Omega-3 Polyunsaturated Fatty Acids for the Prevention and Treatment of Colorectal Cancer

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Abstract

Although the preventability estimate for colorectal cancer (CRC) is more than 50%, CRC remains the second most common cause of cancer mortality in the UK. A potential prevention strategy for CRC prevention is use of omega-3 polyunsaturated fatty acids (O3FAs) through dietary guidance (fish intake) and/or supplementation with purified O3FAs. This thesis advances the precision use of O3FAs in the prevention of CRC, using a biomarker-driven, epidemiological approach and a parallel translational, mechanistic study to delineate the potential benefits of O3FAs based on host, tumour, and gut microbiota factors.

A comprehensive literature review outlined the pre-existing evidence for the use of O3FAs in the treatment and prevention of CRC. It identified potential strategies based on host (low plasma O3FA levels, ethnicity, male sex), tumour (proximal colon, MSI high tumours), and microbiota factors.

An observational study within the UK Biobank (UKBB) (n=121,650 participants) found that dietary O3FA intake (oily fish and fish oil supplements [FOS]) predicted plasma O3FA levels, with FOS use roughly equivalent to a weekly portion of oily fish.

Mean plasma O3FA levels of 0.51 mmol/L were associated with a 12% reduction (hazard ratio 0.88 [95% CI 0.80-0.97]) in CRC risk within UKBB participants (n=248,487, of which there were 2,756 CRC cases). Increasing plasma docosahexaenoic acid levels were associated with a reduced risk of CRC in male participants and with reduced proximal colon cancer risk.

A parallel *in vitro* colonic fermentation model explored the interaction between O3FAs and the fibre inulin on short chain fatty acid (SCFA) production, revealing a mean increase of 12.9 mmol/L in total SCFA production at 24 hours, which was associated with SCFA-producing microbiota within the model.

This thesis works towards a targeted approach for the use of O3FAs in CRC prevention based on host (males), tumour (proximal colon) and microbiome (O3FA-inulin interaction) factors.

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List of Abbreviations

25(OH)-D	25-hydroxyvitamin D
5 HEPE	5-hydroxyeicosapentaenoic acid
AA	Arachidonic acid
ACF	Aberrant crypt focus
ADR	Adenoma detection rate
AEs	Adverse events
AF	Atrial fibrillation/flutter
AHA	American Heart Association
ALA	Alpha linolenic acid
ANOVA	Analysis of variance
ANZCTR	Australian New Zealand clinical Trials Registry
APC	Adenomatous polyposis coli
ASCEND	A Study of Cardiovascular Events iN Diabetes
BCFA	Branched chain fatty acids
BMI	Body mass index
BRAF	B-raf proto-oncogene
C2	Acetate
C3	Propionate
C4	Butyrate
C5	Valerate
C6	Hexanoate
C7	Enanthic acid
C8	Caprylic acid
CA	Conventional adenoma
CALGB	Cancer and Leukaemia Group B trial
CEA	Carcinoembryonic antigen

CI	Confidence interval
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CLO	Cod liver oil
COX	Cyclooxygenase
CRC	Colorectal cancer
CRCLM	Colorectal cancer liver metastasis
CRP	C-Reactive protein
CV	Coefficient of variation
DFS	Disease free survival
DHA	Docosahexaenoic acid
dMMR	DNA mismatch repair-deficient
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
EE	Ethyl ester
EDTA	Ethylenediaminetetraacetic acid
ELOVL	Elongase of very-long fatty acid
EMT	Eicosapentaenoic Acid for Treatment of Colorectal Cancer Liver Metastases
EMT2	Eicosapentaenoic Acid for Treatment of Colorectal Cancer Liver Metastases Trial 2
EN	Enteral feeds
EPA	Eicosapentaenoic acid
EPIC	European Prospective Investigation into Cancer
FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 2
FAP	Familial adenomatous polyposis

FAs	Fatty acids
FFA	Free fatty acid
FFQ	Food frequency questionnaire
FIT	Faecal immunochemical t*est*
FO	Fish oil
FOS	Fish oil supplement
$FOXP3^{+}$	Forkhead box protein P3
GC	Gas chromatography
GC-FID	Gas chromatography flame ionisation detector
GI	Gastrointestinal
GLOBOCAN	Global Cancer Observatory
GPCRs	G protein-coupled receptors
GPS	Glasgow Prognostic Score
HDI	Human development index
HPFS	Health Professionals Follow up Study
HR	Hazard ratio
HRT	Hormone replacement therapy
HSD	Honest significant difference
IBD	Inflammatory bowel disease
iC4	Isobutyrate
iC5	Isovalerate
iC6	Isocaproic acid
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-17A	interleukin 17A
IQR	Interquartile range

IRR	Incidence rate ratio
ISRCTN	International Standard Randomised Controlled Trial Number
IU	International Units
IV	Intravenous
IVs	Instrumental variables
JPHCBPS	Japan Public Health Centre Based Prospective Study
KRAS	Kristen rat sarcoma virus
LA	Linolenic acid
LOS	Length of stay
LPS	Lipopolysaccharide
LTB	Leukotriene
LTB_5	Leukotriene B_5
MCCS	Melbourne Collaborative Cohort Study
MCP	Microbial cell preparation
MENAC	Multimodal Intervention for Cachexia in Advanced Cancer Patients Undergoing Chemotherapy
MET	Metabolic equivalent tasks
MO3FA	Marine omemega-3 polyunsaturated fatty acids
MR	Mendelian randomisation
MSI	microsatellite instability
MSS	Microsatellite stable
NBOCA	National Bowel Cancer Audit
NCBI	National Centre for Biotechnology Information
NCCTG	North Central Cancer Treatment Group
NHS	Nurses' Health Study

NHS BCSP	National Health Service Bowel Cancer Screening Programme
NF-kB	Nuclear transcription factor Kb
O3FA	Omega-3 polyunsaturated fatty acids
O3i	Omega-3 index
O6FA	Omega-6 polyunsaturated fatty acids
OCP	Oral contraceptive poll
OFN	Oxygen free nitrogen
OMICC	Omega-3 Fatty Acid for the Immune Modulation of Colorectal Cancer
ONS	Oral nutritional supplement
OPA	Orthophosphoric acid
OR	Odds ratio
OS	Overall survival
OTU	operational taxonomic unit
OXIPN	oxaliplatin-induced peripheral neurotoxicity
PCoA	Principle coordinate analysis
PGE_2	Prostaglandin E_2
PGE-M	Prostaglandin E_2 metabolite
POCs	post-operative complications
POD	Postoperative day
PN	Parental nutrition
POCs	Post operative complications
POD	Post operative day
PREPARE	PReventaion Using EPA Against colorectal Cancer
PROTEIN	PeRioperative Omega Three and the Effect on ImmNity
PUFAs	Polyunsaturated fatty acids

QC	Quality control
QoL	Quality of life
RBC	Red blood cells
RCT	Randomised control trial
REDUCE-IT	Reduction of Cardiovascular Events With Icosapent Ethyl–Intervention Trial
RNA	Ribonucleic acid
RR	Risk ratio
SACN	Scientific Advisory Committee on Nutrition
SCFA	Short chain fatty acid
SD	Standard deviation
SeAFOod	Systematic Evaluation of Aspirin and Fish oil polyp prevention trial
SMHS	Shanghai Men’s Health Study
SP	Serrated polyp
SPRI	Solid Phase Reversible Immobilisation
SNP	Single nucleotide polymorphism
SWLHC	Swedish Women’s Lifestyle and Health cohort
TCPS	Tennessee Colorectal Polyp Study
TG	Triglyceride
Total FAs	Total fatty acids
TNF-α	Tumour necrosis factor alpha
TG	Triglyceride
UK	United Kingdom
UKBB	United Kingdom Biobank
UMIN	University hospital Medical Information Network
UTI	Urinary tract infection

VITAL	VITamin D and Omega-3 TriaL
Vit D	Vitamin D
WCC	White cell count
WHO	World Health Organisation
WCRF	World Cancer Research Fund

Chapter 1 Introduction

1.1 Omega-3 Polyunsaturated fatty acids for the prevention and treatment of colorectal cancer

With the increasing burden of colorectal cancer (CRC), there is a need to develop prevention and treatment strategies that are: simple, safe, well-tolerated and cost effective; such as dietary guidance and chemopreventative agents.

The bioactive omega-3 polyunsaturated fatty acids (O3FAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in oily fish and in fish oil supplements (FOS) have been shown to have anti-inflammatory and anti-cancer activity in laboratory studies (1). However, epidemiological and clinical data evaluating O3FA dietary intake and interventions are equivocal, due in part to methodological weaknesses; including a lack of dietary data validated through O3FA blood levels and/or the inclusion of FOS use within analyses (2).

Despite these limitations there have been signals demonstrating the differential effects of O3FAs in CRC prevention and treatment. These signals can be characterised according to individual (host) factors such as: dietary intake, plasma O3FA levels, sex, ethnicity and tumour factors such as: the location of cancer within the colorectum [right/proximal colon] and specific molecular subtypes (deficient mismatch repair [dMMR], microsatellite instable [MSI] high) (2).

Mechanistic data from human studies have demonstrated that O3FAs increase the abundance of short chain fatty acid (SCFA) producing bacteria in the colonic microbiome (3), however it is has not yet been determined

whether O3FAs lead to an increase in SCFAs, which have direct anti-CRC activity within the colorectum; presenting a potential therapeutic strategy.

This thesis explores the potential differential effects of O3FAs in the prevention and treatment of CRC according to host, tumour and microbiome factors through a biomarker-driven, epidemiological approach and a parallel translational, mechanistic study.

1.2 Colorectal cancer

1.2.1 Epidemiology

Globally, CRC ranks third for cancer incidence and second for cancer mortality (4). The 2020 Global Cancer Observatory (GLOBOCAN) report, outlined a significant burden of CRC worldwide, with more than 1.9 million new CRC cases and 935,000 deaths; representing one in ten cancer cases and deaths in 2020 (4). The geographical distribution of CRC is highest in Europe, Australia, New Zealand and North America, reflecting the association between specific lifestyle and/or dietary factors with CRC risk (4). The burden of CRC is projected to increase to 3.2 million new cases and 1.6 million deaths by 2040, with 81% of cases predicted to occur in countries with a high or very high human development index (5). This is likely due to an increase in risk factors within those populations, such as: obesity, sedentary lifestyle, processed meat and alcohol consumption (5).

In the United Kingdom (UK), CRC is the second commonest cause of cancer death; there were approximately 42,000 new CRC cases per year between 2016 to 2018 (6). Within Europe, there is a concerning increase in the incidence of CRC in adults aged 20 to 49 years, with a staggering rise in CRC incidence (cases per 100,000) from 1.7% between 1990 to 2004, to

7.9% between 2004 to 2016 (7). Despite CRC being the third most common cancer for both men and women within the UK, there is a slight male predominance of the disease; with women accounting for 44% of cases between 2016 to 2018 (6).

CRC survival is dependent on stage, which describes the extent of the disease, with stage I limited to the lining of the colon, whereas stage IV describes CRC that has extended through the colon and metastasised to distant organs, such as the liver or lung. Five year survival rates for stage I were reported at 90.9% compared to only 10.5% for stage IV (8). Survival rates have remained relatively static over the last decade with net survival rates at 1- and 5- years (regardless of age and stage) in England of 78.3% and 58.4% respectively (9).

1.2.2 CRC risk factors

The preventability estimate for CRC is significant and believed to be 50 to 60%, with several review articles outlining preventative strategies focusing on nutrition, lifestyle factors (such as smoking and alcohol reduction) and screening (10-13).

Hereditable factors account for approximately 35% of CRC risk, with 29% of the UK population having a first- or second-degree relative with CRC. Hereditary cancer syndromes including Lynch syndrome, familial adenomatous polyposis (FAP) and other polyposis syndromes account for 5 to 10% of all CRC diagnoses (14). Inflammatory bowel disease (IBD), (ulcerative colitis [UC] and Crohn's disease [CD]), which cause chronic inflammation, are associated with approximately 1 to 2% of all CRC cases, with a higher incidence in patients with UC (15-17).

Screening is an important tool in CRC prevention, the National Health Service (NHS) bowel cancer screening programme is offered every two years to men and women aged 60 to 74 years. Since 2019 it has consisted of the faecal immunochemical test (FIT), following which individuals with an abnormal FIT test are offered a colonoscopy (18, 19). The number of patients diagnosed with CRC via the screening programme (excluding the first wave of the COVID-19 pandemic) has increased since 2015, with 3,532 diagnosed in 2019 (20). The screening age is planned to be gradually lowered to 50 years (20).

There is a strong association between specific lifestyle factors and CRC risk, including smoking and alcohol use (21, 22). Medications including regular aspirin use and hormone replacement therapy (HRT) have been associated with reduced CRC risk (23, 24). In addition, several systematic reviews have highlighted the association between CRC risk and obesity (25-28).

Diet and nutrition are increasingly important risk factors for CRC, which are largely modifiable. The 2018 '*Diet, nutrition, physical activity and colorectal cancer*' report by the World Cancer Research Fund (WCRF) Report and American Institute for Cancer Research, summarised evidence for the prevention of CRC (29). It reported '*strong*' evidence that: physical activity, consuming wholegrains, dietary fibre, and dairy produce decreased the risk of CRC, whereas the consumption of red and processed meat, alcohol, being overweight, increased CRC risk. There was '*limited, but suggestive*' evidence that consuming foods containing vitamin C, fish, vitamin D and multivitamin supplements may decrease the risk of CRC, whereas a low consumption of non-starchy vegetables and fruit, in addition to the

consumption of foods containing haem iron, were associated with an increased risk (29).

Diet and nutrition have an important role in the composition of an individual's gut microbiome, which can also be associated with CRC risk and presents a potential strategy for CRC prevention, which is discussed in greater detail within this thesis (**section 1.3**) (11, 30).

1.2.3 Colorectal anatomy and function

The colorectum is approximately 150 cm long and consist of three main parts: the proximal (right) colon which consists of the caecum, appendix, proximal colon, hepatic flexure, and transverse colon; the distal (left) colon which consists of: the splenic flexure, descending and sigmoid colon; and the rectum (**figure 1.1**).

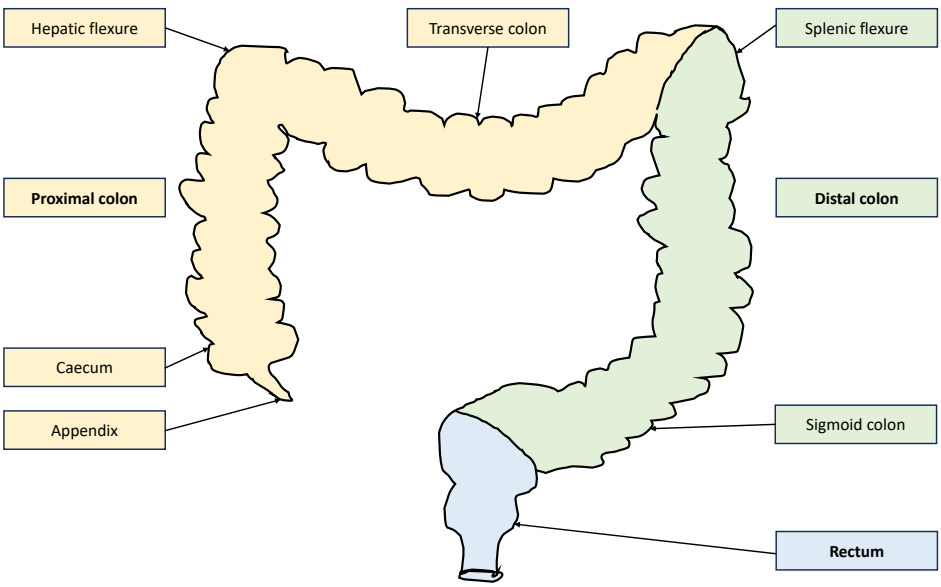


Figure 1.1 Anatomical regions of the colon.

The anatomical regions of the colon have different embryological origins and anatomical blood supplies. Consequently, they are associated with different

histological tumour subtypes, clinical features, and risk factors; in addition to disease progression and survival (31-35).

The rates of CRC incidence differ according to location in the colorectum, a UK study of the National Cancer Registration Analysis Service found that between 2001 and 2017, among patients of screening age (60-74 years), 43.6% were proximal (caecum to descending colon) and 56.4% were distal tumours (sigmoid to rectum) (36). Whereas, a US study of the National Cancer Institute's Surveillance, Epidemiology, and End Results program and the Centres for Disease Control and Prevention's National Program of Cancer Registries from 2015 to 2019, reported incidence according to subsite of 39% proximal colon (caecum to transverse colon, excluding appendix cancer), 24% distal colon (splenic flexure to sigmoid colon) and 30% rectal cancers (37).

Overall the colorectum has four main functions: fluid and electrolyte homeostasis; the absorption of nutrients including SCFAs produced by the colonic microbiota; the formation and propulsion of colonic content, and defecation (38). Different aspects of these functions take place within specific anatomical regions of the colon. The proximal colon, specifically the caecum, is responsible for carbohydrate fermentation and the absorption of water and electrolytes, whereas the majority of sodium absorption takes place in the transverse colon. A small amount of protein fermentation occurs with the descending colon, which in addition to the sigmoid colon acts a conduit for faeces to the rectum, where they are stored until defecation (39).

1.2.4 Pathogenesis of colorectal cancer

CRC is a heterogenous disease with the molecular pathogenesis of CRC potentially determining the chemopreventative activity of O3FAs in the treatment and prevention of CRC in specific subsites (location within the colorectum) and according to molecular subtypes.

CRC is a disease of the colonic mucosa; most CRCs develop from a benign colorectal precursor lesion; a polyp. The polyp to carcinoma sequence was first described in 1990 by Fearon and Vogelstein, who described a multi-hit genetic model for colorectal tumorigenesis through the accumulation of genetic mutations and the inactivity of tumour suppressor genes (40). Recent advancements in our understanding of the molecular pathophysiology of CRC has led to the description of three main molecular pathways:

1. The chromosomal instability (CIN) pathway also known as the '*traditional*' or '*conventional*' pathway. It accounts for approximately 60 to 85% of all CRCs (31, 41). It is characterised by the adenoma-carcinoma sequence due to mutations that inactivate the adenomatous polyposis coli (APC) tumour suppressor gene, leading to an overaction of the Wnt/B-catenin signalling pathways causing abnormal cell proliferation and the development of adenomas (described as conventional adenomas [CAs]). Mutations in the Kirsten ras homolog (KRAS) oncogene promote adenoma growth and progress to CRC due to inactivation of tumour protein 53 (TP53) tumour suppressor gene. Distal colon cancers are predominately CIN-high tumours and exhibit KRAS mutations.
2. The microsatellite instability (MSI) pathway accounts for approximately 15 to 30% of all CRCs (41). It is characterised by

mutations in DNA mismatch repair genes (dMMR) which cause instability in microsatellite regions (MSI-high). These tumours are hypermutated (they have an increased mutation burden) and therefore respond well to immunotherapy. Proximal colon cancers are predominately MSI high (41, 42).

3. The serrated or CpG island methylator phenotype (CIMP) pathway accounts for approximately 10 to 15% of CRCs which occur due to serrated polyps (41, 43). Serrated (saw-toothed) polyps are a heterogenous group and include hyperplastic polyps, sessile serrated adenomas, or traditional serrated adenomas. They are associated with mutations in the BRAF oncogene and can lead to CRC through two different pathways. One pathway leads to mutations in MMR genes and leads to an MSI-high phenotype while the other pathway leads to mutations in TP53 and other oncogenic pathways including Wnt signalling which result in microsatellite stable (MSS) tumours (41, 43).

In summary, proximal colon cancers are associated with serrated adenomas and/or mucinous adenocarcinomas which have a flat morphology and therefore maybe more difficult to detect on colonoscopy. Consequently, they may present at a later stage compared to distal colon cancers – which are associated with conventional adenomas and have a more easily detectable polypoid morphology. With regards to clinical risk factors, proximal colon cancer predominantly affects those who are older, and female compared to distal colon cancer cases, who are typically younger and predominantly male (31, 32). The differences between proximal and distal cancers are summarised in **figure 1.2**.

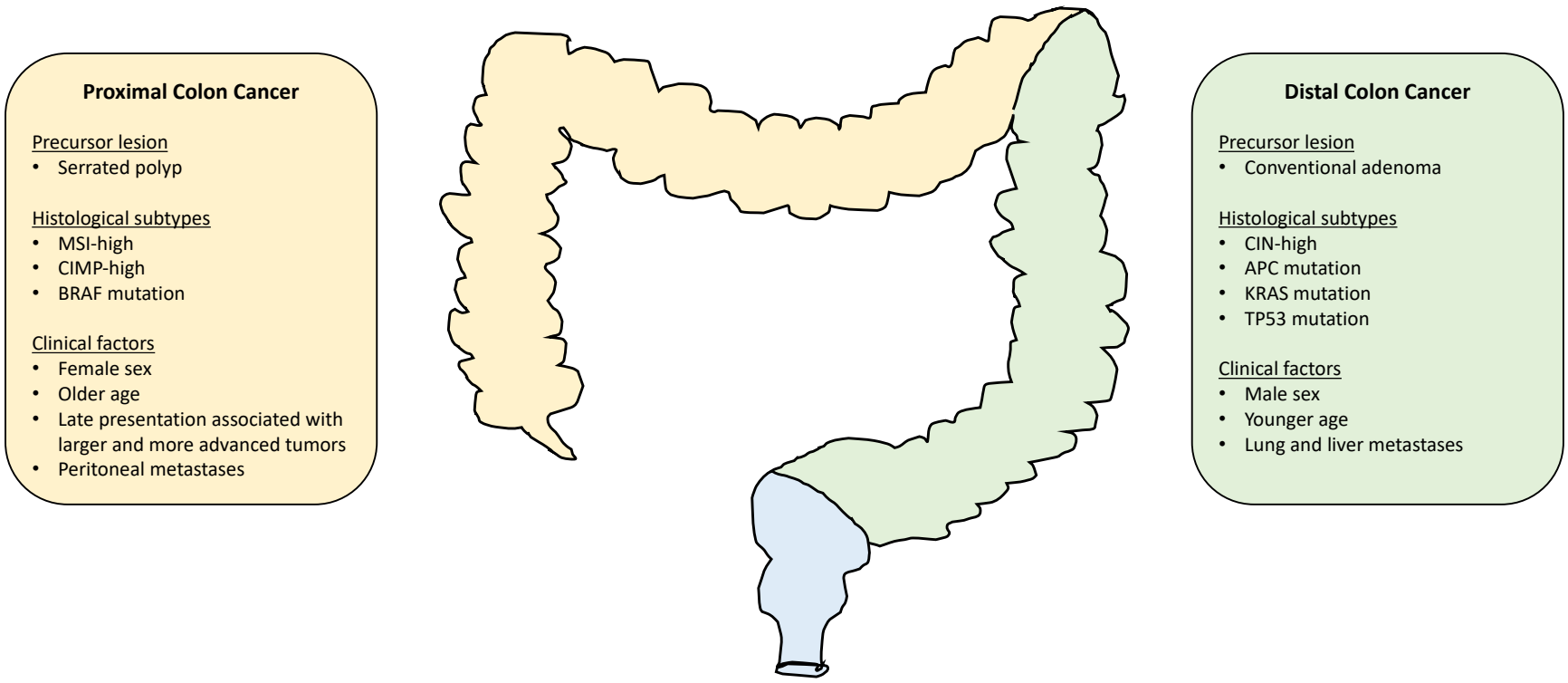


Figure 1.2. Differences between proximal and distal colon cancer.

1.3 The colonic microbiota

The colonic microbiota is associated with the pathogenesis of CRC and is a potential target for CRC prevention and treatment, as outlined in several review articles (44-47). The colonic microbiota consists of several trillions of microbes including bacteria, viruses, fungi, and protozoa, that encode 100 times more unique genes than the human genome. The composition of the colonic microbiota is determined by individual genetic, environmental (obesity, smoking, exercise) and dietary factors (fibre, processed and red meat, O3FAs) (11, 30).

Microbiome analysis techniques utilising molecular sequencing (DNA) to identify organisms using next generation sequencing (NGS), in contrast to traditional culture techniques, has led to an increase in our knowledge and understanding of the composition and function of the colonic microbiome. Bacterial microorganisms are identified according to a taxonomy that utilises the following hierarchy: Phylum, Class, Order, Family, Genus, Species (48). Different microbial analysis techniques (16S rRNA, shotgun metagenomics) can identify organisms at different levels of this hierarchical structure. The functions of the colonic microbiota include the facilitation of nutrient formation and absorption, the production of SCFAs and the maintenance of the homeostatic conditions of the gut and immune function (46).

The colonic microbiota consists of anaerobic bacteria, mainly gram-positive species (lactobacilli and streptococci) and is affected by pH and bile salt concentration. The four main bacterial phyla are *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria*. There is regional diversity within the colonic microbiome, with differences in microbiota associated with the functions of each anatomical region of the colon (**section 1.2.3**). There is a

higher prevalence of microbiota responsible for carbohydrate metabolism within the caecum, whereas the microbiota responsible for protein metabolism are more prevalent in the descending colon (39).

The term '*dysbiosis*' defines the alteration of a microbial community, resulting in decreased bacterial diversity, inflammation and the impairment of the colonic mucus layer (46). The causal relationship between the colonic microbiota and CRC carcinogenesis is unclear. It is difficult to establish whether dysbiosis causes the development of CRC or if it is a consequence of CRC itself; as the tumour may exert changes in the colonic microbiota (49, 50). Mechanisms by which the colonic microbiota are associated with CRC carcinogenesis include: chronic inflammation, immune dysregulation; increased levels of lipopolysaccharide (LPS) endotoxin, secondary bile acids, the metabolism of dietary components and genotoxin production (30, 51, 52). Several microbiota are associated with CRC and include: *Bacteroides fragilis*, *Streptococcus gallolyticus, Enterococcus faecalis* and *Escherichia coli*, *Fusobacterium nucleatum*, *Parvimonas*, *Peptostreptococcus*, *Porphyromonas, Prevotella, Shigella, Campylobacter* and *Streptococcus gallolyticus* (46, 53-55). Whereas a decrease in microbiota including: F*aecalibacterium*, *Blautia, Clostridium, Bifidobacterium* and *Roseburia* are associated with increased CRC risk (46, 53-55).

CRC patients have been shown to have a lower abundance of the SCFA (specifically butyrate) producing genera, in addition to low bacterial diversity within faecal samples and colonic mucosa; compared to healthy controls (45, 54, 56). Furthermore, specific microorganisms have been associated with different stages of CRC and specific molecular subtypes (46, 57).

1.4 Colonic fermentation

The colonic microbiota obtain their energy through a process called fermentation, defined as the anaerobic metabolism of substrates including non-digestible carbohydrates (NDC) and proteins. The fermentation process produces waste products including: SCFAs, branched chain fatty acids (BCFAs), carbon dioxide, methane, hydrogen, ethanol, succinate, and lactate (58, 59).

Substrates that reach the colon are those that are resistant to hydrolysis by enzymes within the upper gastrointestinal tract and can therefore be metabolised by hydrolytic enzymes produced by colonic microbiota (60). Such substrates include carbohydrates, proteins, bile, mucus and both epithelial and microbial dead cells (61). The most common type of fermentation, saccharolytic, takes place within the caecum and proximal colon, where there is greater availability of substrates, specifically NDC. Proteolytic fermentation occurs in the distal colon and utilises amino acids, producing BCFAs, phenols and amines (59, 60, 62).

1.5 Short chain fatty acids

The production of SCFAs, through colonic fermentation are of particular interest due to their potential role in CRC prevention (11, 63, 64) (**section 1.5.2**). SCFAs are carboxylic acids containing 1 to 6 carbons (**figure 1.3**). The main SCFAs produced through colonic fermentation are acetate (C2), propionate (C3) and butyrate (C4), they account for 95% of total SCFA production, with small amounts of other SCFAs including: valerate (C5), hexanoate (C6), isobutyrate (iC4) and isovalerate (iC5), also produced (62).

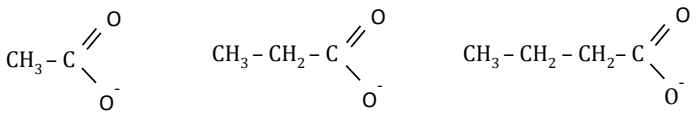


Figure 1.3 The structure of the short chain fatty acids, acetate, propionate, and butyrate.

The different types of fermentation reflect the total amount of SCFAs within specific regions of the colon, with an estimated 70 to 140 millimolar (mM) in the proximal and 20 to 70 mM in the distal colon (65, 66). A study of sudden death victims, with 4 hours of death, included measurements of pH and SCFA concentrations in the colon in addition to portal, hepatic and peripheral venous blood (62). It reported a mean pH of 5.6 in the caecum, 5.7 in the ascending, 6.2 in the transverse, 6.6 in the descending and 6.3 in the sigmoid colon. Molar ratios (the percentage of acetate + propionate + butyrate) of acetate (57%), propionate (22%) and butyrate (21%) were similar in the proximal and distal colon. However, ratios in peripheral blood were 91, 5 and 4% for acetate, propionate, and butyrate respectively, indicating the metabolism and use of SCFAs by specific tissues. A lower pH, reflected a greater total SCFA concentration (mmol/kg), with 131 in the caecum, 123 in the ascending, 117 in the transverse, 80 in the descending and 100 mmol/kg in the sigmoid colon (62).

SCFA production is determined by the abundance and type of microbiota present in the colon, colonic transit time, and the type and amount of substrate fermented (67). Acetate production has the highest luminal concentration as pathways of production are shared among microbiota, whereas propionate and butyrate production is more substrate specific and associated with specific microbiota (68, 69).

1.5.1 Pathways of SCFA production

A series of different pathways lead to the production of SCFAs, with different microbiota associated with specific pathways and genes required for individual SCFA synthesis (**figure 1.4**). The potential to increase SCFA production by modulating the colonic microbiome through probiotics and prebiotics is discussed in **section 1.6.5**.

To summarise the process of SCFA production (**figure 1.4**), NDC are hydrolysed by microbiota into oligosaccharides and then monosaccharides, which are converted into phosphoenolpyruvate, by either the Embden-Meyerhof-Parnas (glycolysis) pathway or the pentose-phosphate pathway. Phosphoenolpyruvate is then converted to pyruvate which in turn is converted to acetyl-CoA which undergoes hydrolysis to form acetate. Microbiota producing acetate through this pathway include *Bacteroides* spp., *Bifidobacterium* spp., *Prevotella* spp. And *Ruminococcus* spp. Acetate can also be formed from carbon dioxide, through the Wood Ljungdahl pathway (*Blautia hydrogenotrophica, clostridium* spp. and *Streptococcus* spp.).

Most butyrate, approximately 85%, is formed through the conversion of acetate to butyrate by butyryl-CoA : acetate CoA-transferase (*Anaerostipes* spp., *Coprococcus catus*, *Eubacterium rectale*, *Eubacterium hallii*, *Faecalibacterium, prausnitzii, Roseburia* spp.). Whereas the remaining 15% of butyrate is formed through the butyrate kinase pathway, some microbiota have the genes for butyrate kinase which enables direct butyrate production (*Coprococcus comes*, *Coprococcus eutactus)*.

Propionate can be converted from pyruvate by the succinate pathway (*Bacteroides* spp., *Phascolarctobacterium succinatutens*, *Dialister* spp.,

Veillonella spp.) and also be produced from lactate through the acrylate pathway (*Megasphaera elsdenii*, *Coprococcus catus)* (58, 70-75).

The pathways of SCFA production and related microbiota are summarised in **figure 1.4**.

1.5.2 Functions and anti-CRC role of SCFAs

There is growing interest in SCFAs in CRC prevention and treatment, with a number of recently published review articles (63, 76). SCFAs have a number of functions relating to metabolic control, appetite regulation, body composition and immune function (59).

Following their production within the colon, SCFAs are absorbed by colonocytes (epithelial cells of the colon) where they are used as an energy source. Remaining levels of propionate and butyrate, which are not metabolised by colonocytes are transported into the portal circulation and used as an energy source for hepatocytes in the liver (72, 77). Butyrate has a specific role in maintaining the colonic epithelium and is the primary energy source for colonocytes. It has a dual role, described as the '*butyrate paradox*', as it can induce proliferation in normal colonocytes and apoptosis in neoplastic cells (59, 78). In addition, butyrate maintains and improves the integrity of the colonic epithelial barrier by regulating tight-junctions between transmembrane proteins of colonic epithelial cells (78). The maintenance of these tight junction proteins is important, as increased permeability of the epithelial barrier is associated with bacterial translocation which can trigger an inflammatory cascade, including activation of nuclear factor kappa B (NF-kB) and inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) (59).

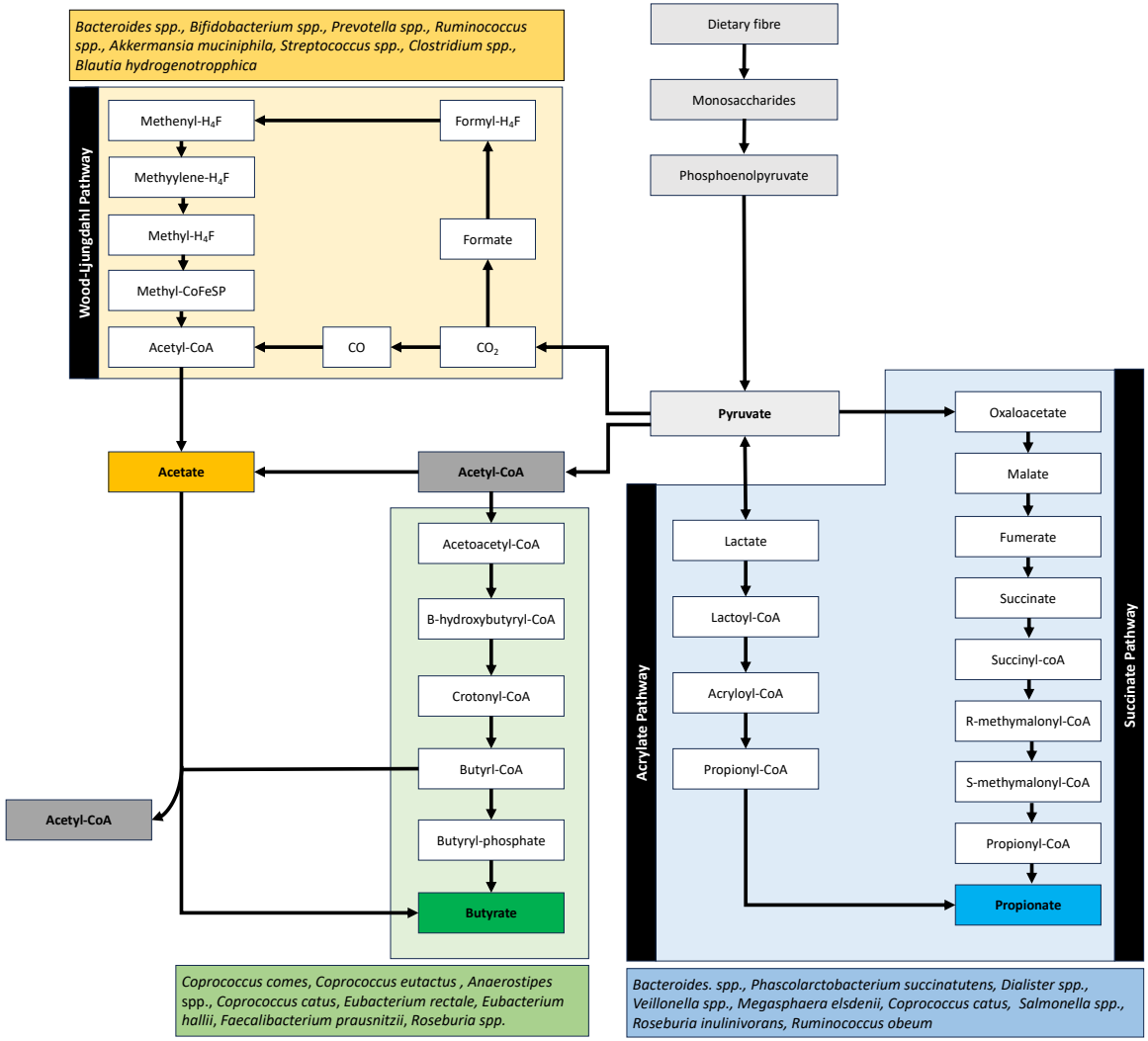


Figure 1.4 Overview of carbohydrate fermentation pathways producing acetate, propionate and butyrate, and associated microbiota.

SCFAs are also able to modulate the inflammatory response, butyrate specifically can inhibit histone deacetylase (HDAC) and the activation of NF-kB in macrophages which contribute to both the immune and inflammatory response, therefore reducing inflammation and CRC (78). Butyrate is also able to induce colonic regulatory T cell (Treg) differentiation (79, 80). Tregs have a role in suppression of inflammatory and allergic response by limiting proliferation of effector CD4+ T cells and increasing intestinal immune homeostasis (80). In addition, SCFAs can also reduce the immune response by signalling through G protein-coupled receptors (GPCRs) which may present a potential therapeutic target (81).

1.6 Omega-3 polyunsaturated fatty acids

1.6.1 O3FAs function, structure, and metabolism

O3FAs are long chain polyunsaturated fatty acids (PUFAs), that have important functions in phospholipid membrane structure and function, cell signalling and lipid metabolism (82, 83).

Fatty acids consist of an aliphatic hydrocarbon chain and a carboxyl group (-COOH) at the start of the chain (the alpha end) and a methyl group ($-CH_3$) at the end of the chain (the omega end). Fatty acids that contain only single bonds between carbons within the chain are saturated fatty acids, whereas unsaturated fatty acids include monounsaturated, (containing one carbon to carbon double bond) or polyunsaturated (containing more than one carbon to carbon double bond). The classification of fatty acids is shown in **figure 1.5.**

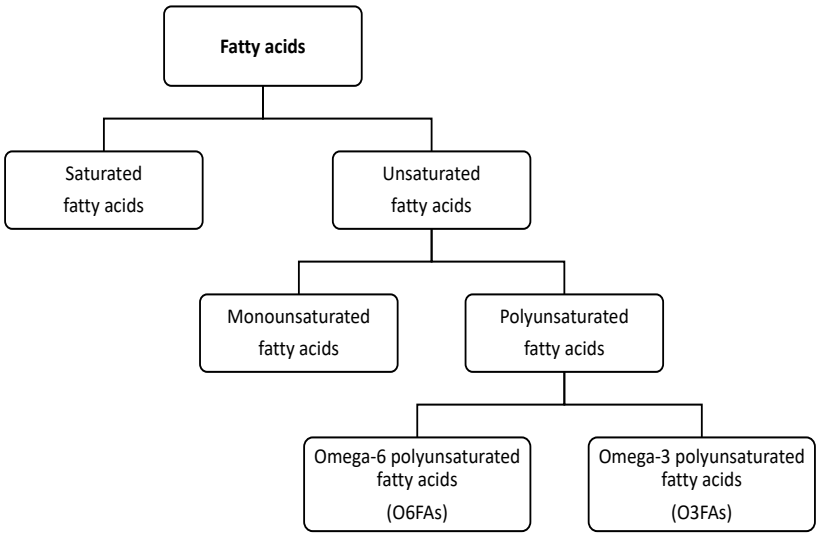


Figure 1.5 Classification of fatty acids.

The main PUFAs are O3FAs and omega-6 polyunsaturated fatty acids (O6FAs), named due to the location of the first carbon to carbon double bond being positioned at the third carbon from the methyl (CH_3) end of the carbon chain (O3FAs) and the sixth carbon (O6FAs). The carbon number in the long chain relates to the number of carbons and the number of double bonds. For example for EPA, C20:5 (ω-3) describes a 20-carbon chain with 5 carbon to carbon double bonds, with the first double bond located at the third carbon from the methyl CH_3 end of the carbon chain (**figure 1.6**) (1).

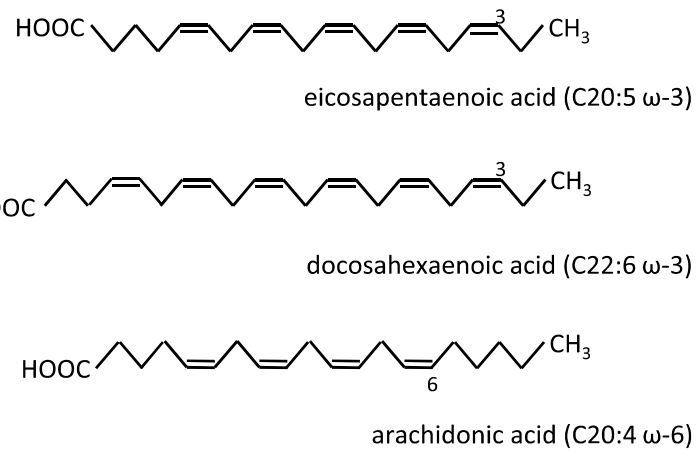


Figure 1.6. Chemical structure of EPA, DHA, and AA.

Both O6FAs and O3FAs are essential PUFAs and must therefore be obtained from the diet. The bioactive forms of O3FAs, EPA and DHA are found naturally in oily fish and/or in FOS. Small amounts of EPA and DHA can be synthesised from the biologically inactive alpha linolenic acid (ALA), found in nuts and seeds, through a series of enzymatic reactions modified by single nuclear polymorphisms (SNPs) in the fatty acid desaturate 1 (FADS1), FADS2 and elongation of very long chain fatty acids (ELOVL) genes which encode for delta-6 desaturase, delta-5 desaturase and elongase enzymes (**figure 1.7**) (84). The O6FA, linoleic acid (LA) found in sunflower and corn oil is abundant in the '*modern western diet*' and can reduce the conversion of ALA to EPA and DHA through substrate competition (85, 86).

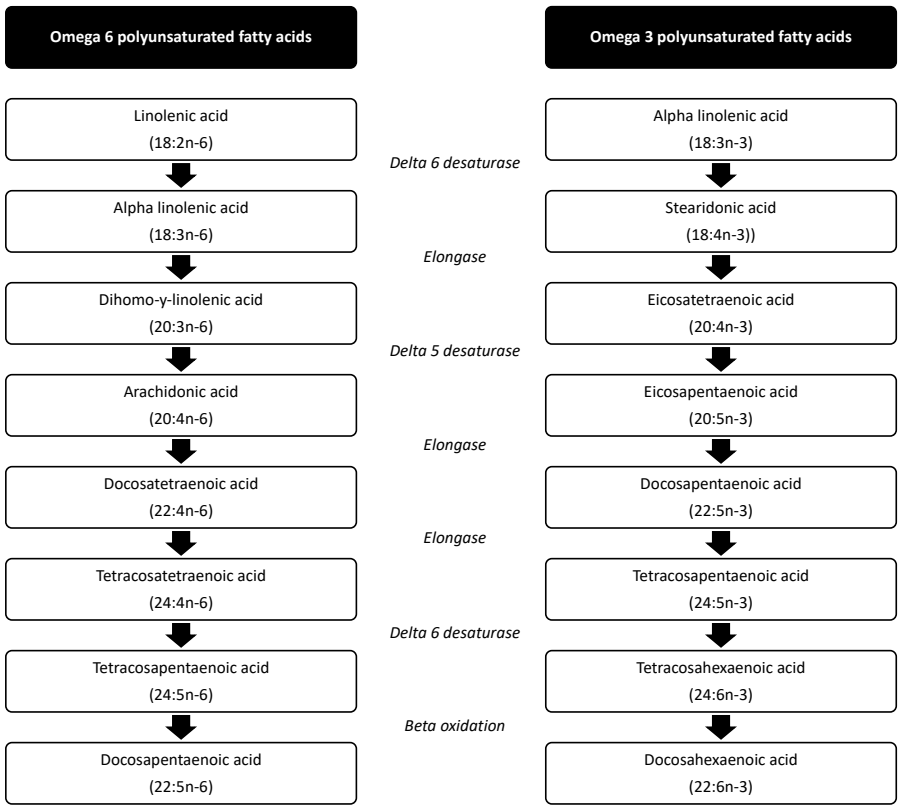


Figure 1.7. PUFA metabolism and enzymatic steps.

