% final concentration of faecal slurry pooled from frozen faecal sample of 2 donors. Values are mean ± SD from 3 replicate wells per condition for solvents and 6 replicate wells for control and chlorogenic acid. Samples were collected and quantified for choline metabolism and TMA production at 0 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h.*

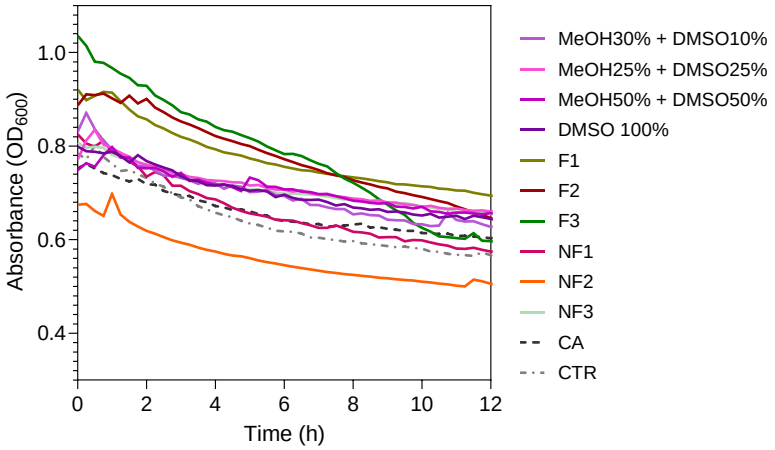
***B**) Growth of phenolic-enriched cultures and solvent controls measured as optical density at 600 nm measured in anaerobic plate reader over 12 h (n = 6 for control and CA, n = 4 for phenolic compounds and n = 3 for solvent controls). The plate-reader was programmed to shake sealed plate for 5 second before measurements taken every 15 minutes. Figure shows means of each condition.*

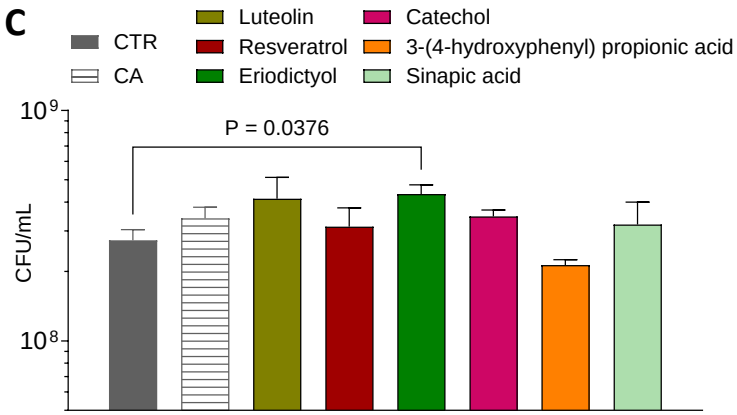
***C**) Viable counts (CFU/mL) of phenolic-enriched cultures were enumerated at 8 h of incubation in each condition grown on Wilkins Chalgren agar, counted after 24 h of incubation in anaerobic environment. Total viable counts were compared using One-way ANOVA with Dunnett's multiple comparison test, significant interactions and p-values are annotated.*

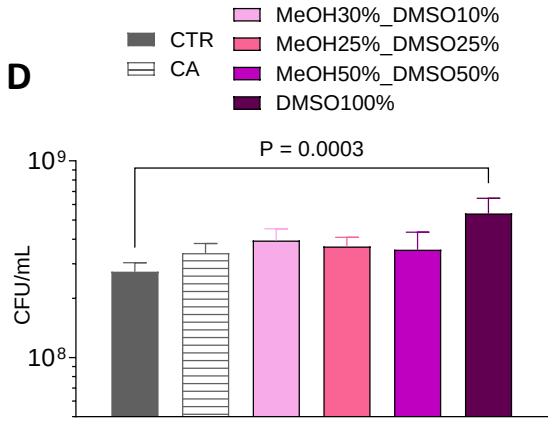
***D**) Viable counts (CFU/mL) of solvent controls compared to chlorogenic acid and untreated control, enumerated as described in C)*

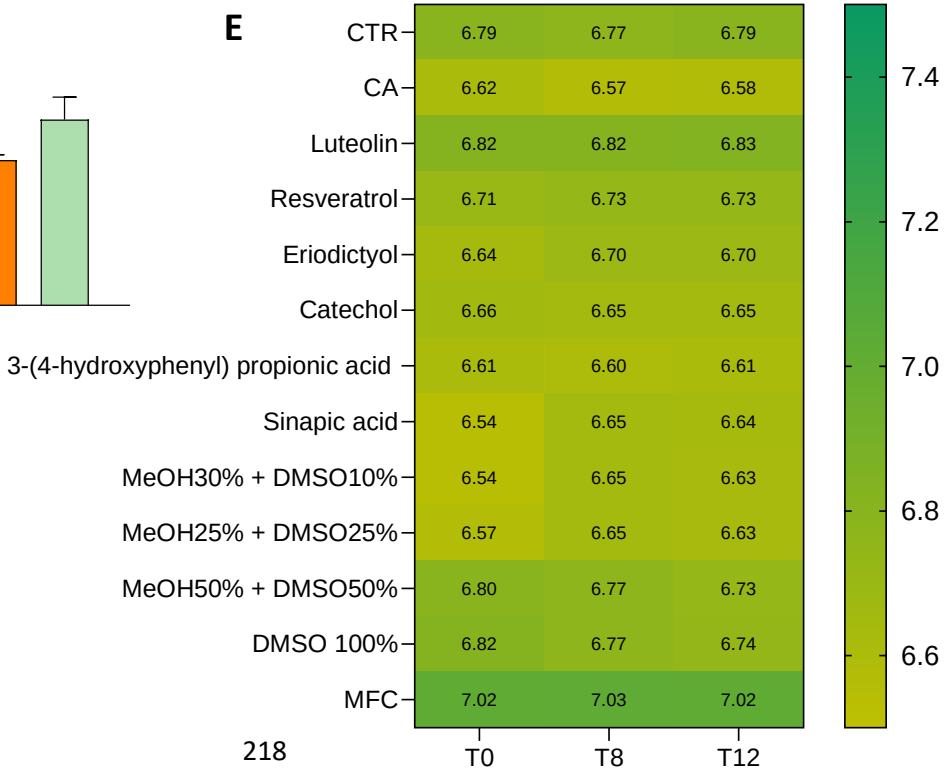
***E**) Heatmap of pH measured at 0 h, 8 h and 12 h in CMHT cultures incubated as described in A)*


A
CTR
CA
MeOH30%_DMSO10%
MeOH25%_DMSO25%
MeOH50%_DMSO50%
DMSO100%
Choline abundance (mol%)
TMA from choline (mol%)
Time (h)
Time (h)


B
Absorbance (OD600)
Time (h)
MeOH30% + DMSO10%
MeOH25% + DMSO25%
MeOH50% + DMSO50%
DMSO 100%
F1
F2
F3
NF1
NF2
NF3
CA
CTR


C
CTR
CA
Luteolin
Resveratrol
Eriodictyol
Catechol
3-(4-hydroxyphenyl) propionic acid
Sinapic acid
P = 0.0376
CFU/mL


D
CTR
CA
MeOH30%_DMSO10%
MeOH25%_DMSO25%
MeOH50%_DMSO50%
DMSO100%
P = 0.0003
CFU/mL


E
CTR 6.79 6.77 6.79
CA 6.62 6.57 6.58
Luteolin 6.82 6.82 6.83
Resveratrol 6.71 6.73 6.73
Eriodictyol 6.64 6.70 6.70
Catechol 6.66 6.65 6.65
3-(4-hydroxyphenyl) propionic acid 6.61 6.60 6.61
Sinapic acid 6.54 6.65 6.64
MeOH30% + DMSO10% 6.54 6.65 6.63
MeOH25% + DMSO25% 6.57 6.65 6.63
MeOH50% + DMSO50% 6.80 6.77 6.73
DMSO 100% 6.82 6.77 6.74
MFC 7.02 7.03 7.02
T0
T8
T12
7.4
7.2
7.0
6.8
6.6

To compare the effectiveness of the flavonoid and non-flavonoid compounds, choline metabolism rate and TMA production rate were calculated as described previously in Chapter 2 and Chapter 3. In **Figure 5-17** panel **A**, none of the controls containing different percentages of solvents had a significant effect on choline metabolism rate (µM/h) compared to control. Sinapic acid showed significantly lower rate of choline metabolism ($p = 0.0259$), as well as eriodictyol, luteolin and resveratrol (all $p < 0.0001$). Some compounds showed significantly lower TMA production rate (luteolin, resveratrol, eriodictyol and CA control, $p < 0.0001$), but catechol significantly increased the rate of TMA production compared to non-treated control ($p < 0.0001$). Controlled conditions supplemented with mixtures of solvents were also effective in reducing TMA production rate (100% DMSO, 50% MeOH/50% DMSO, and 25% MeOH/25% DMSO) with p values reported in panel **A**. However, these changes in TMA production rate for solvent controls did not result in significantly higher maximum TMA produced from choline, as seen in panel **B**, and did not significantly affect the final choline concentration compared to control. All compounds except for catechol significantly decreased the maximum TMA produced from choline and eriodictyol, luteolin and resveratrol also inhibited choline metabolism which resulted in significantly higher final choline concentration for these three compounds.

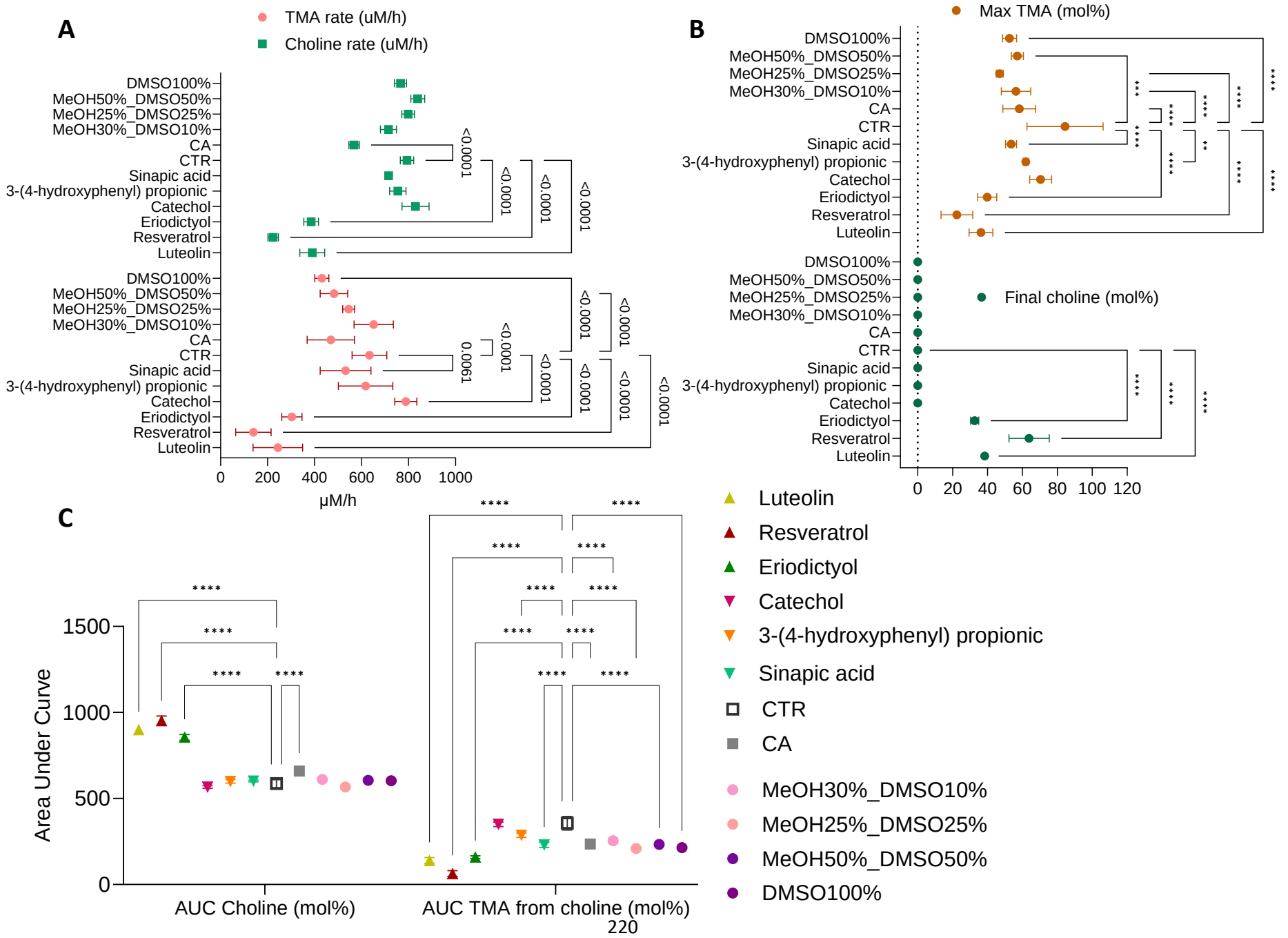
Panel **C** in **Figure 5-17** shows the AUC calculated from choline metabolism and TMA production kinetic curves. Luteolin, eriodictyol and resveratrol showed significantly higher AUC of choline metabolism compared to control as well as AUC of TMA production (all $p < 0.0001$). Non-flavonoid compounds sinapic acid and 3-4HPPA resulted in a significant reduction of AUC for TMA concentration, but similar effects were observed with all solvent supplemented conditions, making it difficult to determine if the effect was due to the presence of solvents or phenolic compounds.

Figure 5-17 The effect of phenolic compounds on choline metabolism and TMA production

***A**) The rate of choline metabolism and the rate of TMA production (µM/h) for CMHT cultures enriched with phenolic compounds, solvent controls and untreated control. P values and interactions were calculated using Two-way ANOVA and with Dunnett's test of multiple comparisons and p values are annotated on the graph.*

***B**) The maximum TMA produced from choline (mol%) and final choline abundance (mol%) in CMHT cultures. P values and interactions were calculated using Two-way ANOVA with Šídák's multiple comparisons test and p values are denoted on the graph as asterisks where ** p<0.01, ***p<0.001, ****p<0.0001.*

***C**) Areas were calculated from kinetic curves displayed and described in **Figure 5-15**. P values and interactions were calculated using Two-way ANOVA and with Dunnett's test of multiple comparisons and p values are annotated on the graph as asterisks where ****p<0.0001.*

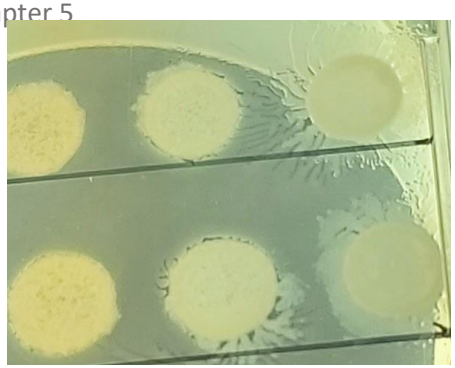

A
TMA rate (uM/h)
Choline rate (uM/h)
DMSO100%
MeOH50%_DMSO50%
MeOH25%_DMSO25%
MeOH30%_DMSO10%
CA
CTR
Sinapic acid
3-(4-hydroxyphenyl) propionic
Catechol
Eriodictyol
Resveratrol
Luteolin
<0.0001
0.0061
0 200 400 600 800 1000
µM/h
B
Max TMA (mol%)
Final choline (mol%)
0 20 40 60 80 100 120
C
Area Under Curve
1500
1000
500
0
****
AUC Choline (mol%)
AUC TMA from choline (mol%)
220
Luteolin
Resveratrol
Eriodictyol
Catechol
3-(4-hydroxyphenyl) propionic
Sinapic acid
CTR
CA
MeOH30%_DMSO10%
MeOH25%_DMSO25%
MeOH50%_DMSO50%
DMSO100%

There were no significant adverse effects of phenolic supplementation on bacterial growth (reported in **Figure 5-16**), however, there were some observed changes in colony morphology shown in **Figure 5-18**. This figure shows sections of Wilkins Chalgren agar plates inoculated with a serially diluted fermentation cultures sampled at 8 h. The Miles and Misra method was used to enumerate total viable count of anaerobic bacteria and there were distinct differences between some conditions observed in more concentrated dilution droplets (dilutions 10^{-1} (most right) to 10^{-4} (most left) are displayed for most of plates). For instance, a large swarming pattern was observed in the control condition, also apparent in the chlorogenic acid supplemented cultures. This growth pattern was inhibited with some of the solvent controls (25% DMSO + 25% MeOH, 10% DMSO + 20% MeOH), but some swarming activity was still observed with 50% DMSO + 50% MeOH and 100% DMSO. For the phenolic compounds, sinapic acid showed low definition of individual colonies in 10^{-4} dilution compared to eriodictyol that showed more distinct separate colonies yet showed visible halos around the most concentrated dilution. The swarming pattern was very apparent in 3-4HPPA, spanning available space between individual droplets and surrounding more concentrated dilutions but merging with others. Catechol supplemented cultures did not show visible swarming but many mixed colonies in 10^{-4} dilution were overlapping and formed small circular halos, appearing 'fuzzier' around the edges. Luteolin only showed swarming border in 10^{-1} dilution and other droplets of dilutions did not show this mixed colony morphology.

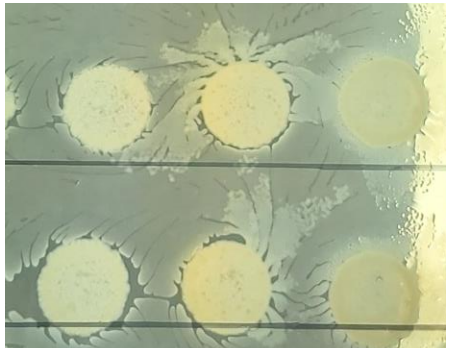
Assessing mixed colony morphology, counting viable colonies, and measuring pH of fermentation cultures enriched with phenolic compounds contributed to elucidating the possible modes of action behind the effects of plant polyphenols on inhibiting TMA production from choline. However, to identify and clarify the mechanisms behind these effects and understand how they could be utilised in clinical settings warrants further research.

Figure 5-18 Sections of Wilkins Chalgren agar plates of CMHT cultures

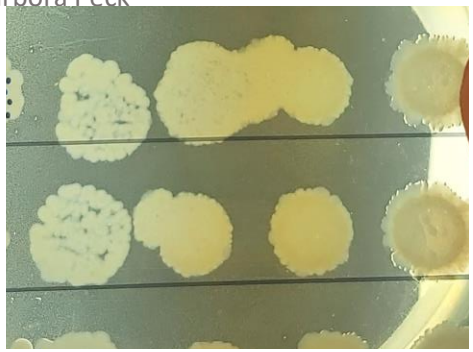
Sections of Wilkins Chalgren agar plates inoculated with a serially diluted fermentation cultures sampled at 8 h using the Miles and Misra method. Sections show more concentrated dilution droplets (dilutions 10^{-1} (most right) to 10^{-4} (most left) for most plates. Each dilution was plated in triplicate per condition. Pictures were taken after the end of the incubation period against a light from colony counter and cropped in PowerPoint to capture at least two replicates from of first three dilutions. All raw photo files are available in the online repository.



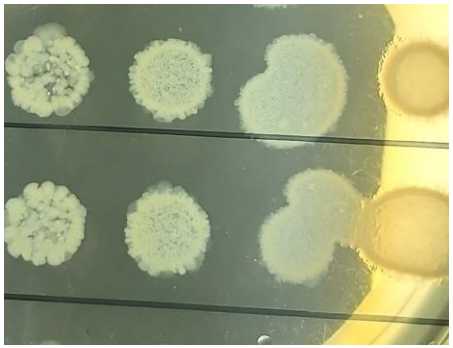
Control



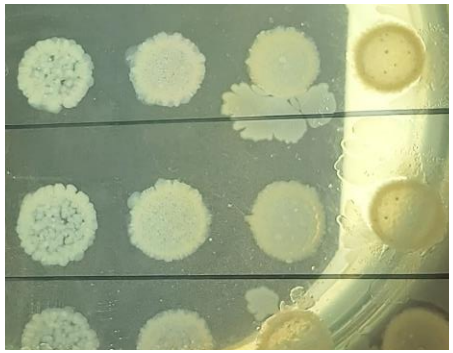
Chlorogenic acid



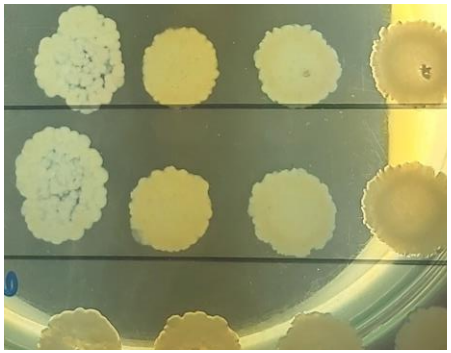
Luteolin



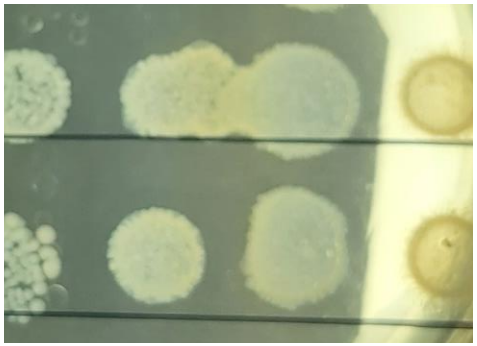
Catechol



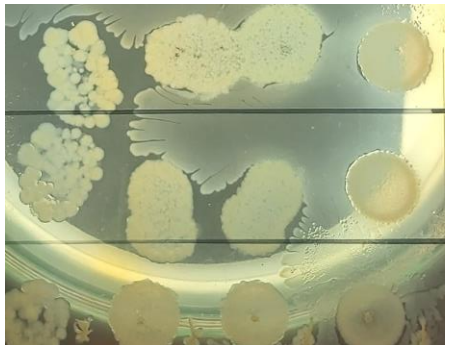
100% DMSO



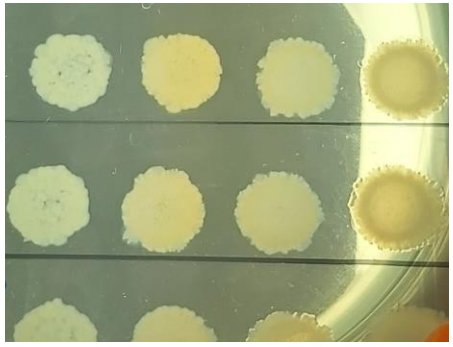
25% DMSO + 25% MeOH



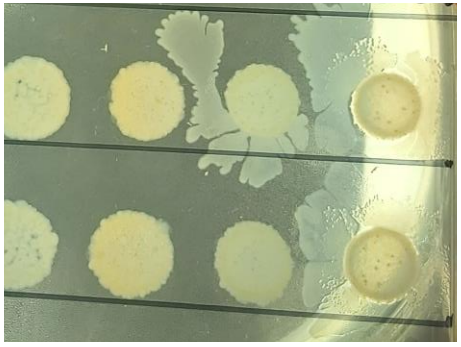
Resveratrol



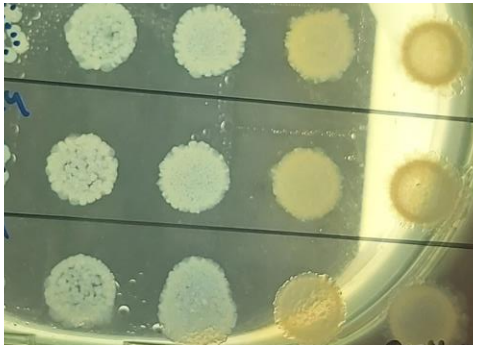
3-4HPPA



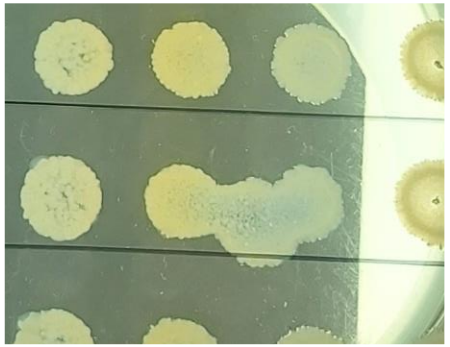
10% DMSO + 30% MeOH



50% DMSO + 50% MeOH



Eriodictyol



Sinapic acid

DISCUSSION

The aim of this work was to identify an effective inhibitor of choline metabolism into TMA and determine the mechanism behind its inhibitory properties. Analysing available literature to select polyphenols for screening their ability to reduce TMA production from choline resulted in identification of several compounds. The identified compounds included berberine, capsanthin or allicin, yet this strategy lacked a systematic approach. Exploring the literature to understand the trends in strategies used for inhibition of choline metabolism to TMA was very useful to identify gaps in evidence and take an alternative approach from others. The approach taken in this thesis was testing individual compounds that are part of effective foods rather than choosing a type of phenolic-rich source and investigating what compounds may be the key players. Furthermore, focusing on pure compounds without sugar moieties allowed for investigation of the structural properties that may be crucial for inhibitory activity of phenolic compounds. Similar approach was taken by Iglesias-Carres and colleagues who tested the inhibitory capacity of compounds found in tea, coffee, and cocoa (228).

Selected compounds included nearly 30 different phenolic acids with a various degree of hydroxylation, methylation, and different lengths of aliphatic chains. These structural properties were considered when testing the inhibitory effects of compounds on choline metabolism to TMA. The presented findings in this chapter confirmed that more complex compounds elicit stronger inhibitory capacity. One possible mechanism behind this effect is that more complex compounds take more time and energy to be broken down by bacterial enzymes, maintaining inhibitory activity for longer. The breakdown of complex polyphenols by the gut microbiota into phenolic acid and other smaller phenolic compounds has been established in numerous studies (141, 165, 166, 171, 176, 309-312). However, the rate of this breakdown is dependent on different factors such as the type of polyphenol, its bioavailability, the food matrix that it is part of, but also some host-dependent functions like enzymatic activity and the composition of the gut microbiota (313-315). Therefore, it is to be expected that the inhibitory capacity of individual polyphenols is majorly influenced by the gut microbial composition of the host, exhibiting large inter-individual variation in the effectiveness of polyphenols as inhibitors of choline metabolism to TMA.

To further test the hypothesis that more complex compounds elicit a stronger inhibitory response of choline metabolism to TMA, 6 representative flavonoid compounds tested in this study (catechin, quercetin, luteolin, 3-hydroxydaidzein, cyanidin and eriodictyol), together with stilbene resveratrol, were among the most effective inhibitors of choline metabolism, particularly luteolin, resveratrol and eriodictyol. In fact, eriodictyol was chosen for further

analysis to determine its mode of action and for testing its impact on bacterial growth in different doses of this compounds, however, this work was not finalised due to limited time and these findings are not included in this thesis. Planned future work included a multi-omics investigation into the potential of eriodictyol to modulate choline metabolism and TMA production both in complex microbial matrices of faecal samples and in single strain model of TMA-producing bacterium to investigate its impact on gene expression and elucidate the modes of action. However, a more in-depth analysis of one of the phenolic compounds was undertaken since one of the aims of this chapter was to use chlorogenic acid as a positive control in fermentation assays. Chlorogenic acid was one of the compounds reported to inhibit choline metabolism in pooled faecal slurries by Iglesias-Carres *et al.* (228). Their findings on the effectiveness of chlorogenic acid were reproduced in this thesis and the ability of chlorogenic acid to inhibit TMA production in higher doses of supplemented choline was confirmed. There were inter-individual differences in the inhibitory response of CA on choline metabolism, most likely stemming from the ability of the microbiota to degrade chlorogenic acid into compounds that show lower inhibitory activity towards choline metabolism.

Furthermore, this process would support the hypothesis that the inhibitory activity of chlorogenic acid is dependent on the microbial composition of the fermentation sample, as it can be broken down into smaller compounds that are no longer effective inhibitors of choline metabolism. In a study by Ma *et al* (316) the proposed microbial degradation of chlorogenic acid by *Sphingomonas* sp strain resulted in caffeic acid and quinic acid as the first metabolites that were then further degraded into protocatechuic acid and shikimic acid. Another report investigated chlorogenic acid metabolites *in-vivo*, identifying caffeic, dihydrocaffeic and m-hydroxybenzoic acids as its breakdown products in rats (317). The microbial degradation of chlorogenic acid has previously been investigated using *in-vitro* colon models resulting in generation of dihydrocaffeic acid, dihydroferulic acid, and 3-(3’-hydroxyphenyl)propionic acid, which accounted for 75–83% of the total catabolites from CA fermentation.

In this thesis, some of the reported breakdown products of chlorogenic acid were tested during the screening of phenolic acids. Structurally similar 3-(4’-hydroxyphenyl)propionic acid was found to significantly affect maximum TMA produced from choline but had no significant effect on choline metabolism rate or TMA production rate compared to untreated control. Dihydrocaffeic acid showed no effect on TMA concentration compared to control but slightly delayed choline metabolism at 6 h. Dihydroferulic acid (also known as 3-(4-Hydroxy-3-methoxyphenyl)propionic acid) was not included in the screening of phenolic compounds but future work should consider testing methylated derivatives of (hydroxyphenyl)propionic acids

and test if this compound was detected by LC-MS full scan analysis as one of the breakdown metabolites of chlorogenic acid as a result of microbial degradation.

Furthermore, supplementing CA and DMB into a single strain model of TMA-producing bacterium revealed some potential mechanisms of action. For instance, *P. mirabilis* growth was significantly inhibited by the phenolic compound yet still grew to an adequate amount that would likely result in choline degradation. Therefore, we could speculate that the mechanism of action could be related to CA binding to the enzyme and preventing choline to be cleaved into TMA by the choline-TMA lyase. However, it could have also been caused by altering gene expression and preventing translation of the *cutC* gene encoding enzyme, resulting in inhibition of choline metabolism. To determine if that was the case, future work should investigate the transcriptome response to supplementing phenolic compounds into TMA producing strains or measure the enzymatic activity by lysing bacterial cells and supplementing polyphenols directly into cell-free extracts.