1.6.2 Sources of O3FAs

The bioactive O3FAs, EPA and DHA are often referred to as '*marine*' as they are primarily sourced from oily fish and FOS. The primary source of O3FAs are algae which are consumed by oily fish who store it in their flesh (87, 88). The current UK recommendations by The Scientific Advisory Committee on Nutrition (SACN) are that adults consume '*at least two portions of fish, of which one should be oily*' a week (89). The basis for many O3FA trials is the American Heart Association (AHA) recommendations of 1 g/d of O3FAs for patients with documented cardiovascular disease through either the consumption of oily fish or an O3FA supplement (90).

There are many sources of oily fish including: salmon, trout, mackerel, herring, sardines, pilchards, and kippers, each with varying amounts of EPA and DHA. The average portion of fish is 140g which is equivalent to 2.8g of O3FAs (89). Non-oily or lean fish, also described as '*white*' fish, include sources such as: cod, haddock, canned tuna and sole, which have a much lower content of O3FAs, approximately 0.4g per 140g portion (89).

In addition to dietary sources, O3FAs are increasingly available as nutritional supplements; with approximately 30% of the UK population using a FOS (91, 92). A 1g FOS capsule available in the UK, provides 0.3g of EPA and DHA per dose which is equivalent to a weekly dose of 2.1g if taken daily (93, 94). It is important to distinguish between FOS and cod liver oil (CLO) which is sourced from the livers of cod, a white fish high in vitamins D and A. A 1g CLO capsule, available in the UK, contains approximately 0.17g of EPA and DHA (95).

1.6.3 Formulations and the absorption of O3FAs

There are different chemical forms of O3FAs including triglyceride (TG), ethyl ester (EE) and free fatty acid (FFA) forms.

Natural dietary sources of O3FAs in the form of oily fish are typically TG form, which means that they are bound to a glycerol backbone. Following oral intake, they enter the stomach where they are emulsified by bile acids. This action allows the pancreatic enzyme lipase, within the small intestine to break down the TG into FFAs. FFAs are then absorbed by enterocytes, where they are re-esterified to TG, before being incorporated into chylomicrons and enter the lymph vessels and blood circulation (96).

Fish oil preparations can include TG, EE and FFA forms. EE forms are produced through the process of re-esterification to produce re-esterified TG bound O3FAs, this increases the concentration of EPA and DHA, allowing them to be manufactured as supplements at high O3FA doses (96, 97). High dose O3FA interventions in the form of EE have/are being examined in randomised control trials (RCTs) (98, 99).

The bioavailability (being the amount of active O3FAs within the systemic circulation) of O3FAs in the form of TG, EE and FFA has been examined in a number of studies by measuring blood O3FA levels (96, 97, 100-102). FFA formulations, which do not need to be broken down by lipase, have the greatest bioavailability (101). Some studies have shown that there is no difference in the bioavailability of TG and EE formulations of O3FAs (100, 103), whereas other have shown that TG formulations are better (102). There is however heterogeneity within studies exploring the bioavailability of O3FA formulations, due to the methods of measurement used to quantify O3FAs.

The epidemiological and clinical evidence O3FAs in CRC prevention and treatment of CRC are discussed in **chapter 3,** in addition to different formulations, dosing and side effects of O3FA supplementation.

1.6.4 Methods of measuring O3FAs

O3FAs can be reported using different measures (individual fatty acids, percentage of O3FA to total fatty acids, omega-3 index, ratio of O6:O3FAs), and within different samples (red blood cell [RBC] membranes, plasma, whole blood), using different quantification techniques (gas-, liquid-chromatography mass spectrometry (GC-MS) (LC-MS), nuclear magnetic resonance (NMR) spectroscopy).

It was previously thought that red blood cell (RBC) membranes were the most accurate representation of the bioavailability of O3FAs over time, as they were not subject to the transient changes in O3FA levels in plasma due to fasting or post-prandial changes following the consumption of O3FAs (104). However, recent RCT data has shown a strong correlation between plasma and RBC, EPA, and DHA levels (105). Some studies report the '*omega-3 index'* (O3i), the percentage of EPA and DHA of total identified PUFAs in RBC membranes. The O3i was first described by Harris and Von Schacky in 2004 as a marker for coronary heart disease, in which they described a threshold of an O3i of ≥8% as being associated with the greatest reduction in cardiovascular risk (106).

In an attempt to reduce the heterogeneity of the reporting of O3FAs within studies, Brenna et al published '*best practices for the design, laboratory analysis, and reporting trials involving fatty acids*' in 2018 and outlined

recommendations for use in the planning, evaluating, and reporting of human studies with fatty acid analyses, including 22 recommendations (107).

O3FAs can be quantified using GC-MS, LC-MS, and NMR spectroscopy. Both GC and LC-MS allow the separation and identification of fatty acids either in a gaseous- (GC-MS), or liquid- mobile phase (LC-MS). Due to the need for sample preparation, both techniques are resource intensive and costly, however they are very sensitive and able to quantify all fatty acids (108, 109). NMR spectroscopy is an alternative technique used to quantify fatty acids according to their nuclear magnetic resonance, using a magnetic field. NMR spectroscopy does not require any sample preparation and therefore allows the high throughput of samples. However, is not as sensitive as GC- or LC-MS and therefore specific fatty acids such as ALA and EPA are unable to be quantified (108, 110, 111).

1.6.5 Anti-colorectal cancer mechanism of O3FAs

The most well understood mechanism by which O3FAs are believed to have anti-CRC activity is though their ability to modulate inflammation, a hallmark of colorectal carcinogenesis (83). EPA modulates cyclooxygenase (COX) 2 dependent synthesis of prostaglandin E_2 which has a role in CRC carcinogenesis, with a large proportion of CRC tumours overexpressing COX-2 (112). Arachidonic acid, derived from LA gives rise to the pro-inflammatory eicosanoid prostaglandin E_2 (PGE_2) through the COX-2 enzyme. Whereas EPA inhibits COX-2 dependent synthesis of PGE_2 by acting as an alternative substrate for COX-2 and therefore reducing the production of PGE_2 in favour of the anti-inflammatory eicosanoid PGE_3, which has a role in resolving inflammation and promoting tissue repair. In addition to PGE_3, O3FAs are implicated in the production of other anti-

inflammatory mediators through the lipoxygenase (LOX) enzyme including both E- and D- series resolvins (113, 114).

Both EPA and DHA can act as direct ligands for G protein coupled receptors (GPCRs) triggering pro-apoptotic signalling of cells including adipocytes and macrophages, therefore reducing inflammation. Other mechanisms by which O3FAs exhibit anti-CRC activity include their ability to alter the membrane dynamics and receptor function of cells altering downstream receptor signalling controlling proliferation and apoptosis. They can also induce cellular oxidative stress, through reactive oxygen species, leading to apoptosis through oxidative damage. O3FAs have also been found to downregulate the activity of specific signalling pathways that promote colorectal carcinogenesis including the Wnt/B-catenin signalling pathway, therefore having the ability to reduce proliferation and increase apoptosis (115).

1.6.6 O3FAs and the colonic microbiota

In addition to the above anti-CRC mechanism of O3FAs, there is increasing evidence that O3FAs modulate the colonic microbiome in favour of SCFA producing bacteria (11, 116, 117). A review article by Costantini et al, outlined the evidence between O3FAs and the colonic microbiome (118).

In summary, human studies of O3FA supplementation have illustrated an increase in SCFA producing bacteria. A case report of a 45 year old healthy male consuming a fish only protein diet (including at least 600 mg daily of O3FAs) for 14 days, reported an associated reversible decrease in species diversity, but an increase in butyrate-producing bacteria (119). Similar findings were reported in a randomised, open-label cross-over trial of 4 g/d

mixed EPA/DHA for 8 weeks followed by a washout period of 12-weeks in healthy volunteers (n=22) (3). They reported no increase in bacterial diversity, but a reversible increase in SCFA producing bacteria including *Bifidobacterium*, *Roseburia* and *Lactobacillus* (3). This same intervention (4 g per day mixed EPA/DHA) for 28 days was used to examine the bioavailability of O3FAs in the ileostomy fluid of CRC patients with a temporary ileostomy. It showed that O3FAs reach the proximal colon with an increase in EPA and DHA concentrations as high as 200 mcg/ml in the intestinal fluid of some patients. The findings were also associated with an increase in known SCFA producing bacteria (120).

Some studies have attempted to quantify SCFA production associated with O3FA supplementation. A lower dose of O3FA (500mg once daily) was evaluated in an RCT examining O3FAs and the dietary fibre inulin (20g once daily) for 6 weeks in 69 participants with low fibre diets. They observed an increase of 1.88 umol/L in serum butyrate levels following the O3FA intervention which did not reach statistical significance (P=0.053), whereas there was an increase of 3.23 umol/L for the inulin intervention (P=0.0004) (121). In addition they found an increase in *Coprococcus* and *Bacteroides* for the O3FA intervention, but no increase in *Bifidobacetreium* or *Lachnospiraceae* as seen for the inulin intervention, which may reflect the low dose of O3FA intervention within the study (121). An O3FA deficient diet was given to C57BL/6 mice who had significantly lower rates of SCFA production, specifically acetate and butyrate compared to control, suggesting that O3FAs are important in maintaining intestinal homeostasis (122).

1.6.7 O3FAs and dietary fibre interaction

The potential probiotic effect of O3FAs by modulating the colonic microbiome towards SCFA producing bacteria and the interaction with prebiotics such as dietary fibre has been explored in a small number of studies as outlined in a recent review article (123).

Dietary fibre is important in CRC prevention as it increases stool bulk, shortens colonic transit time, and therefore reduces exposure to bile acids and faecal carcinogens; in addition to its role in SCFA production (123). The current UK recommendations are that adults increase their dietary intake of fibre to 30g per day (124).

Dietary fibre can be classified according to its solubility (soluble and insoluble) and fermentability. Insoluble fibres include wheat bran and cellulose. Soluble fibre is further classified into viscous (pectin) and non-viscous (inulin) (125). Inulin is a soluble non-viscous fibre and is naturally found in artichoke, asparagus, garlic, onion, leeks and industrially obtained from chicory roots (126). Inulin is a well-studied prebiotic that has been shown to increase SCFA levels (127, 128).

The interaction between O3FAs and dietary fibre has been evaluated in a small number of epidemiological studies which have shown a positive interaction, including a study of 96,354 Seventh-Day Adventists, which found that '*pesco-vegetarians*', who consumed high amounts of fibre and oily fish had a lower risk of CRC compared to those following other diets (vegan, vegetarian, non-vegetarian) (129-131). The interaction between dietary fibre and O3FAs is also discussed in **Chapter 3, Section 3.5.4**.

The interaction between O3FAs and different dietary fibres has also been examined in a number of animal studies. Administration of inulin and O3FAs in non-obese diabetic mice was found to restore gut barrier integrity and increase microbial diversity including an increase in the abundance of *Bifidobacteria* and a decrease in pro-inflammatory bacteria including *Bacteroides intestinalis* and *Streptococcus* (132). The interaction between fish oil and both pectin and cellulose were examined in azoxymethane induced rats, in which there was a significant increase in apoptotic cells and cell per crypt columns in rats fed fish oil and pectin within the proximal and distal colon, compared to fish oil and cellulose. This suggests a possible mechanism of O3FAs in apoptosis rather than cell proliferation in addition to a specific interaction with soluble fibres (133). A later study by the same group confirmed that mouse colonocytes treated with DHA and butyrate had higher rates of apoptosis compared to butyrate alone, by triggering apoptosis involving mitochondrial calcium loading (134).

1.7 O3FAs and the interplay between diet, host, tumour, and microbiome

CRC is a heterogenous disease with the interplay between diet, host, tumour, and colonic microbiome implicated in its pathogenesis; presenting potential targets for CRC prevention and treatment (**figure 1.8**).

There is a potential role for O3FAs in modifying each of these factors. A comprehensive literature review in **chapter 3** outlines and summarises the evidence for a targeted approach for O3FAs in CRC prevention and treatment, **chapters 4 and 5** examine the potential for O3FAs to modulate diet, host, and tumour factors in CRC prevention. **Chapters 6 and 7** examine the potential for O3FAs to modulate the colonic microbiota in favour of SCFA

production, in addition to examining the potential probiotic-prebiotic relationship between O3FAs and different types of dietary fibre.

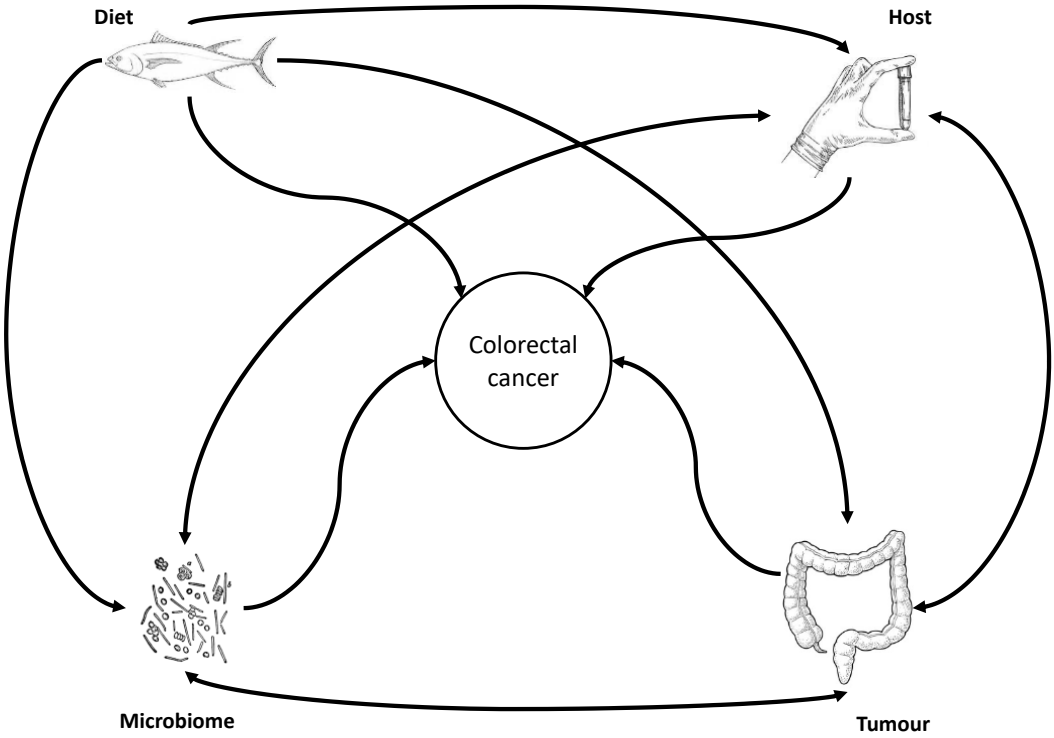


Figure 1.8 The interplay between diet, host, tumour and colonic microbiota factors in CRC and the potential for O3FAs in modifying each of these factors in CRC prevention and treatment.

Chapter 2 Aims and Hypothesis

2.1 Aims and approach

The overall aim of my research leading to this thesis was to advance the precision use of O3FAs in the prevention and treatment of CRC, using a biomarker-driven, epidemiological approach and a parallel translational, mechanistic study. This was achieved through four distinct studies, designed to identify the role of O3FAs in CRC prevention and treatment, according to host, tumour, and gut microbiota factors.

The title of the four studies were:

1. Omega-3 polyunsaturated fatty acids in colorectal cancer prevention and treatment (**chapter 3**).
2. The relationship between oily fish intake, fish oil supplement use and plasma polyunsaturated fatty acid levels in UK Biobank participants (**chapter 4**).
3. The relationship between plasma omega-3 polyunsaturated fatty acid levels and colorectal cancer risk in UK Biobank participants (**chapter 5**).
4. The interaction between omega-3 polyunsaturated fatty acids and dietary fibre on short chain fatty acid production within an *in vitro* colonic fermentation model (**chapters 6 & 7**).

2.1.1 Omega-3 polyunsaturated fatty acids in colorectal cancer prevention and treatment

I first undertook a literature review of clinical studies and observational data, to delineate the potential benefits of O3FAs in the prevention and treatment of CRC (based on host, tumour, and gut microbiota factors), in addition to summarising ongoing clinical trials and highlighting current gaps in the literature.

The rationale for the literature review was based on the publication of several large-scale randomised controlled trials of pharmacological delivery of O3FAs, and prospective cohort studies of dietary O3FAs, investigating the relationship between O3FAs and CRC risk, conducted over the last decade. In addition, to producing an updated review of the literature, following publication of a 2011 review '*Omega-3 fatty acids for the treatment and prevention of colorectal cancer*' by the Hull group (1).

The aims of the literature review were to: 1) review clinical and observational data regarding O3FAs for the prevention and treatment of CRC; 2) delineate potential signals for the benefit from O3FAs in the prevention and treatment of CRC based on host, tumour, and microbiota factors and 3) highlight the current gaps in the literature.

The objectives to achieve these aims were to:

1. Examine studies of O3FAs in colorectal polyp prevention.
1. Examine primary prevention randomised intervention trials of O3FAs.
2. Examine the epidemiological evidence regarding dietary O3FAs and CRC risk and survival.
3. Examine the use of O3FA interventions in the surgical management of CRC and colorectal cancer liver metastases (CRCLM).
4. Examine the use of O3FA interventions as a combination treatment with chemo(radio)therapy.
5. Examine the use of O3FA interventions in the management of advanced CRC including cachexia.
6. Outline future research priorities addressing O3FAs in the prevention and treatment of CRC.

Following the literature review I identified the need for a comprehensive population-based study which examined dietary O3FA intake, validated through measurements of O3FA blood levels and included FOS use data, in addition to considering factors such as CRC and tumour subsite risk.

2.1.2 Studies within the UK Biobank

In order to undertake a comprehensive population-based study that addressed the methodological weaknesses of the pre-existing observational data exploring O3FAs and CRC risk, I conducted two studies within the UK Biobank (UKBB).

The UKBB is a well-characterised prospective cohort of over a half a million individuals in the UK, which includes comprehensive dietary data, as well as data on nutritional supplement use. Between 2021 and 2023, the UKBB released data on plasma fatty acid levels for approximately two thirds of the UKBB population, providing a unique opportunity to explore further the relationship between dietary and supplement O3FA intake and blood levels of O3FAs. The two studies are described below.

2.1.2.1 The relationship between oily fish intake, fish oil supplement use and plasma polyunsaturated fatty acid levels in UK Biobank participants

I first examined the relationship between oily fish intake, FOS use and plasma O3FA levels within the UKBB population, to establish whether O3FA intake (oily fish and FOS use) predicted plasma O3FA levels. This study was conducted using the first tranche of plasma fatty acid levels for approximately a third of the UKBB population.

The hypothesis that oily fish intake and FOS use predict plasma fatty acid levels within the UKBB was tested.

The objectives of the study were to examine the relationships between the following within the UKBB:

1. Oily fish intake and FOS use.
2. Oily fish intake and plasma fatty acid levels.
3. FOS use and plasma fatty acid levels.
4. Oily fish intake and FOS use with plasma fatty acid levels.

2.1.2.2 The relationship between plasma omega-3 polyunsaturated fatty acid levels and colorectal cancer risk in UK Biobank participants

Once I had established that O3FA dietary data (oily fish intake and FOS use) predicted plasma O3FA levels, I used this data to examine O3FA plasma levels in relation to CRC risk within the UKBB. In addition to examining if there were any differential effects of O3FAs according to CRC subsite and sex.

The hypothesis that increasing plasma O3FA and DHA levels reduce overall CRC risk was tested, in addition to increasing plasma O3FA and DHA levels being associated with reduced CRC tumour subsite, specifically the proximal colon.

The objectives were to examine the following within the UKBB:

1. Plasma total O3FAs and DHA levels in relation to overall CRC risk.
2. Plasma O3FA and DHA levels in relation to CRC subsite risk; proximal colon, distal colon, and rectal cancer risk.

2.2 The interaction between omega-3 polyunsaturated fatty acids and dietary fibre on short chain fatty acid production within an *in vitro* colonic fermentation model

In parallel to this epidemiological approach, I conducted a study examining the potential mechanism by which O3FAs have anti-CRC activity via the colonic microbiome.

A potential mechanism of O3FAs is that they modulate the colonic microbiome increasing SCFA producing bacteria, that in the presence of fibre (substrate for SCFA production) increase SCFA production (3, 120, 121). However, it is not known whether O3FAs directly increase SCFA production. Therefore, O3FAs were added to an *in vitro* colonic fermentation model to investigate the effects on SCFA production in addition to examining different dietary fibres for SCFA production, to explore a potential O3FA-fibre interaction.

The following hypothesis were tested using an *in vitro* colonic fermentation model:

1. O3FAs increase SCFA production within an *in vitro* colonic fermentation model
2. O3FAs interact differently with specific dietary fibres within an *in vitro* colonic fermentation model.
3. O3FA exposure is associated with changes in the bacterial content within an *in vitro* colonic fermentation model

The objectives were to investigate the effects of O3FAs within an *in vitro* colonic fermentation model through:

1. SCFA production, measured by gas chromatography-flame ionisation detection (GC-FID).
2. Their interaction with different dietary fibre substrates (inulin, wheat bran and pectin) on SCFA production.
3. The changes in bacterial content within the *in vitro* colonic fermentation model using shotgun metagenomics.

Chapter 3 Omega-3 polyunsaturated fatty acids in colorectal cancer prevention and treatment

3.1 Introduction

This chapter consists of a narrative literature review linking clinical and observational studies to relevant mechanistic work and highlights the current gaps in understanding of how best to utilise purified O3FA formulations and optimise dietary O3FA intake guidelines for the prevention and treatment of CRC according to host, tumour, and gut microbiota factors. The aims and objectives of the literature review are outlined in **chapter 2 section 2.1.1**.

Experimental studies have consistently demonstrated the anti-inflammatory and anti-CRC activity of the two most abundant marine-derived, O3FAs, EPA and DHA (1). However, human observational data linking dietary O3FA intake and blood levels with reduced CRC risk are inconclusive (135). Over the last decade, several large-scale, prospective cohort studies, and randomised controlled trials (RCTs) have investigated the relationship between O3FA intake and colorectal neoplastic risk, as well as post-diagnosis CRC outcomes. There have also been advances in the understanding of the complex mechanisms underlying the anti-cancer activity of O3FAs, particularly the acceptance that O3FAs likely act through indirect effects on the gut microbiome and the host anti-tumour immune response, as well as direct activity on cancer cells themselves (30).

In 2011 the Hull group published '*Omega-3 polyunsaturated fatty acids for the treatment and prevention of colorectal cancer*' (1). It received 375 citations and outlined the current literature for the use of O3FAs in the treatment and prevention of CRC. The evidence at the time consisted mainly

of *in-vivo* pre-clinical animal studies and mucosal biomarker studies; with limited available evidence in the form of observational and clinical trial data. Therefore, this review reflects the current research landscape of the use of O3FAs in the prevention and treatment of CRC which includes better quality evidence in the form of clinical studies and observational data.

3.2 Methods and search strategy

Studies were identified through PubMed and MEDLINE using the following medical subject heading (MeSH) terms: '*fatty acids*', '*fish oils*', '*polyunsaturated fatty acids*', '*omega 3*', '*eicosapentaenoic acid*', '*docosahexaenoic acid*', '*EPA*', '*DHA*' and '*colorectal cancer*', '*colorectal neoplasms*', '*liver neoplasms*', '*colorectal cancer metastases*'. An extensive review of the literature of O3FAs and CRC prevention and treatment was published in 2011 by the Hull group (1). Therefore, only papers published in English between 2011, and August 2021 were included. Ongoing clinical trials were identified by searching the following clinical trials registries; ClinicalTrials.gov, International Standard Randomised Controlled Trial Number (ISRCTN) Registry, World Health Organisation (WHO) International Clinical Trials Registry Platform, Australian New Zealand clinical Trials Registry (ANZCTR) and the University hospital Medical Information Network (UMIN) Clinical Trials Registry.

3.3 Colorectal polyp studies

The classification of polyps based on histology and molecular pathological characteristics (**chapter 1, section 1.2.4**) has led to the understanding that CRC chemoprevention agents are likely to have differential activity against different polyp subtypes (136, 137). Studies with colorectal polyp outcomes are summarised in **table 3.1.**

The original demonstration of the chemopreventative effects of EPA in FAP patients was based on reduction of the number and size of rectal CAs (138). This study led to the phase 3 seAFOod Polyp Prevention Trial, which compared free EPA 2 g daily and aspirin 300 mg daily *versus* placebo in a 2x2 factorial RCT in individuals stratified as 'high risk' at screening colonoscopy for subsequent surveillance colonoscopy at one year (103). Although the primary outcome (the adenoma detection rate [ADR] – the presence or absence of at least one polyp at surveillance colonoscopy) was null for both interventions, there were important secondary findings. EPA users had an overall lower number of left-sided colorectal polyps (incidence rate ratio [IRR] 0.75 [0.60-0.94]) and CAs (IRR 0.86 [0.74-0.99]) at follow-up, in keeping with the findings of the earlier FAP study (103, 138). In contrast, aspirin was associated with a larger treatment effect, with reduced risk of all colorectal polyps (IRR 0.78 [0.68-0.90]), which included right-sided colon polyps (IRR 0.73 [0.61-0.88]) and SPs (IRR 0.46 [0.25-0.87]) (103).

In the seAFOod trial, there was a suggestion of an increase in right-sided colon polyps (IRR 1.02 [0.85-1.22]) and SPs in the active EPA arms (IRR 1.44 [0.79-2.60]) (103). This is counter-intuitive to observational data on CRC risk associated with high dietary marine O3FA intake that suggested a reduced risk of proximal CRCs and MSI-high tumours (139-144). Together, these findings may reflect the differential activity of O3FAs at different stages of colorectal carcinogenesis. Alternatively, the observations from the seAFOod trial could be a spurious finding given the small numbers of SPs reported in this RCT (EPA users n=30, non-EPA users n=22) (103).

A pre-specified sub-study inside '*The VITamin D and OmegA-3 TriaL*' (VITAL) RCT (145) – identified 777 participants that self-reported the finding of one or more colorectal polyps at colonoscopy during follow-up (146). Participants in the active O3FA group, who had a low plasma EPA+DHA level (% total FAs) at baseline, had a reduced risk of CA (OR 0.76 [0.52-1.02], P=0.03), as did African-American participants (OR 0.59 [0.35-1.00]) (146). These findings were mirrored in the association of O3FA use and the risk of myocardial infarction in the main VITAL trial (145). However, participant numbers in these secondary analyses were small. It is unclear whether lower O3FA levels in African American participants in VITAL reflects genetic variation in fatty acid metabolism and/or differences in dietary fatty acid intake, or both (86, 147). Ethnicity was not formally reported in the seAFOod trial but the trial population consisted overwhelmingly of White British individuals (unpublished data) (103).

Table 3.1 Randomised controlled trials and observational studies of colorectal polyp risk and O3FA interventions or dietary intake.

RCTs	Study	Study population	Treatment groups size	Intervention	O3FA assessment	Findings*,†
	Song et al. 2020 (146) **[VITAL]**	Healthy US population Women ≥55 & men ≥50 yrs, 71.3% non-Hispanic white, 20.2% African American	active=12,933, placebo=12,938	1g/d O3FA ethyl esters (460mg EPA, 380mg DHA) (also vit D 2,000 IU/d in 2x2 factorial design) median FU 5.3 yrs	Baseline FFQ Plasma O3FA levels‡	↔ **CA** OR 0.98 (0.84-1.29) **or SP** OR 1.05 (0.84-1.29) ↓ **CA in those with low baseline O3FA plasma level** (<2.5% plasma O3i) OR 0.76 (0.57-1.02) ↓ **CA in African American participants** OR 0.59 (0.35-1.00) **Median RBC % EPA change** from baseline at 12 months 0.7 (IQR 0.2,1.1) in active EPA arms(105)
	White et al. 2019 (148) **[TCPS]**	Participants with a history of colorectal adenoma, 40-80 yrs Genotype rs174535 SNP of FADS1	active=70 placebo=71	2.5g/d (1395mg EPA & 1125mg DHA) for 6 months	No dietary assessment RBC O3FA levels	↔ rectal epithelial cell proliferation ↓ **urinary PGE-M in women at 3 months in active group** (not seen at 6 months) ↔ **urinary PGE-M related to *FADS1* genotype** ↑ **RBC O3FA by** 76%§ in O3FA group vs 6% in placebo group, P=<0.0001
	Hull et al. 2018 (103) **[seAFOod]**	'High risk' after screening colonoscopy, (≥3 colorectal adenomas with at least one ≥10 mm in diameter, or ≥5 colorectal adenomas <10 mm in diameter) 55-73 yrs	active=356 Placebo=353	2g/d free EPA (also aspirin 300mg/d in 2x2 factorial design) for 12 months	EPIC FFQ before and after intervention RBC & rectal mucosal O3FA levels	↔ **ADR** RR 0.98 (0.87-1.12) ↓ **left-sided colorectal adenoma** IRR 0.75 (0.60-0.90) ↓ **CA** IRR 0.86, (0.74-0.99), ↔ **serrated colorectal adenoma** IRR 1.44, (0.79-2.60) **Median RBC % EPA absolute change** from baseline at 12 months 1.0 (IQR 0.2, 1.9) in active EPA arms

* Statistically significant increase(↑), statistically significant reduction (↓), no change **(↔)** in relative risk of O3FA vs placebo/control group, more than (**>**), more than or equal to (**≥**), less than (**<**), less than or equal to (**≤**),aberrant crypt foci (**ACF**), adenoma detection rate (**ADR**), arachidonic acid (**AA**), conventional adenoma (**CA**), confidence interval (**CI**), controls (**ctrls**), days (**d**), Dietary (**D**), eicosapentaenoic acid (**EPA**), European Prospective Investigation into Cancer and nutrition (**EPIC**), fatty acid desaturase 1 (**FADS1**), food frequency questionnaire (**FFQ**), follow up (**FU**), grams (**g**), Health Professionals Follow Up Study (**HPFS**), international units (**IU**), incidence rate ratio (**IRR**), interquartile range (**IQR**), milligram (**mg**), National Health Service Bowel Cancer Screening Programme (**NHS BCSP**),Nurses' Health Study (**NHS**), odds ratio (**OR**), omega-3 fatty acid (**O3FA**), omega-3 index (**O3i**), plasma (**P**), prostaglandin E_2 (**PGE_2**), prostaglandin E_2 metabolite (**PGE-M**), polyunsaturated fatty acids (**PUFAs**), quintile, red blood cells (**RBC**), serrated polyp (**SP**), single nucleotide polymorphisms (**SNPs**), Systematic Evaluation of Aspirin and Fish Oil polyp prevention trial (**SeAFOod**), Tennessee Colorectal Polyp Study (**TCPS**), tertile (**T**) vitamin D (**vit D**), The VITamin D and OmegA-3 TriaL (**VITAL**), years (**yrs**).

† Figures in brackets are the 95% confidence interval, unless stated otherwise.

‡ Plasma O3FA levels in 60% of participants of each group at baseline with a further 1,583 participants providing a further sample at one year.

§ % of total membrane fatty acids.

Observational studies	Cohort	Cohort information	Study numbers	PUFA assessment	Findings
	Wang et al. 2020 (149)	**NHS, NHS II & HPFS** Female nurses and male health professionals Median FU 20 yrs	n=4,517 CA=493, SP=316	Validated FFQ RBC PUFA levels	↔ **CA risk** (OR 1.03, 0.93-1.14) and **SP risk** OR 0.98, 0.86-1.12) related to RBC O3FA levels **no association with location in colorectum**
	He et al. 2018 (150)	**NHS, NHS II & HPFS** Female nurses and male health professionals FU 18-20 years Mean age 60.2 yrs 95% white	n=141,143, non-polyp participants=119,676, CA=9,212, SP=7,945, synchronous CA & SP=2,382	Validated FFQ No PUFA assessment	↓ **SP risk with O3FA intake** [Q1 vs Q4] OR 0.90 (0.84-0.96) ↓ **CA risk with O3FAs intake** OR 0.89 (0.84-0.95) No relationship between O3FAs intake and polyp location or size (P for heterogeneity >0.05)
	Mo et al. 2018 (151)	**UConn Health clinical study of ACF** Mean age 57 yrs 92% Caucasian	Proximal colonic lesion cases=72 (proximal ACF=28, proximal polyp=44) ctrls=36	70-item Block FFQ No PUFA assessment	↑ **proximal colon neoplasia risk with O3FA intake** [mean intake cases 0.872g/1,000Kcal vs 0.746g/1,000Kcal] [cases vs ctrls] OR 2.10 (1.11-3.97) ↑ **ACF risk with O3FA intake** [ACF vs ctrl] OR 2.29 (1.02-5.13) ↔ **polyp risk with O3FA intake** [polyp case vs ctrl] OR 1.76 (0.98-3.18)
	Rifkin et al. 2017 (152)	**TCPS** Screening colonoscopy 40-75 yrs 90.5% White, 7.6% African American	**Adenoma cases=904, polyp-free ctrls=835	108-item FFQ (not validated for PUFA assessment) RBC PUFA levels	↔ **advanced adenoma with RBC EPA levels** [T3>0.55% vs T1 0%] OR 0.41 (0.16-1.05) ↓ **all adenomas & RBC DPA** levels OR 0.64 (0.41-1.00) ↑ **all adenomas & RBC AA** levels OR 1.66 (1.05-2.62)
	Cottet et al. 2013 (153)	**French EN3-EPIC Cohort** Female members of a national health insurance scheme 40-65 yrs Average FU 6.6 yrs	Cases=328, ctrls=619	208-item validated FFQ RBC PUFA levels (not correlated with dietary intake)	↔ **colorectal adenoma with RBC EPA levels** [T1 <0.89% vs T3 1.18%] OR 0.88 (0.62-1.25) ↓ **advanced adenoma with increased RBC EPA levels** OR 0.50 (0.27-0.93) ↓ **advanced adenoma with increased RBC DHA levels** [T1 6.10% vs ≥ 7.21%); OR 0.50 (0.26-0.96)
	Murff et al. 2012 (154)	**TCPS** Screening colonoscopy 40-75 yrs 90.5% White, 7.6% African American	**Adenomatous polyp cases=1597, hyperplastic polyp cases=544, polyp-free cases=3166	108-item FFQ (not validated for PUFA assessment) Urinary PGE-M	↓ **adenoma risk with O3FA intake in women only** [Q1 0.012g/d vs Q5 0.232g/d]; OR 0.67 (0.47-0.97)

** The term Adenoma includes CA (tubular, tubulo-villous and villous) and SP (including traditional serrated adenoma).

Ongoing studies of primary polyp prevention are described in **table 3.2** and include an RCT (NCT03806426) of free EPA 2 g daily, with a primary endpoint of the total number of polypectomies (of lesions >5mm) in the rectal stump of post-colectomy FAP patients over a two-year period, which plans to recruit 204 participants (155). The study was estimated to complete in January 2024 and no results have as yet been published. Other clinical studies exploring O3FA intervention with relevance to colorectal polyp prevention are PREPARE & OMICC, which are both using 4 g daily of pure EPA ethyl ester (EE) in individuals with a prior history of removal of at least one colorectal adenoma (99), and individuals with histologically-confirmed localised CRC or an advanced colorectal mass/polyp, for which surgical or endoscopic resection is planned respectively (156). The primary endpoint of both trials is the O3FA composition in colorectal tissue following 28-30 days of intervention. Secondary outcomes include changes to the gut microbiome (PREPARE) and changes to the gene expression profile of the resected tumour (OMICC). Colorectal polyp recurrence is not a specified secondary outcome in either of these small, short-term studies (**table 3.2**) (99, 156). The OMICC Study was withdrawn in November 2021 due to a lack of enrolment, while PREPARE completed in February 2024 and the results are awaiting publication.

Only one observational study has addressed an association between dietary intake of marine O3FAs and risk of colorectal polyp, which included stratification for polyp subtype (150). This large US study (n=141,143 with follow up for 18 to 20 years) found that increased intake of marine O3FAs was associated with lower risk of both CA ($P<0.001$) and SP ($P=0.004$) (150). However, a smaller study inside the same prospective cohort did not observe

any relationship between RBC O3FA (as the % total FAs) and risk of either CA (n=493, median EPA % 0.44) or SP (n=316, median EPA % 0.43) (149).

Two independent observational studies have reported that the RBC EPA level (as % total FAs) equal to or above the third tertile (reported as 1.18% and 0.55% in the separate studies) is associated with an approximately 50% reduced risk of advanced colorectal adenoma (defined as a polyp ≥10 mm, or with high grade dysplasia, or with any villous component) (152, 153). However, neither study analysed O3FA supplement use or included analysis according to colorectal polyp subtype. The difference in EPA content (as a % of total FAs) between these studies highlights heterogeneity in study populations that makes interpretation of the relationship between tissue O3FA levels and neoplastic risk challenging, even when detailed dietary data are available (**table 3.2**) (149, 152, 153).

By contrast with the observational studies described above, a small case-control study found an association between dietary O3FA intake and increased proximal colonic polyp risk (OR 1.76 [0.98-3.18]), as well as aberrant crypt focus (ACF) incidence (OR 2.29 [1.02-5.13]) (151). Subsequent observational studies of dietary O3FA intake and RCTs of O3FA supplementation will require standardised measurement of blood and/or tissue O3FA levels to understand the relationship between the steady-state O3FA level, post-intervention change in O3FA status and subsequent neoplastic risk.

The Tennessee Colorectal Polyp Study (TCPS) reported levels of the stable urinary metabolite of PGE_2 (11-alpha-hydroxy,9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid; also known as PGE-M) in order to explore a

mechanistic link between O3FA intake and colorectal polyp risk based on the inhibitory activity of EPA on the COX isoforms (157, 158). This case-control study found that women (n=68 cases, 290 controls) with the highest intake of dietary O3FAs (median intake 0.232 g/d) had reduced adenomatous polyp risk (OR 0.67 [0.47-0.97], P-trend=0.01), which correlated with reduced urinary PGE-M levels (r= –0.18, P=0.002) (154). Urinary PGE-M was further evaluated as a biomarker of O3FA activity in a double-blind, placebo-controlled RCT (n=141) of 2.5 g daily of mixed O3FAs in TCPS participants with a history of colorectal adenoma, who were stratified by *FADS1* genotype (rs174535). There was no change in rectal epithelial call proliferation, regardless of *FADS* genotype (159). However, a decrease in urinary PGE-M was observed at 3 months, but not 6 months, in participants allocated to O3FAs, with the effect limited to females (148).

Table 3.2 Registered ongoing clinical trials of O3FA interventions.

Study††	Design	Population	PUFA dose	Treatment duration	Primary Outcome	PUFA content analysis	Current status at July 2023
Effect of EPA-FFA on polypectomy in FAP *NCT03806426* *(155)*	Double blind placebo controlled RCT	Individuals with a diagnosis of FAP (aim to recruit n=204)	2g/d Free EPA	2yrs	Total number of polypectomies (lesions >5mm)	Not stated	Recruiting
EPA for Metastasis Trial 2 (EMT2) NCT03428488 (160)	RCT	Patients undergoing curative liver resection surgery for CRCLM (aim to recruit 448 participants)	4g/d EPA EE	Prior to resection for up to 4yrs	Progression-free survival	RBC EPA levels	Recruiting
Fibre and Fish Oil Supplements for the Prevention of Colorectal Cancer *NCT04211766* (161)	Randomised cross over double-blind trial	Healthy volunteers who consume <20g/d of fibre, post-menopausal women (aim to recruit n=30)	Not stated	30d	Change in mRNA expression profiles in the exfoliated transcriptome in stool	Not stated	Completed, no results posted
Multimodal Intervention for Cachexia in Advanced Cancer Patients Undergoing Chemotherapy (MENAC) NCT02330926 (162)	RCT	Advanced cancer patients undergoing chemotherapy (aim to recruit 240 participants) 4 groups: Standard care; Nutritional supplements and advice; Home-based self-assisted exercise program; Ibuprofen	EPA 2g/d & DHA 1g/d (ONS or capsules)	6wks	Change in body weight	Not stated	Completed, no results posted
Omega-3 Fatty Acid for the Immune Modulation of Colorectal Cancer (OMICC) NCT0366S1047 (156)	Prospective, double-blind, placebo controlled RCT	Patients with CRC or colorectal mass or polyp suspected to be a cancer or advanced adenoma undergoing surgical resection or interventional endoscopy (aim to recruit n=36)	4g/d EPA EE	Up to 30d	Change in O3FA composition of colorectal tissues	Fatty acid analysis of biopsy tissue	Withdrawn due to no recruitment
PeRioperative Omega Three and the Effect on ImmNity (PROTEIN) *NCT03598413* (163)	RCT	Patients with CRC undergoing laparoscopic resection (aim to recruit n=50)	ONS 200ml, 1.42g of EPA and DHA per bottle	7d pre & 7d post-op	Changes in phagocytosis of E.coli, S.Aureus and Candida measured with flow cytometry	Not stated	Completed, no results posted
PRevention Using EPA Against colorectal Cancer (PREPARE) *NCT04216251* *(99)*	Interventional clinical trial	Individuals with a history of colorectal adenoma (aim to recruit 80 participants)	4g/d EPA EE	8-12wks	Change in O3FA composition of colorectal tissues	Fatty acid analysis of biopsy tissue	Completed, no results posted

††Days (**d**), docosahexaenoic acid (**DHA**), ethyl ester (**EE**)eicosapentaenoic acid (**EPA**), grams (g), grams per day (**g/d**), free fatty acid (**FFA**), omega-3 polyunsaturated fatty acids (**O3FAs**), oral nutritional supplement (**ONS**), polyunsaturated fatty acids (**PUFAs**), post-operation (**post-op**), pre-operation (**pre-op**), randomised control trial (**RCT**), red blood cells (**RBCs**), weeks (**wks**), years (**yrs**).

3.4 Primary prevention: randomised intervention trials which include data on CRC outcomes

Two large 2x2 factorial RCTs have explored the effect of 1 g mixed O3FAs (460 mg EPA, 360 mg DHA) daily and either vitamin D (2,000 international units daily, VITAL) or aspirin (300 mg daily, The ASCEND trial) *versus* olive oil placebo, on cardiovascular and overall cancer risk. Both RCTs included CRC data (145, 164).

The VITAL trial studied a healthy population (n=25,781) of men and women aged ≥50 and ≥55 years, respectively. During a median follow up of 5.3 years, there was no reduction in the primary outcome measures of major cardiovascular event (HR 0.92 [0.80-1.06], P=0.24) or risk of invasive cancer diagnosis (HR 1.03, [0.93-1.13], P=0.56). However, secondary analyses demonstrated a reduction in several vascular outcomes including myocardial infarction (HR 0.72 [0.59-0.90]) in the treatment group. Secondary analysis of cancer outcomes included only a small number of incident CRC cases (54 in the O3FA group *versus* 44 in the placebo group), with no significant difference observed between O3FA users and non-users (HR 1.23 [0.83-1.83]) (145). However, VITAL and its ancillary polyp study pinpointed specific characteristics (African-American ethnicity, low baseline plasma EPA+DHA level) associated with cardiovascular (decreased myocardial infarction risk) and polyp prevention benefit from the O3FA intervention (146).

The ASCEND trial included 15,280 diabetic participants aged ≥40 years, 96.5% of the study population was of White ethnicity. During a mean follow up period of 7.4 years, there was no reduction in the primary outcome of first serious vascular event (RR 0.97, P=0.55). There was no statistically significant reduction in the secondary endpoint of gastrointestinal (which

included colorectal) cancer risk (2.9% vs 3.2%, RR 0.90 [0.75-1.07]) in the active O3FA group (164).

To test the chemoprevention efficacy of O3FAs in specific ethnic groups and stratify individuals for a low baseline O3FA level, a very large study would be required. It will be critical for all future studies to collect a core dataset to include ethnicity, genetic profile, and standardised blood O3FA measurements to allow subsequent individual participant meta-analysis. An alternative approach would be to analyse on-trial, and possibly post-intervention, cancer (including CRC) outcomes (using national cancer registry data when possible) from published large cardiovascular RCTs by individual participant meta-analysis (165, 166).

3.4.1 Insights from measurement of blood O3FA levels

Measurement of blood O3FA levels in large RCTs has provided some insight into baseline O3FA levels and variability of the individual response to O3FA interventions. However, the research field continues to be hampered by heterogeneity in choice of O3FA biomarkers (individual fatty acids, O3i, O3:O6FA ratio) and sample types tested (RBCs, plasma, whole blood), which make comparisons between studies difficult.

Blood O3FA levels were measured in only a small proportion of participants in the VITAL and ASCEND trials with the O3i (the combined % EPA and DHA of total FAs in RBC membranes), reported in 200 (0.8%) and 152 (1%) participants respectively (145, 167). Plasma %EPA+DHA was reported in a larger subgroup of the VITAL trial study population (n=15,353 at baseline, n=1,538 at 1 year) (105). There was a strong correlation between the RBC and plasma %EPA+DHA (Spearman correlations: EPA 0.67, DHA 0.73

[P<0.001]) (105). Despite a 54.7% increase in the mean plasma %EPA+DHA in the treatment group, both baseline (2.7%) and 1-year (4.1%) mean plasma %EPA+DHA values were significantly lower than the suggested 8% threshold value, above which reduced cardiovascular risk is observed (168). The median RBC %EPA+DHA in the treatment group was 7.82% [IQR, 6.36-8.5%] but the proportion of participants with a threshold value of ≥8% was not reported (105, 145, 168, 169). In contrast, the ASCEND trial, which evaluated the same daily dose of O3FAs as VITAL, measured EPA+DHA in whole blood spots (blood blotted and then dried on paper). Whole blood %EPA+DHA increased by 32.5% (95% CI 26.3 – 39.1%) in the intervention group (baseline EPA+DHA 7.1% and follow-up 9.1% [the number of participants with a %EPA+DHA of ≥8% was also not reported]) (164). The concept of a threshold O3FA value has gained some credence for interpretation of baseline cardiovascular risk linked to O3FA levels and assessment of benefit from O3FA supplementation for cardiovascular risk reduction (168). A similar biomarker-driven approach to cancer risk and benefit could be taken when sufficient % O3FA level data are available from intervention trials.

Future RCTs should include blood O3FA biomarker assays to allow comparison of O3FA exposure across trials. Blood spots provide a practical method of collecting large scale sample sets. However, the cost of O3FA measurement through gas or liquid chromatography mass spectrometry will continue to limit large-scale testing (107). The importance of uniform measurement of O3FA levels for comparison of RCTs has been highlighted by the REDUCE-IT trial of 4 g of EPA-EE (Icosapent ethyl), which demonstrated a significant reduction in the composite primary outcome of cardiovascular death, nonfatal myocardial infarction, nonfatal stroke,

coronary revascularization, or hospitalization for unstable angina (98), unlike the VITAL and ASCEND trials that were null for the primary cardiovascular outcome (145, 164). The difference in RCT outcomes may be explained by the respective dose and formulation of O3FAs that were evaluated (4 g pure EPA-EE daily compared with 460 mg EPA and 360 mg DHA-EE daily). However, systemic bioavailability of EPA in REDUCE-IT was measured as the absolute serum EPA level (µg/mL), which does not allow comparison with relative (%) fatty acid content data from the VITAL and ASCEND trials (98).

3.4.2 Formulation of O3FA supplements

There has still not been a direct clinical comparison of the anti-CRC activity of EPA alone compared with the combination of EPA and DHA. Current understanding of mechanism(s) of action of EPA and DHA suggests that they share multiple anti-inflammatory and plasma membrane modulatory properties. However, synergistic, rather than additive, anti-cancer activity of EPA and DHA needs formal exclusion. There is currently no RCT support for the concept that EPA is a ‘universal donor’ and can be converted to DHA in humans, who are supplemented with pure EPA (166, 170, 171).

There is a large literature regarding differences in bioavailability of O3FAs as the triglyceride, EE or as the free fatty acid in short-term human dosing studies (172). The seAFOod trial provided the opportunity to compare dose-equivalents of EPA as the free fatty acid and in triglyceride form during a 12-month intervention (103). There was no statistically significant difference in tissue (RBC and rectal mucosa) EPA incorporation, adverse events (AEs) or compliance between free EPA and triglyceride formulations (103). In general, recent large RCTs have confirmed excellent tolerability of O3FAs across a wide daily dose range (98, 103, 145, 164).

3.4.3 Risks associated with O3FA dosing

A recent meta-analysis of RCTs investigating the use of O3FA for cardiovascular disease prevention found an overall increased risk of atrial fibrillation/flutter (AF) associated with O3FA supplementation (EPA alone, as well as combination EPA and DHA) compared with placebo (IRR 1.37 [1.22-1.54], P=<0.001) (173). Although the relationship between O3FA use and the risk of AF was highlighted by the REDUCE-IT trial of EPA-EE 4 g daily, which reported a significant increase in risk of AF in the EPA arm (3.1%) compared with placebo users (2.1%, P=0.004), particularly in those with a past history of AF (98), increased AF risk was also apparent in trials of lower-dose O3FA supplements, including ASCEND (164).

The REDUCE-IT trial also highlighted a small, but statistically significant, increase in serious bleeding risk associated with EPA-EE 4 g daily compared with placebo (3.4% *versus* 2.6%) in individuals taking concomitant anti-thrombotic treatment (98, 174).

It remains unclear whether the risk profile of O3FA supplementation is similar in individuals at risk of CRC, or with CRC, to those with established cardiovascular disease. However, CRC and atheromatous vascular disease share several predisposing factors and often co-exist (175).

The strength of these randomised intervention trials include their large scale and long follow up times. These studies were primarily conducted to examine cardiovascular outcomes but included cancer outcomes including CRC within their secondary outcomes. Therefore, they were not powered for the purpose of investigating CRC prevention. In addition, the study populations consisted of participants who were much younger than the population at risk

of CRC, which is generally older. There are also limitations such as dose and formulation of the O3FA intervention in addition to comprehensive measurements of O3FA levels within the trials. Blood O3FA measurements provide an objective assessment of compliance, in addition to valuable insights of the bioavailability of O3FAs, which may reflect the treatment effect. Nonetheless, these studies provide important insights into the feasibility of investigating O3FA interventions within potential future large scale clinical trials, with regards to the design and consideration of O3FA intervention doses and formulations.

3.5 Epidemiological evidence that dietary O3FAs reduce CRC risk

Despite their inherent limitations, cohort studies with large study populations and long follow-up, are a valuable resource given the time and financial constraints of performing RCTs with CRC outcomes and the methodological challenges of randomised assessment of diet and O3FA supplements (see **table 3.3** for a summary and details of all studies since 2011).

The 2018 '*Diet, nutrition, physical activity and colorectal cancer*' report by the World Cancer Research Fund (WCRF) Report and American Institute for Cancer Research concluded that there is '*limited, but suggestive*' evidence that fish consumption decreases CRC risk (29). The latest data in the Continuous Update Project (18 studies, 1994-2014) included large US cohorts (NHS & HPFS, 2014) and the European Prospective Investigation into Cancer and Nutrition study (EPIC, 2013) (139, 176). A dose-response meta-analysis in the WCRF report (11 studies [1999-2014], 10,365 cases) found an inverse association (RR 0.89 [0.80-0.99]) between fish intake and CRC risk. However, in sensitivity analysis when the EPIC study (2013)

(n=4,355 cases, 40% of the study population) was excluded, there was no significant relationship (RR 0.94 [0.82-1.07]) (135, 176).

Within the last decade, five other meta-analyses have been published (177-181). These essentially meta-analyse the same group of studies, (four studies included data from 1994 to 2014 and only three studies included the US cohort data) (177-181). Not surprisingly, all concluded that there was no association between dietary O3FA intake and reduced CRC risk (177-181). One (the only one to include the 2020 EPIC study) did a meta-analysis of blood O3FA levels (RBC, plasma and serum) from five original studies (1,553 cases) and found that high blood O3FA levels were inversely associated with overall CRC risk (RR 0.79 [0.65-0.98]) (181). There was a dose-response relationship with CRC risk decreasing by 4% for every 1% increase in blood O3FA levels (181). Supporting these data, a recently published pooled analysis of 15 prospective cohort studies (n=4,248 cancer deaths), which examined RBC and/or plasma O3FA levels, found that EPA, DPA, DHA and EPA+DHA levels were all associated with reduced cancer-specific mortality (EPA level and cancer mortality HR 0.91 [0.85-0.96]) (182).

3.5.1 Measurement of dietary O3FA intake and blood O3FA levels

O3FA exposure has been assessed through the variable use of food frequency questionnaires (FFQ). The evaluation of O3FA intake in individual FFQs differs, with only marine sources high in EPA and DHA quantified in some studies (Nurses' Health Study [NHS] & Health Professionals Follow-Up Study [HPFS] cohorts) (139-141, 183); while other studies include assessment of intake of the biologically-inactive 18-carbon O3FA, ALA, sourced from nuts and seeds (Women's Health Initiative & The Rotterdam Study) (184, 185). Other factors influencing O3FA exposure include: the

geographical variability in the quantity of fish available for consumption, per capita fish consumption (highest in Oceania and lowest in Africa, with fish consumption increasing with economic development) (135, 186); and different types/species of fish in the diet (oily [O3FA content highest in mackerel, salmon, herring] or other white fish intake). For example, one case-control study found an inverse association between CRC risk and fish/seafood intake (predominantly oily fish) (OR 0.56 [0.39-0.80]) (187), whereas another found white fish intake was associated with increased CRC risk (OR 1.13 [1.06-1.20]) – perhaps due to the majority of white fish being either fried, cooked in butter or smoked (188). Smoked fish contains nitrosamines, similar to processed meats, which have carcinogenic potential, a potential confounder when evaluating fish intake (135).

Unfortunately, not all FFQs and dietary assessment tools have been validated for the assessment of O3FA intake by O3FA biomarker measurements. In addition, FFQ data may be subject to recall, documentation, and measurement bias (189, 190). Newer FFQs and dietary tools, such as electronic 24-hour dietary recall, may help to quantify O3FA intake more accurately, if validated using RBC or whole blood O3FA levels (191-193). Unfortunately, few observational O3FA studies have included dietary tool validation, to date (**table 3.3**) (139, 142, 194, 195).

The EPIC study published in 2020 (n=521,324) found that total fish intake was inversely proportional to CRC risk, with a weekly intake of 100 to 200 g of fatty or lean fish (a single portion of fish is considered 140 g) associated with 7% lower risk of CRC. A ‘dose’-response was observed for fatty fish intake (142). This weekly ‘dose’ of fish is similar to the current UK Scientific Advisory Committee on Nutrition (SACN) guideline, which recommends

intake of two portions of fish per week (one of which is oily), which equates to approximately 2.8 g of O3FAs (89). A nested case-control study within the EPIC study observed no relationship between plasma O3FA (% of total FAs) and CRC risk (142) However, there appeared to be a (statistically insignificant) reduced risk of proximal colon cancer (OR 0.55 [0.27-1.11]) in individuals with higher levels of plasma total O3FAs (EPA + DPA + DHA), compared with distal (OR 1.54 [0.77-3.08]) colon cancer (142). A further nested case-control biomarker study within the EPIC cohort (n=1,069 CRC cases, median follow-up 6.4 years) that did not analyse dietary PUFA intake, evaluated RBC PUFA levels and CRC risk (196). It found that increasing % EPA was associated with decreased CRC risk (OR 0.75 [0.62-0.92] per 1 mol%) (196). However, in contrast to the earlier EPIC publication (142), increasing EPA (as % of total FAs) was associated with a reduced risk of distal colon (0.63 [0.43-0.91]) and rectal cancer risk (OR 0.67 [0.48-0.94]) (196).

Two other observational studies have examined CRC risk according to plasma O3FA (% of total FAs), neither of these studies found an association between EPA and CRC risk, however statistically significant associations for other fatty acids were reported (194, 195). Docosapentaenoic acid (DPA) (% of total FAs) [which is not thought to be a bioactive O3FA], were associated with reduced CRC risk (P-trend=0.04) and an inverse association between DHA and rectal cancer risk was also found (p-trend=0.006) (195). Whereas, ALA content was associated with reduced colon cancer risk (OR 0.41 [0.23-0.73]), but not rectal cancer risk (OR 1.70 [0.84-3.43]).(194) It is possible that these findings are spurious reflecting small study numbers and multiple testing **(table 3.3**) (142, 194, 195).

One report from the large US Nurses' Health Study (NHS) and Health Professionals Follow-Up Study (HPFS) (n=123,529, 24-26 years follow up) reported the relationship between predicted RBC EPA and DHA content and CRC risk using a prediction model derived from dietary data and historical RBC values (% of total FAs) in cohort participants (139). Higher predicted RBC EPA was associated with increased distal CRC risk in women (HR 1.38, P=0.04) and decreased rectal cancer in men (HR 0.62, P=0.04) (139). An increase in distal colon cancer risk, not limited to a specific sex difference has also been reported by others (142, 143, 197, 198) (**figure 3.1**). Other studies have also reported sex differences in the relationship between O3FA intake and CRC risk, however it is difficult to interpret these findings given relatively small study sizes (143, 177, 199). Improved mechanistic understanding of the anti-CRC activity of O3FAs is needed to interpret possible sex-specific differences in the relationship between O3FA intake and CRC risk. Valid mechanistic hypotheses include alteration of oestrogen metabolism and sex hormone signalling by O3FAs.

More recently, in an attempt to exclude the inherent bias of traditional observational studies, several Mendelian Randomisation (MR) studies have attempted to examine the relationship between PUFAs and CRC risk using genetic variants (instrumental variables [IVs]) to predict PUFA levels (200-203). The FADS gene cluster (FADS1/2), in which there are multiple single nucleotide polymorphisms (SNPs), controls the rate-limiting step for conversion of O6FA and O3FA precursors LA and ALA into AA and EPA, respectively (**chapter 1, section 1.6.1**). However, several of the IVs used within MR studies adopt the same SNP to test different PUFAs. For example, rs174547 is used as the IV for AA, ALA and DPA levels (200, 202). There are also difficulties in interpreting MR studies that stem from limited

understanding of nutrient (PUFA)-gene interactions, given that substrates and products with opposing inflammatory and neoplastic activity (O3FAs and O6FAs) are linked to the same PUFA-metabolising genes (FADS and ELOVL enzymes) (146).

Table 3.3 Epidemiological studies of CRC risk and fish as O3FA intake.

Cohort‡‡	Demographics	Follow up	Dietary assessment	Intake	CRC outcomes	PUFA levels	Subsite analysis
Nguyen et al. 2020 **Shanghai Men's Health Study (SMHS)** (204)	N=59,986 (876 CRC cases) Men 40-74yrs	Mean 9.8yrs	Validated 87-item FFQ	**Marine O3FA intake** Q4 vs Q1§§	↔ **CRC risk & O3FA intake** HR 1.09 (0.87-1.36)***		
Shin et al. 2020 **Swedish Women's Lifestyle and Health (WLH) cohor**t (205)	N=48,233 (344 CRC cases) Women 29-49yrs	Median 21.3yrs	Self-administered validated 80-item FFQ	**O3FA intake** Q4 1.55-22.66g/d vs Q1 0.12-<1.08g/d]	↔ **CRC risk & O3FA intake** HR 0.97 (0.72-1.32)		↔ **colon cancer risk & fish-oil derived O3FA intake** HR 1.25 (0.84-1.87) ↓ **rectal cancer risk & fish oil derived O3FA intake** HR 0.67 (0.42-1.08) ↑ **rectal cancer risk & ALA intake** HR 1.61 (0.98-2.66) ↓ **rectal cancer risk & DHA intake** HR 0.59 (0.37-0.96)
Aglago et al. 2020 **European Prospective Investigation into Cancer and Nutrition (EPIC)** (142)	n=476,160 (6,291 CRC cases) 35-70yrs	Median 14.9yrs	Validated centre-specific questionnaires. Fish and fish products estimated using the US Department of Agriculture Nutrient Database previously matched with the EPIC food list validated	**Total fish intake** Q5 >51.3g/d vs Q1 <9.07g/d,	↓ **CRC risk & total fish intake** HR 0.88 (0.80-0.96)	Nested case-control study (n=461 cases, n=241 ctrls) ↔ **plasma O3FA levels & overall CRC risk or CRC risk according to subsite** (proximal, distal & rectal CR) **Inverse but not statistically significant trend for proximal CR** OR 0.55 (0.27-1.11) **compared**	↓ **proximal colon cancer risk & fatty fish intake** HR 0.81 (0.70-0.95) ↓ **colon cancer risk & O3FA (EPA+DPA+DHA) intake** HR 0.85 (0.75-0.96) ↔ **rectal cancer risk & O3FA (EPA+DPA+DHA) intake** HR 0.91 (0.77-1.08) ↓ **colon cancer risk & EPA intake** HR 0.87 (0.77-0.98) ↓ **colon cancer risk & DPA intake** HR 0.83 (0.73-0.94) ↓ **distal colon cancer risk & DPA intake** HR 0.82 (0.68-1.00)

‡‡Statistically significant increase (↑), statistically significant reduction (↓), no change (↔), more than (**>**), more than or equal to (**≥**), less than (**<**), less than or equal to (**≤**), colorectal cancer (**CRC**), controls (**ctrls**), day (**d**), docosahexaenoic acid (**DHA**) , disease-free survival (**DFS**), eicosapentaenoic acid (**EPA**), European Prospective Investigation into Cancer and nutrition (**EPIC**), food frequency questionnaire (**FFQ**), fish oil (**FO**), follow-up (**FU**), grams per day (**g/d**), hazard ratio (**HR**), Health Professionals Follow up Study (**HPFS**), Japan Public Health Centre Based Prospective Study (**JPHCBPS**), Melbourne Collaborative Cohort Study (**MCCS**), microsatellite instability (**MSI**), microsatellite stable (**MSS**), milligrams (**mg**), Nurses' Health Study (**NHS**), Nurses' Health Study II (**NHSII**), odds ratio (**OR**), omega-3 fatty acid (**O3FA**), Overall survival (**OS**), plasma levels (**PL**), polyunsaturated fatty acid (**PUFA**), quartile (**Q4**), quintile (**Q5**), recurrence free survival (**RFS**), Singapore Chinese Health Study (**SCHS**), Shanghai Men's Health Study (**SMHS**), sub-site analysis (**SSA**), Swedish Women's Lifestyle and Health (**SWLH**), Tertile (**T**) versus (**vs**), years (**yrs**), Women's Health Initiative (**WHI**), Swedish Women's Lifestyle and Health (**SWLH**), United Kingdom Dietary Cohort Consortium (**UKDCC**).

§§ Where tertiles (**T3 vs T1**), quartiles (**Q4 vs Q1**) or quintiles (**Q5 vs Q1**) of PUFA intake appear without values, they were not available in the primary article.

*** Figures in brackets relate to 95% confidence intervals unless states otherwise.

						with distal CR OR 1.54 (0.77-3.08)	↓ **colon cancer risk & DHA intake** HR 0.87 (0.77-0.99)
Bradbury et al. 2020 **UK Biobank** (206)	n=475,581 (2,609 CRC cases) 40-69yrs	Average 5.7yrs	Touchscreen FFQ (207) & web based 24 hour dietary data questionnaire Spearman's correlation coefficients for nutrients in 24 hour and interviewer questionnaire 0.5 to 0.9	**Mean fish intake** Q4 39g/d vs Q1 6g/d **Oily fish mean intake** Q4 22g/d vs Q1 1g/d	↔ **CRC risk & total mean fish intake** HR 0.96 (0.86-1.07) ↔ **CRC risk & oily fish mean intake** HR 0.87 (0.74-1.02)		↔ **total fish intake & colon cancer risk** HR 0.93 (0.77-1.11) or **rectal cancer risk** HR 0.93 (0.77-1.13) ↔ **total fish intake & proximal cancer risk** HR 0.93 (0.77-1.11) or **distal cancer risk** HR0.93 (0.77-1.13)
Sellem et al. 2019 **NutriNet-Sante Cohort** (208)	n=44,039 (190 digestive cancer cases) aged >18yrs & access to the internet	2009-2017	Web-based self-administered FFQ, 24-hour dietary records. Mean daily nutrient intakes estimated using French food composition tables (>3,300) items (209)	**O3FA intake** Q5 vs Q1	↔ **digestive (colon, rectum, liver, stomach) cancer risk & O3FA intake** HR 1.02 (0.62-1.70), ↑ **digestive CR & O3FA in those with <median Vitamin C intake** [HR 1.57 (0.82-2.98) P-trend=0.5], ↔**digestive cancer risk & O3FA in those with <median fruit and vegetable intake** HR 1.10 (0.55-2.19) ↔ **in digestive cancer risk & O3FA intake in those with >median fruit & vegetable or Vitamin C intake**		
Butler et al. 2017 **Singapore Chinese Health Study (SCHS)** (194)	n=350 CRC cases (211 colon, 139 rectal)	Median 3.3 yrs	Validated 165-item FFQ (210)	**ALA** Q4 >3.8 µmol/dL vs Q1 <1.9 µmol/dL **EPA** Q4 >5.1 µmol/dL vs Q1 <2.4 µmol/dL **DHA** Q4 >35.2 µmol/dL vs Q1 <17.6µmol/dL	↓ **colon cancer risk & ALA** ↓ **colon cancer risk & LA** OR 0.43 (0.23-0.82)	**Plasma fatty acid composition (**µmol/dL) **EPA colon** cases 3.52 vs ctrls 3.48, **rectal** cases 3.48 vs ctrls 3.52 **DHA colon** cases **24.04** vs ctrls 23.68, **rectal** cases 25.02 vs ctrls 25.82 **ALA colon** cases **2.50** vs ctrls 2.88, **rectal** cases 2.78 vs ctrls 2.58	↓ **ALA & colon cancer risk** OR 0.41 (0.23-0.73) ↔ **EPA & colon cancer risk** OR 0.93 (0.54-1.62) ↔ **EPA & rectal cancer risk** OR 1.89 (0.42-1.91) ↔ **DHA & colon cancer risk** OR 1.11 (0.59-2.07) ↔ **DHA & rectal cancer risk** OR 0.72 (0.32-1.66)

Song et al. 2017 **Nurses' Health Study (NHS) & Health Professionals Follow up Study (HPFS)**††† (183)	n=1,659 CRC cases	Median 10.4yrs	Validated 116 to 131-item semi-quantitive dietary FFQ (211)completed every 4 years (NHS, NHSII & HPFS), 4 fish and seafood items. Nutrient composition data based on US Department Agriculture Nutrient Database FO supplement use included in calculation of O3FA, EPA, DHA intake Validated FFQ derived O3FA intake with plasma O3FA levels (r=0.58)	**O3FA intake** <0.10g/d vs >0.30 g/d]	↓ **CRC specific mortality & O3FA intake** HR 0.59 (0.35-1.01) ↔ **All-cause mortality & O3FA intake** HR 0.95 (0.77-1.21) ↓ **CRC specific mortality & increased O3FA intake after diagnosis** [increase of ≥0.15g/d vs decrease of ≥0.15g/d] HR 0.30 (0.14-0.64) ↓ **All-cause mortality by increasing O3FA intake after diagnosis** HR 0.87 (0.62-1.21)		
Song et al. 2016 **NHS & HPFS** (140)	n=173,229 (614 CRC cases)	NHS 1984-2010 HPFS 1986-2010		**Marine O3FA intake** ≥0.35g/d vs <0.15g/d	↔ **CRC risk & O3FA intake** HR 0.85 (0.67-1.09) ↓ **High FOXP3⁼ T Cell tumours & O3FA intake** HR 0.57 (0.40-0.81) ↔ **Low FOXP3⁼ T Cell tumours & O3FA intake** HR 1.14 (0.81-1.60)		
Song et al. 2015 **NHS & HPFS** (141)	n=1,125 (MSS CRC cases=941) (MSI CRC cases=184)	2010		**Marine O3FA intake** ≥0.30g/d vs <0.10g/d	↔ **CRC risk & O3FA intake** HR 0.87 (0.72-1.05) ↓ **MSI high CRC & O3FA intake** HR 0.54 (0.35-0.83) ↔ **MSS CRC & O3FA intake** HR 0.97 (0.78-1.20)		
Song et al. 2014 **NHS & HPFS** (139)	n=123,529 (1,469 CRC cases)	24-26yrs		**Women fish intake** ≥40g/d vs <15g/d, **O3FA**	↔ **CRC risk in women & fish intake** HR 1.02 (0.86 - 1.20)	Prediction model of RBC predicted PUFA levels using previously	↑ **distal colon cancer risk & fish intake in women** HR 1.36 (1.00-1.85)

†††Nurses' Health Study (**NHS**) included 121,701 nurses recruited between the ages of 30-55years, Health Professionals Follow up Study (**HPFS**) included 51,519 male healthcare professionals aged 40-75 years.

				intake ≥0.30g/d vs <0.15g/d **Men** **fish intake** ≥46g/d vs <16g/d, **O3FA intake** ≥0.41g/d vs <0.16g/d	**or O3FA intake** HR 1.03 (0.89-1.20) ↔ **CRC risk in men & fish intake** HR 0.88 (0.72 - 1.08) **or O3FA intake** HR0.89 (0.70-1.13) ↔**CRC risk & O3FA intake for 12-16 years before diagnosis in men** HR 0.76 (0.52-1.10)	measured RBC PUFA levels ↑ **distal colon cancer risk & predicted erythrocyte EPA in women** HR 1.38 (1.02-1.86) ↓ **rectal cancer risk & predicted erythrocyte EPA in men** HR 0.62 (0.40-0.94) ↓ **rectal cancer risk & predicted erythrocyte DHA in men HR** 0.61 (0.40-0.93)	↓ **rectal cancer risk & fish intake in men** HR 0.60 (0.39-0.93)
Navarro et al. 2016 **Women's Health Initiative (WHI)** (131)	n=134,017 (1,952 CRC cases), postmenopausal women 50-79yrs	Mean 11.7yrs	WHI 122-item FFQ (212) WHI nutrient database has over 140 nutrient values including: O3FAs, total EPA from food sources	**O3FA intake** Q5 >1.90g/d vs Q1 <80g/d	↔ **CRC risk & O3FA intake** HR 0.90 (0.74-1.09) ↓ **CRC risk & highest DHA+EPA intake** [>0.18g/d] **& lowest soluble fibre intake** [<2.8g/d] HR 0.59 (0.40-0.88)		
Hodge et al. 2015 **Melbourne Collaborative Cohort Study (MCCS)** (195)	n=41,514 27-76yrs recruited 1990-1994	Average 9yrs	121 item FFQ (fish, steamed, grilled or baked) (213) fatty acid data g/d to 2 decimal places of 1044 Australian foods	**O3FA intake** Q5 vs Q1 median O3FA intake ctrls 1.19g/d vs 1.18g/d cases	↔ **CRC risk & dietary O3FA intake** [Q5 vs Q1 cases median O3FA intake ctrls 1.19g/d vs 1.18g/d cases] HR 1.07 (0.76-1.50) ↔ **CRC risk & plasma O3FA levels** HR 0.77 (0.55-1.08)	Study of plasma O3FA levels [n=4,205 including 393 CRC cases] [ctrls 6.46% vs cases 6.36% total FAs] ↔**CRC risk & plasma total O3FA** HR 0.77 (0.55-1.08) ↓**CRC risk & plasma DPA** HR 0.71 (0.49-1.01)	↔ **colon cancer risk & plasma total O3FA** HR 1.00 (0.87-1.14) ↔ **rectal cancer risk & total plasma O3FA** HR 1.00 (0.84-1.19) ↓ **rectal cancer risk & plasma DHA** HR 0.78 (0.65-0.93)
Kraja et al. 2015 **The Rotterdam Study** (130)	n=4,967 (222 CRC cases) >55yrs	Median 14.6yrs	170-item semi-quantitative FFQ nutrition estimates based on Dutch Food Composition Tables. Validation study of 80 participants FFQ vs multiple food records for fat and fibre, Pearson co-	**O3FA intake** 0.7g/d vs 1.5g/d	↑ **CRC risk & O3FA intake** HR 1.44 (1.02-2.04) ↑ **CRC risk & O3FA intake in those with <median dietary fibre intake** [HR 1.96 (1.20-3.19) *O3FA from non-marine sources*		

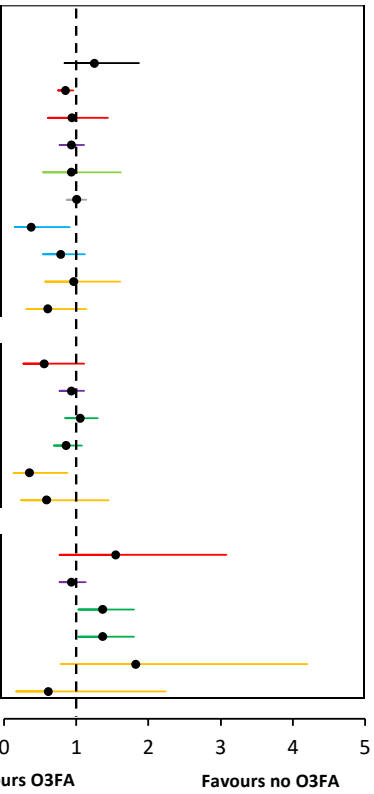
			efficient 0.62 for PUFAs (214)				
Kantor et al. 2014 **Vitamins and Lifestyle Cohort** (199)	n=68,109 (488 CRC cases) 50-76yrs	Completed questionnaire 200-2002 and followed through 2008 Average FU 6.7 yrs	WHI 120 item FFQ. Divided into quartiles based on serving size frequency. University of Minnesota's Nutrition Coding Centre Database used to convert FFQ into EPA & DHA intake.	**FO supplement average 10-year use high use** of ≥4 days/week over ≥3yrs vs no use, **Dietary EPA** Q4 >0.09g/d vs Q1 <0.02g/d	↔**CRC risk & FO supplement average 10-year use** HR 0.51 (0.26-1.00) ↔ **CRC risk & dietary EPA** [HR 0.99 (0.74-1.33)		↓ **colon cancer risk & high FO supplement use** HR 0.37 (0.15-0.91) ↔ **rectal cancer risk & high FO supplement use** HR 0.98 (0.35-2.69)
Theodoratou et al. 2014 (188)	2062 CRC cases & 2776 ctrls	Recruited between 1999-2006	Dietary assessment using Scottish Collaborative Group FFQ version, nutrient intake calculated using the UK National Nutrient Databank	**O3FA median intake** in controls 2.3g/d vs cases 1.1g/d	↓ **CRC risk & O3FA intake** [forward step regression mode OR 0.91 (0.86-0.98) & backward step regression mode OR 0.93 (0.87-0.99 ↑ **CRC risk & white fish intake** OR 1.13 (1.06-1.20)		
Key et al. 2012 **United Kingdom Dietary Cohort Consortium (UKDCC)** (215)	n=565 CRC cases & 1,951 ctrls	Latest dates of complete FU for cancer incidence & vital status in each cohort	Pooling 4 & 7d food diary data in 7 prospective UK cohort studies [EPIC Norfolk, EPIC Oxford, Guernsey Study, Medical Research Council National Survey of Health and Development, Oxford Vegetarian Study, UK Women's Cohort Study and Whitehall II], food diary data coded to give nutrient intakes based on national food table diet	**O3FA intake** 2.08g/d vs 1.30g/d in men 1.63g/d vs 1.02g/d in women	↔ **CRC & O3FA intake in men & women** OR 0.82 (0.59-1.13)		
Banque et al. 2012 (187)	n=245 CRC cases & 490 ctrls	2007-2009	142 dietary-items FFQ nutrients calculated by multiplying for each item the daily frequency by the	**PUFA median intake** ctrls 23.09g vs cases 19.02g, **fish & seafood median intake** ctrls 130.50g vs	↓ **CRC risk & PUFA intake** [OR 0.57 (0.38-0.86)., ↓ **CRC risk & fish + seafood intake** OR 0.56 (0.39-0.80)		

			amount contained in 100g of each food item. Portion size & nutrient content determined by food consumption tables	cases 110.07g**, oily-fish median intake** ctrls 57.78g vs cases 22.41g	↓ **CRC risk & oily-fish intake** OR 0.62 (0.34-1.12)		
Turati et al. 2012 (216)	n=4154 cases & 1953 ctrls	1992-1996	Average weekly frequency of consumptions of 78 specific food groups. Total energy intake computed from FFQ using Italian food composition database (217)	**EPA + DHA intake** <250mg/d vs ≥250mg/d, ALA intake <0.5% vs >0.5% of total energy intake	↔ **CRC risk & EPA + DHA intake** OR 0.99 (0.81-1.21) ↑ **CRC risk & ALA intake** OR 1.15 (0.99-1.33)		
Sasazuki et al. 2011 **Japan Public Health Centre Based Prospective Study (JPHCBPS)** (143)	n=1,268 cases 45-74yrs	Average FU 9.3yrs	Validated 138-item FFQ, 19 items of fish and shellfish. Daily O3FAS, EPA, DHA calculated using a fatty acid composition table. Validity assessed in subsamples within the cohort, spearman rank correlation coefficients EPA 0.38 (165)	**O3FA intake in women** 0.42/d vs 1.92 /d **O3FA intake in men** 0.49g/d vs 2.18g/d	↓ **CRC risk & O3FA intake in women** RR 0.60 (0.31-1.14), ↔ **CRC risk & O3FA intake in men** RR 0.96 (0.57-1.61)		↓ **proximal CRC risk & O3FA intake in men** RR 0.35 (0.14-0.88) ↔ **distal colon or rectal cancer risk & O3FA intake in men & women**

3.5.2 Colorectal cancer subsites and molecular pathogenesis

The large observational studies of dietary O3FA intake have provided important insights into altered CRC risk according to the location of the tumour in the colorectum. Three separate studies have suggested possible reduced risk of proximal colon cancer and increased risk of distal and/or rectal cancer risk in individuals with highest O3FA intake but failed to reach statistical significance (**figure 3.1**) (139, 142, 143). Differential effects of O3FA exposure on tumours in different parts of the colorectum may be explained by differences in the molecular pathogenesis of CRCs. One large observational study has reported that high dietary O3FA intake was associated with a significant reduction in risk of CRCs displaying MSI, whereas there was no association between O3FA intake and overall CRC risk (**table 3.3**) (140). DNA mismatch repair-deficient (dMMR) CRCs, which display MSI, are more prevalent in the proximal colon. Therefore, preferential activity of O3FAs against proximal colonic tumours could be explained by anti-cancer activity against dMMR tumours. The hypothesis that O3FAs augment the host anti-tumour immune response against MSI tumours is supported by the observation that O3FA intake is associated with reduced risk of CRCs with a prominent regulatory T cell infiltrate (identified by forkhead box protein P3 [$FOXP3^{+}$] staining) (**figure 3.2**) (140, 141) .

	Study*	N	Follow up	Assessment of O3FA intake / levels†	HR
Overall colon cancer risk	Shin et al. 2020 [SWLH cohort]	48,233	Median 21.3 yrs	MO3FA intake	1.25 (0.84-1.87)
	Aglago et al. 2020 [EPIC]	476,160	Median 14.9 yrs	O3FA intake (EPA+DPA+DHA)	0.85 (0.75-0.96)
				Plasma O3FA level (EPA+DPA+DHA)	0.94 (0.61-1.44)
	Bradbury et al. 2020 [UK Biobank]	475,581	Mean 5.7 yrs	Total fish intake	0.93 (0.77-1.11)
	Butler et al. 2017 [SCHS]	350	Median 3.3 yrs	Plasma EPA level	0.93 (0.54-1.62)
	Hodge et al. 2015 [MCCS]	41,514	Mean 11.7 yrs	Plasma 03FA level	1.00 (0.87-1.14)
	Kantor et al. 2014 [Vitamins & Lifestyle Cohort]	68,109	Mean 6.7 yrs	10-yr fish oil use (≥4 d/wk and ≥3 yrs)	0.37 (0.15-0.91)
				Total EPA+DHA intake	0.78 (0.57-1.61)
	Sasazuki et al. 2011 [JPHCHPS]	1,268	Mean 9.3 yrs	MO3FA intake in men	0.96 (0.57-1.61)
				MO3FA intake in women	0.60 (0.31-1.14)
Proximal colon cancer risk	Aglago et al. 2020 [EPIC]**	476,160	Median 14.9 yrs	Plasma O3FA level	0.55 (0.27-1.11)
	Bradbury et al. 2020 [UK Biobank]**	475,581	Mean 5.7 yrs	Total fish intake	0.93 (0.77-1.11)
	Song et al. 2014 [NHS & HPFS]‡	123,529	24-26 yrs	MO3FA intake in men	1.05 (0.85-1.30)
				MO3FA intake in women	0.86 (0.69-1.08)
	Sasazuki et al. 2011 [JPHCHPS]**	1,268	Mean 9.3 yrs	MO3FA intake in men	0.35 (0.14-0.88)
				MO3FA intake in women	0.59 (0.24-1.45)
Distal colon cancer risk	Aglago et al. 2020 [EPIC]	476,160	Median 14.9 yrs	Plasma O3FA level	1.54 (0.77-3.08)
	Bradbury et al. 2020 [UK Biobank]	475,581	Mean 5.7 yrs	Total fish intake	0.93 (0.77-1.13)
	Song et al. 2014 [NHS & HPFS]	123,529	24-26 yrs	MO3FA intake in men	1.43 (0.97-2.11)
				MO3FA intake in women	1.36 (1.03-1.80)
	Sasazuki et al. 2011 [JPHCHPS]	1,268	Mean 9.3 yrs	MO3FA intake in men	1.82 (0.79-4.20)
				MO3FA intake in women	0.61 (0.17-2.24)



*Docosahexaenoic acid (**DHA**), docosapentaenoic acid (**DPA**), eicosapentaenoic acid (**EPA**), European Prospective Investigation into Cancer and nutrition (**EPIC**), Health Professionals Follow up Study (**HPFS**), Japan Public Health Centre Based Prospective Study (**JPHCBPS**), marine omega-3 fatty acid intake (**MO3FA**), Melbourne Collaborative Cohort Study (**MCCS**), Nurses' Health Study (**NHS**), Omega-3 fatty acid (**O3FA**), Singapore Chinese Health Study (**SCHS**), Shanghai Men's Health Study (**SMHS**), Swedish Women's Lifestyle and Health cohort (**SWLH**), years (**yrs**), week (**wk**)

† Assessment of O3FA includes: fish intake, fish oil supplement use and/or plasma O3FA levels

**Proximal colon cancers include International Classification of Diseases for Oncology codes C18.0-C18.5: caecum, appendix, ascending colon, hepatic flexure, transverse colon and splenic flexure

‡ Proximal colon cancer cases identified from medical records reviewed by a study physician blinded to exposure information who confirmed CRC diagnosis and extracted information on anatomic location

	Study	N	Follow up	Forms of O3FA	HR
Rectal cancer risk	Shin et al. 2020 [SWLH cohort]	48,233	Median 21.3 yrs	MO3FA intake	0.67 (0.42-1.08)
	Aglago et al. 2020 [EPIC]	476,160	Median 14.9 yrs	O3FA intake (EPA+DPA+DHA)	0.91 (0.77-1.08)
	Bradbury et al. 2020 [UK Biobank]	475,581	Mean 5.7 yrs	Total fish intake	0.93 (0.77-1.13)
	Butler et al. 2017 [SCHS]	350	Median 3.3 yrs	Plasma EPA level	1.89 (0.42-1.91)
	Hodge et al. 2015 [MCCS]	41,514	Mean 11.7 yrs	Plasma 03FA level	1.00 (0.84-1.19)
	Kantor et al. 2014 [Vitamins & Lifestyle Cohort]	68,109	Mean 6.7 yrs	10-yr fish oil use (≥4 days/week and ≥3yrs)	0.98 (0.35-2.69)
				Total EPA+DHA intake	1.22 (0.67-2.21)
	Song et al. 2014 [NHS & HPFS]	123,529	24-26 yrs	MO3FA intake in men	0.79 (0.51-1.22)
				MO3FA intake in women	1.06 (0.76-1.48)
	Sasazuki et al. 2011 [JPHCHPS]	1,268	Mean 9.3 yrs	MO3FA intake in men	1.07 (0.51-2.26)
				MO3FA intake in women	1.62 (0.61-4.32)

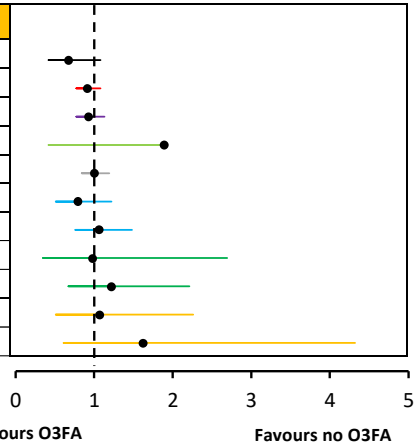


Figure 3.1 Epidemiological studies of CRC risk and O3FA intake according to CRC subsite.

The effect size (HR) is represented by the black marker with the 95% CIs denoted by a line. Subgroup findings within each individual study are grouped by colour.