It should be noted that the purpose of the screening of the phenolic compounds was to identify structural impacts on inhibitory activity. However, this work would benefit from testing this activity in multiple donors to determine if these structure-dependent effects are reproducible across multiple microbial matrices. Furthermore, an in-depth analysis of possible cytotoxic effects or pH altering properties was carried out only for a subset of compounds that showed an effect, while many of the screened compounds could have showed a decrease in TMA production due to modulation of the gut microbiota. Therefore, if the experiments were to be repeated, bacterial growth through plating or optical density should be assessed to determine if inhibition of metabolism is due to lower bacterial abundance. However, these experiments were extremely laborious despite the high-throughput format of the *in-vitro* colon model, rendering any further analysis alongside these experiments very challenging. The number of samples generated per timepoint that had to be analysed for choline and TMA concentration also contributed to the limitations of this work.

Evaluation of bacterial viable counts in faecal samples following polyphenol supplementation revealed that most compounds did not exhibit cytotoxic effects towards total anaerobic bacteria. However, enumerating total anaerobes might not provide sufficient insight into the specific inhibitory effects towards TMA-producing bacteria, therefore, effective polyphenols should be incubated in single strain model of TMA producing bacterium, like what was performed with chlorogenic acid in this study. Previous studies reported antimicrobial properties of polyphenols, capable of selectively inhibiting the growth of specific bacterial species or strains while promoting the proliferation of beneficial bacteria (304, 318, 319). Thus, the outcomes of this analysis could provide insights into the potential of polyphenols to

modulate gut microbiota composition towards a healthier profile, and even specifically target TMA-producing commensals in the gut. The effects of polyphenol compounds on the colony morphology should also be considered when assessing the anti-microbial effects of polyphenol compounds. The changes in morphology could be reflective of selective inhibitory effects towards certain bacteria and therefore changing the absolute abundance of other microbes. These effects could be assessed by performing metagenomics analysis of these samples before and after phenolic supplementation. The inhibitory effects of polyphenols are dependent on the structure and function of the gut microbiota, as shown with chlorogenic acid and its ability to be degraded by *D. desulfuricans* and pooled faecal samples, but not by *P. mirabilis*. Future work should include analysis of microbial community and identifying what species and genera might be responsible for CA degradation. This could be determined by detection of breakdown products in the metabolome of complex microbial samples and upregulation of bacterial genes expressed in response to CA supplementation, indicating the responsible enzymes involved in the CA breakdown by individual TMA-producing strains.

Additionally, assessment of colon model pH over time in response to polyphenol supplementation in non-externally buffered models may elucidate whether alterations in pH contribute to the observed inhibition of microbial metabolism. Polyphenols can exhibit pH-modulating effects due to their acidic nature, or by providing additional carbon source from the catabolism of polyphenols, releasing hydrogen ions as a by-product of fermentation, and decreasing pH (144, 306, 311, 320, 321). Either way, this mechanism may potentially create an environment less favourable for TMA-producing bacteria or cause an inhibition of choline metabolism due to low pH. This effect has been observed in this thesis (in Chapter 4 with pH adjusted and non-adjusted probiotic supernatant) and in our research group, but the mechanisms behind that effect have not been explored. Therefore, changes in pH levels could serve as an indicator of the mechanisms underlying polyphenol-mediated inhibition of TMA production and should be measured.

Overall, future work into the effects of polyphenols on microbial metabolism of choline and other TMAO precursors is needed for designing novel dietary interventions for reducing TMAO-associated cardiovascular risks. The outcomes of this study and the follow-up investigation may inform the development of personalized dietary strategies aimed at modulating the gut microbiome toward a lower TMA-producing phenotype.

CONCLUSION

This chapter identified effective polyphenols able to inhibit TMA production from choline after selecting an approach to test individual pure compounds based on gaps in published literature.

Screening of individual compounds revealed the importance of structural properties of phenolic compounds in inhibitory activity. The findings revealed that more complex polyphenolic compounds exhibit stronger inhibitory capacity on choline metabolism to TMA, dependent on the inter-individual variation in gut microbiota composition. The inhibitory effects of chlorogenic acid were confirmed in a complex faecal matrix and in individual TMA-producing bacteria, however, the effect was dependent on the microbiota-induced breakdown of chlorogenic acid into less effective compounds, limiting its efficacy.

Further research is necessary to validate these findings across multiple donors and explore molecular mechanisms. This includes examining the inter-individual differences in polyphenol metabolism and the impact of polyphenols on gene expression and enzymatic activity in TMA-producing bacteria. Additionally, understanding the potential cytotoxic effects and pH-modulating properties of polyphenols will provide deeper insights into their inhibitory mechanisms, aiding a more personalised approach based on the resident gut microbiota. Overall, this study offers a foundation for developing personalized dietary interventions to modulate the gut microbiome and potentially reduce TMAO-associated cardiovascular risks.

Chapter 6

General discussion

SUMMARY OF MAIN FINDINGS

The overall aim of the research in this thesis was to investigate the relationship between TMAO status of individuals and their capacity to produce TMA based on their gut microbial composition, and how this knowledge can help develop strategies for modulating microbial TMA production from choline using probiotics and polyphenols. The main findings are summarised as follows:

- Measuring TMAO in plasma and urine in human study cohort revealed good correlation between plasma and urinary TMAO levels in individual samples, with occasional elevated TMAO concentrations. No individuals had consistently elevated TMAO levels, but many showed high variation in TMAO levels leading to identification of distinct stable and changeable groups, determining their TMAO status.
- Testing the hypothesis that the capacity to form TMA from choline by the gut microbiota correlates with TMAO status uncovered no relationship between plasma and urinary TMAO levels and capacity for conversion of choline to TMA measured using *in-vitro* fermentation colon models.
- Metagenomic analysis revealed similar gut microbiota compositions among most participants with different ratios of major genera, except for BERI 26, who had low microbial diversity but similar TMAO responses to others.
- Relationships were identified between specific microbial species and both *in-vivo* TMAO levels and *in-vitro* TMA production. Most participants had a similar abundance of key genes encoding enzymes for TMA formation/degradation, showing little correlation between these enzymes and *in-vivo* TMAO status.
- Choline-TMA lyase was not particularly abundant and showed a weak yet significant correlation with plasma TMAO concentration but not with urinary TMAO levels. Capacity to produce TMA from choline *in-vitro* correlated with several EC numbers and specific protein families.
- The findings did not support the hypothesis that the mixed-strain Bio-Kult supplement effectively reduces TMA production from choline in a complex faecal matrix of 7 individual donors using *in-vitro* human colon models.
- Some individual Bio-Kult strains and different doses of the supplement tested in a pooled faecal sample suggested that optimisation of the mixture could have an impact on TMA production capacity.

- Screening for effective polyphenols that inhibit TMA production from choline by testing individual pure compounds revealed the importance of structural properties of phenolic compounds in inhibitory activity.
- More complex polyphenolic compounds exhibited stronger inhibitory capacity on choline metabolism to TMA, with effects dependent on individual gut microbiota composition. The inhibitory effects of chlorogenic acid reported in published literature were confirmed but were limited by microbial metabolism into less effective compounds.

IMPACT AND IMPLICATIONS

One of the aims of this thesis was to investigate changes in plasma and urinary TMAO levels over time to establish TMAO status of participants in the BERI study cohort. There were no individuals in the BERI cohort that had consistently elevated TMAO concentration, but some participants displayed high variation in their TMAO levels. This led to determining TMAO status based on the stability and variability of plasma and urinary TMAO measurements over time, rather than stratification of these continuous variables or dichotomisation into high and low producers of TMAO. This approach resulted in a distinct stable and changeable groups of participants based on their coefficient of variation of TMAO levels measured at the six independent occasions.

This is a novel approach of differentiating participants based on their TMAO status and has the potential to assess the relationships between the variation of TMAO levels and the structure and function of the gut microbiota. Using a median or a mean value to categorise a participant would limit the observations and would most likely result in average plasma TMAO levels reported by others in a healthy population. Indeed, this study highlights the importance of measuring TMAO concentration at multiple separate occasions if it is to be used as a marker or a prognostic determinant of disease. Moreover, the benefit of assessing both urinary and plasma TMAO levels and the abundance of their precursors *in-vivo* gave an insight into the complexity of the multi-organismal mechanism behind TMAO production.

To understand the relationship of TMAO status with the capacity to produce TMA determined by the gut microbiota form and function, the microbial metabolism of TMAO precursor choline was investigated. The faecal microbiota was tested for ability to metabolise choline using *in-vitro* fermentation colon models, and production of TMA was measured over the incubation period to establish the rates of substrate metabolism and the percentage of conversion of choline into TMA. Despite the different outputs of this investigation determining the capacity to produce TMA from choline by individual participants, this analysis also revealed that most

participants were able to metabolise choline. The inter-individual differences arose from the maximum TMA percentage that was produced from choline and the time it took to metabolise it. Perhaps due to these differences, testing the hypothesis that the capacity of the gut microbiota to form TMA from choline correlates with TMAO status revealed no relationship between plasma and urinary TMAO levels and *in-vitro* conversion of choline to TMA. This could mean that choline was not the primary substrate responsible for plasma TMAO levels or that multiple substrates were consumed that resulted in some elevated measurements of plasma and urinary TMAO in some individuals.

Furthermore, the relationship between the *in-vitro* metabolism of choline and the levels of other precursors in plasma, urine and faecal samples has not been previously explored and these associations should be further tested for their correlations with the capacity to produce TMA and other metabolites from other TMAO precursors. This could be performed using *in-vitro* colon models or employing *in-silico* models to simulate substrate metabolism by microbial communities determined by sequencing technologies (322).

Metagenomic analysis showed most participants had a similar gut microbiota composition, except for BERI 26, who showed a low diversity and overall density of microbes yet had similar *in-vivo* and *in-vitro* TMAO response to others. Relationships were found between specific microbial species and both *in-vivo* TMAO levels and *in-vitro* TMA production suggesting their contribution to these processes or their influence on the microbiota function. Most participants had similar abundance of key genes encoding enzymes for TMA formation and degradation, with enzymes like betaine reductase and sarcosine/dimethylglycine N-methyltransferase being abundant across the BERI cohort. Other enzymes were transiently present in the cohort and their presence was correlated with the abundance of some of the major species. Choline-TMA lyase was not particularly abundant and showed a weak yet significant correlation with plasma TMAO concentration but not with urinary TMAO levels.

The capacity to produce TMA from choline measured *in-vitro* was correlated with several EC numbers, suggesting that more than just choline-TMA lyases might be responsible for the inter-individual differences in choline metabolism rate or maximum percentage of TMA produced from choline. The correlations of betaine reductase with both TMA production rate and choline metabolism rate could indicate possible role of this enzyme in determining the capacity to produce TMA from choline but this process would have to be further investigated as our previous research did not show activity of this pathway (4, 323). Protein families involved in methylated amine transport like betaine/carnitine/choline transporter or choline kinase were highly abundant, and many EC numbers correlated with species presence. This study provides the most in-depth analysis of the abundance of pathways contributing to TMA

production and utilisation in a human cohort, together with insights into their correlation with TMAO status and capacity to produce TMA *in-vitro*. However, it is important to remember that gene presence does not always equate to enzymatic activity, therefore these interactions should be further explored using *in-vitro* models. This multi-omics approach opens doors for targeted interventions aimed at modulating gut microbiota to influence TMAO levels and associated cardiovascular outcomes.

The investigation of different strategies for their ability to modulate TMA production identified probiotics and polyphenols as possible targets. Testing the efficacy of the mixed-strain probiotic supplement Bio-Kult in reducing TMA production from choline underscores the challenges in developing effective dietary interventions to modulate the gut microbiota. Number of different control measures were employed to determine the efficacy of the supplement and the strains within it. The findings suggest that optimising the composition of the probiotic supplement may lead to finding an effective strategy for influencing TMA production capacity, contributing to future development of microbiota-targeted interventions. Furthermore, utilising single-strain models of TMA-producing bacteria could enlighten the mechanisms behind the effects of probiotics which has been identified as a crucial aspect limiting the application of microbiome-targeted strategies for decreasing TMAO levels (108, 109, 208, 209, 324). This novel approach to exploring the impact of probiotics on TMA production could lead to the development of personalised approaches to ameliorate cardiovascular risk.

The screening of polyphenols for their inhibitory effects on TMA production from choline highlights the potential of dietary compounds to modulate TMAO levels. The structural properties of phenolic compounds and their differential effects on TMA production highlight the importance of assessing individual purified compounds that are contained within effective extracts, powders, and other phenolic-rich sources. This work contributed to the available evidence from interventions focused on dietary components and other foodstuffs with complex matrices, elucidating the role of the structural properties of phenolic compounds in their efficacy to reduce production of TMA. These findings could be a stepping stone to developing targeted interventions using both purified compounds and extracts with high content of polyphenols, aiming to translate these findings into *in-vivo* effect on TMAO production.

In conclusion, these research findings offer novel insights into the complex relationship between TMAO levels of individuals, their gut microbiota composition, and the effectiveness of dietary interventions in modulating TMA production. This sets a foundation for future research investigating the feasibility of using microbiota-targeted therapies to reduce

production of TMA, contributing to finding a sustainable treatment for elevated TMAO levels in at-risk individuals.

LIMITATIONS AND FUTURE RESEARCH

This study had several limitation arising from the used methodology, shortage of time and the broad scope of the project. There were multiple limitations connected to the use of *in-vitro* colon models for studying the metabolism of dietary components like choline by the human gut microbiota. *In-vitro* models may not fully capture the complexity of the gut microbiota as only the culturable microbes can proliferate successfully, obstructing the insight into the metabolism of methylated amines that occurs *in-vivo*, leading to an oversimplification or misrepresentation of the metabolism by the gut microbiota. The diversity and variability of gut microbial populations in different individuals detected through sequencing methods may not be translated into functional differences detectable by *in-vitro* models due to this reductionistic effect.

It is also important to keep in mind the difficulty of conducting reproducible and repeatable colon model experiments with such high number of factors influencing the process. Despite the batch nature of the models used in this study, many factors can impact individual fermenters, increasing variation between replicates of the same treatment or repeated fermentation of the same sample. These include factors such as pH, oxygen levels, dilution factor, homogeneity of the starter material, and nutrient availability of the inoculated sample. These environmental factors shape the metabolic activities of the gut microbiota and have a direct influence on many of the characterising variables used to determine the *in-vitro* capacity to produce TMA from choline.

These experiments are also not representative of the dynamic nature of the gut and the feeding habits associated with human diet. Food is eaten in meals, not dietary components and therefore the *in-vitro* responses to nutrient challenges have limited translatability into responses *in-vivo* where the microbiome-host interactions increase the complexity. Similar to this, any dietary interventions explored *in-vitro* are limited by the short duration of the experiments, decreasing the exposure that may be necessary to elicit changes or result in lasting effects. Furthermore, *in-vitro* models used in this study are limited by the starting conditions used for fermentation of dietary components. These may not represent the spatial distribution of microbial populations along the different regions of the colon as they mimic the conditions of the distal colon and are inoculated with faecal samples.

Moreover, the complexity of investigating gut microbiota-targeted agents in different colon models was highlighted during testing of probiotics and polyphenols on their effect on TMA production. This investigation showed that multiple factors can affect the performance of potential probiotic strains and these findings should be interpreted with caution. Further research into the mechanisms of action of polyphenols and probiotics is paramount to design effective strategies that can be investigated in human interventions. Overall, these limitations restrain the impacts of the findings from this study and further research with improved methodologies is crucial for enhancing the applicability of *in-vitro* models in studying choline metabolism by the human gut microbiota.

On the same note, the dataset originating from samples from the BERI study brings with it its own set of limitations related to human study interventions that have been discussed at length in published literature (103, 111, 114, 187, 208, 210, 325-328). One of the main issues highlighted is the fact that this study was not designed to investigate the changes in TMAO levels and their relationship with the gut microbiota form and function. This makes it challenging to compare results to other studies that were specifically designed with these outcomes in mind and may result in lack of definitive conclusions arising from this study. For instance, the effect of dietary habits on TMAO levels must be considered when designing studies and collecting samples, and dietary intake of TMAO precursors should be carefully controlled for accurate assessment. This would improve the ability to determine the effectiveness of different dietary strategies on reducing TMA production. Evaluation of studies is very important for identifying gaps in findings and designing future work that needs to be carried out to be able to make clear conclusions. The following research should be considered to overcome the limitations of this thesis and further investigate the effects of polyphenols and probiotics on TMA production:

- Screening of faecal sample donors for their capacity to produce TMA and/or their TMAO status would allow for a more targeted investigation of how these individuals respond to supplementation of probiotics and polyphenols.
- To minimize the impact of factors influencing TMAO levels prior to measurements, intake of TMAO precursors should be limited or at least controlled/replicated for repeated measurements of plasma and urinary TMAO levels. This could involve collecting a 3-day food diary prior to each study day to establish both habitual intake of TMAO substrates and account for increased fasting TMAO levels due to consumption of TMAO precursors.
- Reduction of TMA production by single probiotic strains, mixtures of different proportions and their supernatants should be further investigated in single-strain

models and in complex microbial matrices from multiple donors as the impact of probiotic strains is often dependent on the existing microbiota of the host. The differences in microbiota before and after intervention should be used to determine possible factors leading to the efficacy of various probiotic formulations.

- Future work should also investigate the effect of probiotics on several other outcomes, such as their persistence in cultures after multiple rounds of faecal inoculation, their effects on expression of *cutC* gene in *P. mirabilis*, the ability of supernatant to alter swarming differentiation of *P. mirabilis* cells or alter the colony morphology. Other outcomes such as the metabolomic profile of spent supernatant should be investigated to elucidate the mechanisms at play.
- The impact of polyphenols on changing microbial signatures in colon model fermenters should be investigated, together with the transcriptome of single TMA-producing bacterium supplemented into faecal inoculum from individual donors.
- Further research is necessary to investigate the effectiveness of polyphenol supplementation across multiple donors and determine the role of inter-individual differences in polyphenol metabolism on their TMA inhibition capacity. The breakdown products of the effective treatments should be quantified and tested to determine if their lack of effect stems from lower concentration or their structural properties.
- Understanding potential cytotoxic effects towards the TMA-producing members of the gut microbiota will provide deeper insights into their mechanisms of action, contributing to the development of personalized approaches based on the resident gut microbiota.

Overall, this research raised solid arguments for the importance of determining TMAO status in individuals and proposed novel approaches for reducing TMA production from choline that should be further investigated to establish a sustainable treatment for elevated TMAO levels in individuals at-risk.

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Appendices

APPENDIX I: METHOD DEVELOPMENT

I.I Optimisation of the batch colon model for use with faecal glycerol stocks

The batch colon model was optimised for investigation of the microbial metabolism of methylated amines by Day-Walsh *et al.* (4) using fresh faecal inoculum. To utilise this model with faecal glycerol stocks, the ability of glycerol-preserved microbiota to metabolise choline was investigated. A frozen glycerol stock was used to inoculate colon model vessels supplemented with choline to reach a final 1% faecal concentration. An autoclaved glycerol stock was used to inoculate choline supplemented control vessel to measure if any non-biological choline disappearance resulted in TMA production. The results of this optimisation step are displayed in **Figure S1**. This figure demonstrates that choline was gradually metabolised over the 24 h of the experiment in the control vessels (glycerol stock with 2 mM choline – dark green line), however, only ~40% TMA from choline was produced within that timepoint. In the autoclaved control where choline was incubated with inactivated glycerol stock (autoclaved for 15 min at 121 °C), choline abundance decreased ~ 20% by 24 h yet no TMA was produced in the vessel (dark grey line close to the x axis).

The utilisation of background choline present in the faecal sample and its conversion to TMA was explored in a 'Blank control' (no choline was supplemented) from a fresh faecal sample and its equivalent glycerol stock. Inoculation was performed as previously described and blank vessels were compared with choline inoculated vessel that had background TMA coming from supplemented choline. TMA decreased in the autoclaved control, suggesting that some spontaneous breakdown of TMA might have occurred (as there is no live bacteria present). Alternatively, this decrease in TMA concentration was caused by dilution of the culture due to buffering with acid and alkali and was not corrected by the equation accounting for volume change over incubation period to calculate concentrations of metabolites in colon model vessels. This background TMA was not detected in the blank vessels that did not have added choline as displayed in **Figure S2** In blank vessel inoculated with fresh faecal sample, background choline concentration decreased over 18 h and resulted in equimolar increase in TMA concentration. This was not the case in the vessel inoculated with a glycerol stock version of the sample (Blank GS) where choline concentration slightly decreased but did not result in any TMA production.

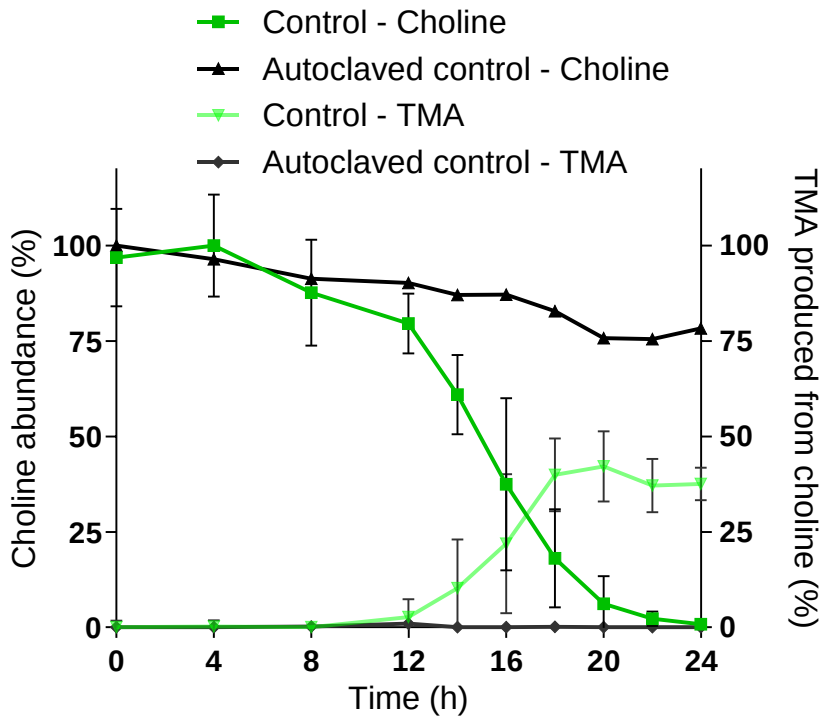


Figure S1 Batch colon model metabolism of choline into TMA by gut microbiota from a faecal glycerol stocks

Choline abundance (%) and TMA produced from choline (mol%) in glycerol stock inoculated control (average of 2 vessels for 2 different samples of the same donor) compared to autoclaved control with inactivated microbiota and 2 mM choline. Samples were collected at T0, 4, 8, 12, 14, 16, 18, 20, 22 and 24 h. Vessels were incubated at 37ºC under constant mechanical stirring, at pH 6.6-7.2 and continuously sparged with N_2 to maintain anaerobic conditions.

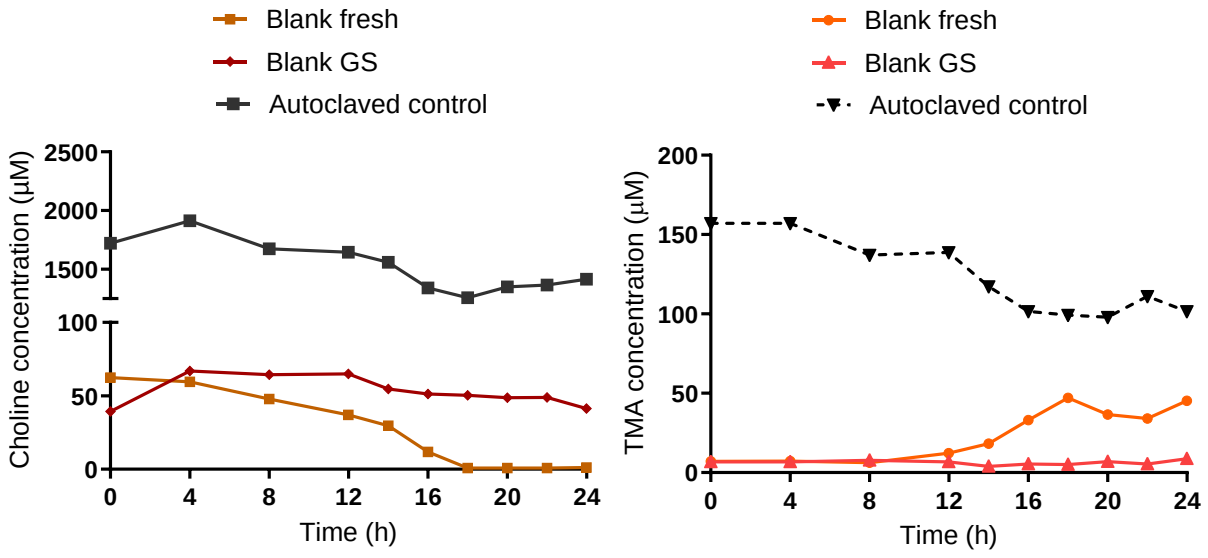


Figure S2 Batch colon model metabolism of background choline into TMA and utilisation of TMA added as a fraction of choline supplemented into inactivated faecal microbiota

Choline concentration (µM) on the left and TMA concentration (µM) on the right-side showing fermentation of choline present in faecal sample – using a blank vessel that had no choline added either to fresh faecal sample or its glycerol stock equivalent. Choline breakdown in autoclaved control was measured (same control as displayed in ***Error! Reference source not found.****, only shown as concentration instead of percentage) in ~2 mM choline supplemented vessel. Samples were collected at T0, 4, 8, 12, 14, 16, 18, 20, 22 and 24 h. Vessels were incubated at 37ºC under constant mechanical stirring, at pH 6.6-7.2 and continuously sparged with N_2 to maintain*

To explain the increased TMA concentration in the Autoclaved control vessel, previous analysis revealed that there is about 5-10% of TMA present in supplemented choline due to either spontaneous breakdown of choline or some other non-biological process as no known bacteria have been able to metabolise choline into TMA in aerobic conditions (how choline substrate was stored). Furthermore, as demonstrated by Day-Walsh *et al.* cited above, this contamination of choline was detected also when choline was dissolved and measured in PBS, and later in other types of media used for choline fermentation. More details are available in the section about quantification of metabolomics data in **section 2.27**.

I.II Optimisation of the growth of Bio-Kult strains to enable inoculation and enumeration of viable cells

To enable inoculation of individual Bio-Kult strains into faecal cultures to observe their effect on TMA production, the characteristics of their growth and the best timepoint to harvest cells for inoculation needed to be established. The individual strains that were further explored were supplied by ADM Protexin in freeze dried form (8 out of 14 strains selected based on availability at the time of investigation). Strains were initially grown to estimated exponential phase in MRS and preserved in cryovials as glycerol stocks, which were used throughout this experimental work. More information on the reagents, consumables, and growing conditions available in Methods. The information about the individual strains is reported in **Table S1** and these strains are predominantly referred to by their strain number. All strains were reported to lack the *cutC* genes that encode TMA lyases involved in the metabolism of choline to TMA.

Table S1 Presumptive probiotic strains from the Bio-Kult supplement used for exploration of single strain effects.

Strain number	Strain name	Protexin® ID
1	*Lactobacillus acidophilus* R20001	PXN® 35™
2	*Lactobacillus paracasei* R20022	PXN® 37™
3	*Lactobacillus rhamnosus* R20031	PXN® 54™
4	*Bifidobacterium longum* R20008	PXN® 30™
5	*Lactobacillus plantarum* R20125	PXN® 47™
6	*Bifidobacterium bifidum* R20032	PXN® 23™
7	*Bacillus subtilis* R20059	PXN® 21™
8	*Streptococcus thermophilus* R20035	PXN® 66™

The growth of Bio-Kult (PRX) strains in MRS medium based on viable count and optical density measurements

The probiotic strains were grown in anaerobic MRS over 12 hours to determine the growth curve and exponential phase used for harvesting cells for inoculation. The first passage of cells grown from glycerol stock was used for inoculation of the growth curve, and the second passage in exponential phase was intended for use in the fermentation experiment. Only single vials of each strain were inoculated (due to time limitations) and to mimic the conditions of the experimental set up.

Figure S3 shows the growth of individual strains from Bio-Kult in MRS medium over 12 h, enumerated on MRS agar (as CFU/mL), with optical density measured at 600nm. At 12 h, most strains still appeared to be in exponential phase. Strains 4 and 6 showed slower growth possibly due to a lower inoculation load with *B. longum* starting at 6.1 ± 0.6x10^5 CFU/mL and *B.*

bifidum at 1.7 ± 0.2x10^5 CFU/mL. These strains were later investigated further for their growth in other media to ensure enough cells were harvested.

To simplify the inoculation process of single strains into faecal cultures at a known final concentration of 10^6 cells/mL, a method for estimating the number of cells based on optical density measurement of harvested probiotic cultures in exponential phase was developed. At 12 h of the previous experiment, all strains were harvested by centrifugation, the supernatant was decanted, and the cell pellet resuspended in 1 mL of sterile pre-warmed anaerobic PBS inside an anaerobic cabinet to create a concentrated stock of unknown quantity of cells. This stock was serially diluted with PBS and both optical density (OD_{600}) and viable count (CFU/mL) of the dilutions measured to derive a standard curve, enabling calculation of the number of cells (CFU/mL) at a given optical density (OD_{600}). The standard curves of each strain are displayed in **Figure S4**, with optical density (OD_{600}) on the x axis and viable count (CFU/mL) on the y axis and interpolated linear regression standard curves with 95% confidence bands. The equations used for calculating the optical density at a concentration of 10^8 CFU/mL are reported in **Table S2**.