3.5.3 Analysis of fish oil supplement use in observational studies

A significant limitation of most observational studies of O3FA intake and CRC risk is the lack of data on concomitant FOS use. FOS are one of the most commonly used nutritional supplements with regular use reported to be as high as 31.2% to 32.6% of the US adult population. The global FOS market is expected to reach 9.88 billion US dollars by 2027 (92, 218-220). Five observational studies have included data on FOS use, only as part of an overall O3FA intake assessment rather than a separate analysis (183, 195, 199, 208, 215). The Vitamin and Lifestyle Study is the only report of the association between the frequency and duration of FOS and CRC risk, although the type and dose of FOS used was not ascertained (199). Those who used FOS for more than three days a week for at least three years had a 49% lower risk of CRC than those who did not, a relationship that did not quite reach statistical significance (p-trend=0.06), perhaps reflecting the limited power of the study (488 CRC cases). Blood O3FA levels were not measured, therefore it is not possible to distinguish the effects of FOS use from dietary O3FA intake on blood O3FA levels (199).

3.5.4 Dietary patterns and interactions

Some observational studies have examined interactions between macronutrients (e.g., fibre) and micronutrients (e.g., vitamin C) in relation to O3FA intake and CRC risk. One study (n=44,039 [190 cases]) found an association between O3FA intake and increased risk of digestive cancer (defined as colon, rectum, stomach, liver, oesophagus, pancreas) in those consuming less than the median fruit and vegetable intake (<434.9 g/d women & <744.1 g/d men), and less than median vitamin C intake (111.2 mg/d women & 116.0 mg/d men) (208). It is unclear what mechanistic hypothesis was being tested in this study (208). Two studies have evaluated

O3FA and dietary fibre intake (130, 131). One (n=4,967 [222 CRC cases]) reported an increased risk of CRC associated with O3FA intake (including non-marine sources) in those with a less than median dietary fibre intake (HR 1.96 [1.20-3.19], P-trend=0.02) and demonstrated a statistically significant positive interaction between dietary fibre and O3FA intake on CRC risk (P-interaction=0.02) (130). The other study (n=134,017 [1,952 CRC cases]), found that higher levels of O3FA intake were associated with a reduced risk of CRC (HR 0.90), with an association between EPA and DHA intakes and CRC risk only in those with lower levels of soluble fibre intake (HR 0.59, P=0.02) (131).

A possible positive interaction between O3FAs and dietary fibre should be explored further, based on preliminary mechanistic data that O3FA intake increases the abundance of SCFA–producing bacteria in human and mouse studies (3, 30, 120, 121). Mechanisms of the potential anti-CRC mechanisms of SCFAs are described in **Chapter 1, section 1.5.2.** The potential for SCFAs to modulate the host anti-tumour immune response which may be relevant to the observation that O3FAs may be preferentially protective against dMMR tumours (30). Analysis of the intestinal microbiome is included in ongoing studies (EMT2, PREPARE) and will help lead to greater understanding of the relationship between O3FAs, the gut microbiome and fibre intake (**table 3.2**) (99, 160).

3.6 Do O3FAs alter post-diagnosis CRC outcomes?

3.6.1 Epidemiological evidence

In the past ten years, there has been increasing focus on the relationship between O3FA intake and post-diagnosis CRC outcomes, in contrast to studies addressing risk of developing CRC. A study of dietary O3FA intake

and CRC outcomes was performed in the NHS and HPFS cohorts (n=1,659). It found that those with a higher intake of O3FA (>0.30 g/d [2.8 servings/week of total fish] vs <0.10 g/d [0.5 serving/week of total fish]) after diagnosis had lower CRC-specific mortality (HR 0.59, P=0.03). Additionally, increased post-diagnosis intake of O3FA by >0.15 g/d vs <0.02 g/d was associated with reduced CRC-specific mortality (HR 0.30 [1.14-0.64]) (183). The mechanistic hypothesis for these findings includes the potential for O3FAs to improve outcomes and safety/tolerability of chemo(radio)therapy, as well as to abrogate cancer cachexia, in addition to direct anti-CRC activity (221).

Another study within the HPFS suggested that men with an O3FA intake of ≥0.41 g/d, for 12 to 16 years prior to diagnosis had (a statistically insignificant) reduced CRC risk (HR 0.76), suggesting a long latency period for O3FA intervention for CRC prevention (139). Ongoing studies including EMT2 (NCT03428488) and MENAC (NCT02330926) will contribute further data on the effect of O3FA supplementation and CRC outcomes (**table 3.2**) (160, 162).

Post-hoc analyses of two adjuvant chemotherapy RCTs (Cancer and Leukaemia Group B trial [CALGB 89803] [n=1,265] and North Central Cancer Treatment Group (NCCTG) phase III trial N0147 [n=1,735]), have highlighted an association between high O3FA intake and post-diagnosis CRC outcomes according to other molecular tumour characteristics (222, 223). Within the CALGB 89803 trial study population, those who consumed dark fish (mackerel, salmon, sardines, swordfish), >1/week for a median follow up of 7 years had longer disease-free survival (DFS) (HR 0.65, P=0.007), recurrence free survival (HR 0.61, P=0.007) and overall survival (OS) (HR 0.68, P-trend=0.04) than those who never consumed dark fish

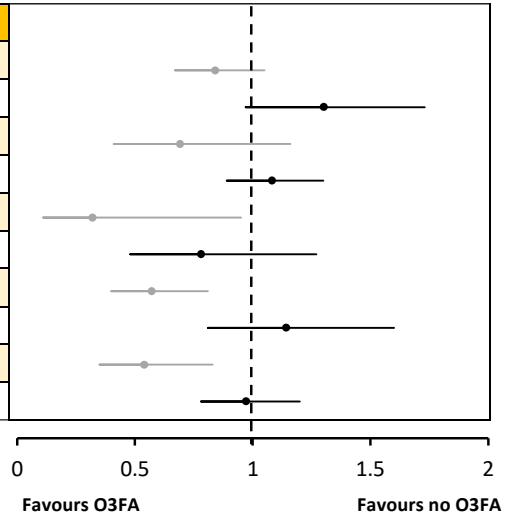
(222). A further analysis found an association between specific CRC subtypes, O3FA intake and survival with DFS being greater in patients with high COX-2 expression in the CRC (n=510) (HR 0.32 [0.11-0.95], P-trend=0.01), if there was greater than median O3FA intake of 0.40 g/d (222). No association between O3FA intake and DFS (P=0.84) or OS (P=0.93) was found in the N0147 trial. However, a statistically insignificant association between high O3FA intake and 3-year survival in participants with KRAS wild-type (HR 0.84, 95% CI 0.67-1.05) and dMMR tumours (HR 0.69 [0.41-1.16]) was observed (**figure 3.2**) (222, 223).

Further prospective studies of the predictive value of the CRC phenotype for benefit from O3FA supplementation or high dietary O3FA intake are required. These should include measurement of O3FA levels and parallel mechanistic studies, with which to confirm the validity of any hypothesis underlying post-hoc analysis of RCT outcomes according to tumour biomarker stratification (144). A recent *in vitro* study reported that COX expression by human CRC cells was associated with relative resistance to EPA (224), an observation which is hard to reconcile with the CALGB 89803 finding that high O3FA intake was associated with larger CRC recurrence risk reduction in individuals with COX-2-high tumours (222).

There has been an overwhelming increase in the epidemiological evidence evaluating dietary O3FAs in the prevention and treatment of CRC. Overall these studies benefit from their large scale and long follow up, which is important in the evaluation CRC risk, as it is a disease that takes time to develop and would be difficult to assess within a clinical trial due to the length of follow up and duration of intervention required. Despite their limitations in the form of measurement and recall bias due to FFQ data, lack of

comprehensive cohort wide blood O3FA measurements and reporting of FOS use, these studies have highlighted important signals of O3FAs in CRC prevention and treatment (reduced CRC risk in males, reduced risk for specific tumour subsite and subtype) which can be used to generate hypotheses and conducte further research.

Study‡	N	Follow up		HR
Song et al. 2019 [NCCTG Phase III trial N0147]	1,105	Median 6.9 yrs	3-year DFS for **KRAS-wildtype** tumours & MO3FA intake	0.84 (0.67-1.05)
	583		3-year DFS for **KRAS-mutant** tumours & MO3FA intake	1.30 (0.97-1.73
	211		3-year DFS for **MMR deficient** tumours & MO3FA intake	0.69 (0.41-1.16)
	1,453		3-year DFS for **MMR proficient** tumours & MO3FA intake	1.08 (0.89-1.30)
Van Blarigan et al. 2018 [CALGB 89803]	163	Median 7 yrs	**High PTGS2 expression** tumours & O3FA intake	0.32 (0.11-0.95)
	347		**Absent/low PTGS2 expression** tumours & O3FA intake	0.78 (0.48-1.27)
Song et al. 2016 [NHS & HPFS]	299	2,895,704-person yrs of follow up	**High FOXP3+ T Cell** tumours & O3FA intake	0.57 (0.40-0.81)
	285		**Low FOXP3+ T Cell** tumours & O3FA intake	1.14 (0.81-1.60)
Song et al. 2015 [NHS & HPFS]	184	NHS (1984-2010) HPFS (1986-2010)	**MSI high CRC** & O3FA intake	0.54 (0.35-0.83)
	941		**MSS high CRC** & O3FA intake	0.97 (0.78-1.20)



‡ Cancer and Leukaemia Group B trial (**CALGB**), colorectal cancer (**CRC**), disease free survival (**DFS**), forkhead box protein P3 (**FOXP3+**), hazard ratio (**HR**), Health Professionals Follow up Study (**HPFS**), Kristen RAt Sarcoma virus (**KRAS**), microsatellite instability (**MSI**), microsatellite stable (**MSS**), North Central Cancer Treatment Group (**NCCTG**), Nurses' Health Study (**NHS**), prostaglandin-endoperoxide synthase 2 (**PTSG2**)

Figure 3.2 Post-trial analyses and epidemiological studies of CRC risk and O3FA intake according to the molecular profile of the CRC.

The effect size (HR) is represented by the black marker with the 95% CIs denoted by a line. Subgroup findings within each individual study are grouped by colour.

3.6.2 O3FA interventions in CRC patients

The use of O3FA supplements in CRC patients has been explored in several RCTs and clinical studies encompassing the full scope of CRC management, from peri-operative management to intervention for cancer cachexia and chemotherapy-induced peripheral neuropathy (**table 3.4**).

O3FA interventions can be administered in different forms including capsules, oral nutritional supplement (ONS) drinks or as nasogastric enteral feeds (EN), in addition to being included as a component of parenteral nutrition (PN) (**table 3.4 & 3.5**). Capsules are generally more acceptable and tolerated better by participants who may be used to taking capsule or tablet forms of medications, in comparison to ONS that may be poorly tolerated due to altered taste (225). However, unlike capsules, ONS provide additional calories and protein which is especially important in patients with cancer related cachexia. Capsule- and ONS-delivered O3FA interventions have been examined in two clinical cross-over trials. One compared compliance of capsules and supplement drinks (5 g/d O3FA) in patients with advanced cancer (n=41), with self-registered compliance being greater in the capsule group ($P<0.01$) but no difference in AEs ($P>0.14$) or in whole blood O3FA concentration between groups (225). In the other study, despite reporting more AEs in the capsule (14/22) vs ONS (10/19) group (n=22 healthy participants, 4 g EPA/DHA), participants stated that they would be more likely to continue taking capsules rather than ONS, if available in the long term (3). Other RCTs have also shown that capsule O3FA interventions are associated with greater compliance than other forms of intervention (ONS, EN, PN) (**table 3.4 & 3.5**) (221, 226).

3.6.3 Biomarkers of O3FAs within clinical studies

Several biomarker studies have showed that colonic tumour tissue has increased levels of tissue O6FAs (measured as % AA of total FAs, O6:O3FA or AA:EPA ratio) and decreased O3FA levels (including EPA) compared to non-neoplastic tissue (227-230).

Three RCTs have embedded PUFA analyses of tumour tissue demonstrating that orally administered O3FAs are incorporated into normal colon, liver, and colorectal cancer liver metastasis (CRCLM) tissue (197, 221, 231). Analysis of normal colonic tissue (n=40) found that EPA was incorporated into both colonic mucosa and muscle layers (P=0.01) after 7-day treatment, unfortunately there was no comparison with paired tumour tissue (197). An RCT of a 72-hour infusion of PN, with or without O3FAs (n=20), found that post intervention tumour tissue had statistically significant lower levels of ALA, EPA, and DHA (measured as % of total FAs) compared with normal tissue, that did not differ according to intervention group. However median time to resection was almost double in the treatment group (10 vs 6 days) which may have accounted for these findings (231). In the phase 2 EMT trial, PUFA analysis (measured as % total FAs) of tumour tissue revealed a significant increase in EPA and DPA (+40%) and a decrease in DHA (-11%) and AA:EPA ratio (-33%) in the treatment (2 g/d free EPA) compared with the placebo group (221, 232).

Six studies (including the three above-mentioned RCTs) measured O3FA blood levels, demonstrating a consistent increase in O3FA levels and decrease in AA level but there were no clear association with clinical outcomes across the different studies **(tables 3.4 & 3.5**) (197, 221, 231, 233-237).

3.6.4 Peri-operative use of O3FAs during surgical management of primary CRC and CRCLMs

EN and PN O3FA supplementation, in combination with other macro- and micro-nutrients has been examined in individuals undergoing primary CRC or CRCLM resection, evaluating clinical outcomes (post-operative complications [POCs], infections, weight and length of stay [LOS]) and pro-inflammatory cytokine levels).

A 2016 meta-analysis (n=694, 11 studies) found that enteral or parental O3FA-enriched nutrition in CRC patients significantly reduced infectious complications (RR 0.63, P=0.004) and hospital stay, in addition to plasma interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) levels. O3FA doses ranged from 0.05 g/kg body weight to 3.3 g/L in PN feed with treatment durations of 5 days pre-surgery to 14 post-operative days (POD) (238). By contrast, a 2020 meta-analysis (n=977, 11 studies [in which only one study (197), from the 2016 meta-analysis was included]) found no significant difference in body mass index (BMI) or serum albumin level, wound infections or pneumonia in post-operative GI cancer patients receiving enteral nutritional therapy including O3FAs (O3FA dose range 0.3 g mixed O3FAs/100mls ONS to 2.2 g/d EPA with durations of 5 days pre-operation to 5 days pre- & 30 days post-operation) (239). The difference in findings between these two meta-analyses may reflect the heterogeneity of study populations, different outcome measures, dosing, and duration of interventions between multiple small studies.

Three RCTs have evaluated the use of O3FA-containing ONS in primary CRC resection patients, with differing doses and durations (**table 3.4**) (234, 240, 241). A Danish RCT of pre-operative ONS (containing 2 g/d EPA & 1

g/d DHA in 200 ml) (n=148), reported an increase in EPA (P<0.001) and production of the anti-inflammatory EPA-derived leukotriene B_5 (LTB_5), with a decrease in AA (P<0.001) and the pro-inflammatory AA-derived leukotriene B_4 (LTB_4) on POD one (EPA & AA measured as % total FAs in circulating granulocyte. However, there was no difference in clinical outcomes (infectious and non-infectious POCs) between the treatment and control groups (P=1.00) (234, 235). Long-term post-trial outcomes of the participants were later evaluated, revealing no OS benefit and an increased risk of all-cause mortality in the treatment group, when adjusted for age, disease severity and use of adjuvant chemotherapy (HR 1.73, P=0.029) (242). The two other RCTs reported no difference in LOS or POCs, however there was a significant increase in post-operative weight recovery in the active treatment group (240, 241). A RCT of 3 g/d EPA TG (in capsules) (n=61) evaluated cachexia-related inflammation using muscle biopsy nuclear factor (NF)-kB protein as a surrogate biomarker and found no difference in muscle inflammation.(226, 243)

O3FA-containing PN has been evaluated in several RCTs of primary CRC resection patients (range 20 to 99 participants) with study durations ranging from one pre-operative day to POD seven (**table 3.4**). Control infusions have included 0.9% saline, soybean- and glutamine-enriched PN, which is likely to have influenced comparison. One study found no significant reduction in POCs, but a reduction in the incidence of systemic inflammatory response syndrome (P<0.05) (244). Another study found an increased rate of POCs including anastomotic leak and urinary tract infection (UTI) (P=0.030) when compared with the administration of saline, which correlated with significantly higher concentrations of interleukin (IL)-6 (P=0.014) and white cell count (WCC) (P=0.029) in the control group (233). Two other RCTs found no

difference in IL-6, C-Reactive protein (CRP),tumour necrosis factor (TNF)-α, or procalcitonin (245) or neutrophil phagocytosis index in the O3FA-containing PN arm (246). Change in phagocytosis of pathogens such as *E.coli* (as a measure of the immune response) is the primary outcome of an ongoing RCT exploring the use of mixed O3FA-containing ONS for 5 days pre-operatively in patients undergoing laparoscopic CRC resection (n=50) (**table 3.2**) (163).

The phase 2 double-blind, placebo controlled EMT trial of 2 g/d free EPA (median duration 30 days) in CRCLM patients awaiting liver resection surgery was a 'window of opportunity' trial of pre-operative dosing, which pre-specified analysis of long-term clinical outcomes (221). It found no difference in tumour cancer cell Ki67 proliferation index (primary outcome) (247). However, there was a signal that EPA may improve OS up to 40 months after surgery (P=0.0985). DFS was improved in the EPA-treated group from 14.7 vs 22.6 months but did not reach statistical significance (P=0.1887) (221). Colorectal cancer progression-free survival is the primary outcome measure of the phase 3 EPA for Metastasis Trial 2 (EMT2) trial (NCT03428477) (recruitment target n=448) evaluating 4 g/d EPA ethyl ester, started pre-operatively and continued up to 4 years post CRCLM liver resection, results are due early 2026 (**table 3.2**) (160).

Table 3.4 Studies of O3FAs in participants with CRC undergoing surgical management.

Study ‡‡‡	Study population	PUFA dose & duration	Primary Outcome	PUFA Assessment	I inflammatory Markers	Results	Compliance
Hogan et al. 2020 (240)	N=121 (ctrl=56, Tx=52)	3.3g EPA & DHA 5d pre-op ONS	LOS		↔ CRP or WCC	↔ **LOS, complications** ↑ **% weight change at day 7,** 0.77% in Tx & -1.5% in ctrl group (P=0.041)	Tx 13 of 20 supplements consumed, ctrl 14 of 20 supplements consumed
Hossain et al. 2020 (226)	n=61 (ctrl=29, Tx=32)	3g/d EPA 5d pre-op & 21d post-op CAPS	Cardiorespiratory fitness, physical strength, lean muscle mass, NF-κB protein expression		↔ NF-kB protein expression, WCC	↔ **cardiorespiratory fitness, physical strength, lean muscle mass, hand grip, anaerobic threshold, LOS, POCs**	Pill counts Tx 87%, ctrl 88% of
Bakker et al. 2020 (233)	n=44 (ctrl=23, Tx=21) elective laparoscopic colon resection	2 IV doses morning & night pre-op of 2 mL/kg infusion (0.2g FO/kg. 10g/FO = 1.25-2.82g EPA & 1.44-3.09g DHA PN	IL-6 in LPS-simulated whole blood cells on POD2	Granulocyte membrane % fatty acid composition ↑EPA, ↓O6:O3FA ratio, AA: EPA ratio in Tx group	↑WCC in Tx group on POD 4 ↔ CRP, IL-10	↔ **IL-6 at POD2** ↑**UTIs, anastomotic leakage & SIRS in Tx group**	
Ma et al. 2015 (245)	n=99 (ctrl=48, Tx=51) gastric & CRC patients	EPA & DHA 8.6-17.2g/L/d 7d post-op PN	Post-op inflammatory response (IL-6, CRP, TNF-α, procalcitonin)		↔ IL-6, CRP, TNF-α & procalcitonin	↓**FFA, triglycerides & HDL in Tx group**	
Cockbain et al. 2014 (221)	n=88 (ctrl=45, Tx=43) 1st or 2nd CRCLM resection	2g/d free EPA Median duration of treatment 30d CAPS	CRCLM Ki67 proliferation index	Tumour tissue as % fatty acid content (% difference from placebo reported) ↑EPA (+40%), ↑DPA (+41%) & ↓AA:EPA ratio (-33%)	Post treatment ↓Urinary PGEM 51.8% lower in Tx than ctrl group	↔ **CRCLM PI** ↔ **OS up to 40 months** in 7/41 deaths in Tx group vs 14/42 in ctrl group ↔ **median DFS** 22.6 in Tx group vs 14.7 months in ctrl group	Mean pill counts Tx 91%, ctrl 94%
Sorensen et al. 2014	n=148 (ctrl=64,	2g/d EPA & 1g/d DHA 200ml ONS	LTB4, 5-HETE, LTB5 and 5-HEPE from	Granulocyte % fatty acids in Tx group d1	% difference from placebo ↑LTB_5 (176%),	↔ **infectious or non-infectious POCs**	Pre-op ONS compliance Tx 65 of 74 patients, ctrl 59 of 74 patients

‡‡‡ Statistically significant increase(↑**),** statistically significant reduction (↓**),** no change **(↔)**, more than (**>**), more than or equal to (**≥**), less than (**<**), less than or equal to (**≤**), adverse events (**AEs**), arachidonic acid (**AA**), capsules (**CAPS**), C-reactive protein (**CRP**), control (**ctrl**), days (**d**), disease free survival (**DFS**), docosahexaenoic acid (**DHA**), docosapentaenoic acid (**DPA**), eicosapentaenoic acid (**EPA**), enteral nutrition (**EN**), fish oil (**FO**)
, grams (**g**), grams per day (**g/d**), high density lipoprotein (**HDL**), intravenous (**IV**), interleukin 6 (**IL-6**), interleukin 8 (**IL-8**), interleukin 10 (**IL-10**), kilograms (**kg**), length of stay (**LOS**), leukotriene B4 (**LTB4**), leukotriene B5 (**LTB5**), linoleic acid (**LA**), liposaccharide (**LPS**), millilitre (**ml**), nuclear factor kappa-light-chain-enhancer of activated B cells (**NF-kB**), omega-6 polyunsaturated fatty acids (**O6FAs**), oral nutritional supplement (**ONS)**, overall survival (**OS**), parental nutrition (**PN**), post-operative (**post-op**), post-operative complications (**POCs**), pre-operative (**pre-op**), proliferation index (**PI**), prostaglandin E_2 metabolite (**PGEM**), quality of life (**QoL**), systemic inflammatory response syndrome (**SIRS**), treatment group (**Tx)**, urinary tract infections (**UTIs**), versus (**vs**), weeks (**wks**), white cell count (**WCC**), 5-hydroxyeicosapentaenoic acid (**5-HEPE**), percentage **(%)**

(197, 234, 235, 242)	Tx=65) CRC surgery	7d pre-op & 7d post-op ONS	stimulated neutrophils Colonic wall content of EPA, DPA & DHA (n=21 Tx group & n=19 ctrl group); 30d POC Survival	pre-op: ↑EPA, DPA, DHA, total O3FA, ↓AA Colonic mucosa as % fatty acid content (% difference from placebo reported) ↑EPA (+147%) ↑total O3FA (+48%) Colonic muscular layer as % fatty acid content (% difference from placebo reported) ↑EPA (+107%)	5-HEPE (306%), ↓LTB_4 (-12%) ↔CRP, albumin	↔ **3-year or 5-year survival, disease recurrence** ↑ **mortality in Tx group** when adjusted for age, stage of disease and adjuvant chemotherapy HR 1.73 (1.06-2.83)§§§	Post-op compliance Tx 17 of 74 patients, ctrl 16 of 74 patients
Aliyaziciogl u et al. 2013 (246)	n=36 CRC patients: 4 groups [ctrl, glutamine, O3FA, O3FA & glutamine]	O3FA 0.1-0.2g/kg/d 7d post-op PN	Neutrophil phagocytosis index, neutrophil adhesivity index, IL-8		↔ mean IL-8 between groups	↑ **mean neutrophil phagocytosis index** in all Tx groups ↑ **mean neutrophil adhesivity index** in all Tx groups	
Stephenso n et al. 2013 (231)	n=15 (ctrl=11, Tx=9) CRCLM	500ml 72-hour infusion (1.5ml/kg/body weight/hour) PN	Fatty acid composition of tumour and liver tissue without tumour	↓ EPA, DHA, LA, O3FA, O6FA:O3FA ratio of tumour tissue (% of total FAs) in both groups compared to liver tissue without tumour	Not reported	↔ **by intervention** Tumour tissue vs normal liver tissue in both groups ↓50% EPA, 40% O3FAs, 12% O6FAs, ↑47% O6:O3FAs	
Zhu et al. 2012 (244)	n=57 (ctrl=28, Tx=29) elderly patients with CRC	FO 0.2g/kg/d**** 7d post-op PN	Infective POCs & SIRS		↓ Il-6, TNF-α, CD4, CD8, CD4/CD8	↔ **Infectious POCs** ↓ **SIRS in Tx group** ↓ **LOS in Tx group**	

§§§ Figures in brackets relate to 95% confidence intervals unless stated otherwise.

**** EPA and DHA doses not reported.

3.6.5 Combination treatment with chemo(radio)therapy

O3FA treatment as an adjunct to chemo(radio)therapy has been evaluated in a number of studies exploring several outcomes including tolerability of chemotherapy, quality-of-life measures, and the systemic inflammatory response (**table 3.5**). A double-blind, placebo-controlled RCT (n=140) in CRC patients receiving chemotherapy evaluated the use of a strain-specific probiotic, (microbial cell preparation, Hexbio®) and 2 g/d of O3FAs (700 mg/d EPA & DHA) over 8 weeks, reporting an improvement in global health status ($P<0.001$) and chemotherapy-related adverse events (diarrhoea, anorexia), in addition to a reduction in IL-6 ($P=0.002$), but no difference in TNF-α or CRP levels (248). However, a statistically significant reduction in the Glasgow Prognostic Score (GPS) (an inflammation-based cancer prognostic marker composed of CRP and albumin) was demonstrated in a RCT of 2.4 g/d O3FA capsules in patients with local advanced rectal cancer receiving neoadjuvant radiotherapy (published abstract only) (249).

One group conducted a series of studies in CRC patients undergoing chemotherapy, evaluating the effects of O3FAs on the systemic inflammatory response and nutritional outcomes. They found that 2 g/d mixed O3FAs increased BMI ($P=0.03$), decreased the CRP/albumin ratio ($P=0.005$), but did not affect pro-inflammatory cytokine levels (IL-6, IL-8 or TNF-α) (250), despite a statistically significant reduction in plasma AA level and increase in EPA level (% total FAs) in the treatment group (237), An increase in time-to-tumour progression between the O3FA and control group [593 *vs* 330 days ($P=0.04$)] was statistically significant despite a small number of progression events (6 vs 5) (251). A further study evaluated 2.5 g/d O3FA, reporting a 35% increase in RBC EPA (as % total FAs) in the treatment group

(P=0.03) but found no difference in treatment related AEs, death, or survival (236).

The potential for O3FAs to reduce the incidence and severity of oxaliplatin-induced peripheral neurotoxicity (OXIPN) was explored in a double-blind placebo-controlled RCT (n=71) of O3FA capsules (640 mg three times daily, DHA 54% EPA 10%). There was a reduction in both incidence (P=0.002) and severity (P=0.001) of OXIPN (252).

In summary, several small single-centre studies suggest that O3FAs may reduce systemic inflammation in addition to improving nutritional and survival outcomes but stop short of providing practice-changing evidence for adjunct O3FA treatment during medical management of CRC patients.

3.6.6 O3FA use in the context of advanced CRC management including cachexia

Systemic inflammation related to cancer is an important factor in the development of cancer-related cachexia (253). A meta-analysis of ONS interventions (dietary counselling; high calorie, high protein, O3FA-enriched ONS [aiming to provide 590 to 600 Kcal, 32-33 g protein & 2-2.2 g EPA O3FA-enriched ONS consumed over a duration of 4 to 12 weeks, improved mean body weight by +1.89 kg (P=0.02) compared to isocaloric ONS controls, in addition to reducing lean body mass loss and improving some aspects of quality of life (254). A study of a O3FA-enriched ONS (16 g protein, 1.1 g EPA, 0.5 g DHA) in patients with advanced or recurrent gastrointestinal cancer undergoing systemic chemotherapy was associated with an increase of skeletal muscle (p=0.0002) and lean body mass (P<0.0001), as well as improved prognosis, in those with a GPS of ≥1

(P=0.0096) (255). This study also highlighted the challenges of conducting clinical studies in participants with advanced cancer, relating to compliance, study retention and long-term follow up, in addition to the potential that ongoing chemotherapy treatment may affect or hinder the evaluation of an intervention. Within the study population, 51/88 participants in the treatment group were excluded due to poor compliance, and overall, 58.8% discontinued due to early satiety and/or altered taste as side effects from systemic chemotherapy (255).

Ongoing studies include the Multimodal - Exercise, Nutrition and Anti-inflammatory medication for Cachexia (MENAC) trial. Advanced cancer patients (n=240) receiving chemotherapy are randomised to four interventions, including 3 g/d O3FA in an ONS (**table 3.2**), with the primary outcome measure of change in body weight (162). Cancer-related cachexia evaluated through L2/L3 vertebral level muscle mass on routine CT imaging is an exploratory end-point of the EMT2 trial (160).

Table 3.5 Studies of O3FAs in participants with CRC undergoing chemoradiotherapy.

Study††††	Study population	PUFA dose & duration	Primary Outcome	PUFA Assessment	Inflammatory Markers	Findings	Compliance
Haidari et al. 2019 (256)	n=96 stage II/III CRC: Ctrl=24, active O3FA & placebo Vit D=24, active Vit D & placebo O3FA=24, active Vit D & O3FA=24	660mg (54mg EPA, 250mg DHA, 26mg other O3FAs) for 8 weeks CAPS	Serum vit D levels, CEA, TNF-α, IL-8, IL-6, IL-1B		↓TNF-α, IL-1b, IL-6, IL-8, in active-O3FA groups ↓NF-kB in active O3FA & Vit D group	↑**25(OH)-D in active Vit D groups** ↓**CEA in all groups** but no significant difference between groups	Pill counts Active O3FA & placebo Vit D n=4 excluded due to <90% compliance
Camargo et al. 2019 (236)	n=56 (ctrl=28, Tx=28) GI cancer patients receiving chemotherapy	2.5g/d O3FA (1g/d EPA & 550mg DHA) for 9wks CAPS	Treatment response and adverse effects	↑35% EPA in RBC (% total FAs) analysis of Tx group	↔ oxidative stress variables (catalase, superoxide dismutase, glutathione peroxidase)	↔ **AEs or treatment response** ↔ **hospitalisation** 6 in ctrl group vs 1 in Tx group RR 0.17 (0.02-1.34)‡‡‡‡	Measured as RBC O3FA (% total FAs)
Silva et al. 2018 (249)§§§§	n=114 (ctrl=59, Tx=59) stage II/III rectal cancer & neo-adjuvant chemoradiotherapy	2.4g/d EPA & DHA for 8wks CAPS	Glasgow prognostic score (CRP & albumin)*****		↓CRP, CRP/albumin ratio	↓**GPS at end of chemoradiation & 4wks later**	
Golkhalkhali et al. 2018 (248)	n=140 (ctrl=70, Tx=70) CRC patients on chemotherapy	2g/d OPU3FA (1.4g EPA & DHA) & MCP for 8wks CAPS	QoL, SEs of chemotherapy, IL-6, TNF-α, CRP		↓Il-6 in Tx group post intervention ↔ CRP, TNF-α	↓**chemotherapy-related adverse events (diarrhoea, dry mouth, taste alteration) in Tx group** ↑**QoL global health status in Tx group**	
Shirai et al. 2017 (255)	n=179 (ctrl=91, Tx=37) advanced or recurrent GI cancer & chemotherapy	16g protein, 1.1g EPA, 0.5g DHA for 6mths ONS	Tumour recurrence and progression, systemic inflammatory response		↔ CRP	↔**in survival between groups** ↑**survival in those with GPS ≥1 in Tx group** ↑**skeletal muscle mass & lean body mass in Tx group**	51/88 in Tx group non-compliant and excluded

†††† Statistically significant increase (↑**),** statistically significant reduction (↓**),** no association **(↔)**, more than (**>**), more than or equal to (**≥**), less than (**<**), less than or equal to (**≤**), arachidonic acid (**AA**), adverse events (**AEs**), *Carcinoembryonic antigen* (**CEA**), C-reactive protein (**CRP**), control (**ctrl**), days (**d**), docosahexaenoic acid (**DHA**), eicosapentaenoic acid (**EPA**), enteral nutrition (**EN**), fish oil (**FO**), Glasgow prognostic score (**GPS**), grams per day (**g/d**), interleukin 6 (**IL-6**), interleukin 8 (**IL-8**), interleukin 10 (**IL-10**), interleukin 17A (**IL-17A**), intravenous (**IV**), microbial cell preparation (**MCP**), months (**mths**), oral nutritional supplement (**ONS)**, oxaliplatin induced peripheral neurotoxicity (**OXPIN**), parental nutrition (**PN**), post-operative (**post-op**), pre-operative (**pre-op**) post-operative complications (**POCs**), quality of life (**QoL**), relative risk (**RR**), treatment group (**Tx)**, Tumour Necrosis Factor alpha (**TNF-α),** vitamin D (**vit D**), *25-hydroxyvitamin D* (**25(0H)-D**) weeks (**wks**)

‡‡‡‡ Figures in brackets relate to 95% confidence intervals unless stated otherwise.

§§§§ Published abstract only NCT02534389.

***** The GPS is an inflammation-based score that is calculated by combining CRP and albumin (indicators of systemic inflammatory response and nutritional status).

Esfashani et al. 2016 (252)	n=71 (ctrl=35, Tx=36) CRC patients receiving oxaliplatin chemotherapy	640mg O3FA tds, during & one month after completing chemotherapy, CAPS	Oxaliplatin induced peripheral neurotoxicity (OXPIN)		↔Il-6 & TNF-α	↓**OXPIN in Tx group**	
Camargo et al. 2016 (251)	n=30 (ctrl=13, Tx=17)	2g/d FO (600mg EPA & DHA) for 9wks CAPS	Death, disease progression, delays & interruptions to chemotherapy, CEA			↔ **in death, disease progression, delays, or interruptions to chemotherapy & CEA** ↑**time to tumour progression in Tx group** (593d vs 330d)	Patient reported & pill counts – figures not reported but all patients complied with protocol
Mocellin et al. 2013 (237)	n=11 (ctrl=5, Tx=6) CRC patients on chemotherapy		IL-6, IL-1B, IL-10, IL-17A, TNF-α, CRP/albumin ratio, PUFA profile	↑% plasma EPA & DHA & ↓AA, O6FA:O3FA ratio in Tx group	Primary outcome	↔ **Il-6, IL-1B, IL-10, IL-17A & TNF-α, albumin** ↓**CRP in Tx group**	
Silva et al. 2012 (250)	n=23 (ctrl=12, Tx=11) CRC patients on chemotherapy		CRP, TNF-α, IL-8 & IL-6		Primary outcome	↔ **CRP, TNF-α, IL-8, IL-6 at baseline & 9wks** ↓**CRP/albumin ratio in Tx group**	Tx 10/11, ctrl 8/12 completed the study or consumed >80% of the capsules

3.7 Main conclusions

This review summarises and synthesises the evidence for the use of O3FAs in the prevention and treatment of CRC. It includes studies published over the last decade, which reflect a considerable amount of new clinical trial and observational data. This review highlights the potential for the use of O3FAs along the entire CRC tumorigenesis pathway; from primary prevention to the treatment of CRC related cachexia. A major paradigm shift has been the signal towards the use of O3FAs in CRC prevention and treatment according to specific host and tumour factors (**figure 3.3**). These signals require further evaluation through more robust and well-designed studies to test these hypotheses as the primary aim.

3.8 Future research priorities

Increased mechanistic understanding of the anti-cancer activity of EPA and DHA is integral to future treatment stratification. There is a need to ensure that mechanistic observations *in vitro* and *in vivo* are confirmed in human studies in order to translate findings into improved patient stratification for O3FA intervention.

The shift towards consideration of O3FAs as immunotherapeutics and that there may be other '*indirect*' mechanism of action of O3FAs such as probiotic effects on the gut microbiome, as opposed to direct activity on cancer cells, should lead to other therapeutic opportunities using O3FAs in combination with other anti-CRC interventions.

Future O3FA trials should include stratification for one or more of these factors with standardised use of blood O3FA measurements. This will be critical for the ability to compare data across RCTs and attempt meta-

analysis, particularly given that 'pharmacological' use of O3FAs always occurs on a background of variable dietary intake of O3FAs. A neglected area of research has been a cost-effectiveness and health economic evaluation of the pharmacological use of O3FAs for primary and secondary prevention of CRC, which should be nested within future intervention studies.

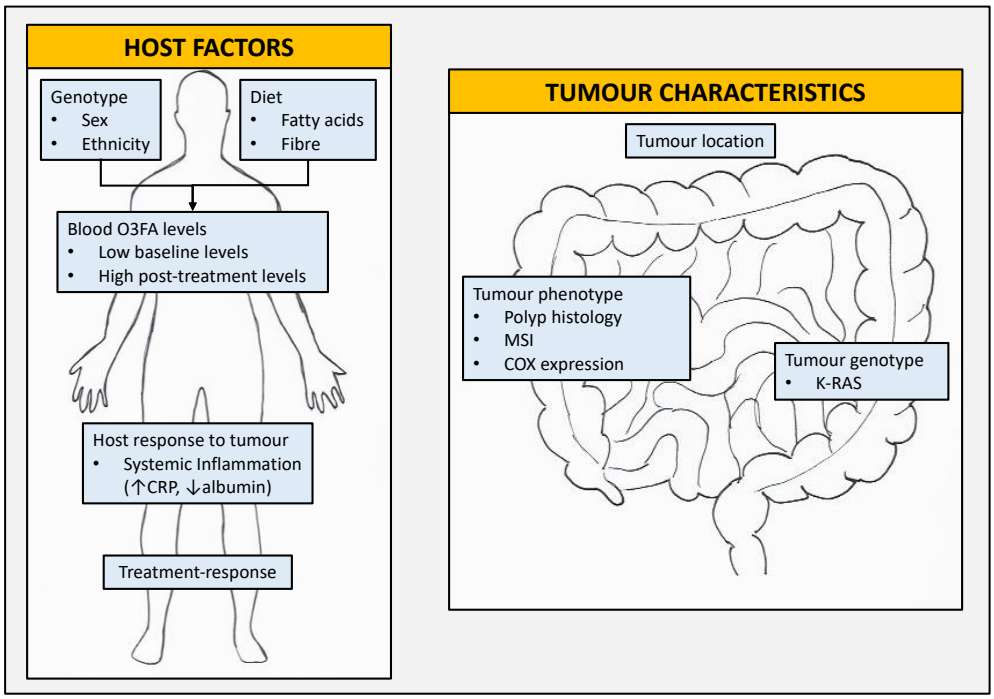


Figure 3.3 Potential predictive and treatment response biomarkers of anti-colorectal cancer activity of O3FAs.

Evaluable biomarkers are classified as either tumour characteristics or host factors, which link to a differential effect of O3FA treatment or altered neoplastic risk associated with dietary O3FA intake. The mechanistic link(s) between genotype, diet, and blood O3FA levels has not been elucidated but could underlie the differences in treatment effect of O3FAs according to ethnicity.

3.9 Latest literature since review published

This review article was current until December 2021, when it was accepted for publication. It was published in February 2022 and has received 19 citations (2). Despite the review being systematic it was not prospectively registered and therefore is a narrative review.

Since its publication there have been additions to the literature identifying potential host and tumour factors associated with the benefits of O3FAs in the prevention and treatment of CRC. A secondary analysis of the SeAFOod polyp prevention trial (n=538), identified that individuals with the I allele of the FADS insertion-deletion (Indel) polymorphism (rs66698963), which is associated with elevated AA levels, were likely to benefit from EPA in colorectal polyp prevention, by competing with AA (257)p. Further validation of a gene (FADS indel rs66698963), diet (oily fish and FOS) interaction is important within other post-hoc trial analyses and/or observational studies (257).

Following the release of the UKBB NMR data, a study estimating the O3i in the UKBB was published. The O3i was measured in 250 random blood samples (from non UKBB participants) in RBCs using CG-MS and in plasma using NMR spectroscopy, from which an estimated O3i for the UKBB was derived (258). The estimated O3i correlated with oily fish intake and FOS use within the UKBB, demonstrating the importance of blood O3FA levels in evaluating diet and supplement use in observational studies (258).

A further study in the UK Biobank (n=253,138) examined both O3FAs and O6FAs as a percentage of total fatty acids (FAs) with 19 cancers including colon and rectal cancer risk (259). The authors reported a decrease in colon

cancer risk associated with increasing quintiles of O3FAs as a % of total FAs (quintile 5 HR 0.87 [0.78-0.97]), but not rectal cancer. Whereas there was a decrease in both colon and rectal cancer risk associated with increasing quintiles of O6FAs as % of total FAs (259). The association between O6FAs as a % of total FAs and decreased cancer risk was observed for 14 other cancers within the analysis, which raises the question regarding multiple testing and type 1 error (259). In addition, despite their models being adjusted, it could be argued that they were not sufficiently adjusted for all clinical risk factors associated with the individual cancers examined within the analysis.

Chapter 4 The relationship between oily fish intake, fish oil supplement use and plasma polyunsaturated fatty acid levels in UK Biobank participants

4.1 Introduction

This chapter examines the relationship between dietary O3FA intake, both oily fish intake and FOS use in relation to plasma PUFA levels in a large subset of the UK Biobank (UKBB) population with available data. The aims and objectives of the study are outlined in **chapter 2 section 2.1.2.1**. The study detailed within this chapter was published in January 2024 (260).

Existing observational evidence linking O3FA intake and health outcomes is often limited to small homogenous studies and is hampered by a lack of robust validation of dietary O3FA intake by blood O3FA levels (usually restricted to a small, nested, case-control validation) (142, 261), and the absence of data on FOS use in the majority of studies, despite widespread supplement use (92, 199, 218). The UKBB is a well-characterised, prospective cohort of 502,441 women and men, aged 40 to 69 years, recruited between 2006 and 2010 in the UK [9]. It includes comprehensive dietary data, as well as data on nutritional supplement use (262). In March 2021, the UKBB released data on plasma PUFA levels for approximately 120,000 UKBB participants using the Nightingale nuclear magnetic resonance (NMR) metabolomics platform (110). There was subsequent release of the second tranche of NMR data by the UKBB in July 2023. The study outlined within this chapter includes the first tranche of NMR data.

A recent study used UKBB plasma PUFA data to derive an estimated '*omega-3 index*' (O3i; the percentage EPA and DHA of total identified PUFAs

in RBC membranes) for UKBB participants (258). Schuchardt and colleagues then investigated the relationship between the estimated O3i and several demographic and lifestyle factors, including fish consumption and FOS use, but did not explore the relationship between dietary O3FA intake and FOS according to the actual plasma O3FA data available in the UKBB.

Herein, I describe the relationship between dietary O3FA intake from oily fish, FOS use and plasma O3FA levels in UKBB participants, in order to define the relationship between dietary and supplemental O3FA intake, as well as their relationship with circulating PUFA levels and clinical/lifestyle factors.