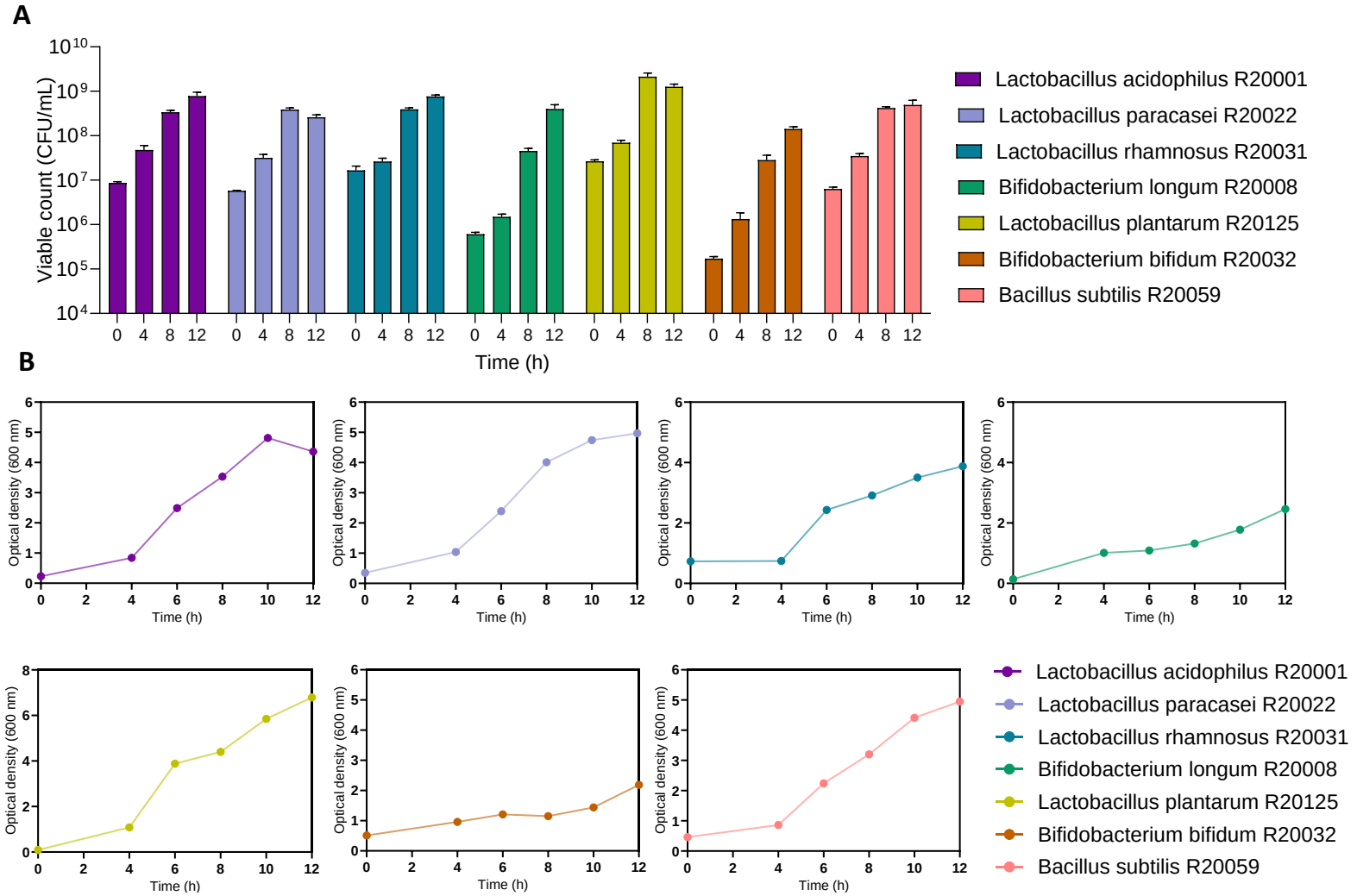


Figure S3 The growth of individual Bio-Kult probiotic strains in MRS broth over 12 h expressed as viable count (CFU/mL) and optical density (at 600 nm)

***A**) Viable count (CFU/mL) of individual probiotic strains on MRS agar following growth in MRS broth over the incubation period (12 h) was extrapolated from pelleted cells diluted in PBS). Values are mean ± SD of three technical replicates. Strain 8 did not grow well enough in 1st passage and was not used during this experiment.*

***B**) Optical density at 600 nm of individual Bio-Kult probiotic strains over 12 h of incubation in MRS broth, measured in 1:10 dilution in PBS and calculated to absolute optical density.*

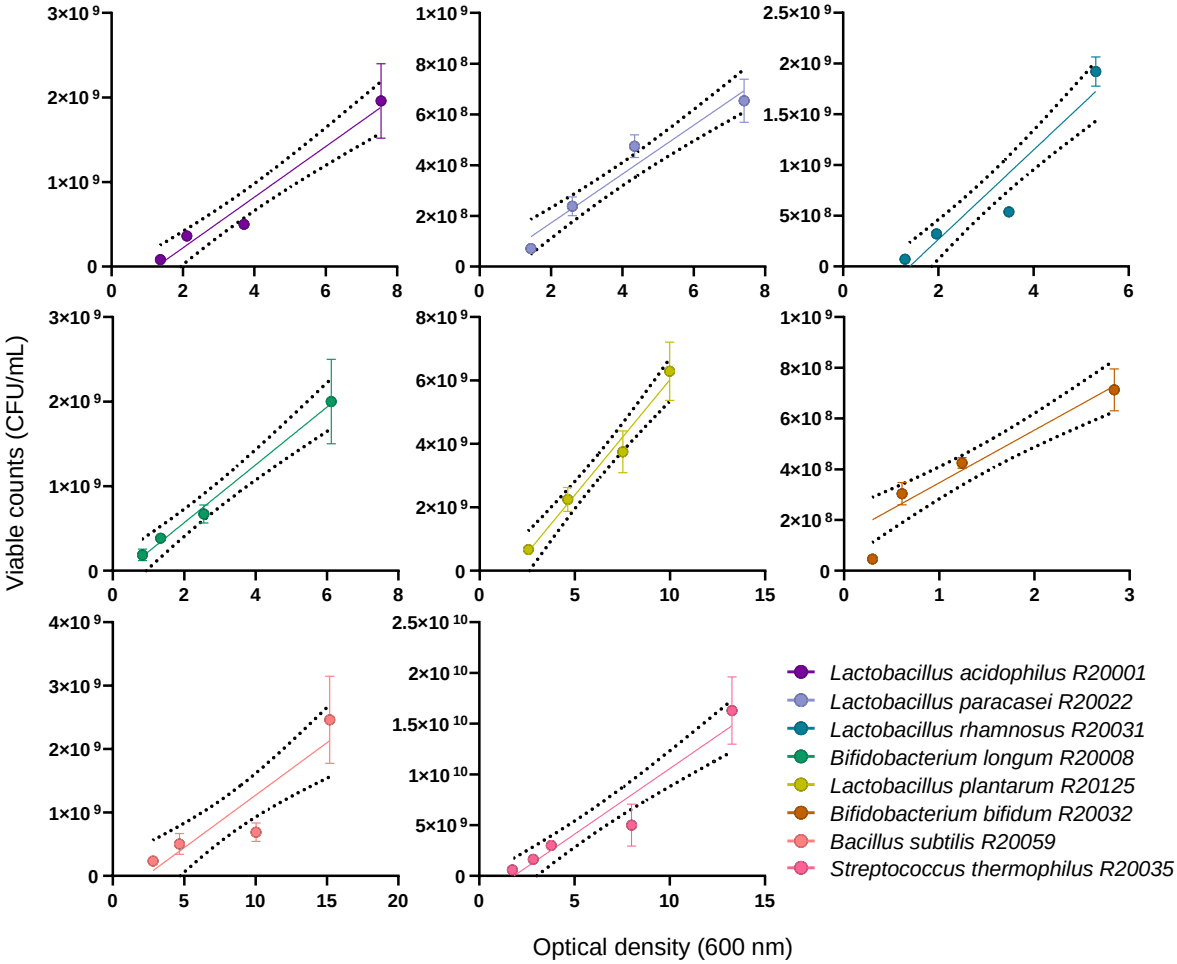


Figure S4 Linear regression curves of Bio-Kult strain stock concentration measured as viable count (CFU/mL) vs optical density (600nm)

Bacterial cell concentration of individual probiotic strains grown in MRS broth; viable count (CFU/mL) is plotted against optical density (OD_{600}). The incubation period was 12-16 h depending on strain. Values are mean ± SD of three technical replicates. Graphs were created in GraphPad Prism 10.1 and represent interpolated linear standard curves with 95% confidence bands.

Table S2 Equations used for calculations of standard curves of viable count (CFU/mL) and optical density (OD_{600}) of probiotic strains.

The equations for the linear trendlines (in Error! Reference source not found.) were used to calculate the OD_{600} = 10^8 cells/mL, and the stock volume (µL) needed to give 10^8 cells/mL.

Strain	Equation	R^2	OD_{600} for 10^8 cells	Mean ± SD of stock volume added to make 1 mL (µL)
1	$3x10^{-9}x + 1.3444$	0.96	1.64	48 ± 13
2	$1x10^{-9}x + 0.3987$	0.95	1.40	44 ± 14
3	$2x10^{-9}x + 1.5548$	0.90	1.75	74 ± 9
4	$3x10^{-9}x + 0.3499$	0.99	3.35	193 ± 81
5	$1x10^{-9}x + 1.8136$	0.98	2.81	68 ± 39
6	$6x10^{-9}x – 0.0442$	0.89	0.56	33 ± 14
7	$5x10^{-9}x + 3.2559$	0.83	3.76	111 ± 36
8	$7x10^{-10}x + 2.1266$	0.92	2.83	141 ± 44

The response of individual strains in different batch culture media and the confirmation of 10^8 cells/mL stock concentration

Figure S5 panel **A** depicts the inoculated concentrations at 0 h (extrapolated from the 10^8 CFU/mL stock), and the concentration of individual strains in three different media after 24 h of incubation. Three types of batch media were tested (CMBB, CMHT and CMB) to determine the medium and model most suitable for enrichment of faecal cultures with individual Bio-Kult strains, that could be used to investigate their effect on TMA production from choline.

The pH stability of fermentation cultures was investigated, reported in panel **B**, since that formed one of the criteria for a suitable fermentation system due to the inhibition of choline metabolism at a lower pH. The pH in CMBB and CMHT media remained stable between 8 h and 24 h of incubation, compared to CMB that decreased in pH by 1.2 ± 1.0 (mean ± SD, n = 8 strains) to a minimum of pH 4.4 in strain 4 (*B. longum*) by 24 h. This decrease was thought to be caused by the metabolism of supplemented glucose to acids and the lower buffering capacity of CMB compared to CMBB and CMHT. CMB was therefore deemed not suitable for use in anaerobic fermentations of probiotic strains without external pH buffering.

The method for estimating the inoculation concentration of probiotic strain using optical density measurement was established in the previous section and described in detail in methods chapter. To confirm the inoculation concentration, the 10^8 CFU/mL stock was enumerated on MRS agar to measure the viable count at the time of inoculation. The measured mean bacterial concentration of 10^8 CFU/mL stocks is plotted in **Figure S5** panel **C**. These stocks were prepared on 4 separate occasions to use for inoculation of different experiments. Relatively consistent viable counts (CFU/mL) were measured for the different strains, establishing the reproducibility of the developed method. There were significant differences in the inoculation loads with strains accounting for 49.8 % of total variation ($p < 0.0001$) and the occasion of stock preparation accounted for 5.6% of total variation ($p = 0.0077$). The individual results of Two-way ANOVA with multiple comparisons test are reported in Supplementary information provided in the online repository.

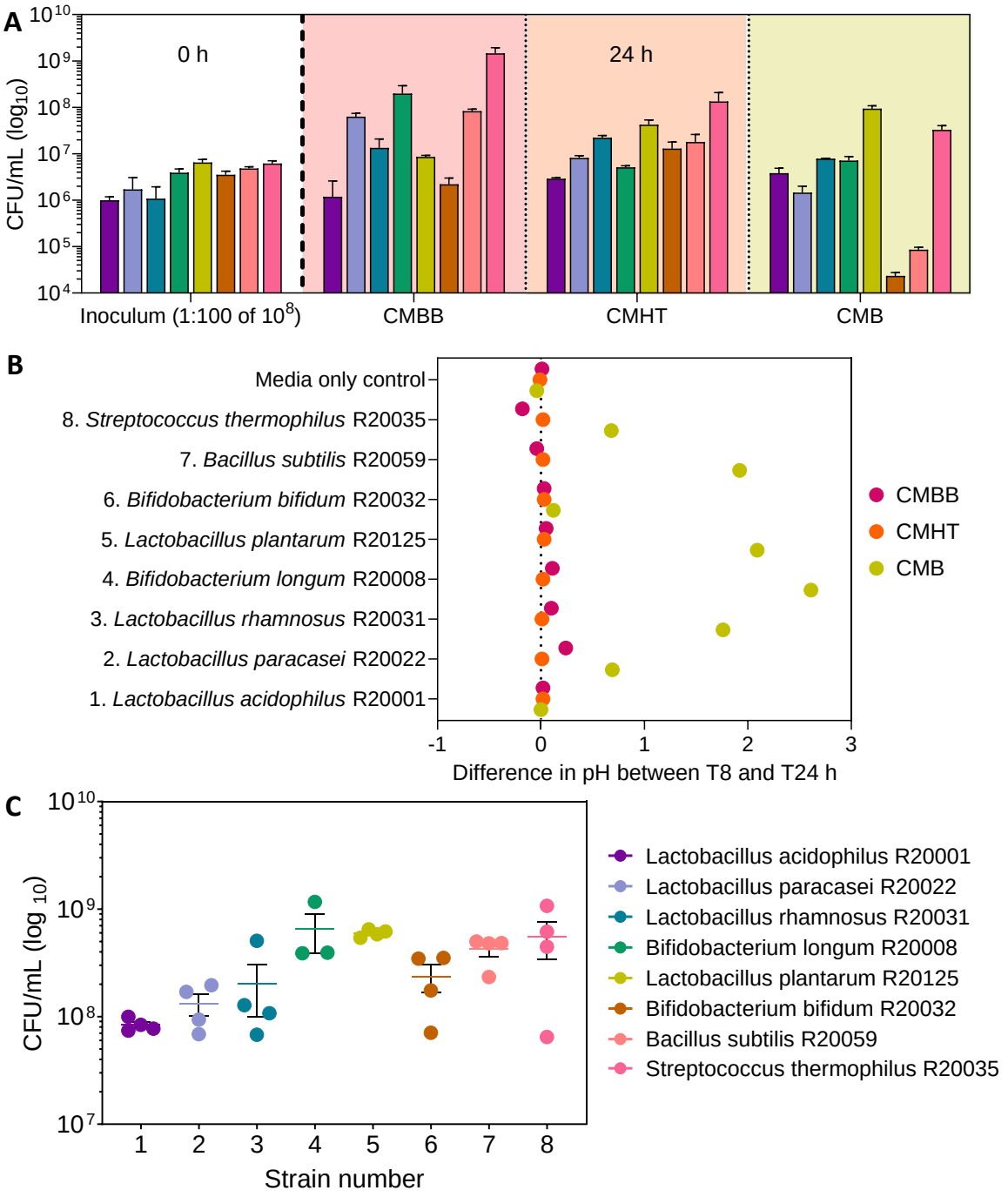


Figure S5 Response of individual Bio-Kult strains incubated in different batch fermentation media for 24 h, and the concentration of viable bacteria in the inoculum stock.

***A**) Viable count (CFU/mL) of individual PRX strains at 0 h (extrapolated from the 10^8 stock – the cultures were inoculated with 1% of strain stock (1:100 dilution of 10^8estimated stock based on OD measurements and equations from **Error! Reference source not found.**)). The response of strains in CMBB, CMHT and CMB at 24 h after incubation was measured by viable count (CFU/mL) on MRS agar. Values are triplicates in a 5 mL spot of most countable dilution (each strain had 1 vial in each media).*

***B**) The difference in pH of individual Bio-Kult (PRX) cultures grown in CMBB, CMHT or CMB, where difference = 8 h – 24 h value (single value for each time point per vial) compared to media only control that had no probiotic strain added.*

***C**) Viable count (CFU/mL) of 10^8 stock of individual Bio-Kult (PRX) strains from 4 independent tests. Strains were grown in MRS (or BHI for strain 4 and 6) over 12-18 hours, then harvested by centrifugation, supernatant decantation, and resuspension in 1 mL of PBS, which was measured by optical density at 600 nm to calculate the OD needed for dilution of stock to 10^8 cells. Mean + SEM viable count (CFU/mL) per strain are displayed.*

I.III Development of a single strain model of a *cutC*-containing bacterium

Methods

The objectives were:

Develop a single-strain model of a *cutC*-containing bacterium that can be used for co-culture experiments with probiotic strains to investigate their effect on the ability of bacteria containing *cutC* genes to metabolise choline into TMA

Halodesulfovibrio aestuarii DSM 17919

Preparation of glycerol stocks

H. aestuarii DSM 17919 was purchased from the DSM collection in freeze-dried form. Strain was inoculated into its recommended media and grown into exponential phase (1-2 days) after which it was decanted into sterile pre-reduced Falcon tubes and centrifuged for 10 min at 4000 × g at 4 °C. Supernatant was decanted and pellet was resuspended in glycerol solution from an orange Cryobead tube and transferred onto the beads. Sealed tube was mixed by inverting and glycerol stock was snap frozen using dry ice and stored at -80 °C.

Culture preparation

For inoculation using the frozen glycerol stock, an insulated container with dry ice was used to transfer stock from the freezer into the anaerobic cabinet where a fresh Hungate tube with 10 mL media (top was briefly unscrewed) had a scoop of frozen culture added using a sterile inoculation loop or a sterile toothpick. Tube was recapped, mixed by inverting and incubated until exponential phase to be subcultured at 2% into fresh media. This 2nd passage was used for inoculation of experiments, unless otherwise stated.

Media preparation

The preferred medium of *H. aestuarii* strain was based on the recommended formula from DSMZ 163 (https://mediadive.dsmz.de/medium/163?bacdive=4161), only modified by replacing sodium thioglycolate with L-cysteine hydrochloride as a reducing agent. The medium was prepared using ingredients in **Table** , by bringing solution A to the boil and then cooling down to room temperature whilst sparging with 100% N_2 gas. Solutions B and C were prepared and then added to solution A, mixture was adjusted to pH 7.0 with NaOH, and distributed under N_2 flow into anoxic Hungate-type tubes. During distribution, the medium was continuously swirled to keep the grey precipitate suspended, otherwise iron oxidised on contact with oxygen, creating black clumpy precipitate. Medium was autoclaved in the

pressure resistant Hungate tubes for 15 min at 121°C. This medium was prepared by the Media lab team at QIB and was used for the growth assays of *H. aestuarii* in Hungate tubes.

Table S3 Halodesulfovibrio medium

Halodesulfovibrio (Postgate) medium		
Solution A:	Amount	Unit
NaCl	25	g
K_2HPO_4	0.5	g
NH_4Cl	1	g
Na_2SO_4	1	g
$CaCl_2$ x 2 H_2O	0.1	g
$MgSO_4$ x 7 H_2O	2	g
Na-DL-lactate	2	g
Yeast extract	1	g
Na-resazurin solution (0.1% w/v)	0.5	ml
Distilled water	980	ml
Solution B:		
$FeSO_4$ x 7 H_20	0.5	g
Distilled water	10	ml
Solution C:		
L-cysteine HCl	0.1	g
Ascorbic acid	0.1	g
Distilled water	10	ml

Growth and choline metabolism assays

To assess growth of *H. aestuarii* in its recommended medium, the 2nd passage from the frozen glycerol stock was inoculated at 2% into Hungate tubes, compared to 2% inoculated into CMBB medium prepared as previously described and decanted into Hungate tubes (10 mL). Growth at 37 °C was estimated using a turbidometer inside the anaerobic cabinet at 0, 4, 6, 8, and 24 h. This experiment was replicated with choline supplemented Hungate tubes of *H. aestuarii* medium, with sampling at 0, 2, 4, 6, 8, 12 and 24 h to assess choline metabolism and TMA production. Due to the high turbidity readings from the previous attempt, cultures were sampled and diluted 1:10 using anaerobic PBS and measured in cuvettes with a spectrophotometer at ambient atmosphere to estimate changes in growth independently from changes in media colour due to iron precipitation.

An experiment was also carried out to understand the growth of the strain in different environments and different media to overcome dark colour of media limiting optical density measurements. For this analysis, *H. aestuarii* medium was inoculated with 2% of *H. aestuarii* 2nd passage in Hungate tubes with or without 2 mM choline. Culture was dispensed into a 96-well plate to measure growth using a plate reader inside an anaerobic cabinet at 37 °C. This control condition using Halodesulfovibrio medium was compared to Halodesulfovibrio medium

mixed with Postgate C in a 2:1 ratio with or without choline, Halodesulfovibrio medium mixed with CMBB in 1:1 ratio or *H. aestuarii* strain inoculated into CMBB only, both with and without choline. These mixtures were aliquoted into the 96-well plate and sterile CMBB and Halodesulfovibrio medium was used as controls. The plate was sealed with a gas permeable adhesive film, incubated inside a plate reader in an anaerobic cabinet and OD readings at 600 nm were taken every 15 min after 30 s of mixing over the course of 24 h.

Colony forming units and standard curve of diluted culture pellet

Finally, Halodesulfovibrio medium solidified with 1.6% agar was used to investigate the ability of *H. aestuarii* cultures to grow on solid media to allow for viable count determination. For this, the 2nd passage was inoculated into 10 mL of Halodesulfovibrio medium, grown for 12-16 h until exponential phase was reached. The culture was then transferred into a sterile Falcon tube and centrifuged for 10 min at 4000 × g at 4 °C to harvest the cells. Supernatant was decanted and the pellet was resuspended in 1 mL sterile PBS. This 100% stock was then further diluted to 50, 25, and 12.5% of original resuspended culture. The optical density of these dilutions was measured using a spectrophotometer at 600 nm (1:10 dilution of diluted stock) and percentages of stock were serially diluted for plating on Halodesulfovibrio agar using the Miles and Misra method for estimating CFU per 8 µL droplets of dilutions (n=3 replicates of each serial dilution per 4 stock dilutions). The viable counts and OD measures of *H. aestuarii* stock (100%), 50%, 25% and 12.5% dilutions were used to calculate a linear regression between viable count and optical density, to allow for estimation of OD of culture that had 10^8 viable cells/mL. This equation was then used to calculate volume of 100% stock of bacteria resuspended in 1 mL that was needed to reach 10^8 cells in 1 mL of PBS that would be used for inoculation of experiments with a known number of cells.

To confirm number of viable cells that were present in the diluted stock, 10^8 stocks were serially diluted and plated on recommended agar each time stocks were used for inoculation, incubated for 24 h inside the anaerobic cabinet at 37 °C.

Citrobacter freundii FC50

C. freundii FC50 strain was obtained from the Narbad Group culture collection (isolated by Dr Fatma Cebeci) and grown aerobically from glycerol stock by Dr Melinda Mayer shaken in an incubator that was set to 37 °C.

Preparation of glycerol stocks

The strain was subcultured into anaerobic nutrient broth and grown into exponential phase inside anaerobic cabinet at 37 °C (~12 hours) after which it was transferred into sterile pre-reduced Falcon tubes and centrifuged for 10 min at 4000 × g at 4 °C. Supernatant was

decanted and the pellet was resuspended in glycerol solution from a blue Cryobead tube and transferred onto the beads. Sealed tube was mixed by inverting and glycerol stock was snap frozen using dry ice and stored at -80 °C.

Culture preparation

For inoculation using the frozen glycerol stock, dry ice was used (as for inoculation of *H. aestuarii*) same as in the previous section. A sterile pipette tip was used for transferring a scoop of frozen stock into fresh anaerobic nutrient broth and the culture was incubated at 37 °C until exponential phase to be subcultured at 2% into fresh media. This 2nd passage was used for inoculation of experiments, unless otherwise stated.

Media preparation

Nutrient broth (**Table**) was prepared from powder using boiling water and autoclaved in a glass Duran bottle for 15 min at 121°C. Medium was dispensed under laminal flow into sterile glass universal bottles. Vials were placed in the anaerobic cabinet for 12-24 h to displace oxygen. CMBB and CMHT media were also prepared as described before and dispensed into sterile glass universal bottles (10 mL media each) inside the anaerobic cabinet to remain anoxic. For preparation of agar plates, nutrient broth was supplemented with 16 g/L of agar powder prior to autoclaving and when cooled down to pouring temperature was dispensed into sterile petri dishes.

Table S4 Nutrient broth

Nutrient broth (Oxoid CM0001)	g/L
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
pH 7.4 ± 0.2 @ 25°C	

Growth and choline metabolism assays

All three types of media (CMBB, CMHT and Nutrient broth) were supplemented with filter-sterilised 1 M choline solution (20 µL to make 2 mM final concentration) to investigate the ability of *C. freundii* to metabolise choline and produce TMA. Cultures were inoculated in triplicate with 2 % of 2nd passage of cells and incubated inside the anaerobic cabinet static for 48 h at 37 °C. During this experiment, optical density readings were made with a spectrophotometer at 600 nm using clean cuvettes and viable count were enumerated on both Nutrient agar and Wilkins-Chalgren agar at 24 and 48 h of incubation.

Desulfovibrio desulfuricans QI0028

Colony forming units and standard curve of diluted culture pellet for *D. desulfuricans QI0028*

Finally, Postgate C medium solidified with 1.6% agar was used to investigate the ability of *D. desulfuricans* cultures to grow on solid media to allow for viable count determination and measurement of optical density of serially diluted resuspended cells harvested during exponential phase (as described for *H. aestuarii*). Briefly, the harvested stock resuspended in 1 mL PBS (100% stock was then further diluted to 50, 25, 12.5, 6.25 and 3.125% of original resuspended culture. The optical density and viable count (CFU/mL) were measured and plotted to calculate the linear regression between viable colonies and optical density readings to allow for estimation of OD of culture that had 10^8 viable cells/mL. To confirm number of viable cells that were present in the diluted stock, 10^8 stocks were serially diluted and plated on recommended agar each time stocks were used for inoculation and incubated for 24 h before counting.

Results

Previously published literature was searched for strains containing *cutC/D* genes that have been reported to produce TMA from choline using *in-vitro* fermentation assays. A comprehensive list of considered strains and those that were chosen due to availability in culture collections and the type of *cut* cluster containing genes encoding choline TMA lyases is displayed in **Table S5**.

Table S5 Strains containing cutC genes that had full genome sequences available for further analysis and were considered based on their availability in culture collection, their type of cut gene cluster and scientific interest.

Species	Strain	NCBI taxon ID	Considered for further analysis (Yes/No)
Oleidesulfovibrio alaskensis	G20	207559	No
Proteus mirabilis	HI4320	529507	Yes
Desulfovibrio desulfuricans	ATCC 27774	525146	Yes
Escherichia fergusonii	UMN026	564	No
Proteus mirabilis	ATCC 29906	525369	Yes
Proteus penneri	ATCC 35198	471881	No
Escherichia coli	MS 200-1	749550	No
Escherichia coli	MS 69-1	749531	No
Klebsiella sp.	MS 92-3	749535	No
Halodesulfovibrio aestuarii	ATCC 29578	1121444	Yes
Proteus mirabilis	BB2000	1266738	No
Citrobacter freundii	NBRC 12681	1006003	Yes
Proteus vulgaris	NCTC 10376	585	No

Different strains of *P. mirabilis* were considered for their representation of type II.a choline TMA-lyase. *P. mirabilis* DSM 4479 was selected due to its fully sequenced genome, published reports of choline metabolism and relevance to human microbiome. Another fully sequenced strain containing *cutC/D* genes, but with no reports of the type of choline TMA-lyase present or the organisation of other genes in the *cut* cluster, was selected – *H. aestuarii* DSM 17919. This strain was chosen due to its origin in salt marshes and the low abundance in the human gut microbiome, giving an opportunity to investigate its ability to adapt to faecal microbiome. The strains of *C. freundii* and *D. desulfuricans* shown in **Table S5** were not readily available from culture collections, therefore phylogenetically similar strains from the local culture collection of the Narbad group at Quadram Institute were chosen for further analysis – *D. desulfuricans* QI0028 and *C. freundii* FC50. The four strains were tested for the following criteria to determine their suitability for use as a single strain choline TMA-lyase (encoded by *cutC* gene) producing model:

i) Ability to consistently grow to sufficient cell density in its defined medium
ii) Ability to grow and survive in CMBB
iii) Ability to metabolise supplemented choline to TMA during anaerobic fermentations
iv) Ability to grow on agar plates to calculate estimated OD_{600} value at 10^8 CFU/mL from linear regression of optical density vs viable count

Candidate strains were excluded if they did not meet these criteria.

Halodesulfovibrio aestuarii DSM 17919

H. aestuarii ATCC 29578 was obtained from the DSM culture collection under accession number 17919 and was rehydrated from freeze-dried pellet in DSMZ medium number 163, a higher percentage salt containing Postgate medium prepared anaerobically in Hungate tubes. The iron content of this medium supplied by ferrous sulfate heptahydrate was quite high at 0.5 g/L. Sulfate reduction by sulfate-reducing bacteria results in iron sulfide precipitation that coloured the medium black, causing inconsistencies in bacterial growth measurements by optical density readings. Precipitate could also be entrapped in biofilms created by this bacterium, further confounding the estimation of bacterial growth by optical density.

These inconsistencies are presented in **Figure S6** panel **A** where optical density was measured at 600 nm of *H. aestuarii* inoculated in DSMZ 163 using different growth vessels and measurement equipment to establish a consistent growth curve of the bacterium. *H. aestuarii* was grown in an anaerobic plate reader in a 96-well format (n = 6) with measurements every 15 minutes and 5 second shaking prior to measurement to disrupt potential biofilms formed.

H. aestuarii was also grown in Hungate tubes, with growth measured by a turbidometer or by taking a sample of the culture and diluting it in anaerobic PBS to measure OD_{600} in cuvettes using a spectrophotometer. However, the measurements of optical density in spectrophotometer were performed in aerobic conditions and values could be overestimated due to iron oxidation and precipitation (causing dark colour of media), further confounding the growth measurements. Despite the differences in the optical density values using the three methods, the overall growth pattern was consistent, showing exponential growth between 4 h and 8 h and plateauing in plate reader and spectrophotometer, but further increased by 24 h using turbidometer, reaching similar OD as the other two methods.

Attempts to grow this strain in other media than DSMZ 163 were not successful (shown in supplementary information online), and viable count enumeration by dilution of harvested stock did not show a linear pattern as displayed in panel **B**. The optical density of stock dilutions was linear, but this was most likely due to the dilution of black precipitate rather than giving a good estimate of bacterial count. Furthermore, choline abundance (mol%) in panel **C** remained consistent over the 24 h of incubation and no substantial amount of choline was converted into TMA. Therefore, this strain was not a suitable candidate for single strain model of *cutC*-containing bacterium.