4.2 Methods

4.2.1 Study approval

Approval for this study was obtained from the UKBB (research ID 73904, **appendix A**).

4.2.2 Assessment of oily fish intake and nutritional supplement use

I examined oily fish intake based on its robust association with increased blood O3FA levels, by contrast with other (lean) fish such as cod, tuna, or haddock (263, 264).

The self-reported frequency of oily fish intake was assessed using a touchscreen food frequency questionnaire (FFQ), reporting typical dietary intake at the time of recruitment (206). I categorised oily fish intake as ‘never’, ‘less than once a week’, ‘once a week’, ‘greater than or equal to twice a week (combining UKBB categories ‘2-4 times a week’, ‘5-6 times a week’ and ‘once, or more, daily’), or ‘unknown’ (combining ‘do not know’ and ‘prefer not

to answer' categories), reflecting the distribution of the data and current UK dietary recommendations for oily fish intake (one portion of oily fish per week) (89). Reproducibility of the touchscreen data has been evaluated in a subset of participants (n≈320,000), who were invited to complete a 24-hour dietary questionnaire on five separate occasions, (April 2009 to June 2012) (207).

Nutritional supplement use was examined using the 'mineral and other dietary supplements use' question inside the touchscreen FFQ (265). Participants were asked if they regularly used any of the following: 'fish oil (including cod liver oil)', 'glucosamine', 'calcium', 'iron', 'selenium', 'none' and 'prefer not to answer'. Participants could select more than one answer. I categorised supplement intake as 'no supplement use', 'FOS', 'other', and 'unknown' (combining 'do not know' and 'prefer not to answer' categories). FFQ data were missing for 447 (0.4%) participants. Nutritional supplement use was also examined using the UKBB 24-hour dietary recall tool to examine the agreement between the FFQ and 24-hour dietary recall for nutritional supplement use (207). Participants were asked 'did you have any vitamin or mineral supplements yesterday, e.g., vitamin C, multivitamins, fish oil, calcium supplement?', which was recorded as 'yes' or 'no'. No data were available on the formulation or dose of supplements.

4.2.3 Plasma fatty acid profiles

A plasma fatty acid profile was available for 121,650 randomly selected UKBB participants using a non-fasted (mean time since last meal 4 hours) plasma sample collected in an ethylenediaminetetraacetic acid-containing tube at either initial UKBB assessment (2006 to 2010) or the first repeat UKBB assessment (2012 to 2013), with a plasma fatty acid profile available for both visits in a smaller subset of participants (n=1,426) (110).

Plasma fatty acid levels were measured using the Nightingale Health NMR-based metabolic biomarker profiling platform (110, 111). The following fatty acid classes were analysed as the absolute plasma concentration in mmol/L; total FAs, total O3FAs, total O6FAs, DHA and LA. NMR spectroscopy allows sample analysis at scale, but this methodology is unable to quantify some individual fatty acids including ALA, AA, docosapentaenoic acid (DPA) and EPA (111, 266). The Nightingale fatty acid panel also includes percentage values of total FAs for total PUFAs, O3FAs, O6FAs, DHA and LA, in addition to the ratio of total O6FAs to O3FAs.

The plasma fatty acid profile for each UKBB participant was linked to touchscreen dietary and nutritional supplement data collected at the same assessment visit.

4.2.4 Other clinical data

The following variables were also used to describe the population: sex; age (years); body mass index (BMI; Kg/m^2); ethnicity (White, Mixed, Asian, Black, Chinese, Other and unknown); alcohol intake (never, once to three times a month, once or twice a week, three to four times a week, daily or most days); current tobacco smoking (yes on most or all days, only occasionally, no); physical activity defined using summed metabolic equivalent task (MET) minutes per week for all activities including walking, moderate and vigorous activity (low [<150 MET x minutes per week], moderate [600 to 3,000 MET x minutes per week] and high [>3,000 MET x minutes per week]; and Townsend socio-economic deprivation index (quintiles of increasing deprivation [least to most deprived]). Menopausal status (pre-menopausal, post-menopausal, unsure because of hysterectomy or other reason,

unknown); use of hormone replacement therapy (HRT) (use [whether the participant had ever used HRT], no use, unknown) and oral contraceptive (OCP) (use [whether the participant had ever used OCP], no use, unknown) were included based on possible sex-specific differences in O3FA levels (267, 268).

4.2.5 Data preparation prior to analysis

Prior to data analysis I prepared the data by defining the study population with an available plasma fatty acid profile level as described in section 4.3.1. I categorising the variables as described in sections 4.2.2 and 4.2.4. Missing/unknown data values were reported and later excluded from the models.

4.2.6 Statistical analysis

I examined the complete UKBB population with available plasma fatty acid data and accompanying touchscreen data on diet and nutritional supplement use (>120,000), so statistical justification of the sample size was not necessary. An arbitrary P value of <0.005 was considered statistically significant given the large dataset and propensity for type 1 error due to multiple testing.

Population characteristics were reported as the mean and standard deviation (SD), or as the percentage (%) value. Plasma fatty acid data were log-transformed when appropriate to allow parametric analysis. The chi-squared test was used to examine the difference between populations with and without plasma fatty acid levels. The Cochran-Armitage test for trend was used to examine the differences in population characteristics according to oily fish intake and nutritional supplement use. A linear regression model was

used to examine the association between plasma fatty acid levels and oily fish intake. One-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) test was used to examine the association between plasma fatty acid levels and nutritional supplement use.

Ordinal logistic regression models were used to analyse multiple factors that predicted the highest quartile of plasma total O3FA levels, plasma DHA levels, and the percentage O3FAs of total FAs. Each model was adjusted for: sex; age at recruitment (≤39, 40-49, 50-59, ≥60 years), BMI (≤24.9, 25.0 to 29.9, ≥30 Kg/m^2), oily fish intake frequency (never, <once a week, once a week, ≥twice a week); supplement use (no supplement use, FOS use, other supplement use); ethnicity (White, Mixed, Asian, Black, Chinese and Other); alcohol intake (never, once to three times a month, once or twice a week, three to four times a week, daily or most days); smoking (non-smoker, occasional smoker, smoker); physical activity (low, moderate, or high); and socio-economic deprivation (quintiles of increasing Townsend deprivation index [least to most deprived]).

To examine the modifying effects of menopausal status (pre-menopausal and post-menopausal), use of hormone replacement therapy (HRT) (use, no use), and oral contraceptive use (use, no use), the models were re-run for females only. Females who answered 'unsure – had a hysterectomy' and 'unsure-other reason' within the 'have you had your menopause?' question were excluded from the model.

Participants with missing covariate data for the adjusted variables were excluded from the models. Variables within each model were examined for co-linearity. Variables with a moderate or high correlation (based on a

variable inflation factor [VIF] >5) were removed and the models were re-derived. All analyses were performed in R Studio version 4.1.2.

4.3 Results

4.3.1 Plasma fatty acid profile data

Plasma fatty acid levels were measured at least once in 121,650 participants (24.5% of the total UKBB population), of which 116,513 had levels measured at the initial assessment visit only, 3,711 had plasma fatty acid levels measured at the first repeat assessment visit only, and 1,426 participants had plasma fatty acid levels measured at both timepoints (**figure 4.1**).

A moderate correlation (r^2 value between 0.52 and 0.57) between initial and repeat assessment plasma fatty acid levels has previously been reported by the UKBB for a cohort of 1,439 participants (our cohort of 1,426 likely reflects subsequent participant drop-out from the UKBB study) (269). I also investigated the absolute difference between paired initial and repeat plasma fatty acid levels. There was a slightly higher mean value for most fatty acid classes, but the very small absolute differences failed to reach statistical significance, except for plasma total FAs (**table 4.1**). Therefore, I used the initial assessment data for individuals who had provided more than one plasma sample (n=1,426) to generate a cohort of 121,650 for subsequent analysis (**figure 4.1**).

Table 4.1 The comparison of plasma fatty acid data at initial and first repeat assessment visits (n=1,426).

	Plasma fatty acid level (mmol/L)[1]		Difference between paired fatty acid values[2]	P value[3]
	Initial assessment	First repeat assessment		
Total FAs	11.84 (2.39)	11.95 (2.36)	-0.013 (-0.022 to -0.003)	0.007
Total PUFAs	4.98 (0.80)	5.02 (0.82)	-0.003 (-0.011 to +0.005)	0.418
Total O3FAs	0.53 (0.22)	0.54 (0.22)	-0.004 (-0.022 to +0.015)	0.685
DHA	0.23 (0.08)	0.24 (0.84)	+0.01 (-0.006 to +0.026)	0.230
Total O6FAs	4.45 (0.68)	4.48 (0.71)	-0.004 (-0.011 to +0.003)	0.295
LA	3.41 (0.68)	3.41 (0.71)	0.006 (-0.004 to +0.016)	0.228

[1] Mean (standard deviation).
[2] Mean and 95% confidence interval of the difference between paired initial and repeat assessment plasma fatty acid levels.
[3] Paired t-test.

Clinical characteristics were compared between the study population with a plasma fatty acid profile (n=121,650) and UKBB participants without a plasma fatty acid profile (n=380,761). The groups were well-matched for all variables although some numerically small differences did reach statistical significance given the size of the groups (**table 4.2**). Therefore, I concluded that the population, for which a plasma fatty acid profile was available, was representative of the whole UKBB population.

Table 4.2 Characteristics of UKBB participants with and without a plasma fatty acid profile.

Variable	Population with a plasma fatty acid profile	Population with no plasma fatty acid profile	P value[5]
Number of participants	**121,650**	**380,761**	
Sex			
Male (%)	55,915 (46)	173,169 (46)	0.50
Female (%)	65,735 (54)	207,592 (55)	
Age[1]			
Age (years)	56.5 (8.1)	56.5 (8.1)	0.51
BMI[1]			
BMI (kg/m^2)	27.4 (4.8)	27.4 (4.8)	0.12
Oily fish intake[2]			
Never	13,161 (11)	41,642 (11)	0.25
<Once a week	39,713 (33)	125,121 (33)	0.16
Once a week	46,030 (38)	142,441 (37)	0.01
≥Twice a week	21,886 (18)	68,337 (18)	0.73
Unknown	860 (1)	3,220 (1)	<0.001
Mineral and dietary supplement use[2]			
None	69,207 (57)	213,368 (56)	<0.001
Other	13,960 (12)	43,966 (12)	0.50
Fish oil	38,036 (31)	117,680 (31)	<0.001
Unknown	447 (<1)	5,747 (1)	<0.001
Menopausal status[3]			
Pre-menopausal	15,423 (24)	48,623 (23)	0.83
Post-menopausal	39,781 (61)	125,599 (61)	0.95
Not sure as had a hysterectomy or for other reason	10,333 (16)	32,559 (16)	0.83
Unknown	198 (<1)	811 (<1)	<0.001
Ever used hormone replacement therapy[3]			
Use	25,026 (38)	78,879 (38)	0.73
No use	40,376 (61)	127,472 (61)	0.94
Unknown	333 (1)	1,241 (1)	0.01
Ever used oral contraceptive pill[3]			
Use	53,036 (81)	167,356 (81)	0.72
No use	12,406 (19)	39,112 (19)	0.86
Unknown	228 (<1)	1,124 (1)	<0.001
Ethnicity			
White	114,844 (94)	357,770 (94)	<0.001
Non-white[4]	6,235 (5)	20,768 (6)	<0.001
Mixed	678 (1)	2,276 (1)	0.23
Asian	2,346 (2)	7,533 (2)	0.27
Black	1,816 (2)	6,242 (2)	<0.001
Chinese	353 (<1)	1,220 (<1)	0.10
Other	1,060 (1)	3,497 (1)	0.13
Unknown	553 (1)	2,223 (1)	<0.001

[1] Mean (standard deviation).
[2] Oily fish/supplement intake was recorded at the same time as the plasma fatty acid levels were measured. Oily fish intake at initial assessment is reported for the population with no plasma fatty acid profile. Figures in brackets are % values of the whole population.
[3] Percentage (%) of female population.
[5] T-test for continuous variables and chi-squared test for categorical variables.

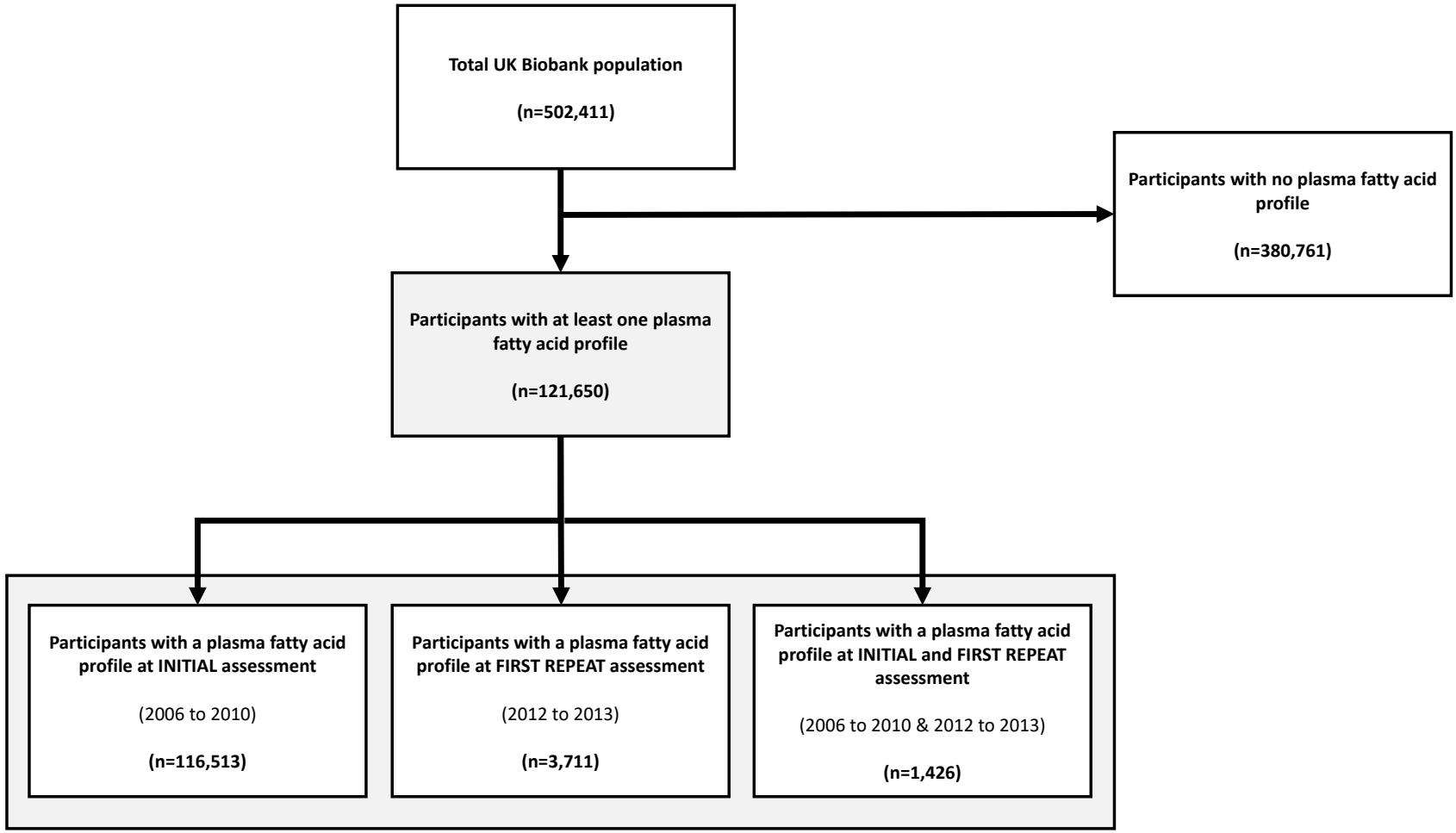


Figure 4.1 Distribution of plasma fatty acid data in the UK Biobank population.

For participants with a plasma fatty acid profile at initial and first repeat assessment, only the initial assessment data were included in the analysis.

4.3.2 Fish oil supplement use

Firstly, I assessed the agreement between the FFQ and 24-hour dietary recall tool for nutritional supplement use. The 24-hour dietary recall data were collected a mean 2.5 (SD 1.7) years after the corresponding FFQ response. There was a good level of agreement (86%) between the FFQ and 24-hour dietary recall data suggesting that FOS supplement use was consistent over time for the majority of participants (**table 4.3**).

Table 4.3 Agreement between nutritional supplement data from the FFQ and corresponding 24-hour dietary recall tool.

		FFQ			
		None	Other	Fish oil	Unknown
24-hour dietary recall[1]	Yes	1,145 (12)	1,380 (66)	4,445 (86)	8 (24)
	No	8,566 (88)	699 (34)	718 (14)	26 (77)
	Total number of participants	9,711	2,079	5,163	34

[1] Participants were asked "did you use vitamin or mineral supplements yesterday". Percentages in brackets.

The 24-hour dietary recall question about vitamin and mineral supplement use (collected between April 2009 and September 2010) was compared with the FFQ mineral and supplement use question collected at initial assessment (2006 to 2010). Paired data were available for 16,987 (13.96%) of the population with a plasma fatty acid profile (n=121,650).

There was good agreement between the FFQ FOS response and 24-hour dietary recall data with 4,445 (86%) individuals, who answered 'yes' to FOS use in the FFQ, also answering 'yes' to the question about supplement use in the previous 24 hours. Conversely, 88% of individuals who stated no supplement use in the FFQ also answered 'no' in the 24-hour recall tool. Lower agreement between the FFQ and the 24-hour dietary recall tool for other supplement use may reflect less regular use of other non-fish oil supplements, such as glucosamine or zinc. For subsequent analyses, I used

the FFQ question to stratify participants as ‘FOS user’, ‘other’ supplement user, or as ‘no supplement use’ (FFQ data were missing for 447 participants [0.4%]).

Almost one third of participants (n=38,036, 31%) used a FOS (**table 4.4**). FOS users had higher oily fish intake than individuals who did not take a supplement (**table 4.4**). This finding was specific for FOS use, as opposed to other supplement use, which is presumably related to shared belief about the health benefits of both dietary and supplemental O3FA intake (**table 4.4**).

Nutritional supplement users were older and more likely female than individuals who did not report nutritional supplement use. Nutritional supplement use (including FOS) use was common in post-menopausal women, including HRT users. Overall, FOS users were distinct from other nutritional supplement users, who were proportionally more likely to be female, premenopausal, of South Asian ethnicity and report lower frequency of alcohol intake (**table 4.4**).

4.3.3 Oily fish intake

I also characterised participants according to oily fish intake (**table 4.5**). Dietary intake data were available for 120,790 (99.3%) participants with a plasma fatty acid profile. The largest proportion of these participants (n=46,030, 38%) reported eating oily fish once a week, in accordance with UK dietary guidelines [14], with 18% (n=21,886) reporting consumption of oily fish at least twice a week (**table 4.5**). Participant characteristics that predicted higher oily fish intake were similar to those associated with FOS use, including female sex, post-menopausal status, and HRT use (**table 4.5**).

Table 4.4 The characteristics of UKBB participants with a plasma fatty acid profile according to nutritional supplement use.

	No supplement use	Other	FOS	P value[4]
Number or participants Percentage of participants (%)	**69,207 (57)**	**13,960 (12)**	**38,036 (31)**	
Sex				
Male (%)	34,651 (50)	4,344 (31)	16,675 (44)	<0.001
Female (%)	34,556 (50)	9,616 (69)	21,361 (56)	
Age[1]				
Age (years)	55.2 (8.2)	57.2 (7.8)	58.7 (7.4)	<0.001
BMI[1]				
BMI (Kg/m^2)	27.6 (4.9)[a]	27.2 (4.9)[b]	27.2 (4.5)[b]	<0.001
Oily fish intake				
Never	8,649 (13)	1,725 (12)	2,728 (7)	<0.001
<Once a week	24,798 (36)	4,086 (29)	10,744 (28)	
Once a week	24,743 (36)	5,360 (38)	15,831 (42)	
≥Twice a week	10,517 (15)	2,729 (20)	8,589 (23)	
Unknown	500 (1)	60 (<1)	144 (<1)	
Menopausal status[2]				
Pre-menopausal	10,245 (30)	1,987 (21)	3,164 (15)	<0.001
Post-menopausal	18,750 (54)	6,121 (64)	14,846 (70)	
Not sure as had a hysterectomy or for other reason	5,497 (16)	1,496 (16)	3,323 (16)	
Unknown	64 (<1)	12 (<1)	28 (<1)	0.32
Ever used hormone replacement therapy[2]				
Use	11,158 (32)	3,986 (42)	9,842 (46)	<0.001
No use	23,263 (67)	5,594 (58)	11,459 (54)	
Unknown	135 (<1)	36 (<1)	60 (<1)	0.04
Ever used oral contraceptive pill[2]				
Use	28,408 (82)	7,678 (80)	16,884 (79)	<0.001
No use	6,047 (18)	1,900 (20)	4,424 (21)	
Unknown	101 (<1)	38 (<1)	53 (<1)	0.45
Ethnicity				
White	65,368 (95)	13,037 (93)	36,207 (95)	<0.001
Non White[3]	3,602 (5)	866 (6)	1,673 (4)	<0.001
Mixed	392 (1)	95 (1)	187 (1)	0.18
Asian	1,438 (2)	345 (3)	514 (1)	<0.001
Black	975 (1)	240 (2)	567 (2)	0.18
Chinese	175 (<1)	51 (<1)	123 (<1)	0.03
Other	622 (1)	135 (1)	282 (1)	0.01
Unknown	237 (<1)	57 (<1)	156 (<1)	0.07
Current tobacco smoking				
No	49,467 (72)	10,451 (75)	28,290 (74)	<0.001
Only occasionally	1,605 (2)	266 (2)	778 (2)	0.002
Yes, on most or all days	5,030 (7)	649 (5)	1,686 (4)	<0.001
Unknown	13,105 (19)	2,594 (19)	7,282 (19)	0.48
Alcohol intake				
Rarely / Never	10,013 (15)	2,346 (17)	5,172 (14)	0.003
One to three times a month	6,362 (9)	1,327 (10)	3,295 (9)	0.007
Once or twice a week	14,475 (21)	2,792 (20)	7,920 (21)	0.54
Three or four times a week	13,381 (19)	2,640 (19)	7,660 (18)	0.003
Daily or almost daily	11,860 (17)	2,265 (16)	6,704 (19)	0.09
Unknown	13,116 (19)	2,590 (19)	7,285 (19)	0.51
Qualifications				
College or University degree	23,276 (34)	4,882 (35)	11,435 (30)	<0.001
Vocational qualifications	7,885 (11)	1,594 (11)	4,977 (13)	<0.001
Optional national exams at ages 17-18 years	7,607 (11)	1,588 (11)	4,176 (11)	0.94
National exams at age 16 years	18,395 (27)	3,723 (27)	9,993 (26)	0.30
None of the above	11,313 (16)	2,040 (15)	7,046 (19)	<0.001
Unknown	731 (1)	133 (1)	409 (1)	0.87
Average household income before tax				
<18,000	12,998 (19)	2,674 (19)	7,845 (21)	<0.001
18,000 to 30,999	14,275 (21)	3,159 (23)	9,210 (24)	
31,000 to 51,999	15,727 (23)	3,111 (22)	8,144 (21)	
52,000 to 100,000	13,232 (19)	2,273 (16)	5,587 (15)	
>100,000	3,745 (5)	558 (4)	1,306 (3)	
Unknown	9,230 (13)	2,185 (16)	5,944 (16)	

[1] Mean with standard deviation shown in brackets.

[2] Percentage values are of females only.
[3] Non-white includes Mixed, Asian, Black, Chinese, and Other
[4] Cochran-Armitage test for trend for categorical variables and ANOVA for continuous variables. [b] Indicates statistically significant difference between no supplement and other and fish oil supplement groups

Table 4.5 The characteristics of UKBB participants with a plasma fatty acid profile according to oily fish intake category.

	Never	<Once a week	Once a week	≥Twice a week	P value[4]
Number or participants Percentage of participants (%)	**13,161 (10.9)**	**39,713 (32.9)**	**46,030 (38.1)**	**21,886 (18.1)**	
Sex					
Male (%)	6,310 (48)	19,039 (48)	20,339 (44)	9,767 (45)	<0.001
Female (%)	6,851 (52)	20,674 (52)	25,691 (56)	12,119 (55)	
Age[1]					
Age (years)	54.1 (8.2)	55.5 (8.0)	57.3 (7.9)	58.3 (7.8)	<0.001
BMI[1]					
BMI (Kg/m^2)	27.8 (5.2)	27.5 (4.8)	27.3 (4.6)	27.3 (4.7)	0.005
Supplement use					
No supplement use	8,649 (66)	24,798 (62)	24,743 (54)	10,517 (48)	<0.001
Other	1,725 (13)	4,086 (10)	5,360 (12)	2,729 (13)	0.006
Fish oil	2,728 (21)	10,744 (27)	15,831 (34)	8,589 (39)	<0.001
Unknown	59 (<1)	85 (<1)	96 (<1)	51 (<1)	0.003
Menopausal status[2]					
Pre-menopausal	2,164 (32)	5,767 (28)	5,358 (21)	2,067 (17)	<0.001
Not sure as had a hysterectomy or for other reason	1,141 (17)	3,188 (15)	4,027 (16)	1,913 (16)	0.48
Post-menopausal	3,526 (52)	11,686 (57)	16,263 (63)	8,117 (67)	<0.001
Unknown	20 (<1)	33 (<1)	43 (<1)	22 (<1)	0.004
Ever used hormone replacement therapy[2]					
Use	2,235 (33)	7,150 (35)	10,271 (40)	5,263 (43)	<0.001
No use	4,575 (67)	13,441 (65)	15,339 (60)	6,814 (56)	<0.001
Unknown	41 (<1)	83 (<1)	80 (<1)	41 (<1)	0.004
Ever used oral contraceptive pill[2]					
Use	5,572 (81)	17,225(83)	20,683 (81)	9,340 (77)	<0.001
No use	1,247 (18)	3,394 (16)	4,927 (19)	2,741 (23)	<0.001
Unknown	32 (1)	55 (<1)	81 (<1)	38 (<1)	0.40
Current tobacco smoking					
No	11,195 (85)	35,267 (89)	41,814 (91)	19,892 (91)	<0.001
Only occasionally	318 (2)	1,063 (3)	1,217 (3)	581 (3)	0.38
Yes, on most or all days	1,639 (13)	3,362 (9)	2,974 (7)	1,402 (6)	<0.001
Unknown	9 (<1)	21 (<1)	25 (<1)	11 (<1)	0.60
Alcohol intake					
Rarely / Never	3,965 (30)	7,365 (19)	7,651 (17)	4,146 (19)	<0.001
One to three times a month	1,749 (13)	5,012 (13)	4,681 (10)	2,154 (10)	<0.001
Once or twice a week	3,294 (25)	10,325 (26)	12,104 (26)	5,342 (24)	0.11
Three or four times a week	2,326 (18)	8,993 (23)	11,519 (25)	5,419 (25)	<0.001
Daily or almost daily	1,809 (14)	7,990 (20)	10,047 (22)	4,800 (22)	<0.001
Unknown	18 (<1))	28 (<1)	28 (<1)	25 (<1)	0.75
Ethnicity					
White	12,162 (92)	37,767 (95)	43,882 (95)	20,494 (94)	0.01
Non White[3]	953 (7)	1,816 (5)	1,922 (4)	1,282 (6)	0.0002
Mixed	77 (1)	215 (1)	233 (1)	148 (1)	<0.001
Asian	640 (5)	676 (2)	619 (1)	330 (2)	<0.001
Black	111 (1)	513 (1)	521 (1)	512 (2)	<0.001
Chinese	21 (<1)	125 (<1)	141 (<1)	58 (<1)	0.31
Other	104 (1)	287 (0.7%)	408 (1)	234 (1)	<0.001
Unknown	46 (<1)	130 (0.3%)	226 (<1)	110 (1)	0.0002
Qualifications					
College or University degree	3,176 (24)	13,048 (33)	15,958 (35)	7,377 (34)	<0.001
Vocational qualifications	1,510 (12)	4,648 (12)	5,503 (12)	2,732 (13)	0.001
Optional national exams at ages 17-18 years	1,258 (10)	4,562 (12)	5,128 (11)	2,408 (11)	0.03
National exams at age 16 years	4,063 (31)	10,936 (28)	11,660 (25)	5,360 (25)	<0.001
None of the above	2,953 (22)	6,196 (16)	7,337 (16)	3,721 (17)	<0.001
Unknown	201 (2)	323 (1)	444 (1)	288 (1)	0.49
Average household income before tax					
<18,000	3,173 (24)	7,162 (18)	8,378 (18)	4,700 (21)	0.07
18,000 to 30,999	2,853 (22)	8,604 (22)	10,177 (22)	4,917 (23)	0.02
31,000 to 51,999	2,851 (22)	9,512 (24)	10,156 (22)	4,418 (20)	<0.001
52,000 to 100,000	1,874 (14)	7,486 (19)	8,311 (18)	3,396 (16)	0.25
>100,000	350 (3)	1,869 (5)	2,361 (5)	1,031 (5)	<0.001
Unknown	2,060 (16)	5,080 (13)	6,647 (14)	3,424 (16)	<0.001

[1] Mean with standard deviation shown in brackets.
[2] Percentage values are of females only.

[3] Non-white includes Mixed, Asian, Black, Chinese, and Other
[4] Cochran-Armitage test for trend for categorical variables and linear regression for continuous variables.

4.3.4 The relationship between oily fish intake and fish oil supplement use

The relationship between dietary oily fish intake and likelihood of FOS use is described in **figure 4.2**. The relationship between oily fish intake and likelihood of FOS use was evident with a clear 'dose-dependent' relationship between increasing frequency of dietary oily fish intake and the proportion taking a FOS, which was not apparent for other supplement use (**table 4.5**). Higher oily fish intake was associated with a larger proportion of FOS users with 8,589 (39%) of individuals, who reported eating oily fish more than or equal to twice a week, using a FOS compared with 21% of those who reported never eating oily fish (P<0.001) (**figure 4.2**). By contrast, increasing oily fish intake frequency was not associated with other supplement use (**figure 4.2 and table 4.5**).

4.3.5 Plasma fatty acid profiles in UKBB participants

The distribution of individual plasma fatty acid values in the 121,650 UKBB participants, who were randomly selected for NMR lipidomic profiling, is shown in **figure 4.3**. Each fatty acid category demonstrated a wide range of values with a positive skew. The distribution of the individual values for the proportion of O3FAs to total FAs was also positively skewed with a corresponding negative skew for the proportion of O6FAs to total FAs (**figure 4.4**). All plasma fatty acid data were log-transformed prior to analysis of the relationship between plasma fatty acid levels and O3FA intake (**figure 4.5**).

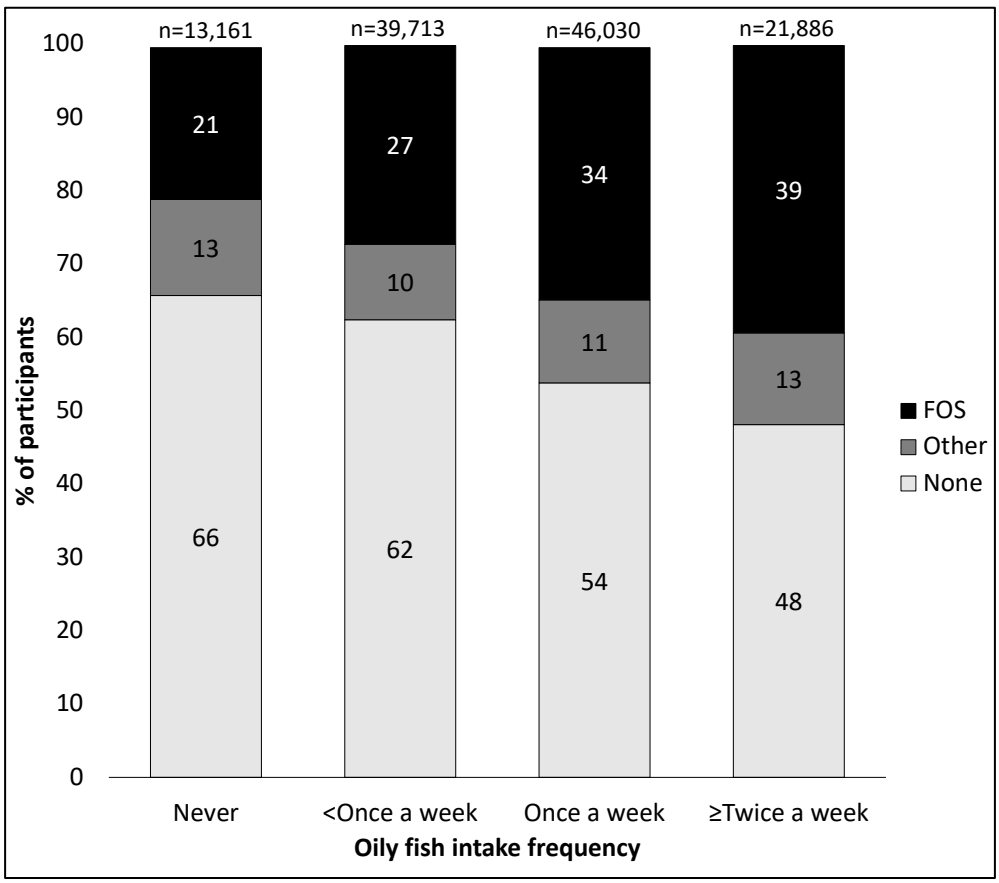


Figure 4.2 Nutritional supplement use according to dietary oily fish intake frequency of the UKBB study population with a plasma fatty acid profile.

See table 4.5 for the actual number of cases in each category including missing data which accounted for <1% of the study population.

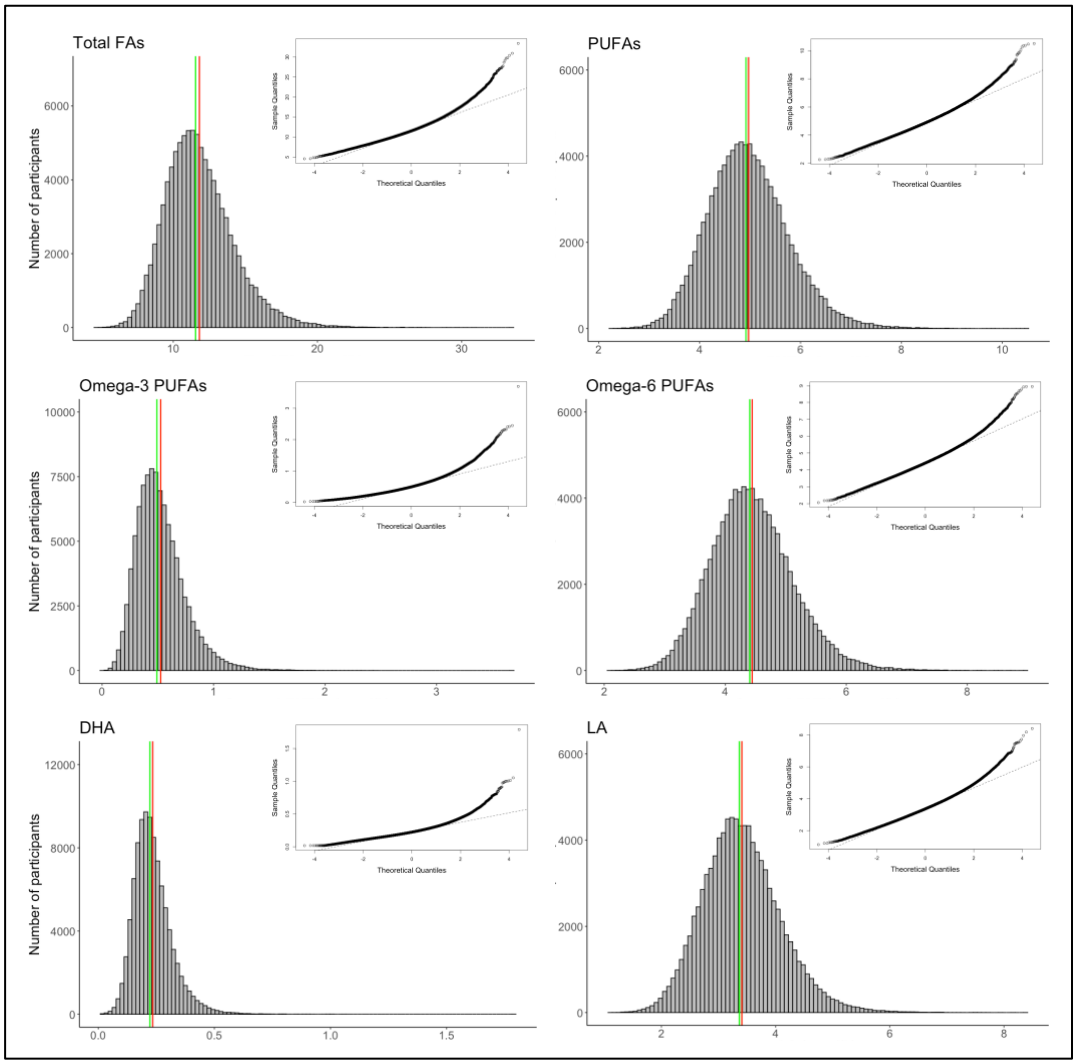


Figure 4.3. Distribution of plasma fatty acid levels in the UK Biobank population with an NMR plasma fatty acid profile (n=121,650).

The X axis for each panel is the fatty acid concentration in mmol/L. The mean plasma fatty acid value for each fatty acid class or individual PUFA is denoted by a red line. The median plasma fatty acid value for each class or individual PUFA is denoted by a green line. In each case, the inset panel is the corresponding Q-Q plot.

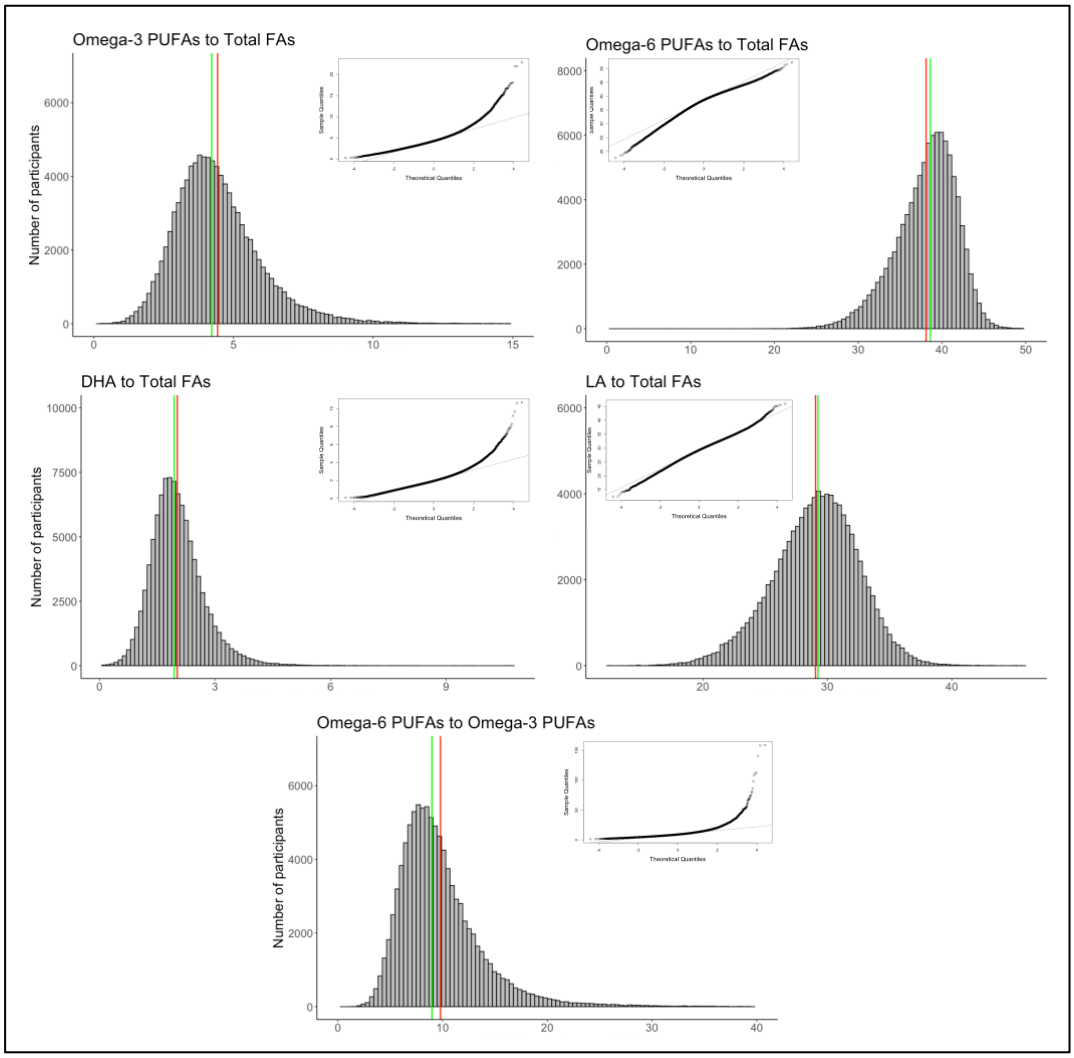


Figure 4.4. Distribution of plasma fatty acids as a proportion of total FAs and the O6FA to O3FA ratio in the UK Biobank population with an NMR plasma fatty acid profile (n=121,650).

The mean plasma fatty acid proportion (%) or ratio value is denoted by a red line. The median plasma fatty acid proportion or ratio value is denoted by a green line. In each case the inset panel is the corresponding Q-Q plot.

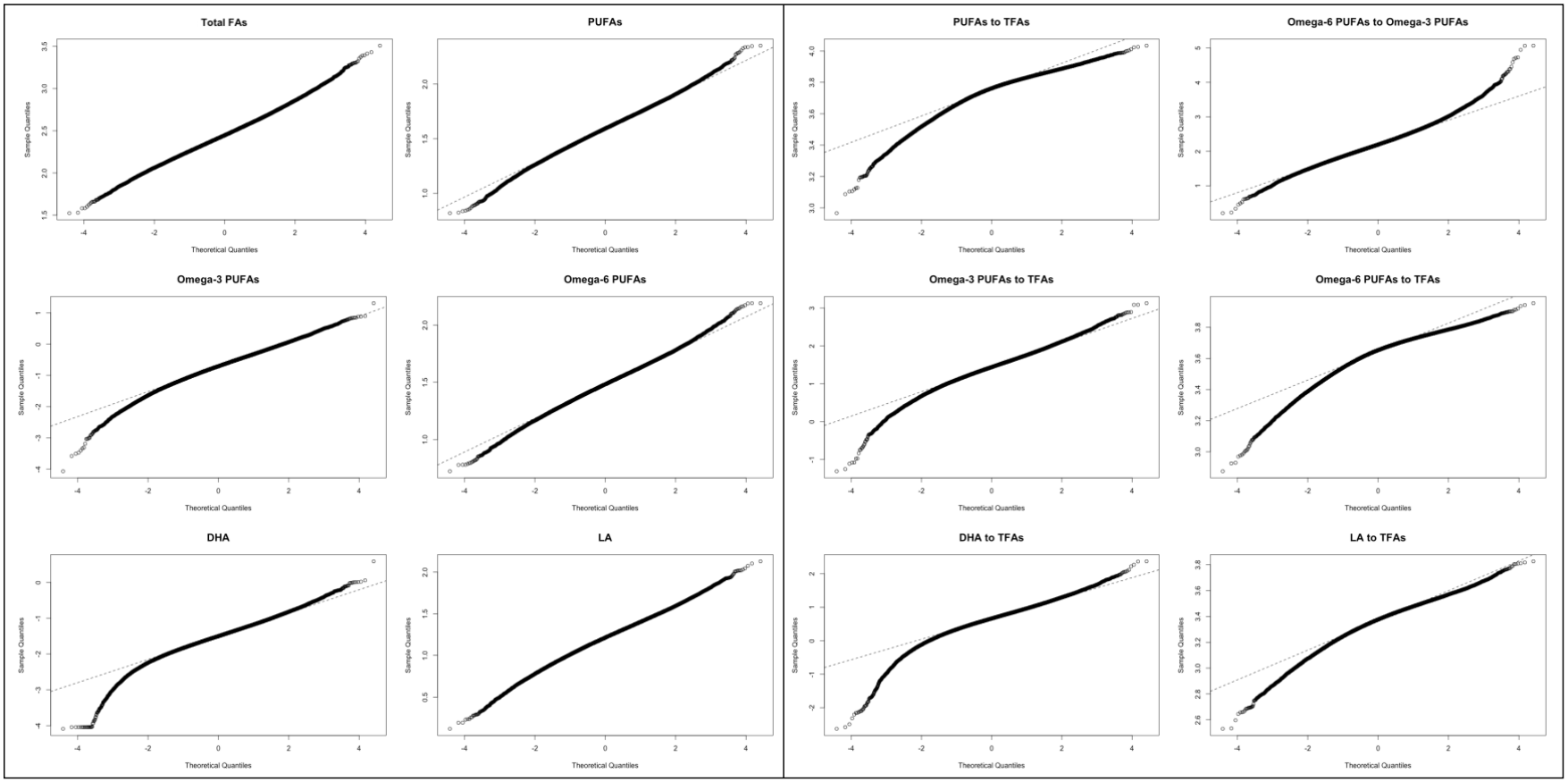


Figure 4.5. Q-Q plots of log-transformed plasma fatty acid data from the UK Biobank population with an NMR plasma fatty acid profile (n=121,650).

4.3.6 The relationship between omega-3 PUFA intake from oily fish and FOS, and plasma fatty acid levels

Increasing oily fish intake frequency was associated with higher mean plasma total PUFAs, as well as the concentration of total O3FAs and DHA, with a clear dose-response relationship in each case (**table 4.6**). This was reflected in a stepwise increase in mean total O3FAs concentration between 0.07-0.1mmol/L and a stepwise increase in mean DHA level between 0.03-0.04 mmol/L across increasing oily fish intake frequency categories (**table 4.6, figure 4.6**). There was a corresponding dose-dependent decrease in plasma total O6FAs and LA concentrations associated with increasing oily fish intake which was smaller than that for O3FAs (**table 4.6**). These findings were mirrored by changes in the individual PUFA class proportions reported against total fatty acids (**table 4.6**). There was an approximate 50% reduction in the O6FAs/O3FAs in individuals who reported eating oily fish ≥ twice a week compared with those who did not eat oily fish (**table 4.6, figure 4.6**).

There was significant overlap between individual fatty acid level values in each oily fish intake category, consistent with wide inter-individual variability in PUFA levels related to other genetic and environmental factors governing plasma PUFA levels, independent of dietary PUFA intake (**figure 4.6**). For example, 2,468 (12.7%) of 21,866 participants who reported eating oily fish ≥ twice a week had a total O3FA level below the median level (0.38 mmol/L) for those who reported never consuming oily fish (**figure 4.6**).

Table 4.6 Plasma fatty acid levels reported as absolute concentration or proportion of total fatty acids according to oily fish intake frequency and supplement use.

	Oily-fish intake category[4]				Supplement use category[5]		
	Never (n=13,161)	**<Once a week** (n=39,713)	**Once a week** (n=46,030)	**≥Twice a week** (n=21,886)	**No supplement use** (n=69,207)	**Other** (n=13,960)	**FOS** (n=38,036)
Total FAs[1]	11.83 (2.47)	11.86 (2.40)	11.88 (2.38)	11.75 (2.35)	11.77 (2.41)[a]	11.87 (2.37)[b]	11.97 (2.36)[c]
Total PUFAs[1]	4.87 (0.80)	4.93 (0.79)	5.01 (0.80)	5.05 (0.81)	4.92 (0.79)[a]	5.03 (0.81)[b]	5.08 (0.81)[c]
Total PUFAs to total FAs[2]	41.67 (3.87)	42.08 (3.71)	42.64 (3.68)	43.44 (3.76)	42.25 (3.78)[a]	42.78 (3.68)[b]	42.82 (3.73)
O3FAs[1]	0.40 (0.17)	0.47 (0.18)	0.55 (0.21)	0.65 (0.26)	0.49 (0.20)[a]	0.52 (0.21)[b]	0.60 (0.23)[c]
O3FAs to total FAs[2]	3.34 (1.09)	3.97 (1.20)	4.61 (1.43)	5.49 (1.87)	4.11 (1.43)[a]	4.37 (1.52)[b]	4.99 (1.64)[c]
DHA[1]	0.18 (0.06)	0.21 (0.07)	0.24 (0.08)	0.28 (0.10)	0.22 (0.09)[a]	0.23 (0.08)[b]	0.26 (0.09)[c]
DHA to total FAs[2]	1.56 (0.47)	1.82 (0.53)	2.09 (0.63)	2.46 (0.81)	1.89 (0.63)[a]	2.00 (0.66)[b]	2.23 (0.71)[c]
O6FAs[1]	4.47 (0.71)	4.46 (0.67)	4.46 (0.68)	4.41 (0.68)	4.43 (0.68)[a]	4.51 (0.69)[b]	4.48 (0.68)[c]
O6FAs to total FAs[2]	38.32 (3.96)	38.12 (3.67)	38.02 (3.55)	37.94 (3.49)	38.14 (3.68)[a]	38.41 (3.56)[b]	37.83 (3.54)[c]
LA[1]	3.47 (0.71)	3.43 (0.67)	3.42 (0.68)	3.34 (0.70)	3.40 (0.68)[a]	3.47 (0.70)[b]	3.42 (0.69)[c]
LA to total FAs[2]	29.55 (3.75)	29.18 (3.39)	28.94 (3.35)	28.56 (3.46)	29.09 (3.44)[a]	29.42 (3.48)[b]	28.73 (3.42)[c]
O6FAs to O3FAs[3]	13.02 (6.16)	10.65 (4.27)	9.13 (3.52)	7.76 (3.02)	10.51 (4.64)[a]	10.03 (4.48)[b]	8.45 (3.27)[c]

[1] Plasma fatty acid levels are reported as mmol/L, mean (standard deviation)
[2] The proportion (as %) of total FAs, mean (SD)
[3] The ratio of total O6FAs to O3FAs, mean (SD)
[4] Participants with oily fish intake data, n=120,790. Linear regression model to the examine the difference between plasma fatty acid levels and proportions across oily fish intake categories. A statistically significant increase was observed for all plasma PUFA levels and ratios according to increasing oily fish intake frequency categories (P <0.001).
[5] Participants with mineral and supplement use data, n=121,203. ANOVA and Tukey's test to examine the difference between plasma fatty acid levels and proportions across supplement categories: [a] P<0.005 for the difference between 'no supplement use' and 'FOS', [b] P<0.005 for the difference between 'no supplement use' and 'other', and [c] P<0.005 for the difference between 'other' and 'FOS'.

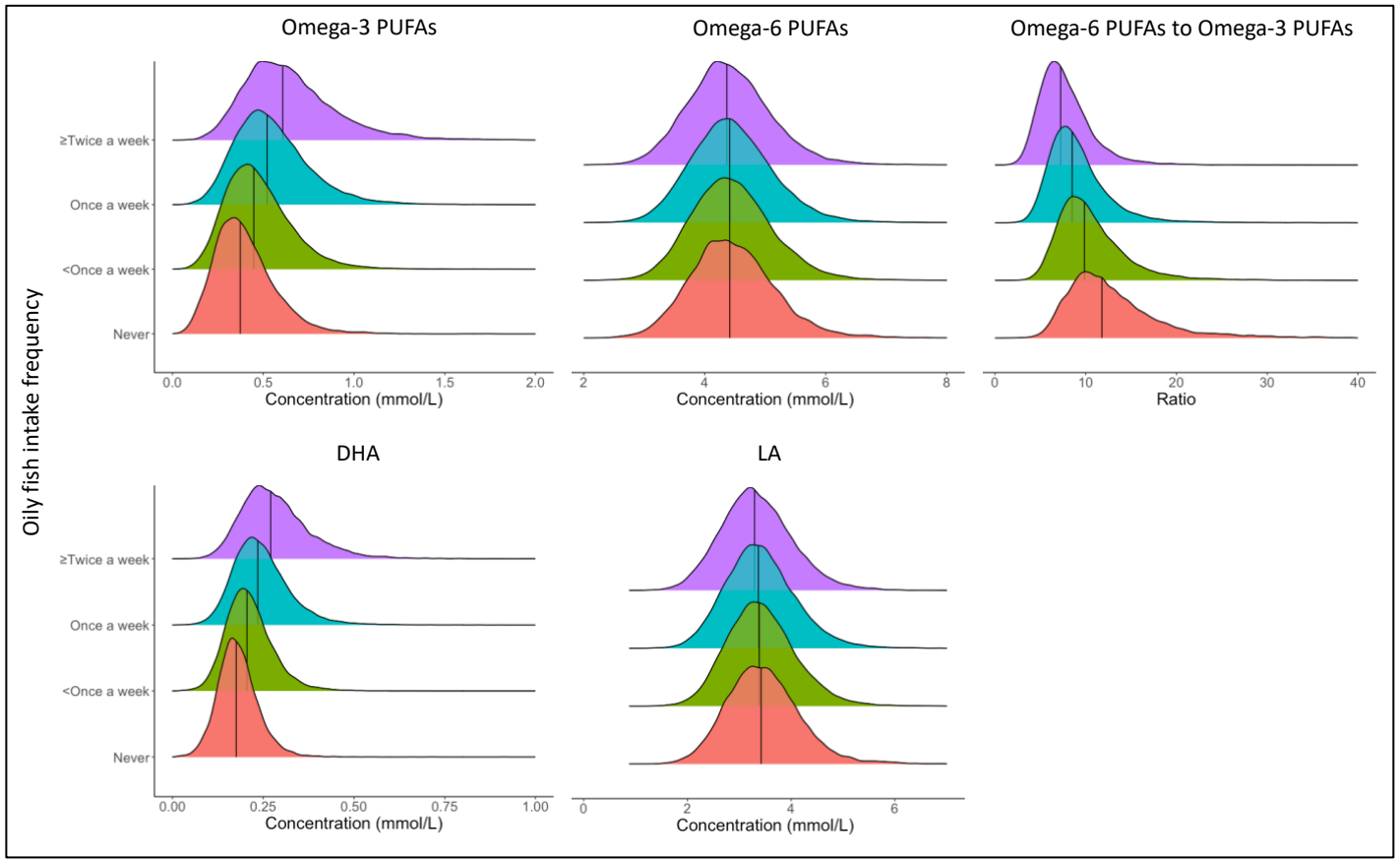


Figure 4.6 Ridgeline plots demonstrating the distribution of plasma fatty acid levels and the ratio of O6FAs to O3FAs values in UKBB participants with oily fish intake data (n=120,790).

The median value for plasma fatty acid concentration (mmol/L) and ratio of O6FAs to O3FAs is denoted by the dark vertical line.

FOS use was associated with higher plasma concentrations of total PUFAs, total O3FAs and DHA than for individuals who did not report any supplement use (**table 4.6**). By contrast to the relationship between oily fish intake frequency and PUFA levels, FOS users also demonstrated higher O6FAs and LA concentrations than individuals who did not report any supplement use (**table 4.6**). However, this association disappeared when O6FAs and LA were recorded as proportions of total FAs (**table 4.6**). Users of other non-FOS supplements had higher O3FA levels than individuals who did not report supplement use, likely due to other supplement users having a higher oily fish intake (oily fish ≥twice a week 20%) compared to non-supplement users (15%) (**table 4.6**).

Given the relationship between oily fish intake and FOS use (**figure 4.2**), I next examined plasma PUFA levels according to both oily fish intake and FOS use to delineate their respective contributions to plasma PUFA levels (**figure 4.7**). Comprehensive summary data on fatty acid levels according to both oily fish intake and FOS use are available in **table 4.7**, in which mean (SD) values are expressed with their respective median (IQR) data. FOS use was consistently associated with an increase in O3FAs, including DHA, and the opposite relationship with the concentration of total O6FAs, including LA (**figure 4.6 & table 4.7**). In general, FOS use was associated with an increase in the median plasma O3FA and DHA level for an individual oily fish intake category to the level associated with the next highest oily fish intake category (**figure 4.6**). For example, participants who never ate oily fish, but used a FOS had a median (IQR) plasma O3FA level of 0.45 [0.35-0.59] mmol/L, compared with those who did not use supplements, but ate oily fish less than once a week, who had a median plasma O3FAs level of 0.42 [0.33-0.54] mmol/L (**table 4.7**).

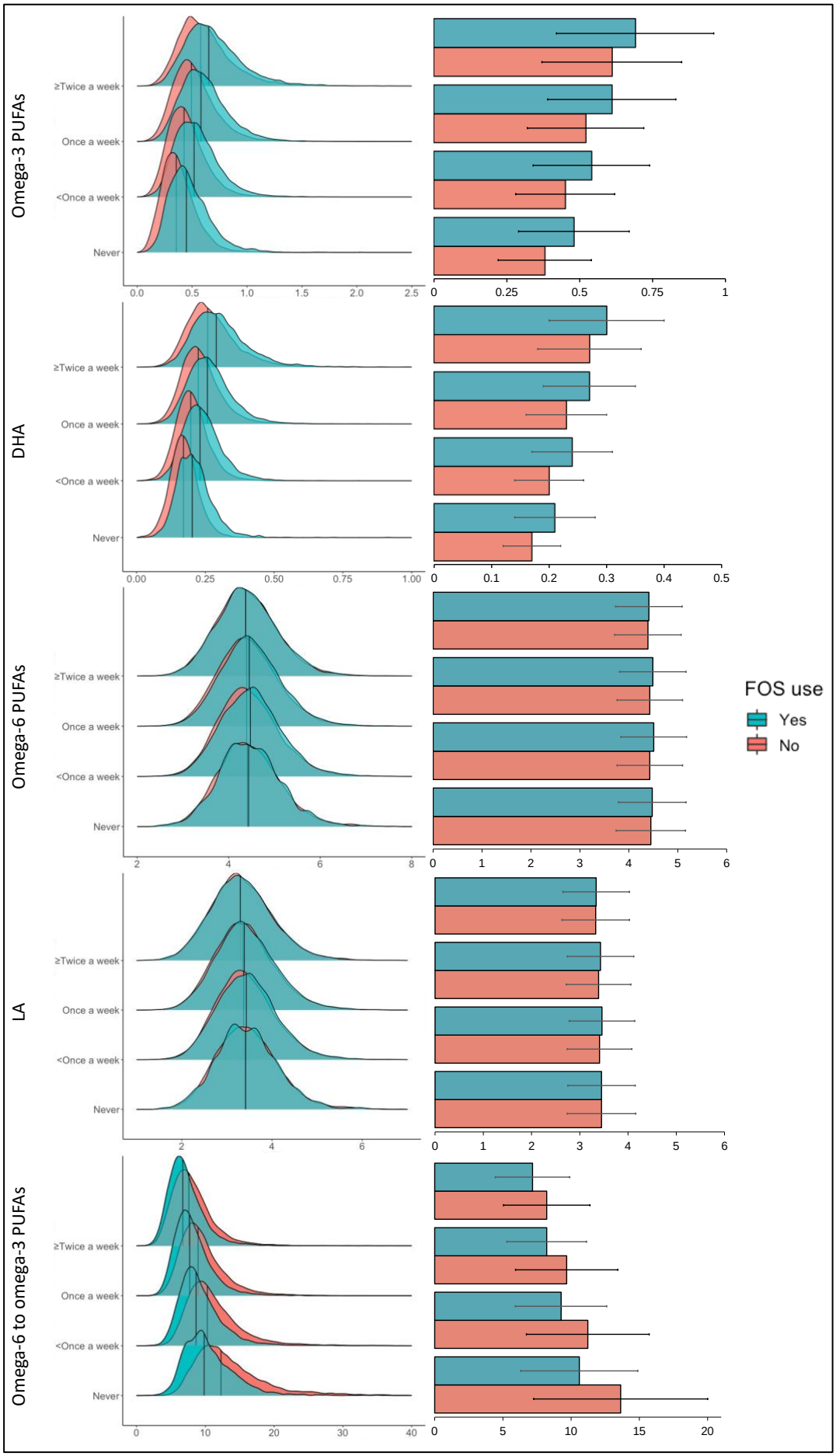

Omega-3 PUFAs
DHA
Omega-6 PUFAs
LA
Omega-6 to omega-3 PUFAs
≥Twice a week
Once a week
<Once a week
Never
FOS use
Yes
No

Figure 4.7 Ridgeline and bar plots showing plasma PUFAs levels according to oily fish intake and FOS use.

The X axis shows the fatty acid concentration in mmol/L or the O6FA to O3FAs ratio. Median values are denoted with a dark line in the ridge line plots. Bar plots are the median values with error bars denoting the interquartile range. Green denotes the population who used a FOS compared with the population that did not use a FOS shaded in red. For plasma fatty acid level and ratio values according to oily fish intake and FOS use, see **table 4.7.**

Table 4.7 Plasma fatty acid classes as the absolute concentration, proportion of total FAs, and the omega-6 to omega-3 PUFA ratio, according to oily fish intake frequency and supplement use.

Plasma fatty acid levels (mmol/L)		Never (n=13,161)[1]			<Once a week (n=39,713)			Once a week (n=46,030)			Twice a week (n=21,886)		
		No supplement use	Other	FOS	No supplement use	Other	FOS	No supplement use	Other	FOS	No supplement use	Other	FOS
Number of participants		8,649 (65.7%)	1,725 (13.1%)	2,728 (20.7%)	24,798 (62.4%)	4,086 (10.3%)	10,744 (27.1%)	24,743 (53.8%)	5,197 (11.3%)	15,831 (34.4%)	10,517 (48.1%)	2,729 (12.5%)	8,589 (39.2%)
Total FAs	Mean[2]	11.78 (2.50)	11.84 (2.38)	11.99 (2.45)[c]	11.78 (2.40)	11.89 (2.44)[b]	12.03 (2.35)[c]	11.78 (2.39)	11.91 (2.33)[b]	12.02 (2.36)[c]	11.70 (2.38)	11.80 (2.34)	11.81 (2.32)[c]
	Median[3]	11.48 (10.04, 13.21)	11.56 (10.18, 13.20)	11.77 (10.28, 13.39)	11.50 (10.11, 13.12)	11.62 (10.22, 13.23)	11.78 (10.40, 13.39)	11.51 (10.12, 13.14)	11.64 (10.29, 13.27)	11.77 (10.40, 13.33)	11.43 (10.07, 13.03)	11.52 (10.17, 13.15)	11.57 (10.22, 13.17)
Total PUFAs	Mean	4.83 (0.80)	4.95 (0.82)[b]	4.96 (0.80)[c]	4.88 (0.78)[a]	4.97 (0.80)[b]	5.05 (0.79)[c]	4.95 (0.79)[a]	5.06 (0.81)[b]	5.10 (0.81)[c]	5.00 (0.81)[a]	5.10 (0.80)	5.11 (0.82)[c]
	Median	4.76 (4.28, 5.30)	4.88 (4.39, 5.42)	4.90 (4.42, 5.46)	4.82 (4.35, 5.35)	4.93 (4.42, 5.44)	5.01 (4.51, 5.53)	4.90 (4.40, 5.44)	5.00 (4.50, 5.56)	5.05 (4.55, 5.58)	4.95 (4.45, 5.49)	5.04 (4.53, 5.61)	5.06 (4.55, 5.62)
O3FAs	Mean	0.38 (0.16)	0.28 (0.15)[b]	0.48 (0.19)[c]	0.45 (0.17)[a]	0.47 (0.18)[b]	0.54 (0.20)[c]	0.52 (0.20)[a]	0.54 (0.20)[b]	0.61 (0.22)[c]	0.61 (0.24)[a]	0.64 (0.24)[b]	0.69 (0.27)[c]
	Median	0.36 (0.27, 0.46)	0.36 (0.28, 0.47)	0.45 (0.35, 0.59)	0.42 (0.33, 0.54)	0.45 (0.34, 0.57)	0.52 (0.40, 0.65)	0.49 (0.38, 0.62)	0.52 (0.41, 0.65)	0.58 (0.46, 0.73)	0.57 (0.44, 0.73)	0.61 (0.47, 0.77)	0.65 (0.51, 0.83)
DHA	Mean	0.17 (0.05)[a]	0.18 (0.05)[b]	0.21 (0.07)[c]	0.20 (0.06)[a]	0.21 (0.06)[b]	0.24 (0.07)[c]	0.23 (0.07)[a]	0.24 (0.08)[b]	0.27 (0.08)[c]	0.27 (0.09)[a]	0.28 (0.09)[b]	0.30 (0.10)[c]
	Median	0.17 (0.14, 0.20)	0.17 (0.15, 0.21)	0.20 (0.17, 0.24)	0.20 (0.16, 0.23)	0.20 (0.17, 0.25)	0.23 (0.19, 0.28)	0.22 (0.18, 0.27)	0.23 (0.19, 0.28)	0.26 (0.21, 0.31)	0.25 (0.21, 0.32)	0.27 (0.22, 0.34)	0.29 (0.23, 0.36)
O6FAs	Mean	4.45 (0.71)	4.56 (0.73)[b]	4.48 (0.69)	4.43 (0.67)[a]	4.50 (0.69)	4.51 (0.67)[c]	4.43 (0.67)[a]	4.51 (0.69)	4.49 (0.68)[c]	4.39 (0.68)[a]	4.46 (0.67)	4.41 (0.68)[b]
	Median	4.40 (3.97, 4.87)	4.51 (4.07, 5.00)	4.44 (4.02, 4.89)	4.39 (4.00, 4.84)	4.46 (4.03, 4.92)	4.48 (4.04, 4.92)	4.39 (3.97, 4.85)	4.47 (4.05, 4.94)	4.45 (4.03, 4.90)	4.35 (3.93,4.80)	4.42 (3.99, 4.89)	4.38 (3.97, 4.83)
LA	Mean	3.45 (0.71)[a]	3.58 (0.75)[b]	3.45 (0.70)	3.41 (0.67)[a]	3.47 (0.69)	3.46 (0.68)[c]	3.39 (0.67)[a]	3.47 (0.69)	3.43 (0.69)[c]	3.33 (0.70)[a]	3.40 (0.69)[b]	3.34 (0.69)
	Median	3.40 (2.97, 3.87)	3.51 (3.08, 4.01)	3.41 (2.99, 3.86)	3.37 (3.00, 3.82)	3.42 (3.00, 3.87)	3.43 (3.00, 3.87)	3.35 (2.93, 3.80)	3.42 (3.01, 3.90)	3.38 (2.97, 3.85)	3.28 (2.85, 3.75)	3.35 (2.92, 3.83)	3.30 (2.87, 3.76)
O6FAs to O3FAs ratio	Mean	13.63 (6.38)	13.86 (6.63)[b]	10.60 (4.29)[c]	11.22 (4.50)[a]	10.83 (4.17)[b]	9.26 (3.35)[c]	9.67 (3.75)[a]	9.29 (3.43)[b]	8.21 (2.92)[c]	8.21 (3.17)[a]	7.84 (2.97)[b]	7.17 (2.73)[c]
	Median	12.23 (9.86,15.69)	12.43 (9.90, 15.71)	9.79 (7.92, 12.30)	10.34 (8.49, 12.90)	9.98 (8.13, 12.44)	8.63 (7.15, 10.70)	8.98 (7.35, 11.15)	8.69 (7.08, 10.66)	7.70 (6.32, 9.48)	7.69 (6.13, 9.65)	7.34 (5.92, 9.17)	6.73 (5.43, 8.42)

Percentage of total FAs (%)		Never (n=13,161)[1]			<Once a week (n=39,713)			Once a week (n=46,030)			Twice a week (n=21,886)		
		No supplement use	Other	FOS	No supplement use	Other	FOS	No supplement use	Other	FOS	No supplement use	Other	FOS
Number of participants		8,649 (65.7%)	1,725 (13.1%)	2,728 (20.7%)	24,798 (62.4%)	4,086 (10.3%)	10,744 (27.1%)	24,743 (53.8%)	5,197 (11.3%)	15,831 (34.4%)	10,517 (48.1%)	2,729 (12.5%)	8,589 (39.2%)
PUFAs	Mean	41.50 (3.90)[a]	42.25 (3.82)[b]	41.82 (3.75)[c]	41.92 (3.73)[a]	42.28 (3.66)	42.40 (3.65)[c]	42.45 (3.69)[a]	42.89 (3.56)	42.84 (3.69)[c]	43.19 (3.78)[a]	43.66 (3.71)	43.66 (3.75)[c]
	Median	42.02 (39.16, 44.26)	42.69 (40.15, 44.84)	42.26 (39.47, 44.52)	42.25 (39.69, 44.59)	42.83 (40.23, 44.78)	42.95 (40.26, 45.00)	42.98 (40.33, 45.04)	43.42 (40.98, 45.34)	43.38 (40.72, 45.45)	43.64 (40.99, 45.78)	44.02 (41.58, 46.21)	44.14 (41.49, 46.26)
O3FAs	Mean	3.17 (0.98)	3.20 (0.99)[b]	2.97 (1.24)[c]	3.75 (1.10)[a]	3.91 (1.13)[b]	4.48 (1.30)[c]	4.35 (1.33)[a]	4.55 (1.37)[b]	5.05 (1.49)[c]	5.20 (1.78)[a]	5.44 (1.80)[b]	5.87 (1.93)[c]
	Median	3.12 (2.51, 3.78)	3.16 (2.55, 3.79)	3.87 (3.15, 4.64)	3.68 (3.01, 4.38)	3.82 (3.14, 4.58)	4.37 (3.61, 5.19)	4.22 (3.46, 5.08)	4.41 (3.64, 5.32)	4.90 (4.03, 5.85)	4.93 (3.98, 6.09)	5.20 (4.20, 6.41)	5.63 (4.54, 6.83)
DHA	Mean	1.49 (0.43)[a]	1.53 (0.43)[b]	1.79 (0.53)[c]	1.74 (0.50)[a]	1.81 (0.51)[b]	2.01 (0.57)[c]	1.99 (0.59)[a]	2.08 (0.60)[b]	2.25 (0.65)[c]	2.35 (0.77)[a]	2.45 (0.78)[b]	2.60 (0.83)[c]
	Median	1.49 (1.22, 1.77)	1.54 (1.25, 1.79)	1.76 (1.45, 2.07)	1.72 (1.42, 2.03)	1.79 (1.49, 2.11)	1.97 (1.64, 2.34)	1.95 (1.60, 2.33)	2.03 (1.69, 2.42)	2.20 (1.82, 2.61)	2.24 (1.82, 2.75)	2.36 (1.92, 2.88)	2.52 (2.05, 3.05)
O6FAs	Mean	38.33 (3.99)[a]	39.04 (3.95)[b]	37.85 (3.80)[c]	38.17 (3.71)[a]	38.37 (3.64)[b]	37.92 (3.59)[c]	38.10 (3.58)[a]	38.34 (3.43)[b]	37.79 (3.51)[c]	37.99 (3.54)	38.22 (3.40)[b]	37.79 (3.45)[c]
	Median	38.82 (35.90, 41.17)	39.54 (36.64, 41.85)	38.32 (35.39, 40.59)	38.69 (35.90, 40.85)	38.91 (36.25, 40.97)	38.49 (35.82, 40.44)	38.61 (36.01, 40.65)	38.87 (36.39, 40.76)	38.29 (35.77, 40.27)	38.47 (35.92, 40.49)	38.63 (36.31, 40.54)	38.24 (35.79, 40.23)
LA	Mean	29.56 (3.73)[a]	30.43 (3.92)[b]	29.00 (3.58)[c]	29.22 (3.38)[a]	29.44 (3.44)[b]	28.98 (3.38)[c]	29.00 (3.33)[a]	29.32 (3.32)[b]	28.71 (3.37)[c]	28.61 (3.46)[a]	28.94 (3.42)[b]	28.38 (3.47)[c]
	Median	29.73 (27.18, 31.97)	30.60 (27.94, 33.15)	29.15 (26.69, 31.43)	29.46 (27.10, 31.54)	29.66 (27.28, 31.80)	29.19 (26.86, 31.30)	29.23 (26.91, 31.29)	29.49 (27.25, 31.57)	28.93 (26.62, 31.02)	28.78 (26.42, 30.98)	29.09 (26.69, 31.29)	28.51 (26.22, 30.75)

[1] Missing data in each oily fish category: Never (n=59) 0.4%, <Once a week (n=85) 0.2%, Once a week (n=259) 0.6%, ≥Twice a week (n=51) 0.2%.
[2] Mean (standard deviation).
[3] Median (interquartile range).
ANOVA and Tukey's test to examine the difference between plasma fatty acid levels and proportions across supplement categories within oily fish intake frequency categories. [a] P<0.005 for the difference between 'no supplement use' and 'FOS', [b] P<0.005 for the difference between 'no supplement use' and 'other', and [c] P<0.005 for the difference between 'other' and 'FOS'.

4.3.7 Factors predicting omega-3 PUFA levels in UKBB participants

I then examined the relationship between plasma O3FA levels and clinical characteristics by building multivariate models that included dietary oily fish intake and supplement use, in addition to other factors that might be associated with plasma O3FA levels. Models were constructed to test the predictive value of these variables for the highest quartile of plasma O3FAs and DHA (both absolute concentration and as a proportion of total fatty acids) (**table 4.8**).

In the multivariate model, FOS use was retained as an independent predictor of higher plasma O3FA levels compared with no supplement use (OR 2.03 [1.97 - 2.09]), but the effect size was modest in comparison with the relationship between plasma O3FA levels and oily fish intake (**table 4.8**). The odds ratio (OR) for the highest quartile of total plasma O3FAs and DHA in FOS users was similar to the OR associated with oily fish intake less than once weekly (**table 4.8**), consistent with the plasma O3FA concentrations observed in these groups (**figure 4.6**).

Consistent with existing literature, increasing age, female sex and alcohol intake were predictive of high plasma total O3FA and DHA levels, whilst there was an inverse relationship with tobacco smoking (**table 4.8**) (139, 270, 271). Although excess body weight was positively associated with the plasma total O3FA and DHA concentration, an inverse relationship was observed after correction for total fatty acid concentrations. This may be explained by hypertriglyceridemia (and hence elevated PUFAs) associated with obesity (272-274) (**table 4.8**). The removal of covariates (alcohol intake frequency, deprivation, age, and exercise) with a VIF > 5 from the model, had no discernible effect on risk estimates.

Increasing alcohol intake was associated with higher plasma levels of O3FAs and the proportion of O3FAs to TFAs, as well as DHA, whereas smoking was inversely related with O3FA levels (**table 4.8**).

To investigate possible female-specific predictive factors, I generated a model restricted to women only (**table 4.9**). There were no marked differences in ORs for individual characteristics compared with the multivariable analysis of the whole study (**table 4.8**). In the female-only model, post-menopausal status was associated with increasing plasma O3FA levels (OR 1.65, 95% confidence interval [1.53-1.78]) (**table 4.9**).

Table 4.8 Factors predicting plasma O3FA and DHA levels in UKBB participants.*

Characteristic	Quartiles of O3FA[1]	Quartiles of O3FA to total FAs[1]	Quartiles of DHA[1]
Age			
≤49 (reference)	1.0	1.0	1.0
50-59	1.64 (1.58 – 1.70)	1.38 (1.33 – 1.43)	1.48 (1.42 – 1.54)
>60	1.92 (1.85 – 2.00)	1.71 (1.64 – 1.77)	1.61 (1.55 – 1.67)
Sex			
Female (reference)	1.0	1.0	1.0
Male	0.57 (0.56 – 0.59)	0.64 (0.62 – 0.66)	0.38 (0.36 – 0.39)
BMI (kg/m^2)			
≤24.9 (reference)	1.0	1.0	1.0
25-29.9	1.29 (1.25 – 1.33)	0.95 (0.92 – 0.99)	0.89 (0.86 – 0.91)
≥30	1.20 (1.14 – 1.23)	0.71 (0.69 – 0.74)	0.58 (0.55 – 0.60)
Supplement use			
No supplement use (reference)	1.0	1.0	1.0
Fish oil use	2.03 (1.97 – 2.09)	2.28 (2.21 – 2.36)	2.16 (2.09 – 2.23)
Oily fish intake			
Never (reference)	1.0	1.0	1.0
<Once a week	1.93 (1.84 – 2.03)	2.34 (2.22 – 2.46)	2.18 (2.07 – 2.29)
Once a week	3.42 (3.25 – 3.60)	5.02 (4.77 – 5.29)	4.36 (4.14 – 4.59)
≥Twice a week	6.66 (6.29 – 7.06)	12.07 (11.37 – 12.82)	9.55 (9.00 – 10.14)
Alcohol frequency			
Never (reference)	1.0	1.0	1.0
Once to three times a month	1.01 (0.96 – 1.07)	0.99 (0.94 – 1.05)	1.06 (1.00 – 1.12)
Once or twice a week	1.17 (1.12 – 1.23)	1.09 (1.04 – 1.14)	1.31 (1.25 – 1.37)
Three to four times a week	1.43 (1.40 – 1.49)	1.31 (1.25 – 1.37)	1.75 (1.67 – 1.83)
Daily or most days	1.64 (1.56 – 1.72)	1.34 (1.28 – 1.41)	2.13 (2.03 – 2.23)
Smoking			
Non-smoker (reference)	1.0	1.0	1.0
Occasional smoker	0.98 (0.90 – 1.07)	0.82 (0.75 – 0.89)	0.86 (0.79 – 0.94)
Smoker	0.69 (0.66 – 0.73)	0.50 (0.47 – 0.53)	0.54 (0.51 – 0.57)
Ethnicity			
White (reference)	1.0	1.0	1.0
Mixed	1.20 (0.98 – 1.47)	1.28 (1.04 – 1.58)	1.30 (1.06 – 1.60)
Asian	1.04 (0.94 – 1.16)	1.02 (0.92 – 1.14)	0.92 (0.83 – 1.02)
Black	1.54 (1.37 – 1.74)	4.05 (3.56 – 4.61)	2.40 (2.12 – 2.71)
Chinese	1.34 (1.03 – 1.75)	1.17 (0.89 – 1.54)	1.38 (1.03 – 1.75)
Other	1.48 (1.27 – 1.72)	1.74 (1.48 – 2.04)	1.58 (1.35 – 1.85)
Exercise[2]			
Low (<600 MET x minutes per week)	1.0	1.0	1.0
Moderate (600 to 3,000 MET x minutes per week)	1.01 (0.95 – 1.08)	0.98 (0.93 – 1.05)	1.01 (0.95 – 1.08)
High (>3000 MET x minutes per week)	0.95 (0.90 – 1.00)	0.93 (0.88 – 0.98)	1.01 (0.95 – 1.07)
Deprivation[3]			
Least deprived Quintile 1 (-6.26 to -3.96)	1.0	1.0	1.0
Quintile 2 (-3.96 to -2.81)	0.96 (0.92 – 1.00)	0.93 (0.90 – 0.97)	0.94 (0.90 – 0.98)
Quintile 3 (-2.81 to -1.36)	0.92 (0.89 – 0.96)	0.91 (0.87 – 0.95)	0.90 (0.86 – 0.94)
Quintile 4 (-1.36 to 1.31)	0.91 (0.87 – 0.95)	0.86 (0.82 – 0.90)	0.89 (0.85 – 0.93)
Most deprived Quintile 5 (1.31 to 10.88)	0.82 (0.79 – 0.86)	0.79 (0.75 – 0.82)	0.80 (0.76 – 0.83)

[1] Odd ratio and 95% confidence intervals.
[2] Summed metabolic equivalent task (MET) minutes per week for all activities including walking, moderate and vigorous activity.
[3] Townsend Deprivation index in fifths

*Model adjusted for characteristics shown, missing data removed from the model (n=97,260).

Table 4.9 Factors predicting plasma O3FA and DHA levels in female UKBB participants.‡‡‡‡‡

Characteristics	Quartiles of O3FA[1]	Quartiles of O3FA to total FAs[1]	Quartiles of DHA[1]
Age			
≤49	1.0	1.0	1.0
50-59	1.59 (1.47 – 1.72)	1.30 (1.20 – 1.40)	1.39 (1.29 – 1.50)
>60	2.11 (1.93 – 2.31)	1.65 (1.51 – 1.81)	1.68 (1.53 – 1.83)
BMI (kg/m^2)			
≤24.9 (reference)	1.0	1.0	1.0
25-29.9	1.20 (1.15– 1.26)	0.91 (0.87 – 0.96)	0.86 (0.82 – 0.90)
≥30	1.04 (0.99 – 1.11)	0.64 (0.60 – 0.67)	0.54 (0.51 – 0.57)
Supplement use			
No supplement use (reference)	1.0	1.0	1.0
Fish oil use	2.07 (1.97 – 2.17)	2.33 (2.22 – 2.44)	2.24 (2.13 – 2.35)
Oily fish intake			
Never (reference)	1.0	1.0	1.0
<Once a week	2.08 (1.92 – 2.25)	2.59 (2.39 – 2.80)	2.38 (2.20 – 2.57)
Once a week	3.76 (3.48 – 4.06)	5.65 (5.22 – 6.11)	4.79 (4.43 – 5.18)
≥Twice a week	7.06 (6.46 – 7.71)	12.76 (11.64 – 13.97)	9.83 (8.98 – 10.75)
Use of oral contraceptive pill			
No use (reference)	1.0	1.0	1.0
Use	0.99 (0.93 – 1.05)	0.97 (0.91 – 1.03)	1.02 (0.96 – 1.08)
Use of hormone replacement therapy			
No use (reference)	1.0	1.0	1.0
Use	1.03 (0.98 – 1.09)	1.05 (0.99 – 1.10)	1.04 (0.99 – 1.09)
Had menopause			
No (reference)	1.0	1.0	1.0
Yes	1.65 (1.53 – 1.78)	1.27 (1.17 – 1.37)	1.46 (1.35 – 1.58)
Alcohol frequency			
Never (reference)	1.0	1.0	1.0
Once to three times a month	1.00 (0.93 – 1.08)	1.00 (0.92 – 1.07)	1.08 (1.00 – 1.16)
Once or twice a week	1.17 (1.10 – 1.24)	1.12 (1.05 – 1.19)	1.36 (1.28 – 1.45)
Three to four times a week	1.41 (1.32 – 1.51)	1.34 (1.26 – 1.44)	1.78 (1.67 – 1.91)
Daily or most days	1.56 (1.46 – 1.68)	1.34 (1.25 – 1.44)	2.11 (1.97 – 2.27)
Smoking			
Non-smoker (reference)	1.0	1.0	1.0
Occasional smoker	0.84 (0.72 – 0.97)	0.72 (0.62 – 0.84)	0.75 (0.64 – 0.86)
Smoker	0.70 (0.64 – 0.76)	0.49 (0.45 – 0.54)	0.53 (0.49 – 0.58)
Ethnicity			
White (reference)	1.0	1.0	1.0
Mixed	1.24 (0.93 – 1.66)	1.26 (0.95 – 1.68)	1.25 (0.94 – 1.67)
Asian	0.90 (0.76 – 1.07)	0.86 (0.73 – 1.03)	0.80 (0.68 – 0.95)
Black	1.24 (1.04 – 1.47)	3.07 (2.55 – 3.69)	1.79 (1.50 – 2.13)
Chinese	1.92 (1.32 – 2.79)	1.66 (1.12 – 2.44)	1.85 (1.26 – 2.71)
Other	1.43 (1.15 – 1.78)	1.64 (1.30 – 2.06)	1.53 (1.22 – 1.91)
Exercise [2]			
Low (<600 MET x minutes per week)	1.0	1.0	1.0
Moderate (600 to 3,000 MET x minutes per week)	0.99 (0.90 – 1.09)	0.92 (0.84 – 1.02)	0.99 (0.90 – 1.09)
High (>3000 MET x minutes per week)	0.96 (0.88 – 1.05)	0.88 (0.81 – 0.97)	0.99 (0.91 – 1.08)
Deprivation[3]			
Least deprived Quintile 1 (-6.26 to -3.96)	1.0	1.0	1.0
Quintile 2 (-3.96 to -2.81)	0.99 (0.93 – 1.06)	0.97 (0.91 – 1.04)	0.99 (0.93 – 1.06)
Quintile 3 (-2.81 to -1.36)	0.95 (0.89 – 1.02)	0.93 (0.87 – 1.00)	0.93 (0.87 – 0.99)
Quintile 4 (-1.36 to 1.31)	0.93 (0.87 – 1.00)	0.88 (0.82 – 0.94)	0.92 (0.86 – 0.98)
Most deprived Quintile 5 (1.31 to 10.88)	0.85 (0.80 – 0.91)	0.80 (0.74 – 0.86)	0.82 (0.77 – 0.88)

[1] Odd ratio and 95% confidence intervals.
[2] Summed metabolic equivalent task (MET) minutes per week for all activities including walking, moderate and vigorous activity.
[3] Townsend Deprivation index in fifths.

‡‡‡‡‡ Model adjusted for female sex and characteristics shown, missing data removed from the model (n=42,704).

4.4 Discussion

I report the largest cohort study of the relationship between dietary oily fish intake, FOS use, and plasma PUFA levels, made possible by the release of the first tranche of plasma NMR data on approximately 120,000 UKBB participants, representing approximately a third of the UKBB population. There was a subsequent release of the second tranche of NMR data in July 2023 that includes coverage of approximately two thirds of the UKBB population. The second tranche of NMR data is included in **chapter 5**.

I found that, although oily fish intake is the strongest predictor of plasma O3FA levels, FOS use contributes significantly to the plasma O3FA concentration in UKBB participants. Given that approximately 20-30% of individuals in UK, Canada, New Zealand, and Australia report FOS use (92, 199, 218, 275-277), I conclude that FOS use could confound the relationship between oily fish intake, plasma O3FA levels and health outcomes, if FOS use is not accounted for. Data unadjusted for FOS use may underlie reports in which the relationship between a disease outcome and dietary O3FA intake is not supported by nested case-control analysis of the same outcome according to blood O3FA levels (142, 261).