Citrobacter freundii FC50

C. freundii was obtained from the Narbad group culture collection and was isolated from the human gut using sinigrin enrichment by Dr Fatma Cebeci. This strain was a facultative anaerobe and was grown in nutrient broth as its recommended medium. **Figure S6** panel **D** shows optical density measured at 0 h, 24 h and 48 h of growth in Nutrient broth, CMBB and CMHT. CMHT showed similar pattern to Nutrient broth with moderate increase in OD at 24 h, compared to CMBB that increased to OD 0.88 ± 0.04 (mean ± SD, n=3). The changes in optical density were reflected in viable count (panel **E**) in these different media measured at 24 h and 48 h of incubation by counting colonies on Nutrient agar and on Wilkins Chalgren agar. Despite CMHT showing a lower viable count at 24 h compared to CMBB and Nutrient broth, there was no difference in viable count between the growth media at 48 h. However, due to Bio-Kult strains exhibiting a faster growth rate, CMHT would not be suitable for co-culture of *C. freundii* and Bio-Kult strains. Finally, panel **F** shows that *C. freundii* did not metabolise choline to TMA in either of the media, deeming it not suitable for use in the single strain model.

Figure S6 Assessing suitability criteria of H. aestuarii DSM 17919 and C. freundii FC50 as a single strain model

***A**) Growth of H. aestuarii in its defined media DSMZ 163 as optical density at 600 nm measured in anaerobic plate reader over 24 h (n = 6), as turbidity in Hungate tubes at 0, 4, 8 and 24 h (n = 3), and as optical density measured in cuvettes (exposed to oxygen when measured) at 0, 2, 4, 6, 8, 12, 14, 16, 18, 20 and 24 h (n = 3). DSMZ 163 media was used as a blank and subtracted.*

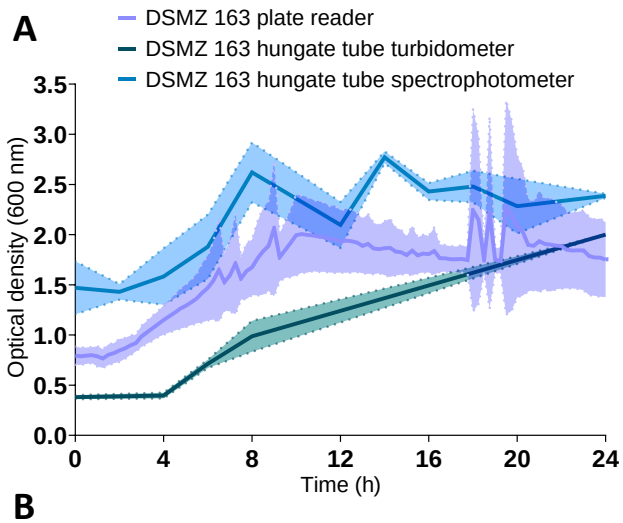
***B**) Viable count (CFU/mL) of H. aestuarii stock that was serially diluted to 50%, 25% and 12.5% of 10 mL 24 h culture pelleted and resuspended in 1 mL anaerobic PBS plated on DSMZ 163 medium solidified with 1.6 % agar and pre-reduced prior to inoculation (n = 3 dilution replicates). Right side of figure depicts the optical density measurements of stock and dilutions used for CFU/mL plating (measured using cuvettes with cultures diluted 1:10 in aerobic environment and then calculated into raw values).*

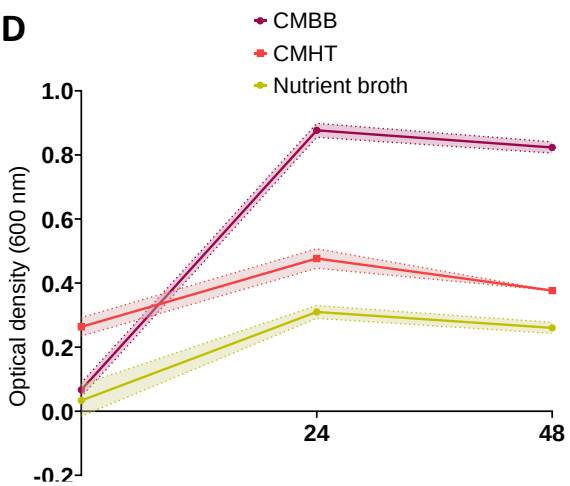
***C**) Choline abundance (mol%) and TMA from choline (mol%) of H. aestuarii cultures supplemented with ~2 mM choline and incubated in anaerobic DSMZ 163 media for 24 h, with measurements taken aseptically from Hungate tubes at 0, 2, 4, 6, 8, 12 and 24 h (n = 3).*

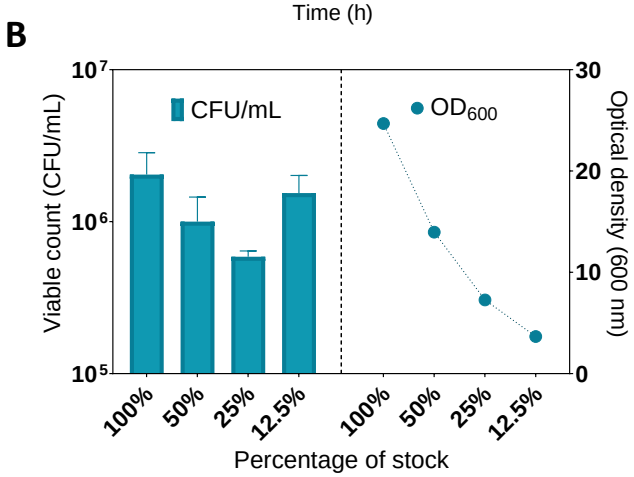
***D**) Growth of C. freundii supplemented with ~2 mM choline in its recommended nutrient broth and in CMBB and CMHT measured by optical density at 600 nm at 0, 24 and 48 h (n = 3 replicates per media type)*

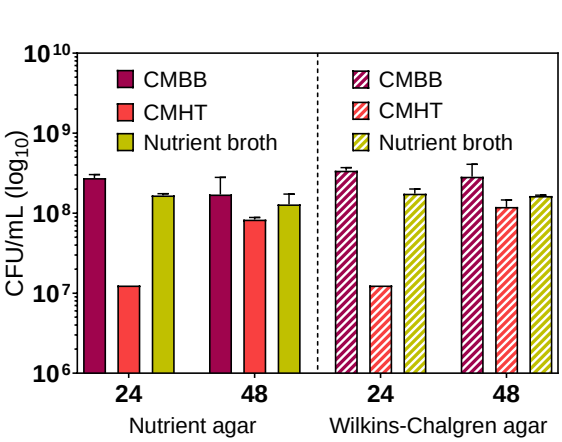
***E**) Viable count (CFU/mL) at 24 and 48 h of incubation in each liquid culture (CMBB, CMHT, Nutrient broth) grown on Nutrient agar (left side) and Wilkins-Chalgren agar (right side), counted after 24 h of incubation in anaerobic environment.*

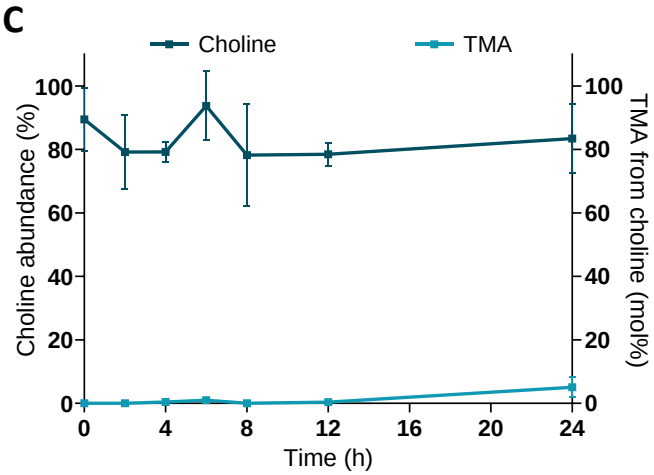
F) Choline abundance (mol%) and TMA from choline (mol%) of *C. freundii* cultures supplemented with ~2 mM choline and incubated in anaerobic CMBB, CMHT and Nutrient broth with measurements taken at 0, 24 and 48 *h.*

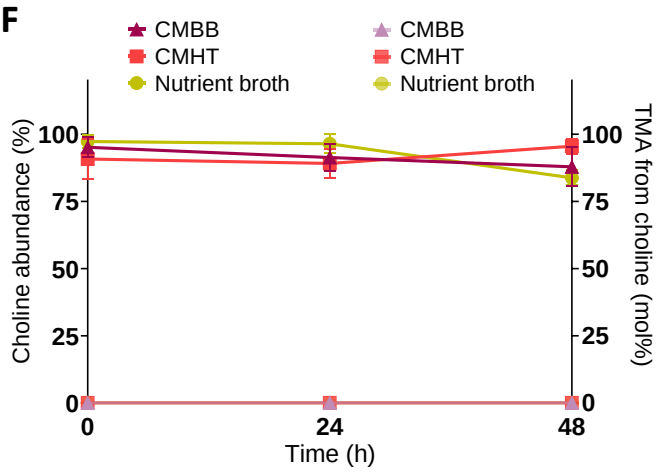

A
DSMZ 163 plate reader
DSMZ 163 hungate tube turbidometer
DSMZ 163 hungate tube spectrophotometer
Optical density (600 nm)
Time (h)


D
CMBB
CMHT
Nutrient broth
Optical density (600 nm)


B
CFU/mL
OD600
Viable count (CFU/mL)
Optical density (600 nm)
100%
50%
25%
12.5%
Percentage of stock


E
CMBB
CMHT
Nutrient broth
CFU/mL (log10)
Nutrient agar
Wilkins-Chalgren agar


C
Choline
TMA
Choline abundance (%)
TMA from choline (mol%)
Time (h)


F
CMBB
CMHT
Nutrient broth
Choline abundance (%)
TMA from choline (mol%)
Time (h)

Desulfovibrio desulfuricans QI0028

D. desulfuricans QI0028 was obtained from the Narbad group culture collection. The culture was grown from an anaerobic glycerol stock by Dr Melinda Meyer, originally isolated from the human gut by Dr Tianqi Li, and the whole genome was sequenced by Dr Lizbeth Sayavedra. This strain was a strict anaerobe and was grown in Postgate C using Hungate tubes as its recommended growth conditions. The presence of *cutC* in the genome was confirmed from searching the Genbank annotated protein sequences from WGS. The initial growth curve was conducted in Postgate C medium using Hungate tubes and measuring turbidity over 36 h. **Figure S7** panel **A** displays the exponential increase in turbidity between 12 h and 22 h and a near plateau from 24 h onwards.

Optical density was also measured during growth of *D. desulfuricans* in CMBB, however there was very little change in OD, therefore viable counts were taken as a more reliable estimate of growth. Three different types of vessels were tested to identify a suitable vessels for single strain model. Panel **B** shows initial inoculation loads of *D. desulfuricans* in CMBB using Hungate tubes, Universal glass bottles and 15 mL plastic Falcon tubes. After 8 h growth, universal bottles had the lowest viable count (CFU/mL), with Hungate and Falcon tubes showing no difference in colony counts (grown on Postgate C agar plates and incubated in anaerobic conditions for 24 h before counting).

However, *D. desulfuricans* failed to consistently grow in Postgate C medium in the first passage inoculated from liquid 'mother' culture stored at 4 °C, often taking 5-7 days to grow to exponential phase, ready to be subcultured. For that reason, an alternative medium used for other anaerobic species, Anaerobic basal broth (ABB), was investigated as an alternative to grow *D. desulfuricans* both in microtiter plates and in Hungate tubes. **Figure S7** panel **C** depicts the growth of *D. desulfuricans* either with or without 2 mM choline supplemented into ABB using anaerobic plate reader. The optical density was measured in a 96-well format (n = 6) with measurements every 15 minutes and 5 second shaking prior to measurement to disrupt potential biofilms formed. Despite choline supplementation increasing optical density readings compared to no choline added, the growth curve shape was similar with a gradual increase between 8 h and 20 h after which growth plateaued, similar to turbidity readings in Postgate C. A further option was growing *D. desulfuricans* in ABB supplemented with or without choline in Hungate tubes measured by a turbidometer as displayed in panel **D**. *D. desulfuricans* grew more slowly in Hungate tubes and appeared to be still increasing in optical density at 36 h of incubation.

The linear regression of optical density vs viable count of serially diluted stock of *D. desulfuricans* was calculated from cell harvested by centrifugation of *D. desulfuricans* in 10 mL Postgate C, which was then resuspended in 1 mL anaerobic PBS. Colonies from *D. desulfuricans* stock dilutions were counted after 24 h of incubation on Postgate C agar. The linear regression curve with 95% confidence bands is displayed in panel **E** and the equation used for calculation of optical density at 10^8 cells per mL was:

$$y = 2 \times 10^{-9}x - 0.2167$$

where y is the optical density of undiluted stock and x is viable count. A concentration of $2x10^8$ cells/mL is equal to an optical density of 0.18. This equation was used to estimate the viable count from the optical density when harvesting cells grown in ABB and when confirmation of inoculation was carried out on ABB agar.

Choline abundance and TMA from choline (mol%) are displayed in panel **F**. Choline was gradually depleted between 6 - 12 h in Postgate C, corresponding to TMA production that remained consistent after 12 h and reached 80.8 ± 24.1 mol% (mean ± SD, n = 3) at 24 h. There were fewer measurement points during *D. desulfuricans* growth in CMBB and all choline was metabolised by 8 h with TMA production reaching 43.8 mol% and 45.1 mol% at 8 h and 12 h, respectively. Choline was then fully converted into TMA by 24 h.

Based on the presented findings, *D. desulfuricans* met all the criteria to be a suitable candidate for single strain model of a *cutC*-containing bacterium.

Figure S7 Assessing suitability of Desulfovibrio desulfuricans QI0028 as a single strain model.