There is excellent agreement between plasma fatty acid levels measured by the Nightingale Health NMR platform and established gas chromatography (GC) methods (110). However, there is a paucity of comparable plasma NMR data, with which to draw direct comparisons with other studies. Similar individual plasma PUFA levels have been reported in a large (>one million) cross-sectional study of plasma samples from the USA (271) and a much smaller study of young Canadians (278), which both employed GC for quantification of fatty acid levels. Concerns about the reliability of plasma

fatty acid measurements related to fluctuations after eating and recent consumption of alcohol (107), have been allayed by more recent studies demonstrating the reliability of repeated plasma O3FA values over time (279, 280).

The literature on population-level circulating fatty acid profiles is also beset by heterogeneity of the blood fraction measured. However, an ancillary study of the 2 x 2 factorial Vitamin D and Omega-3 (VITAL) Trial, which explored the effect of 1 g mixed O3FAs (460 mg EPA and 360 mg DHA) daily and/or vitamin D_3 (2000 IU) daily *versus* placebo in a healthy US population (n=25,781) on cardiovascular and invasive cancer risk, confirmed excellent agreement between fatty acid levels measured in plasma and red blood cell membranes (105). I note that plasma O3FA levels are comparable to whole blood and RBC O3FA levels reported in a global survey of 298 studies of healthy adults (281).

Consistent with several previous cohort studies (139, 142, 270), the strongest predictor of O3FA levels in UKBB participants was oily fish intake, with a clear ‘dose-response’ relationship, which was similar to observations from a double-blind, randomised trial that utilised a capsule intervention approximating to the EPA and DHA content of a portion of oily fish (282), as well as other cross-sectional observational studies (283, 284).

Delineation of the separate contributions of FOS use and dietary oily fish intake to circulating O3FA levels in the UKBB has provided unique insight into the relative importance of these sources of O3FAs, with the caveat that the UKBB does not contain data on the dose, formulation, or frequency of FOS use. A typical prescription 1 g FOS capsule that is available in the UK

(Omacor®), provides 0.3 g of EPA + DHA per dose which is equivalent to a weekly dose of 2.1 g (if taken daily). By contrast, one portion of oily fish is estimated to contain 2.8 g O3FAs. I report plasma O3FA levels for FOS users that are similar to values in UKBB participants who ate oily fish more than once a week, consistent with similar mixed O3FA intake from daily supplement use and marine O3FAs exposure from eating one portion of oily fish per week. The UKBB findings suggest a larger effect size of FOS use than a much smaller study of the EPIC Norfolk cohort (n=4,949), which reported that the effect of FOS use (including cod liver oil) on plasma O3FA levels was equivalent to eating one-third of a portion of oily fish per week (91).

I appreciate that the relationship between FOS use and plasma O3FA levels may be underestimated by inclusion of cod liver oil (CLO) use in the UKBB FOS category given the lower content of marine O3FAs in CLO (a 1 g CLO capsule contains roughly 0.17 g of EPA and DHA combined). Ideally, future studies should include strategies to collect FOS use data that includes frequency of use, formulation, and dose, in addition to distinguishing FOS from CLO use.

Another limitation of the study is the inability of the NMR technique to distinguish and measure several, key individual PUFAs that are measurable by gas chromatography methods, including EPA, its metabolite DPA, and its O6FA counterpart arachidonic acid.

It is recognised that several other biological factors are associated with O3FA levels apart from O3FA intake (285). The large UKBB cohort has confirmed findings from other, independent studies that age, female sex, and alcohol

intake are associated with higher blood O3FA levels (267, 268, 271, 286), with a negative association with BMI (274). Similar conclusions have been reported by Schuchardt and colleagues for the estimated O3i value, which was derived from the original UKBB plasma PUFA data (258).

This study was possible due to the strength of the UKBB cohort which include the release of the NMR metabolic biomarker data in addition to comprehensive data on clinical and lifestyle factors. The overall strengths of the UKBB cohort lie in its scale and duration. It includes over half a million of volunteers, recruited between the ages of 40 to 69, from 2006 to 2010. It is powered to detect both common and complex diseases over time, and is linked to both cancer and death registries (262).

As with any cohort study there are limitations relating to the UKBB cohort, including '*health volunteer'* selection bias and the representativeness of the UKBB cohort to the general population. A study by Fry et al in 2017 compared the socioeconomic and health-related characteristics of UKBB participants with the general population, they found that UKBB participants were more likely to be older, female and live in less socioeconomic deprived areas than non UKBB participants (287).

Other limitations relate to the potential for confounding; a risk in all observational studies. However, the comprehensive data on clinical and lifestyle factors within the UKBB means that it is possible to include potential co-variates and adjust for them within models. Overall, I found low levels of missing data within the UKBB, with higher rates of missing data for certain variables such as 'average household income before tax' which may relate to data collected later into the study or specific variables that maybe more

time consuming for participants. There are also issues relating to measurement bias and reliability of the data as some data such as diet and lifestyle factors (smoking, alcohol) are self-reported, in addition the data is collected at specific timepoints and may not reflect potential changes to diet and lifestyle factors that may occur since recruitment. Nevertheless, there are two forms of dietary based questionnaire within the UKBB, the touchscreen and the 24-hour dietary recall data which improves the reliability of the data (207). In addition, specific dietary factors such oily fish intake and FOS use can also be examined using the NMR metabolic biomarker data, which is an objective measure; although it only reflects a specific time point.

4.5 Conclusion

Dietary O3FA intake and FOS use predict plasma O3FA levels in UKBB participants. FOS use was common in UKBB participants and is associated with higher O3FA levels than in those who do not use a FOS. Data on FOS use should be integral to the analysis of all epidemiological data, which examine the link between dietary O3FA intake, blood levels of fatty acids and health outcomes.

Chapter 5 The relationship between plasma omega-3 polyunsaturated fatty acid levels and colorectal cancer risk in UK Biobank participants

5.1 Introduction

Having established that dietary O3FA intake, in the form of oily fish and FOS use predicts O3FA plasma levels in the UKBB within **chapter 4**, this chapter examines the relationship between plasma O3FA levels and CRC risk in UKBB participants. The aims and objectives of the study are outlined in **chapter 2, section 2.1.2.2**.

In summary, observational data linking dietary O3FA intake (mainly from oily fish) and reduced CRC risk are inconclusive (29). Limitations of the methodology used to measure dietary O3FA intake could explain this discrepancy; these include recall bias inherent in dietary intake assessment tools and the confounding effect of FOS use, which is often not considered (207, 260). In addition, observational studies of the relationship between blood O3FA levels and CRC outcomes have been limited to small, nested case-control studies (139, 142, 194, 195).

The UKBB contains data about CRC diagnosis, and since July 2023 has plasma fatty acid levels at initial assessment to the study for approximately two-thirds of the cohort (110, 288). Therefore, I investigated the relationship between plasma O3FA levels and CRC risk during UKBB follow-up.

The majority of previous observational studies of O3FA intake or blood O3FA levels and CRC outcomes have considered CRC as a single cancer entity, despite evidence that clinical risk factors and the molecular pathogenesis of

cancers differ in a site-specific manner along the large bowel from proximal colon to rectum (**chapter 1, section 1.2.3**) (32, 33). However, a few studies have investigated colorectal site-specific cancer risk and oily fish intake providing preliminary evidence that dietary O3FA intake is associated with a larger effect size for risk reduction of proximal colon cancer than distal tumours, possibly linked to the predilection for defective dMMR tumours in the proximal colon (139, 141-143). Therefore, I also examined the relationship between plasma O3FA levels and CRC risk stratified for primary tumour location.

5.2 Methods

5.2.1 The UK Biobank

The UKBB study is described in **chapter 4, section 4.2.1**. Approval for this study was obtained from the UKBB (research ID 73904) (**Appendix A**).

5.2.2 Study population

I excluded participants with: prevalent cancer at recruitment (n=38,573) (International Classification of Diseases 10th revision [ICD-10] codes [C00-C97 & D37-D48], participants with *in situ* [D00-D09] and benign neoplasms [D10-D36] were included in the analysis), inflammatory bowel disease (IBD) (K50.0, K50.1, K50.8, K50.9, K51.0-K51.9) (n=6,803), appendix cancer (C18.1) (n=119) and carcinoid syndrome (E34.0) (n=87). Participants without available plasma data were excluded from the study (n=201,103), in addition to participants with incomplete data on clinical co-variates (n=15,704), leaving a study population of 234,598 participants (**figure 5.1**).

For participants with plasma fatty acid data at initial and repeat assessment visits (n=14,391), I used the initial plasma fatty acid profile as described in

the previous analysis of dietary and supplement O3FA intake in UKBB participants (**chapter 4, section 4.2.3**) (260).

5.2.3 Assessment of outcome

Incident CRC cases were identified using data linked from national cancer registries provided by the Medical Research Information Service in England and Wales, and The Information Services Division in Scotland (289). The latest cancer registry record linked to the UKBB was on the 1st June 2022 (290). CRC cases were recorded according to ICD-10 codes C18-C20. A CRC subsite analysis was performed using the following categorisation: proximal colon cancers were defined as tumours occurring in the caecum, ascending colon, hepatic flexure or transverse colon (C18.0, C18.2-C18.4); distal colon cancers were defined as tumours occurring at the splenic flexure, in the descending and/or the sigmoid colon (C18.5-C18.7) and rectal cancers were defined as tumours occurring at the rectosigmoid junction and/or in the rectum (C19, C20). Overlapping lesions of the colon (C18.8), unspecified colon cancers (C18.9) and cancers that had more than one subsite code or duplicate codes were excluded from the analysis.

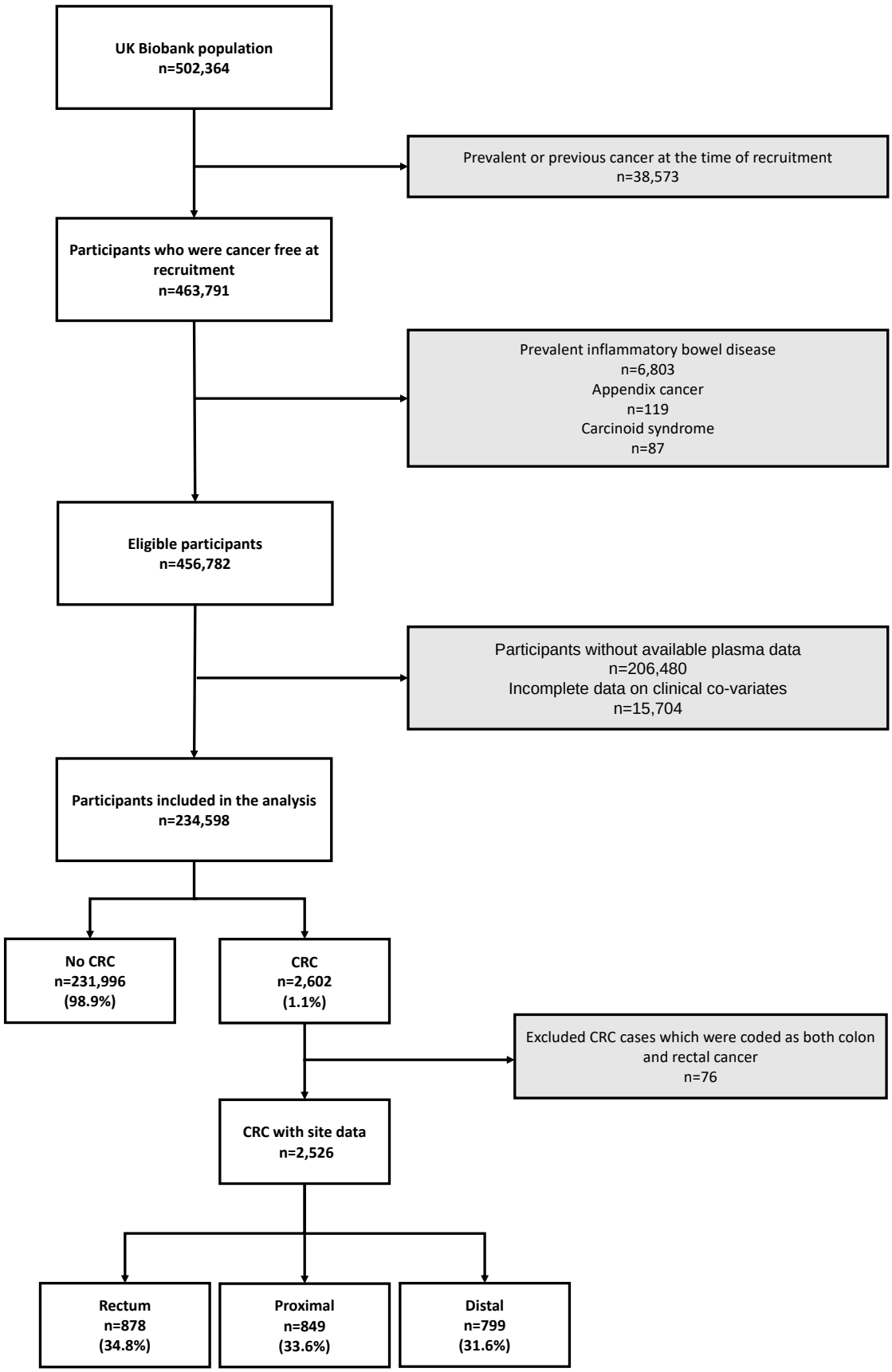


Figure 5.1 Flow diagram describing derivation of the study population.

5.2.4 Assessment of exposure

Plasma fatty acid levels were available for 274,120 participants at initial and repeat assessment visits [10, 17]. I analysed total O3FAs and DHA as the absolute concentration (mmol/L). I also analysed the ratio of O3FAs to O6FAs (O3/O6 ratio), which I calculated from available UKBB data.

5.2.5 Covariates and potential confounders

Data on the following variables were collected from initial assessment. Variables included: age (years); body mass index (BMI; Kg/m^2) (≤24.9, 25-29.9, ≥30); oily fish intake (never, <once a week, once a week, ≥twice a week, unknown), FOS use (use, no use, unknown); processed meat intake (never, <once a week, once a week and ≥twice a week); alcohol intake (never, once to three times a month, once or twice a week, three to four times a week, daily or most days); aspirin & NSAID use (regular use, no use); diagnosis of ischaemic heart disease (ICD-10 codes I20-I25) (yes, no); current tobacco smoking (smoker, occasional smoker, non-smoker); family history of CRC (fist degree relative having had CRC [mother, father or sibling], no family history); history of bowel cancer screening (yes, no), ethnicity (White, Mixed, Asian, Black, Chinese, Other) and Townsend socio-economic deprivation index (quintiles of increasing deprivation [least to most deprived]). Menopausal status (pre-menopausal, post-menopausal, unsure because of hysterectomy or other reason, unknown); use of hormone replacement therapy (HRT) (use [whether the participant had ever used HRT], no use, unknown) and oral contraceptive (OCP) (use [whether the participant had ever used OCP], no use, unknown) were included based on possible sex-specific differences in O3FA levels (267, 268).

5.2.6 Statistical analysis

Population characteristics were described as the percentage (%) or mean and standard deviation (SD), as appropriate.

To ensure that I did not introduce bias by excluding the population described (**section 5.2.2**), I examined the difference between the covariates described in **section 5.2.5**, for participants who were excluded and included in the analysis, using the Cochran-Armitage test for trend.

To analyse risk according to CRC subsite (proximal colon, distal colon, rectal) the study population without CRC were randomly assigned into three separate control populations to allow for comparison using the set.seed () function in R Studio (version 4.1.2), which allows the generation of random data sets from the data.

Plasma fatty acid levels were described as median and interquartile range (IQR), due to the distribution of the data (260).

Separate Cox proportional hazards regression models were used to calculate the hazard ratio (HR) and 95% confidence interval (CI) for the individual associations between plasma O3FA or DHA level, and the ratio of O3FAs to O6FAs (O3/O6 ratio), and CRC risk. O3FA and DHA levels, in addition to the O3/O6 ratio were included within the models as increasing tertiles, to reflect the distribution of the data. All models were adjusted for the covariates defined in **section 5.2.5**, however I did not adjust for oily fish intake or FOS use, given that I have previously shown they predict O3FA levels (260).

To examine potential sex differences, I conducted sensitivity analyses for male and female participants separately. The female-only models were adjusted for menopausal status (pre-menopausal, post-menopausal), HRT (use, no use) and OCP use (use, no use). Separate sensitivity analyses stratified for sex were not undertaken for the CRC subsite analysis, given the small numbers.

A P value of <0.05 was considered statistically significant. All analyses were performed in R Studio version 4.1.2.

5.3 Results

During a mean follow up of 13.4 years (SD 0.8) there were 2,602 (1.1%) incident CRC cases in 234,598 participants with plasma fatty acid data (**figure 5.1**).

The differences between UKBB participants included within the study population and those excluded from the analysis are shown in **table 5.1**.

The main difference I observed between participants included and excluded from the study population was the rate of bowel cancer screening. Higher rates were reported in the included study population (70.2%) compared to participants excluded from the analysis (64.4%), which may reflect those participants with prevalent cancer at the time of recruitment who were excluded from the study population.

With the exception of bowel cancer screening, the proportion of participants as a percentage was very similar between participants included and excluded from the study population, within a large number of the covariables.

However, when the groups were compared, I observed statistically significant differences; these findings are likely to reflect the size of the populations and type 1 error. For example, 11.1% of the study population reported never eating oily fish, compared to 10.8% of the excluded population, this percentage difference of 0.3% between the groups is unlikely to be of clinical significance on a population level, however it resulted in a statistically significant finding ($P<0.001$).

I therefore accepted the study population and did not use any additional statistical techniques such as multiple imputation for missing data given the large study numbers and similarity in the proportions of co-variable data.

Table 5.1 Differences in co-variables between the study population and UKBB participants excluded from the analysis.

Variable	Study population	Excluded participants	P value[1]
Number of participants (% of total population)	**234,598**	**267,766**	
Sex			
Male	108,887 (46.4)	120,180 (44.9)	<0.001
Female	125,711 (53.6)	147,586 (55.1)	
Age			
Mean age at initial assessment (SD) (years)	56.35 (8.07)	56.68 (8.11)	<0.001
BMI (Kg/m^2)			
<25.0	74,357 (31.7)	85,634 (32)	0.03
25.0 to 29.9	101,464 (43.3)	112,696 (42.1)	<0.001
≥30	58,777 (25.1)	66,331 (24.8)	0.02
Missing data	0	3,105 (1.2)	<0.001
Aspirin & NSAID use			
Use	62,934 (26.8)	71,496 (26.7)	0.32
Non use	171,664 (73.2)	196,270 (73.3)	
Diagnosis of ischaemic heart disease			
Yes	24,291 (10.4)	27,906 (10.4)	0.44
No	210,307 (89.6)	239,860 (89.6)	
Family history of CRC			
Fist degree relative with CRC	25,560 (10.9)	29,055 (10.9)	0.62
No family history / unknown	209,038 (89.1)	238,711 (89.1)	
Ever had bowel cancer screening			
No	69,956 (29.8)	85,530 (31.9)	<0.001
Yes	164,642 (70.2)	172,514 (64.4)	<0.001
Missing data	0	9,722 (3.6)	<0.001
Oily fish intake			
Never	25,980 (11.1)	28,835 (10.8)	<0.001
<Once a week	77,381 (33)	87,458 (32.7)	0.02
Once a week	88,119 (37.6)	100,333 (37.5)	0.51
≥Twice a week	41,928 (17.9)	48,251 (18)	0.18
Missing data	1,190 (0.5)	2,889 (1.1)	<0.001
FOS use			
Use	43,890 (18.7)	48,557 (18.1)	<0.001
No use	190,708 (81.3)	214,506 (80.1)	<0.001
Missing data	0	4,703 (1.8)	<0.001
Processed meat intake			
Never	21,105 (9)	25,674 (9.6)	<0.001
<Once a week	70,067 (29.9)	82,232 (30.7)	<0.001
Once a week	69,400 (29.6)	76,590 (28.6)	<0.001
≥Twice a week	74,026 (31.6)	81,040 (30.3)	<0.001
Missing data	0	2,230 (0.8)	<0.001
Current tobacco smoking			
No	210,382 (89.7)	237,702 (88.8)	<0.001
Yes, only occasionally	6,195 (2.6)	7,536 (2.8)	<0.001
Yes, on most or all days	18,021 (7.7)	21,208 (7.9)	0.002
Missing data	0	1,320 (0.5)	<0.001
Alcohol intake			
Rarely / Never	43,946 (18.7)	54,666 (20.4)	<0.001
One to three times a month	26,177 (11.2)	29,659 (11.1)	0.36
Once or twice a week	61,693 (26.3)	67,564 (25.2)	<0.001
Three or four times a week	55,439 (23.6)	59,974 (22.4)	<0.001
Daily or almost daily	47,343 (20.2)	54,402 (20.3)	0.23
Missing data	0	1,501 (0.6)	<0.001
Ethnicity			
White	223,587 (95.3)	232,661 (86.9)	<0.001
Non-white	11,011 (4.7)	14,084 (5.3)	
South Asian	493 (0.2)	537 (0.2)	0.47
Black	4,330 (1.8)	5,549 (2.1)	<0.001
Chinese	3,429 (1.5)	4,629 (1.7)	<0.001
Other	732 (0.3)	841 (0.3)	0.92
Mixed	2,027 (0.9)	2,528 (0.9)	0.003
Missing data	0	21,021	<0.001
Deprivation			
Least deprived Quintile 1	49,019 (20.9)	51,329 (19.2)	<0.001
Quintile 2	48,487 (20.7)	51,861 (19.4)	<0.001

Quintile 3	47,444 (20.2)	52,904 (19.8)	<0.001
Quintile 4	45,703 (19.5)	54,645 (20.4)	<0.001
Most deprived Quintile 5	43,945 (18.7)	56,403 (21.1)	<0.001
Missing data	0	624 (0.2)	<0.001
Menopausal status[2]			
Pre-menopausal	30,575 (24.3)	33,454 (22.7)	<0.001
Post-menopausal	75,042 (59.7)	90,326 (61.2)	<0.001
Not sure as had a hysterectomy or for other reason	19,932 (15.9)	22,959 (15.6)	0.03
Unknown	162 (0.1)	847 (0.6)	<0.001
Hormone replacement therapy use[2]			
Use	47,817 (38)	56,084 (38)	0.85
No use	77,528 (61.7)	90,295 (61.2)	0.009
Unknown	366 (0.3)	1,207 (0.8)	<0.001
Oral contraceptive pill use[2]			
Use	101,974 (81.1)	118,393 (80.2)	<0.001
No use	23,401 (18.6)	28,111 (19)	0.004
Unknown	336 (0.3)	1,082 (0.7)	<0.001

[1] T-test for continuous variables and chi-squared test for categorical variables.
[2] Percentage (%) of female population.

Population characteristics of participants with and without CRC are shown in **table 5.2**. Compared to participants without CRC, those who developed CRC were more likely to be male, older, have a higher BMI, have a family history of CRC, be of White ethnicity and have higher frequency intakes of processed meat and alcohol (P<0.001) (**table 5.2**). Aspirin and NSAID use were higher in participants with CRC, which might reflect shared risk factors for CRC and IHD (**table 5.2**). With regards to O3FA dietary intake, participants with and without CRC were most likely to consume oily fish once a week, however there was a statistically significant difference between the groups which is likely due to the large study population (37.5 vs 39.5%, P=0.04), there was no significant difference in FOS use between participants with and without CRC (18.7 vs 19.7%) (**table 5.2**). There was no association between smoking, or deprivation, and overall CRC risk (**table 5.2**). Female participants with CRC were more likely to be post-menopausal and use HRT which could be due to the fact that participants with CRC were generally older (**table 5.2**).

Table 5.2 Population characteristics of participants with and without CRC.

Variable	No CRC	CRC	P value[1]
Number of participants (% of total population)	**231,996 (98.9)**	**2,602 (1.1)**	
Sex			
Male	107,381 (46.3)	1,506 (57.9)	<0.001
Female	124,615 (53.7)	1,096 (42.1)	
Age			
Mean age at initial assessment (SD) (years)	56.31 (8.07)	60.33 (6.82)	<0.001
BMI (Kg/m^2)			
<25.0	73,698 (31.8)	659 (25.3)	<0.001
25.0 to 29.9	100,259 (43.2)	1,205 (46.3)	0.002
≥30	58,039 (25)	738 (28.4)	<0.001
Aspirin & NSAID use			
Use	62,188 (26.8)	746 (28.7)	0.03
Non use	169,808 (73.2)	1,856 (71.3)	
Diagnosis of ischaemic heart disease			
Yes	23,908 (10.3)	383 (14.7)	<0.001
No	208,088 (89.7)	2,219 (85.3)	
Family history of CRC			
Fist degree relative with CRC	25,178 (10.9)	382 (14.7)	<0.001
No family history / unknown	206,818 (89.1)	2,220 (85.3)	
Ever had bowel cancer screening			
No	162,946 (70.2)	1,696 (65.2)	<0.001
Yes	69,050 (29.8)	906 (34.8)	
Oily fish intake			
Never	25,714 (11.1)	266 (10.2)	0.17
<Once a week	76,551 (33)	830 (31.9)	0.24
Once a week	87,091 (37.5)	1,028 (39.5)	0.04
≥Twice a week	41,457 (17.9)	471 (18.1)	0.79
Missing data	1,183 (0.5)	7 (0.3)	0.11
FOS use			
Use	43,377 (18.7)	513 (19.7)	0.19
No use	188,619 (81.3)	2,089 (80.3)	
Processed meat intake			
Never	20,941 (9)	164 (6.3)	<0.001
<Once a week	69,375 (29.9)	692 (26.6)	<0.001
Once a week	68,587 (29.6)	813 (31.2)	0.06
≥Twice a week	73,093 (31.5)	933 (35.9)	<0.001
Current tobacco smoking			
No	208,035 (89.7)	2,347 (90.2)	0.40
Yes, only occasionally	6,123 (2.6)	72 (2.8)	0.73
Yes, on most or all days	17,838 (7.7)	183 (7.0)	0.23
Alcohol intake			
Rarely / Never	43,482 (18.7)	464 (17.8)	0.25
One to three times a month	25,940 (11.2)	237 (9.1)	<0.001
Once or twice a week	61,050 (26.3)	643 (24.7)	0.07
Three or four times a week	54,839 (23.6)	600 (23.1)	0.50
Daily or almost daily	46,685 (20.1)	658 (25.3)	<0.001
Ethnicity			
White	221,055 (95.3)	2,532 (97.3)	<0.001
Non-white	10,941 (4.7)	70 (2.7)	
South Asian	482 (0.2)	11 (0.4)	0.03
Black	4,311 (1.9)	19 (0.7)	<0.001
Chinese	3,407 (1.5)	22 (0.8)	0.01
Other	726 (0.3)	6 (0.2)	0.57
Mixed	2,015 (0.9)	12 (0.5)	0.03
Deprivation			
Least deprived Quintile 1	48,468 (20.9)	551 (21.2)	0.74
Quintile 2	47,954 (20.7)	533 (20.5)	0.83
Quintile 3	46,952 (20.2)	492 (18.9)	0.10
Quintile 4	45,186 (19.5)	517 (19.9)	0.63
Most deprived Quintile 5	43,436 (18.7)	509 (19.6)	0.29
Menopausal status[2]			
Pre-menopausal	30,432 (24.4)	143 (13)	<0.001
Post-menopausal	74,251 (59.6)	791 (72.2)	<0.001

Not sure as had a hysterectomy or for other reason	19,771 (15.9)	161 (14.7)	0.31
Unknown	161 (0.1)	1 (0.1)	
Hormone replacement therapy use[2]			
Use	47,315 (38)	502 (45.8)	<0.001
No use	76,940 (61.7)	588 (53.6)	<0.001
Unknown	360 (0.3)	6 (0.5)	
Oral contraceptive pill use[2]			
Use	101,128 (81.2)	846 (77.2)	<0.001
No use	23,153 (18.6)	248 (22.6)	<0.001
Unknown	334 (0.3)	2 (0.2)	

[1] T-test for continuous variables and chi-squared test for categorical variables.
[2] Percentage (%) of female population.

Increasing plasma O3FA levels were associated with reduced overall CRC risk, with the greatest effect size for the second tertile (range 0.43-0.61 mmol/L) of total O3FAs, HR 0.88 (95% CI 0.80-0.97, P=0.008) (**figure 5.2**). There was a reduced risk associated with the highest tertile of plasma O3FA levels, however there appeared to be a plateau effect when considering the findings associated with the second tertile, (HR 0.91 [95% CI 0.83-1.00], P=0.06). These findings may suggest that there is an optimal plasma level associated with reduced CRC risk but may also reflect the wider range of plasma O3FA levels in the third tertile (0.61 to 4.77 mmol/L) (**figure 5.2**).

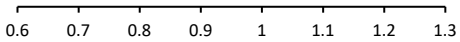
A similar pattern was observed for DHA levels and overall CRC risk, with the strongest relationship observed for the second tertile (range 0.20-0.27 mmol/L), (HR 0.89 [0.80-0.98]) (**figure 5.2**). An inverse association was also observed for participants in the third tertile for plasma DHA levels (HR 0.91 [0.82-1.00]), P=0.06) (**figure 5.2**). There was no statistically significant association between the O3/O6 ratio and overall CRC risk (**figure 5.2**).

I then examined the relationship between CRC risk and plasma O3FA levels according to CRC tumour location. There was an inverse relationship between plasma O3FA and proximal colon cancer risk, with the strongest association observed for the second tertile (HR 0.82 [0.69-0.97]). There was a dose response for increasing tertiles of DHA and proximal colon cancer

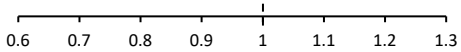
risk (**figure 5.2**). The relationship was specific for proximal colon cancer risk, as I observed no relationship between plasma O3FA, DHA or the O3/O6 ratio and distal colon or rectal cancer risk.

When I examined models adjusted for sex (**figure 5.3**), O3FAs were associated with reduced overall CRC risk in male participants, with a larger effect for the second tertile (0.84 [0.74-0.95]) compared to the third tertile (0.89 [0.78-1.00]). I observed a concentration response for increasing tertiles of DHA, with the second tertile being non-statistically significant but suggestive of reduced CRC risk (HR 0.91 [0.81-1.03]) and the third tertile of DHA being significantly associated with reduced CRC risk (HR 0.86 [0.75-0.98]). These findings were specific for males with no relationship between plasma O3FA, DHA levels or the O3/O6 ratio and overall CRC risk observed for female participants (**figure 5.3**)

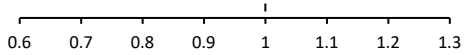
Overall CRC	Tertiles	Range (mmol/L)	Median (IQR) (mmol/L)	Participants n=234,598	Cases n=2,602	Hazard ratio[1] (95% confidence interval)	P value
Omega-3 PUFAs	T1	0.00 to 0.42	0.34 (0.28, 0.38)	77,713 (33.1)	860	Reference	
	T2	0.42 to 0.61	0.51 (0.47, 0.56)	78,267 (33.4)	831	0.88 (0.80-0.97)	**0.008**
	T3	0.61 to 4.77	0.77 (0.67, 0.93)	78,618 (33.5)	911	0.91 (0.83-1.00)	**0.06**
DHA	T1	0.00 to 0.20	0.16 (0.14, 0.18)	77,782 (33.2)	913	Reference	
	T2	0.20 to 0.27	0.23 (0.21, 0.24)	78,245 (33.4)	814	0.89 (0.80-0.98)	**0.02**
	T3	0.27 to 2.12	0.33 (0.29, 0.39)	78,571 (33.5)	875	0.91 (0.82-1.00)	**0.06**
O3/O6 ratio	T1	0.00 to 0.10	0.08 (0.07, 0.09)	77,835 (33.2)	786	Reference	
	T2	0.10 to 0.13	0.11 (0.10, 0.12)	78,313 (33.4)	878	0.99 (0.90-1.09)	0.83
	T3	0.13 to 0.80	0.15 (0.14, 0.18)	78,450 (33.4)	938	0.97 (0.88-1.07)	0.51



Proximal colon cancer	Tertiles	Range (mmol/L)	Median (IQR) (mmol/L)	Participants n=78,180	Cases n=849	Hazard ratio[1] (95% confidence interval)	P value
Omega-3 PUFAs	T1	0.00 to 0.43	0.34 (0.28, 0.38)	25,829 (33.0)	277	Reference	
	T2	0.43 to 0.61	0.51 (0.47, 0.56)	25,844 (33.1)	262	0.84 (0.71-1.00)	**0.05**
	T3	0.61 to 3.13	0.77 (0.68, 0.93)	26,507 (33.9)	310	0.84 (0.71-1.00)	**0.05**
DHA	T1	0.00 to 0.20	0.16 (0.14, 0.18)	25,865 (33.1)	308	Reference	
	T2	0.20 to 0.27	0.23 (0.21, 0.25)	25,932 (33.2)	262	0.82 (0.69-0.97)	**0.02**
	T3	0.27 to 1.32	0.33 (0.29, 0.40)	26,383 (33.7)	279	0.76 (0.64-0.90)	**0.002**
O3/O6 ratio	T1	0.00 to 0.10	0.08 (0.07, 0.09)	25,774 (33.0)	237	Reference	
	T2	0.10 to 0.13	0.11 (0.10, 0.12)	26,075 (33.4)	291	1.06 (0.89-1.26)	0.53
	T3	0.13 to 0.72	0.15 (0.14, 0.18)	26,331 (33.7)	321	1.01 (0.85-1.20)	0.91



Distal colon cancer	Tertiles	Range (mmol/L)	Median (IQR) (mmol/L)	Participants n=78,131	Cases n=799	Hazard ratio[1] (95% confidence interval)	P value
Omega-3 PUFAs	T1	0.00 to 0.43	0.33 (0.28, 0.38)	25,855 (33.1)	260	Reference	
	T2	0.43 to 0.61	0.51 (0.47, 0.56)	26,112 (33.4)	269	0.93 (0.78-1.10)	0.41
	T3	0.61 to 4.77	0.77 (0.67, 0.93)	26,164 (33.5)	270	0.90 (0.75-1.07)	0.24
DHA	T1	0.00 to 0.20	0.16 (0.14, 0.18)	25,761 (33.0)	275	Reference	
	T2	0.20 to 0.27	0.23 (0.21, 0.25)	26,276 (33.6)	250	0.89 (0.75-1.06)	0.20
	T3	0.27 to 2.12	0.33 (0.29, 0.39)	26,094 (33.4)	274	0.96 (0.80-1.15)	0.63
O3/O6 ratio	T1	0.00 to 0.10	0.78 (0.07, 0.09)	25,966 (33.2)	262	Reference	
	T2	0.10 to 0.13	0.11 (0.10, 0.12)	26,110 (33.4)	266	0.90 (0.75-1.06)	0.21
	T3	0.13 to 0.76	0.15 (0.14, 0.18)	26,055 (33.3)	271	0.84 (0.70-1.00)	0.05



Rectal cancer	Tertiles	Range (mmol/L)	Median (IQR) (mmol/L)	Participants n=78,211	Cases n=878	Hazard ratio[1] (95% confidence interval)	P value
Omega-3 PUFAs	T1	0.00 to 0.42	0.34 (0.28, 0.38)	26,003 (33.2)	297	Reference	
	T2	0.42 to 0.61	0.51 (0.47, 0.56)	26,289 (33.6)	278	0.89 (0.76-1.06)	0.19
	T3	0.61 to 4.16	0.77 (0.68, 0.93)	25,919 (33.1)	303	0.98 (0.83-1.16)	0.85
DHA	T1	0.00 to 0.20	0.16 (0.14, 0.18)	26,133 (33.4)	307	Reference	
	T2	0.20 to 0.27	0.23 (0.21, 0.25)	26,013 (33.3)	278	0.97 (0.83-1.15)	0.76
	T3	0.27 to 1.68	0.33 (0.29, 0.40)	26,065 (33.3)	293	1.04 (0.87-1.23)	0.68
O3/O6 ratio	T1	0.00 to 0.10	0.78 (0.07, 0.09)	26,069 (33.3)	261	Reference	
	T2	0.10 to 0.13	0.11 (0.10, 0.12)	26,107 (33.4)	300	1.07 (0.90-1.26	0.45
	T3	0.13 to 0.80	0.15 (0.14, 0.18)	26,035 (33.3)	317	1.09 (0.92-1.29	0.31

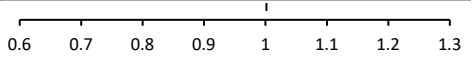
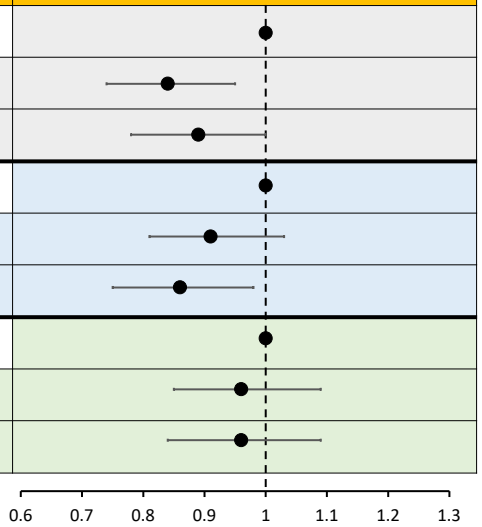


Figure 5.2 The association between plasma fatty acid levels and CRC risk.

[1] Models adjusted for age (years); BMI (<24.9, 25-29.9, >30 Kg/m^2); processed meat intake (never, <once a week, once a week and ≥twice a week); alcohol intake (never, once to three times a month, once or twice a week, three to four times a week, daily or most days); aspirin & NSAID use (regular use, no use); diagnosis of ischaemic heart disease (yes, no), smoking (smoker, occasional smoker, non-smoker); family history of CRC (mother, father or sibling having had CRC, no family history); history of bowel cancer screening (yes, no), ethnicity (White, Mixed, Asian, Black, Chinese, Other); and deprivation (quintiles of increasing deprivation [least to most deprived]).

The hazard ratio is represented by the black marker with the 95% CIs denoted by a line.

Overall CRC Males	Tertiles	Range (mmol/L)	Median (IQR) (mmol/L)	Participants n=108,887	Cases n=1,506	Hazard ratio[1] (95% confidence interval)	P value
Omega-3 PUFAs	T1	0.00 to 0.43	0.33 (0.27, 0.38)	41,726 (38.3)	593	Reference	
	T2	0.43 to 0.61	0.51 (0.46, 0.55)	36,441 (33.5)	471	0.84 (0.74-0.95)	**0.005**
	T3	0.61 to 4.77	0.76 (0.67, 0.92)	30,720 (28.2)	441	0.89 (0.78-1.00)	**0.06**
DHA[4]	T1	0.00 to 0.20	0.16 (0.14, 0.18)	46,742 (42.9)	660	Reference	
	T2	0.20 to 0.27	0.23 (0.21, 0.24)	34,699 (31.9)	473	0.91 (0.81-1.03)	0.13
	T3	0.27 to 2.12	0.33 (0.29, 0.40)	27,446 (25.2)	373	0.86 (0.75-0.98)	**0.02**
O3/O6 ratio	T1	0.00 to 0.10	0.08 (0.06, 0.09)	39,463 (36.2)	505	Reference	
	T2	0.10 to 0.13	0.11 (0.10, 0.12)	36,878 (33.9)	504	0.96 (0.85-1.09)	0.52
	T3	0.13 to 0.80	0.15 (0.14, 0.18)	32,546 (29.9)	497	0.96 (0.84-1.09)	0.51



Overall CRC Females	Tertiles	Range (mmol/L)	Median (IQR) (mmol/L)	Participants n=124,936	Cases n=1,089	Hazard ratio[2] (95% confidence interval)	P value
Omega-3 PUFAs	T1	0.00 to 0.43	0.34 (0.28, 0.39)	35,733 (28.6)	266	Reference	
	T2	0.43 to 0.61	0.51 (0.47, 0.56)	41,555 (33.3)	357	0.96 (0.82-1.13)	0.61
	T3	0.61 to 3.22	0.77 (0.68, 0.94)	47,648 (38.1)	466	0.98 (0.84-1.15)	0.84
DHA	T1	0.00 to 0.20	0.17 (0.15, 0.19)	30,799 (24.7)	253	Reference	
	T2	0.20 to 0.27	0.23 (0.22, 0.25)	43,255 (34.6)	338	0.88 (0.73-1.02)	0.08
	T3	0.27 to 1.47	0.33 (0.29, 0.39)	50,882 (40.7)	498	0.98 (0.83-1.15)	0.79
O3/O6 ratio	T1	0.00 to 0.10	0.08 (0.07, 0.09)	38,095 (30.5)	280	Reference	
	T2	0.10 to 0.13	0.11 (0.10, 0.12)	41,170 (33.0)	373	1.06 (0.91-1.24)	0.48
	T3	0.13 to 0.72	0.15 (0.14, 0.18)	45,671 (36.6)	436	0.99 (0.85-1.16)	0.91

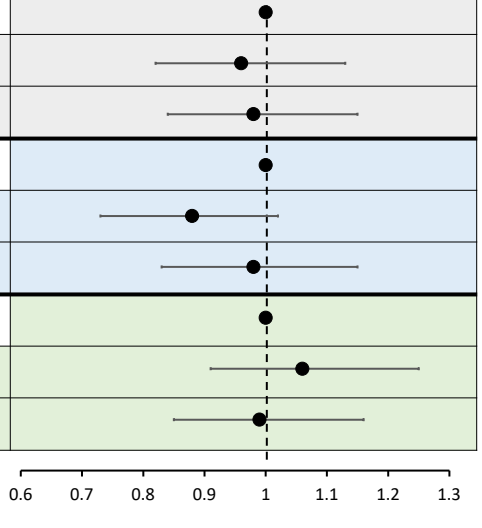


Figure 5.3 The associations between plasma fatty acid levels and overall CRC risk in male and female participants.

[1] Models adjusted for age (years); BMI (<24.9, 25-29.9, >30 Kg/m^2); processed meat intake (never, <once a week, once a week and ≥twice a week); alcohol intake (never, once to three times a month, once or twice a week, three to four times a week, daily or most days); aspirin & NSAID use (regular use, no use); diagnosis of ischaemic heart disease (yes, no), smoking (smoker, occasional smoker, non-smoker); family history of CRC (mother, father or sibling having had CRC, no family history); history of bowel cancer screening (yes, no), ethnicity (White, Mixed, Asian, Black, Chinese, Other); and deprivation (quintiles of increasing deprivation [least to most deprived]).

[2] Model adjusted for the variables outlined above, in addition to menopausal status (pre-menopausal, post-menopausal, not sure as had a hysterectomy or for other reason), use of hormone replacement therapy (use, no use), use of oral contraceptive pill (use, no use).

The hazard ratio is represented by the black marker with the 95% CIs denoted by a line.

5.4 Discussion

Within this large prospective cohort study, plasma O3FA levels of 0.42 to 0.61 mmol/L were associated with an 12% reduction (HR 0.88 (0.80-0.97]) in overall CRC risk.