A*) Growth of D. desulfuricans in Postgate C medium over 36 h using turbidity as an indicator of growth in anaerobic Hungate tubes taking readings at 0, 2, 4, 6, 8, 10, 12, 14, 22, 24, 30 and 36 h (n = 3).*

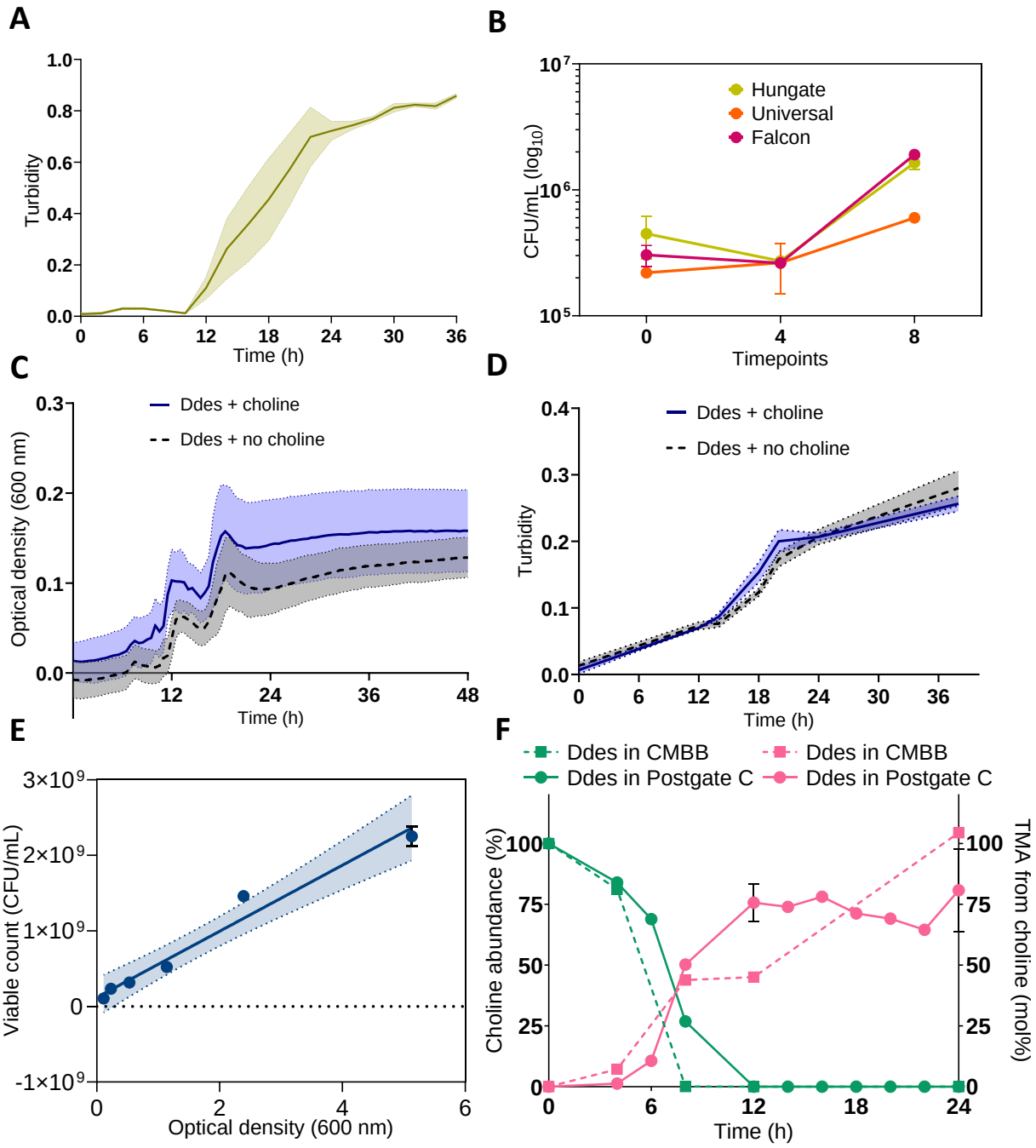
B*) Viable count (CFU/mL) of D. desulfuricans incubated in CMBB with ~2 mM choline in triplicates (using three different types of vessels due to inconsistencies in growth – Hungate tubes, Universal glass bottles, and plastic 15 mL Falcon tubes). Cultures were diluted in pre-reduced PBS and all dilutions were plated at inoculation, 4 h and 8 h. Colonies were counted after 24 to 36-hour incubation on Postgate C medium solidified with 1.6% agar and pre-reduced before inoculation.*

C*) Growth curve of D. desulfuricans (Ddes) in Anaerobic basal broth (ABB) with or without ~2 mM added choline grown over 48 hours, with optical density measurements at 600 nm every 15 minutes in anaerobic atmosphere at 37° C. Uninoculated ABB was used as blank and subtracted.*

D*) Growth curve of D. desulfuricans (Ddes) in Anaerobic basal broth (ABB) with or without ~2 mM added choline grown over 38 hours in anaerobic Hungate tubes and measured by turbidity readings taken at 0, 12, 14, 18, 20, 24 and 38 h. Readings were taken inside anaerobic cabinet using uninoculated ABB as blank.*

E) Linear regression of viable count (CFU/mL) and optical density readings of diluted *D. desulfuricans* stock harvested at late exponential phase (~20 h growth). Viable count (CFU/mL) of *D. desulfuricans* stock diluted to 50, 25, 12.5, 6.25 and 3.125 % of pelleted 10 mL Postgate C culture resuspended in 1 mL anaerobic PBS, grown on Postgate C agar for 24 h and plotted against the optical density of the stock dilutions; Values are mean ± SD of three technical replicate viable counts per dilution vs single measure of optical density. Linear regression curve interpolated from scatter plot with 95% confidence bands was calculated *using GraphPad Prism 10.1.*

F) Choline abundance (mol%) and TMA from choline (mol%) of *D. desulfuricans (*Ddes) cultures supplemented with ~2 mM choline and incubated in anaerobic CMBB or Postgate C broth with measurements taken at 0, 4, 6, 8, 12, 14, 16, *18, 20, 22 and 24 h for Postgate C (n = 3) and 0, 4, 8, 12 and 24 for CMBB (n = 1 replicate).*


A
Turbidity
Time (h)
B
CFU/mL (log10)
Hungate
Universal
Falcon
Timepoints
C
Optical density (600 nm)
Ddes + choline
Ddes + no choline
Time (h)
D
Turbidity
Ddes + choline
Ddes + no choline
Time (h)
E
Viable count (CFU/mL)
Optical density (600 nm)
F
Ddes in CMBB
Ddes in Postgate C
Ddes in CMBB
Ddes in Postgate C
Choline abundance (%)
TMA from choline (mol%)
Time (h)

Proteus mirabilis DSM 4479

P. mirabilis ATCC 29906 was obtained from the DSM culture collection under accession number 4479 and was rehydrated from a freeze-dried pellet into aerobic Nutrient broth and grown for 24 h in aerobic conditions. This culture was subcultured into anaerobic Nutrient broth at 2% and grown anaerobically at 37 °C, and then harvested in exponential phase (6 h) by centrifugation and resuspended in glycerol solution supplied in Cryobead tubes. The stock was snap frozen using dry ice and stored at -80 °C. The glycerol stock was used for inoculation of the first passage using a scoop of frozen stock or one bead as inoculum. Usually, a 2 % subculture (2^{nd} passage) was used for experimental investigations unless an accurate bacterial count was needed, then a 2^{nd} passage was harvested at exponential phase and a diluted stock of 10^8 cells/mL was used as the inoculum.

Figure S8 panel **A** shows the growth curve of *P. mirabilis* in Nutrient broth and CMBB over 24 h, using optical density measurements at 600 nm. *P. mirabilis* in Nutrient broth showed a limited increase in optical density, reaching 0.33 ± 0.02 at 24 h (mean ± SEM, from 4 separate experiments of n=3), compared to 0.78 ± 0.01 in CMBB (mean + SD, n=3). Preparation of *P. mirabilis* stock to calculate the linear regression of viable count and optical density is shown in panel **B**, where the viable count (CFU/mL) of a serially diluted pellet of 12 h culture resuspended in anaerobic PBS was counted on Nutrient agar (displayed on left y axis) after 24 h incubation, and the optical density of diluted stock (plotted on right y axis) was measured using cuvettes in a spectrophotometer at 600 nm. Undiluted stock reached an optical density of 1.82 ± 0.55 (mean ± SEM from 2 separate preparations) and a viable count (CFU/mL) of 1.1 ± 0.2x10^8 (mean ± SD, n =3).

Panel **C** represents the linear regression of viable count and optical density with 95% confidence bands. The equation used for calculating the OD at 10^8 cells/mL was:

$$y = 1 \times 10^{-8}x + 0.4569$$

Where x is the optical density and y is the viable count (CFU/mL). An OD of 1.46 was equal to 10^8 CFU/mL, and the average OD of undiluted stock was 3.46 ± 2.87 (mean ± SD, n =4). The mean volume of stock added to make 1 mL of 10^8 cells was 454.8 ± 200.1 µL (mean ± SD, n =4), with the variation stemming from the volume of original culture harvested by centrifugation. Higher volume of culture was often grown to yield higher mass of pellet. The cultures were harvested between 4 and 12 h of growth, since investigation of the viable count of stock harvested at different time points of the growth showed no difference as seen in panel **D**.

Panel **E** focuses on the growth of *P. mirabilis* in CMBB supplemented with 5 mM choline when inoculated with 1% of 24 h culture grown in Nutrient broth with or without 2 mM choline as a stimulant to induce choline metabolism and observe the effect on growth rate. There was a slight delay of growth in induced *P. mirabilis* compared to non-induced, however, the exponential phase lasted around 2 h for both conditions, showing rapid growth upon exposure to choline.

Choline abundance (mol%) and TMA produced from choline (mol%) are displayed in panel **F**. Choline was rapidly depleted between 2 - 6 h in CMBB corresponding to gradual TMA production that remained consistent after 8 h and reached 73.0 ± 15.0 mol% (mean ± SD, n = 5) at 24 h. *P. mirabilis* growth in Nutrient broth was delayed by 2 h and choline was nearly fully metabolised by 8 h (5.5 ± 4.4 mol% remaining; mean ± SD, n = 6), with TMA production reaching 71.0 ± 11.1 mol% (mean ± SD, n = 6) at 24 h.

Overall, *P. mirabilis* met all the criteria for being a suitable candidate for a single strain model of a *cutC* containing bacterium. Furthermore, with *P. mirabilis* strain DSM 4479 being widely studied, its genome fully sequenced, and published primer pairs targeting its *cutC* gene, it was chosen for investigation of its interactions with Bio-Kult strains and their effect on the ability of *P. mirabilis* to metabolise choline into TMA in co-culture experiments.

Figure S8 Assessing the suitability of Proteus mirabilis DSM 4479 as a single strain model.

***A**) Growth of P. mirabilis in nutrient broth and in CMBB supplemented with ~2 mM choline incubated in anaerobic conditions over 24 h with optical density readings taken at 0, 4, 6, 8 and 24 h. Values are Mean ± SD (n = 3)*

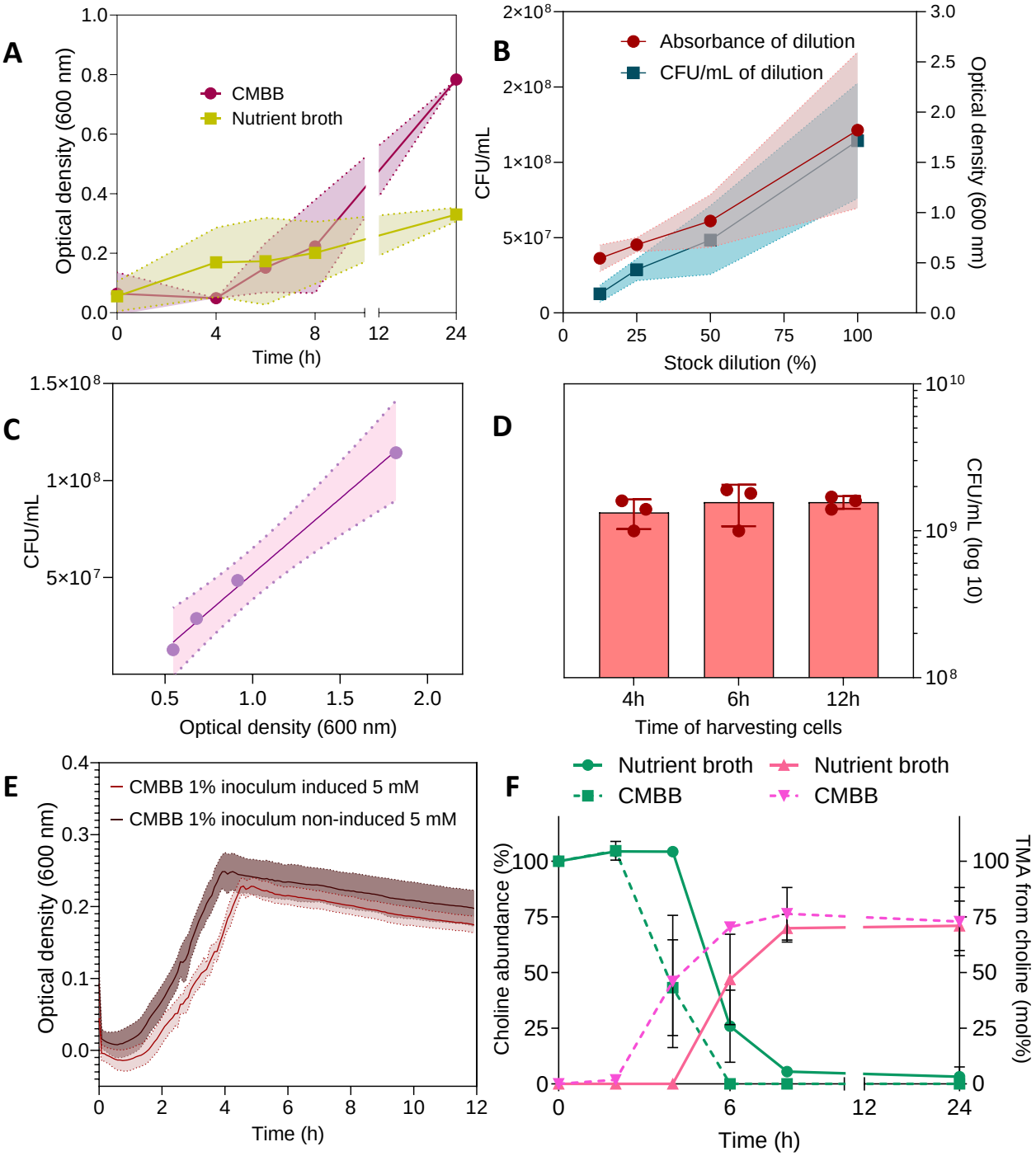
***B**) Viable counts (CFU/mL) of P. mirabilis stock that was serially diluted to 50%, 25% and 12.5% of 10 mL 12 h culture pelleted and resuspended in 1 mL anaerobic PBS plated on Nutrient broth solidified with 1.6 % agar and pre-reduced prior to inoculation (n = 3 dilution replicates). Right Y axis depicts optical density measurements of stock and dilutions used for viable count plating (measured using cuvettes with cultures diluted 1:10 in aerobic environment and then calculated into raw values). This dilution was repeated on two separate occasions.*

***C**) Linear regression of viable count (CFU/mL) and optical density readings of diluted P. mirabilis stock harvested at stationary phase (8-12 hours or overnight). P. mirabilis stock diluted as described in B) and viable count determined after 24 h incubation of agar plates in anaerobic environment. Values are means of two separate dilution curves and three technical replicate plate counts per dilution. Linear regression curve interpolated from scatter plot with 95% confidence bands was calculated using GraphPad Prism 10.1.*

***D**) Viable count (CFU/mL) of *P. mirabilis* stock that was harvested at three different time points from replicate 2nd passage of *P. mirabilis* cells. Cultures (10 mL) were harvested by centrifugation, resuspend in sterile pre-reduced PBS and inoculated on Nutrient agar after 4, 6 and 12 h of growth in *Nutrient broth. Values are Mean ± SD (n = 3 dilution replicates).*

***E**) Growth of 1% P. mirabilis inoculum in CMBB supplemented with 5 mM choline, inoculum was a subculture from stationary phase Nutrient broth P. mirabilis culture either induced or non-induced with 2 mM choline at the beginning of growth. Optical density was measured using an anaerobic plate reader at 600 nm every 15 min for 12 h. Values are Mean ± SD (n = 6 replicates).*

F) Choline abundance (mol%) and TMA from choline (mol%) of *P. mirabilis* cultures supplemented with ~2 mM *choline and incubated in anaerobic CMBB and Nutrient broth with samples taken at 0, 2, 4, 6, 8*


A
Optical density (600 nm)
CMBB
Nutrient broth
Time (h)
B
CFU/mL
Absorbance of dilution
CFU/mL of dilution
Optical density (600 nm)
Stock dilution (%)
C
CFU/mL
Optical density (600 nm)
D
CFU/mL (log 10)
4h
6h
12h
Time of harvesting cells
E
Optical density (600 nm)
CMBB 1% inoculum induced 5 mM
CMBB 1% inoculum non-induced 5 mM
Time (h)
F
Nutrient broth
CMBB
Nutrient broth
CMBB
Choline abundance (%)
TMA from choline (mol%)
Time (h)