This is the first study to examine CRC risk using cohort wide plasma levels within a large epidemiological study. Plasma O3FA levels were examined as an objective measure of dietary O3FA intake. The rationale for this approach was based on previous work (**chapter 4**) that demonstrated, dietary O3FA intake in the form of oily fish intake and FOS use predicts plasma O3FA and DHA levels within the UKBB (260).

Previous data examining plasma O3FA levels and CRC risk has relied on small, nested case control studies (142, 291, 292). Two metanalyses examining the relationship between O3FA levels and overall CRC risk have been published – both consisting solely of case-control studies (181, 293).

One analysis published in 2020, of five studies (published 2005 to 2019) included 1,553 cases among 6,837 participants. It reported a pooled relative risk ratio (RR) for the highest versus lowest blood O3FA levels of 0.79 (0.64-0.98). A dose-response analysis of 808 cases among 2,302 participants reported a RR 0.96 (0.92-1.00) for every 1% increase in blood O3FA levels (181). The authors also used a random-effects cubic spline model to examine a nonlinear relationship between CRC risk and dietary O3FA intake, reporting a suggestive association between dietary DHA and EPA intake and CRC risk which did not reach statistical significance (P=0.06), with a suggestion that an intake of approximately 0.4 g per day of EPA and 0.5 g per day of DHA were associated with the greatest reduction in RR for CRC

(181). Their findings suggest that there is an optimal O3FA dose for CRC prevention, this being roughly equivalent to a combined weekly dose of EPA/DHA of 6.3 g, which is approximately two portions of oily fish a week. It is difficult to fully translate the findings from the metanalysis to my study. However, I previously showed that median plasma O3FA levels for participants in the UKBB who consumed oily fish ≥twice a week and did not use a FOS were 0.57 mmol/L (IQR 0.44, 0.73) and I observed a reduction in overall CRC risk for median plasma O3FA levels of 0.51 mmol/L (0.47, 0.56). This suggests that the plasma O3FA levels associated with CRC risk reduction in the UKBB, reflect dietary intake of O3FA within the metanalysis.

In contrast, a 2023 metanalysis of 7 studies (published 2005 to 2021), including 2,086 cases among 7,570 participants, examined the relationship between blood levels of fatty acids, including O3FAs, and overall CRC risk. However, within the analysis of O3FA levels it appears the authors only included 5 studies (1,303 cases among 6,399 participants), in which they reported no association with CRC risk (OR 0.85 [0.53-1.37]) (293). The authors did not identify the five studies that they included within their analysis of O3FA blood levels, however the seven studies they included within the analysis of blood fatty acids are the same five studies included in the independent 2020 metanalysis (181). In addition, they included a study by Zhang et al 2015 (no reference) and an additional analysis by Aglago et al. within the EPIC study, published in 2021 that included 433 CRC cases and 433 matched controls (294), the other analysis by Aglago et al. included within both metanalyses is from 2020 and includes 461 CRC cases and 461 matched controls (142). It is unclear whether the studies by Aglago et al. include the same the cases and controls and are therefore potentially included twice within the analysis.

The work by Aglago et al. included in the metanalysis described above is from a nested case-control study within the large-scale epidemiological study EPIC (n=476,160, including 6,291 CRC cases) which reported an inverse relationship between dietary O3FA intake (total fish intake) and CRC risk (HR 0.88 [0.80-0.96]). Despite this finding the case-control study reported no association between plasma O3FA levels and CRC risk; likely reflecting a lack of power within the case-control study to determine an association (142).

The differences observed between the metanalyses for blood O3FA levels and CRC risk, are likely to reflect the lack of power and heterogeneity of the studies included within the analyses, particularly given that when they considered dietary intake of O3FAs in the wider study populations, both EPA and DHA were associated with reduced CRC risk. This highlights the strengths of my study in its ability to analyse cohort wide O3FA levels.

I observed a larger effect size for the second tertile of plasma O3FA and DHA levels associated with CRC risk. This could reflect the distribution of plasma O3FA and DHA levels within the second and third tertiles. I observed a wider distribution of levels within the third tertile for both O3FAs. In addition, there are likely to be other factors beyond dietary intake and the variables adjusted for within the model, which may determine plasma O3FA and DHA levels.

The reduced risk of CRC associated with the second tertile of both plasma O3FA and DHA levels suggest a plateaux effect and suggests that there is an optimal amount of O3FA intake; and not a "*more is better*" approach. The second tertile of plasma DHA levels reflects the plasma DHA concentration of participants who consumed oily fish ≥twice a week and therefore signals that consumption of oily fish twice a week could confer the maximum level of

CRC prevention from dietary O3FA intake. This level of regular fish intake is similar to that recommended by several national Nutrition Advisory Committees (295), including the UK Scientific Advisory Committee on Nutrition which recommends eating two portions of fish per week, one of which should be of oily fish (89).

My findings suggest that there is a non-linear plasma O3FA level and CRC risk; which has also been observed in relation to other health outcomes. A non-linear relationship between plasma EPA (P=0.0004) and all-cause mortality was identified in a pooled analysis of 17 prospective cohort studies (n=15,720 deaths), using a restricted cubic spline technique; the non-linear association was specific for EPA levels (182). A study within the UKBB examined the association between 168 NMR metabolites and overall cancer risk in 83,290 UKBB participants of which 10,214 had incidental cancer. They reported a reduced risk of overall cancer risk associated with O3FAs (HR 0.93 [0.90-0.95]), in addition they undertook restrictive cubic splines plots to examine a non-linear relationship, and reported a L shaped relationship suggestive of a threshold effect for O3FAs (P non-linear <0.001) (296). Plasma O3FA levels as mmol/L were not reported in the study (296).

I observed sex differences associated with plasma O3FA and DHA levels with overall CRC risk, reporting a significant relationship for male participants. A similar association for reduced overall CRC risk and O3FA dietary intake in males has been observed in other cohort studies (142, 143, 177). A study in the NHS and HPFS cohorts reported an inverse trend for CRC risk and O3FA intake for 12 to 16 years before diagnosis in males, which did not quite reach statistical significance (HR 0.76 [0.52-1.10]) (139). In addition, a study in the Japan Public Health Centre Based Prospective

Study reported a reduced risk of O3FA dietary intake and proximal colon cancer risk in men only (143). However, these findings may be due to low power (n=1,268 cases of which n=213 and n=281 for proximal and distal colon cancer respectively in men, and n=204 and n=125 for proximal and distal colon cancer respectively in women) or due to a lack of association (143).

The potential benefits for O3FAs in CRC risk in men may be due to males having lower baseline plasma O3FA levels. I previously found that when adjusted for dietary O3FA intake within the UKBB, males were more likely to have lower plasma O3FA (OR 0.57 [0.56-0.59]) and DHA levels (0.38 [0.36-0.39]) compared to females. This is likely related to male participants having lower rates of oily fish intake, with only 45% of participants consuming oily fish ≥twice a week or more being males, compared to 55% being females, in addition to 44% of FOS consumers being male (**chapter 4, section 4.3.7**).

I found no association for plasma O3FA or DHA levels with CRC risk in female participants when adjusted for female sex factors including menopausal status, HRT and OCP use. Further mechanistic understanding is required to evaluate the sex specific differences observed for O3FA intake/levels and CRC risk.

Within the analysis, I examined CRC risk according to tumour subsite, reporting a decrease in proximal colon cancer risk associated with high plasma O3FA and DHA levels. The plasma DHA level was more strongly associated, and a concentration-response was observed. The response I observed for DHA may reflect the lower variability in levels seen within the

model which ranged from 0.27 to 1.37 mmol/L for the third tertile, compared to 0.27 to 2.12 in the model examining overall CRC risk (**figure 5.2**).

Several other cohort studies have reported an association between dietary O3FA intake and reduced proximal colon cancer risk, but failed to reach statistical significance (**chapter 3, section 3.5.2**) (EPIC, Nurses' Health Study and Health Professionals Follow Up Study, Japan Public Health Centre Based Prospective Study) (139, 142, 143). This is possibly due to a lack of power due to the number of cases according to subsite included within those prior analyses.

Other studies have demonstrated a reduced risk associated with specific CRC molecular subtypes, including dMMR tumours which display MSI and have a predilection for the proximal colon (**chapter 3, section 3.6.1**) (141). These tumours are associated with chronic inflammation, mediated by prostaglandin E_2 and display high T-cell proliferation (140, 297). A potential hypothesis for the reduced risk observed with O3FAs is their ability to promote the host anti-tumour immune response and may explain why I observed no effect for distal colon or rectal cancer risk (140, 141). A limitation of the UKBB data is that beyond CRC subsite identified according to ICD-10 code, I was unable to characterise subsite according to stage, grade and/or the molecular pathology of CRC.

The strengths of this study include the use of cohort wide plasma O3FA and DHA levels, which I previously demonstrated correlated with dietary O3FA intake in the form of oily fish and FOS use (260). I included a large number of CRC cases, which added power to the study, and meant that the analysis of CRC risk could be conducted according to subsite. Limitations of the study

reflect that plasma O3FA, and DHA levels were collected at a specific point in time, mainly being at the initial assessment visit and therefore may have been measured several years prior to CRC diagnosis. In addition, there are limitations with the NMR data which does not include ALA or EPA levels and therefore DHA was used as a surrogate for bioactive O3FAs. Due to the demographics of the UKBB population (predominantly White ethnicity [94.6%]) (287), I was unable to undertake any further analyses according to ethnicity, due to a lack of power.

Potential future work and considerations relating to the study are discussed in **chapter 8, section 8.5**.

5.5 Conclusion

Plasma O3FA levels of 0.42 to 0.61 mmol/L were associated with reduced CRC risk within the UKBB, suggesting an optimal O3FA plasma level associated with CRC risk reduction. I identified an association between plasma O3FA / DHA levels and CRC in male participants (host factor), in addition to a reduced risk associated with proximal colon cancer (tumour factor).

Chapter 6 The interaction between omega-3 polyunsaturated fatty acids and dietary fibre on short chain fatty acid production within an *in vitro* colonic fermentation model

6.1 Introduction

This chapter details a study investigating whether O3FAs increase SCFA production within the colon, as a potential anti-CRC mechanism. A batch static *in vitro* colonic fermentation model (consisting of sealed glass fermentation bottles under anaerobic conditions), was used to carry out the study, due to cost and its ability to facilitate high-throughput evaluation (298, 299). The background to the study, including colonic fermentation and pathways of SCFA production are outlined in **chapter 1, sections 1.3 to 1.5**.

In summary, evidence exists that oral dosing of O3FAs modulates the colonic microbiota in favour of SCFA-producing bacteria (3, 120, 121). An ileostomy study conducted by the Hull Group, demonstrated that O3FAs reach the proximal colon in concentrations of up to 200 mcg/ml (120). However, it is not known whether the ability of O3FAs to modulate the colonic microbiome in favour of SCFA producing bacteria, leads to an increase in SCFA production. In addition, the interaction between O3FAs and different types of dietary fibre (soluble, insoluble) was explored to determine how their relationship may potentiate SCFA production.

6.2 Methods

The aims and objectives of the study are detailed in **chapter 2, section 2.2**. The primary and secondary hypotheses and outcomes are summarised below.

6.2.1 Primary hypothesis & outcome measure

The primary hypothesis was that O3FAs increase SCFA production within an *in vitro* colonic fermentation model. The primary outcome was the total SCFA level (mmol/L) (the sum of acetate [C2], propionate [C3] and butyrate [C4]) at 24 hours.

6.2.2 Secondary hypothesis & outcome measures

The following secondary hypotheses were tested:

- Do O3FAs interact differently with specific dietary fibres within an *in vitro* colonic fermentation model? This was measured as the total SCFA level at 24 hours.
- Is O3FA exposure within an *in vitro* colonic fermentation model associated with changes in the bacterial content? This was measured using shotgun metagenomics (**chapter 7**).

6.2.3 Ethical approval

Ethical approval from the University of Glasgow College of Medicine Medical Veterinary and Life Sciences was received on December 8th, 2021 (Application number 200210044; **Appendix B**).

6.2.4 Participants

Healthy volunteers were invited to take part in the study. Poster advertisement was placed in the University of Glasgow campus and the University of Glasgow online social network service (Yammer), to identify potential volunteers.

All volunteers were asked to avoid eating fish for 2 days prior to faecal collection. The recruitment poster, patient information sheet and consent form are included within **Appendices C, D & E.**

Participants were included in the study if they met the following criteria:

- Healthy volunteers (see exclusion criteria below);
- Able to give informed consent;
- Able to understand and comply with the requirements of the study, as judged by the Investigator.

Participants were excluded from the study if they:

- Were vegetarian and/or vegan;
- Were pregnant or aiming to get pregnant;
- Used fish oil supplements (including cod liver oil) regularly (greater than or equal to three times per week);
- Used non-steroidal anti-inflammatory (NSAID) medications, aspirin, laxatives and/or antibiotics in the 3 months prior to sample collection;
- Had a diagnosis of inflammatory bowel disease;
- Had a diagnosis of irritable bowel syndrome;
- Had undergone a previous colonic or small bowel resection;
- Had undergone a cholecystectomy;
- Were currently smoking;
- Were unable to provide written informed consent.

6.2.5 O3FA concentrations

The O3FA concentrations tested within the *in vitro* colonic fermentation model were based on a clinical study in which oral dosing of 4 g/d of O3FA

capsules, containing mixed EPA/DHA (50:50), for 28 days led to an increase in EPA and DHA concentrations as high as 200 mcg/ml, in the intestinal fluid of some patients with a temporary ileostomy (120). This same O3FA capsule intervention was tested within the model, with each 500 mg of capsule oil containing 241 mg of EPA triglyceride and 241 mg DHA triglyceride. O3FA concentrations were chosen to reflect the range of EPA and DHA concentrations observed in the ileostomy fluid of the participants receiving the O3FA intervention (120). The following O3FA concentrations were used: 1 mcg/ml (0.05 mg into 50 ml reaction volume), 25 mcg/ml (1.25 mg into 50 ml reaction volume) and 50 mcg/ml (2.5 mg into 50 ml reaction volume).

6.2.6 Dietary fibres

Dietary fibres were chosen to reflect the different categories of dietary fibre, insoluble (wheat bran), soluble viscous (pectin) and soluble non-viscous fibre (inulin). Dietary fibres were pre-weighed and added to the 100 ml fermentation bottles. Inulin (Beneo-Orafti) was tested as two different concentrations, 0.01 mg/ml (500 mg in 50 ml reaction volume) and 0.02 mg/ml (1 g in 50 ml reaction volume), natural wheat bran (Holland and Barrett) and pectin from apple (Sigma Aldrich) were tested at a concentration of 0.01 mg/ml (500 mg in 50 ml reaction volume). The concentrations used corresponded to a bolus intake of approximately 6 g of fibre diluted into 300mL of colonic contents (300). The current UK dietary recommendations for fibre intake are 30 g per day, therefore the amount of fibre within the reaction volume was roughly equivalent to what would be expect after a meal (301).

6.2.7 Sample size calculation

The primary aim of this study was to test whether O3FAs within an *in vitro* colonic fermentation model lead to an increase SCFA production, in addition to generating hypothesis about the interaction between O3FAs and different dietary fibres. A sample size calculation from some preliminary experiments was conducted to ensure there was enough power to obtain meaningful data within the feasibility of time and resource constraints.

The sample size calculation was based on preliminary experiments conducted using faecal samples from three volunteers. Within these experiments the capsule oil was added directly to the fermentation media as described (**section 6.2.11**). The sample size calculation was based on an O3FA concentration of 50 mcg/ml and an inulin concentration of 0.01 mg/ml.

Preliminary experiments demonstrated a mean percentage increase of 22% (SD 20.23) in the total SCFA level at 24 hours, when O3FA 50 mcg/ml + inulin (0.01 mg/ml) was added to the *in vitro* colonic fermentation model, compared to the inulin only (0.01 mg/ml) reaction. On this basis, I estimated that 10 participants were required to demonstrate a 20% increase in the total SCFA level at 24 hours for O3FA 50 mcg/ml & inulin (0.01 mg/ml) within the fermentation model, with 80% power at a significance level of 0.05.

6.2.8 Faecal sample collection and preparation

Stool samples were collected using in-house collection kits consisting of a sealed 0.7 L plastic container, with the addition of AnaeroGen™ 3.5 L Sachet (Oxoid) within a plastic bag (**see Appendix F** for instructions given to participants). Samples were processed with 2 hours of collection. A faecal slurry (32% weight/weight [wt/wt]) was prepared, by homogenising the faecal

sample on its own for 1 minute using a hand blender (Bosch MSM6300GB), following which 48 g of faeces were vortexed in 150 ml of oxygen-free nitrogen (OFN) purged sodium phosphate buffer, (pH 7.0, 37°C) for 3 minutes. It was then strained trough nylon mesh to remove particulate material. A 1 ml aliquot of faecal slurry for DNA analysis and 0.5 ml of faecal slurry in 1 ml of RNAlater™ (Therma Fisher Scientific) for RNA analysis were collected and stored at -80°C.

6.2.9 Solutions used within the fermentation process

The following solutions were used to create the fermentation media. They were each made in advance and stored at 4°C prior to use.

- **Buffer solution** (100 ml), 0.4 g of ammonium bicarbonate, CH_5NO_3 (Sigma-Aldrich) and 3.5 g of sodium bicarbonate, $NaHCO_3$ (Sigma-Aldrich) were added to 100 ml of distilled water, dH_2O.
- **Macromineral solution** (100 ml), 0.57 g pf di-sodium hydrogen phosphate anhydrous, Na_2HPO_4 (VWR chemical), 0.62 g of potassium dihydrogen orthophosphate KH_2PO_4 (Fisher Scientific) and 0.06 g of magnesium sulphate heptahydrate, $MgSO_4.7H_2O$ (Fisher Scientific) were added to 100 ml of distilled water, dH_2O.
- **Micromineral solution** (100 ml), 13.2 g of calcium chloride dehydrate, $CaCl_2.2H_2O$ (Sigma-Aldrich), 10 g of manganese (II) chloride tetrahydrate, $MnCl_2.4H_2O$ (Sigma-Aldrich), 1 g of cobalt (II) chloride hexahydrate, $CoCl_2.6H_2O$ (Thermo Fisher Scientific) and 8 g of iron (III) chloride hexahydrate, $FeCl_3.6H_2O$ (Acros) were added to 100 ml of distilled water, dH_2O.
- **Mucin and bile acids** (200 ml), 2 g of mucin from porcine stomach (type 2) (Sigma) and 1.52 g bile acids from porcine (Sigma) were added to 200

ml of distilled water, dH_2O. This was then aliquoted into 10 ml and stored in universal containers at 20°C prior to usage.

The **reducing solution** was made on the day of the experiment. To make the reducing solution (100 ml), 4 ml of sodium hydroxide, NaOH (1M) was added to 0.63 g of sodium sulphide nonahydrate, $Na_2S.9H_2O$ (Sigma-Aldrich), 0.63 g of L-cysteine hydrochloride monohydrate, $C_3H_{17}NO_2S$ HCL H_2O (VWR) and made up to 95 ml of distilled water, dH_2O.

The **sodium phosphate buffer** was made in advance of the experiment and stored at 4°C. To make the sodium phosphate buffer (100 ml), 39 ml of solution (a) was added to 61 ml of solution (b), the pH was then adjusted to 7.0 using 1M NaOH. To make solution (a), 0.91g of potassium dihydrogen phosphate, KH_2PO_4 was added to 100 ml of distilled water, dH_2O (Fisher Scientific). To make solution (b), 1.19 g sodium hydrogen phosphate, Na_2HPO_4 was added to 100 ml distilled water, dH_2O (VWR)

6.2.10 Fermentation media

The fermentation medium was prepared on the day of the experiment. To produce 1 L of fermentation medium the following were combined in a 2 L conical flask: 2.25 g tryptone (Oxoid), 450 ml of distilled water, 112.5 mcl micromineral solution, 225 ml bicarbonate buffer solution, 225 ml macromineral solution and 1.125 ml 0.1% resazurin to indicate anaerobic conditions. The fermentation media was then boiled on a heat plate to sterilise it and remove any dissolved oxygen. A colour change from blue to pink indicated that the fermentation media was prepared, it was then cooled under OFN until it reached 37°C. A 10 ml aliquot of mucin and bile acids were

then added to the fermentation media which was adjusted to a pH 7 with 6 M HCl. The media was then kept at 37˚C within a water bath until it was ready for use.

6.2.11 Fermentation method

Each 100 ml fermentation bottle (**figure 6.16**) contained 43 ml of fermentation media at pH 7.0 and 2 ml of reducing solution, in addition to the pre-weighed fibres and the O3FA oil. The O3FA capsule oil was extracted from the capsules using a needle and syringe. Care was taken to avoid oxidation of the capsule oil by limiting its exposure to air and extracting it immediately prior to it being added to the model. The O3FA capsule oil was weighed within a pipette tip and then added to the fermentation media and reducing solution, which was already placed within the fermentation bottle.

Each bottle was then sealed with a self-sealing septum and gas-tight crimp top. After full decolouration of the fermentation media (pink to clear), 5 ml of faecal slurry was injected into each fermentation bottle (total volume 50 ml). Each bottle was then de-gassed using OFN for 2 minutes. All bottles were incubated in a shaking water bath at 37 ˚C, 60 strokes per minute.

6.2.12 Sampling

Each fermentation bottle was sampled at 0, 8 and 24 hours, using a needle and syringe through the self-sealing septum at each timepoint. The timepoints were chosen to reflect baseline (0 hours), mid fermentation (8 hours) and late fermentation (24 hours). Samples were collected (1 ml aliquot) at 0, 8 and 24 hours and stored at -20˚C for SCFA analysis (1 ml into 333 mcl of 1M of NaOH to stabilise the SCFAs) within a 3 ml universal

container, long chain PUFA (LC-PUFA) analysis (1 ml at 0, 8 and 24 hours), RNA analysis (0.5 ml at 8 and 24 hours) and DNA analysis (1 ml at 24 hours), all stored at -80˚C in 1.5ml Eppendorf tubes, in addition to a 1 ml aliquot (0, 8 and 24 hours) for pH measurement stored in a 3 ml universal container. The aliquot for pH measurement was processed immediately using a pH meter 7020 (Electronic Instruments Ltd, Chertsey, Surrey, England).

6.2.13 Short chain fatty acid analysis

6.2.13.1 Short chain fatty acid extraction

To allow quantitation of each SCFA, an external standard curve and internal standard were used to calculate SCFA concentrations within each sample. The external standard contained known concentrations of acetic acid (C2), propionic acid (C3), butyric acid (C4), valeric acid (C5), caproic acid (C6), enanthic acid (C7), caprylic acid (C8), isobutyric acid (iC4), isovaleric acid (iC5), and isocaproic acid (iC6) all of which were contained in 2 M NaOH to prevent loss of SCFAs. The concentration of each SCFA within the external standard was calculated by dividing the measured weight in g of the individual SCFA by the molar mass of the individual SCFA in 200 ml. To account for losses of SCFA in the standards and samples an internal standard of 2-ethyl butyric acid in 2 M NaOH was also added to all samples (**table 6.1**).

Table 6.1 External standards and internal standards.

	Acid Name	Molar Mass (g/L)	Measured weight (g)	Calculated Molarity (mmol/L)
External standards	Acetic acid	60.05	2.2039	183.5
	Propionic acid	74.08	1.9746	133.3
	Butyric acid	88.11	1.8453	104.7
	Valeric acid	102.13	1.7659	86.5
	Caproic acid	116.16	1.7349	74.7
	Enanthic acid	130.18	1.6838	64.7
	Caprylic acid	144.21	1.6618	57.6
	Iso-butyric acid	88.11	1.7965	102
	Iso-valeric acid	102.13	1.7579	86.5
	Iso-caproic acid	116.156	1.1704	50.4
Internal standard	2- ethyl butyric acid	116.16	1.7189	74.0

Six external standards were created by adding different concentrations of external standard made up to 800 mcl with distilled water, 100 mcl internal standard, 100 mcl orthophosphoric acid (OPA), and 3 ml diethyl-ether, per sample (**table 6.2**). OPA was added to lower the pH and increase the dissociation constant, this caused the SCFAs to become soluble in the organic phase rather than in the aqueous phase when diethyl ether was added, allowing the SCFAs to be extracted. The sample was then vortexed for one minute on an IKA shaker at a speed of 1500 rpm, the ether layer was removed (containing the SCFAs), 3 ml of diethyl-ether added and the sample vortexed for a further one minute. This procedure was repeated for a total of four times.

To extract the SCFAs from the fermentation samples, the same process was used with 800 mcl of fermentation slurry supernatant instead of the 800 mcl standard and water mix, which was combined with 100 mcl internal standard and 100 mcl OPA.

Table 6.2 Standards for standard curve.

Standard number	External standard (mcl)	Water (mcl)	Internal standard (mcl)	OPA (mcl)	Final Volume (mcl)
1	10	790	100	100	1000
2	25	775	100	100	1000
3	50	750	100	100	1000
4	100	700	100	100	1000
5	200	600	100	100	1000
6	300	500	100	100	1000

6.2.13.2 Gas chromatography – flame ionisation detector

Measurement of SCFAs was performed using an Agilent 7820A GC system. The carrier gas was nitrogen. One mcl of the sample was injected by the injector to the inlet. The initial oven temperature was 80˚C, with the temperature increasing by 15˚C to 210˚C and held for 1 minute, with a maximum oven temperature of 230˚C. The equilibrium time was 1 minute, and the total oven run time was 10.67 minutes. The sample was vaporised by the inlet, which had a temperature of 230˚C and a split flow of 40 ml/min at 0.5 minutes.

A splitless column, in which the time separation occurred, was used (Agilent GC column DB-WAX [AG125-7012], inner diameter 0.53mm, length 15m, film 1.00mcl). Two phases took place within the column, a mobile phase where the sample reached the column and a stationary phase where the sample was separated based on volatility, boiling and polarity. The sample then reached the flame ionisation detector (FID) which responded to the analytes within the sample as they occurred by changing its electrical output. The base temperature of the FID was 250˚C, with a hydrogen flow of 30 ml/min and an air flow of 400 ml/min. A chromatogram was produced from the retention time in minutes (the time to reach the detector) and the amount of the analyte reaching the detector (millivolts).

There were regular solvent washes of both ether and methanol between samples to minimise contamination and maintain the integrity of the syringe. Data was then processed in Microsoft Excel 2023.

6.2.13.3 Calculations to quantify SCFAs and quality controls

A number of quality controls were used to process data from the GC-FID. These included:

1. The coefficient of variation (CV%) of the internal standard across all samples with a value of <10% accepted. A higher value identified that there may have been an issue with SCFA extraction method or the
2. GC-FID for a specific sample.
3. The curve of the external standards was used to assess SCFA extraction, with a coefficient of determination (R^2) value of 0.99 accepted.
4. There was a periodic injection of external standard 4 (100 mcl of external standard) during the run of samples to monitor the CV% of standard 4 throughout the run, with a value of <10% accepted.

The concentration of SCFAs within the samples was calculated by first determining the response factor using the following equation.

Equation 6.1 Response factor.

Response factor = (external standard peak area / internal standard peak area) x (internal standard concentration / external standard concentration)

This response factor was then used to calculate the sample concentration using the following equation.

Equation 6.2 Sample concentration.

Sample Concentration = (sample area ratio / response factor) x internal standard concentration

6.2.14 Statistical analysis

Data were expressed as mean and standard deviation (SD). The total SCFA level was the sum of acetate (C2), propionate (C3) and butyrate (C4) at a specific time point (0, 8 and 24 hours). SCFA levels were expressed as mmol/L. SCFA levels of the O3FA concentrations +/- dietary fibres were compared to control and expressed as a percentage change (%). A paired t-test was used to compare SCFA levels and the percentage change between fermentation reactions. A P value of <0.05 was considered statistically significant. All analyses were performed in R Studio version 4.1.2.

6.3 Results

6.3.1 Preliminary experiments

A series of preliminary experiments were conducted to facilitate the method development for the study. These experiments were conducted using faecal samples provided by four volunteers, three of which also participated in the final study (V1, V2, V3). The specific aspects of method development are detailed below.

The control described the fermentation model on its own, which was inoculated with the faecal slurry but did not contain other additives in the form of O3FAs or fibre. Additions of O3FAs to the fermentation reactions were described according to their concentration (for example O3FA 1, 25, 50 mcg/ml), fibre additives were described according to the fibre on its own

without O3FAs (for example, inulin [0.01mg/ml] only), and the fermentation reactions that included both O3FA and fibre were described according to the O3FA concentration, and the fibre tested (for example O3FA 50 mcg/ml & inulin 0.01 mg/ml) .

6.3.1.1 O3FA concentrations

The O3FA concentrations used were based on the findings from preliminary experiments in which O3FA concentrations of 1, 50, 100 and 200 mcg/ml were tested. Within the preliminary experiments I observed a decrease in total SCFA production in O3FA concentrations at 100 and 200 mcg/ml. This could have been due to over saturation of O3FA within the *in vitro* colonic fermentation model, or that the optimal O3FA concentration for SCFA production was achieved at a lower concentration within the model (50 mcg/ml).

The mean total SCFA level at 24 hours from the preliminary experiments (n=4), reflecting the different O3FA concentrations (with and without inulin 0.02 mg/ml) tested is shown in **figure 6.1**. It illustrates that the mean total SCFA level was highest for the O3FA 50 mcg/ml concentration (25.47 [10.83] mmol/L) compared to the O3FA 200 mcg/ml concentration (22.92 [11.11] mmol/L). However, the mean total SCFA level for the O3FA 100 mcg/ml concentration were much lower (12.01 [14.78] mmol/L) and possibly reflects the fact that these results were based on two experiments in comparison to the four experiments that generated the data shown for the other O3FA concentrations.

Within the preliminary experiments the O3FA capsule oil was solubilised within ethanol to create a solution. However, the ethanol appeared to

positively influence SCFA production, as the ethanol control (23.25 [8.41] mmol/L) demonstrated an increase in total SCFA production at 24 hours above the control (14.63 [7.43] mmol/L) (**figure 6.1**). In response to these findings, the O3FA capsule oil was added directly to the fermentation media as described in **section 6.2.1.1** and the experiment repeated.

6.3.1.2 Dietary fibres

Inulin was initially chosen as a fibre to investigate within the model as it is soluble, highly fermentable and a known prebiotic (302). Inulin was tested at two different concentrations, as following the preliminary experiment findings it appeared that a higher inulin concentration (0.02 mg/ml) may have been over stimulating SCFA production within the model, reaching a steady state at 24 hours across all the fermentations regardless of the O3FA concentration (**figure 6.1**). Further preliminary experiments tested inulin at a lower concentration (0.01 mg/ml) and found differences in SCFA production, based on increasing O3FA concentrations in the presence of inulin.

6.3.1.3 Intra-experimental variability

I initially conducted the preliminary experiments, testing different experimental conditions (O3FA concentrations +/- inulin) in duplicate. The findings from the duplicate fermentations demonstrated good concordance in pH and SCFA levels (**figure 6.2**). Therefore, to allow for different experimental conditions to be tested within the model, no replicate fermentations were conducted.

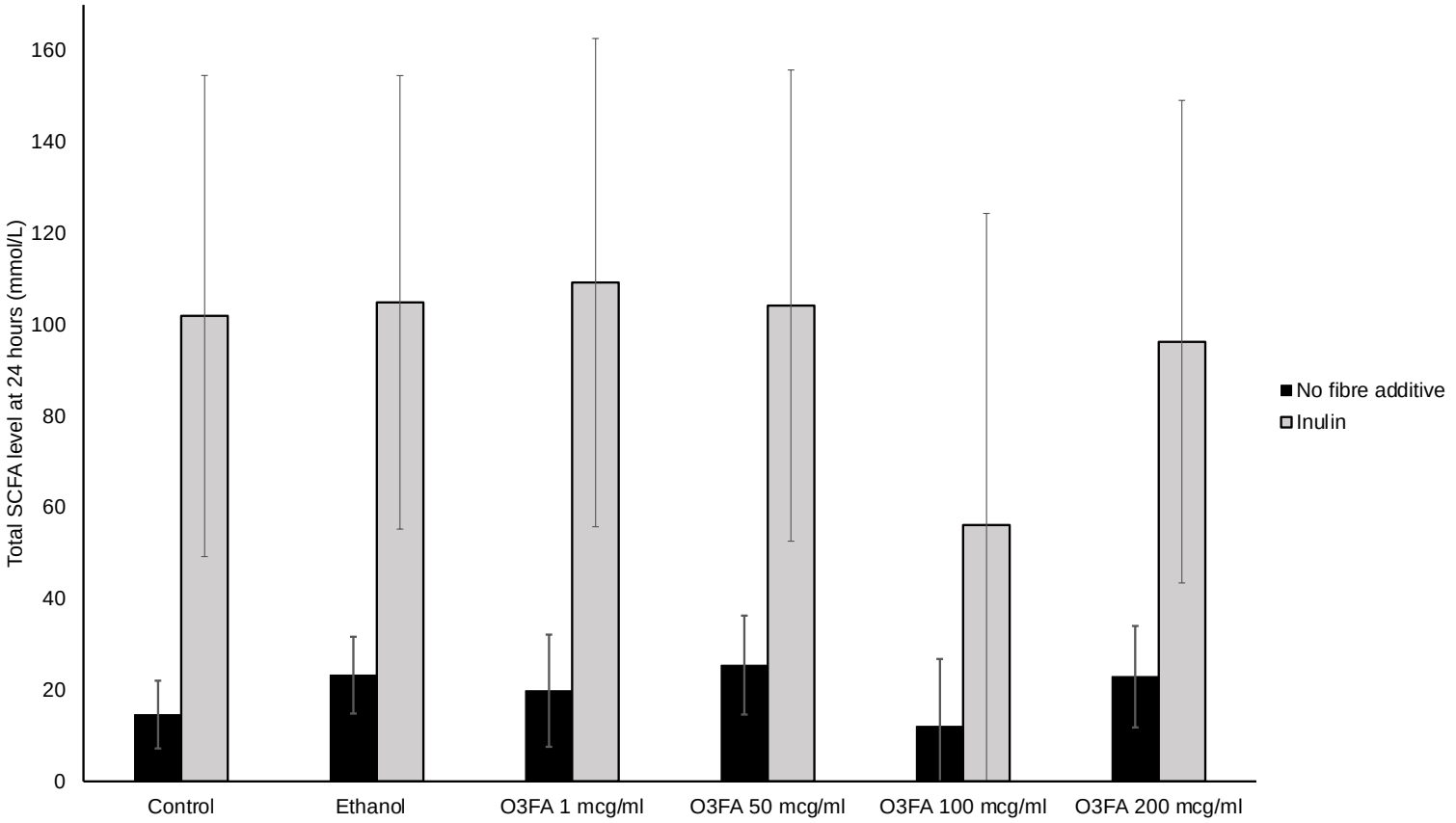


Figure 6.1 Bar chart illustrating the total SCFA level at 24 hours from preliminary experiments.

Mean values shown and error bars show standard deviation. The O3FA capsule oil was solubilised in ethanol n=4, except for the O3FA 100 mcg/ml concentration which was conducted in n=3 and the capsule oil added directly to the fermentation reaction. Within the preliminary experiments, ethanol was added to the model on its own to examine its effect on SCFA production.

6.3.1.4 Inter-experimental variability

In order to evaluate inter-experimental variability, I conducted the same experiment using a faecal sample from the same volunteer one week apart, with no dietary modification. The findings demonstrated similar levels of total SCFA production, reflecting the reliability of the *in vitro* colonic fermentation model (**figure 6.3**).

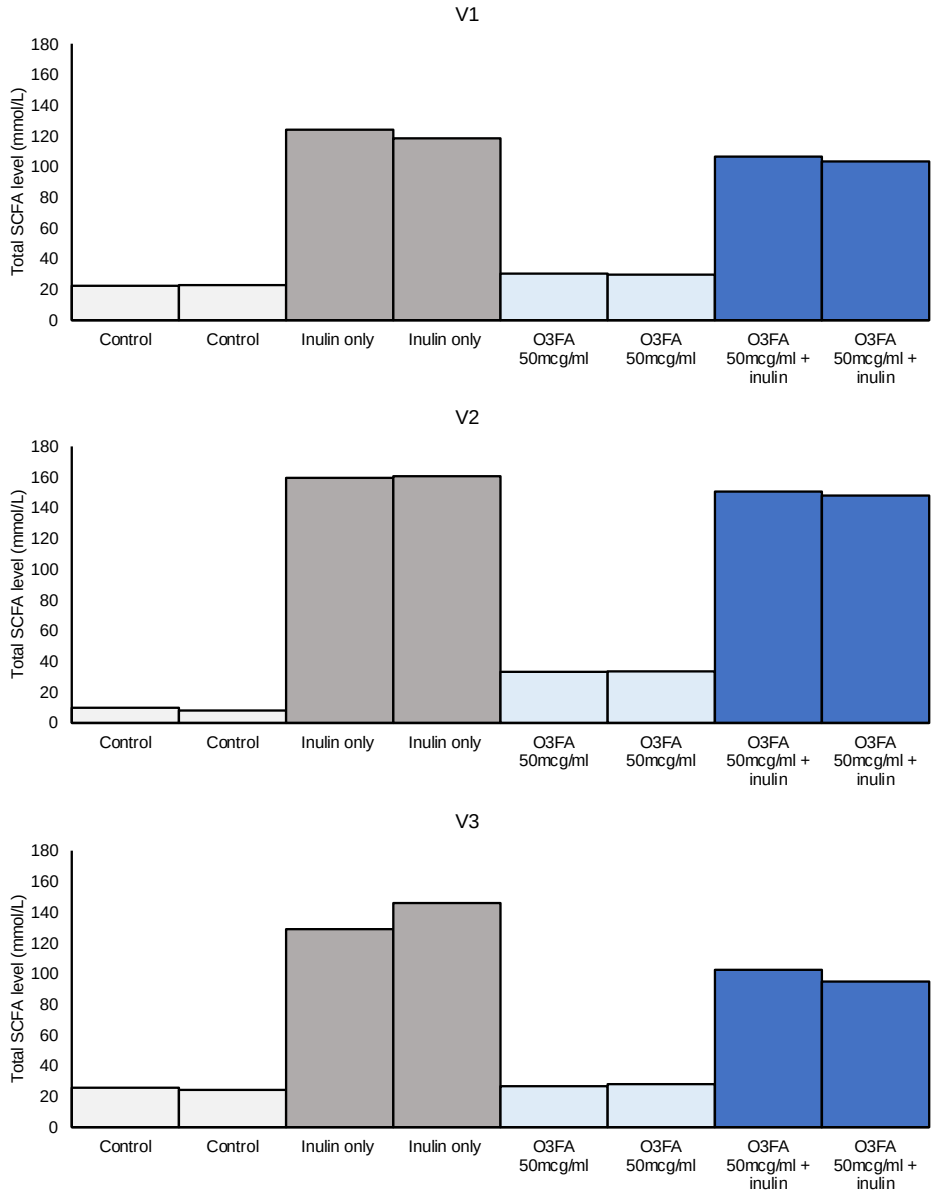


Figure 6.2. Inter-experimental variability in duplicate fermentation reactions for individual volunteers.

Control, inulin only (0.02 mg/ml), O3FA (50 mcg/ml), O3FA (50 mcg/ml) & inulin (0.02 mg/ml) shown.

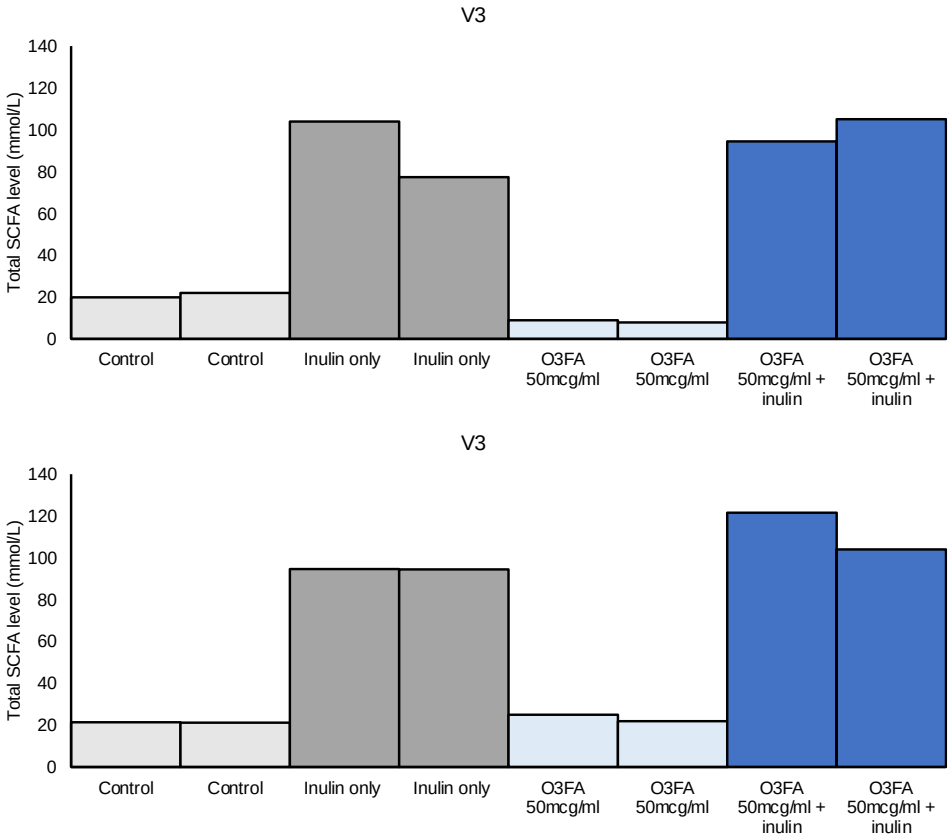


Figure 6.3 Intra-experimental variability within experiments conducted using samples from the same volunteer.

Control, inulin only (0.02 mg/ml), O3FA (50 mcg/ml), O3FA (50 mcg/ml) & inulin (0.02 mg/ml) shown.

6.3.1.5 Standard curve to calculate SCFAs

In order to calculate the concentration of SCFAs within the extracted fermentation samples, a standard curve was first calculated from the external and internal standard concentrations used to create the standard curve (**table 6.2**).

In order to calculate the standard curve, the concentrations of individual SCFA corresponding to dilutions were calculated. Each of the dilutions of external standard 0, 10, 25, 50, 100, 200 and 300, were divided by the volume of the standard water mix (800 mcl) (**table 6.2**) and then multiplied by the concentration of the corresponding individual SCFA in mmol/L in 200 ml (**table 6.1**). For example, for standard 4, a dilution of 100 mcl of external standard, was divided by the standard water mix of 800 mcl and then multiplied by the concentration of the individual SCFA in mmol/L. For acetate (C2) this was [(100/800) x 183.5] = 22.9 mmol/L.

The ratio of the peak area of the SCFA to the peak of the internal standard area was then calculated by dividing the peak area of the individual SCFA measured by the GC-FID by the area of the internal standard within the sample prepared for that individual standard. The concentration of the SCFA was then plotted against the ratio of the peak to internal standard area. A linear slope was then calculated and a R^2 value to >0.99 accepted. Standard curves for acetate, propionate and butyrate are shown in **figure 6.4**.

To quantify the SCFAs within the extracted fermentation samples. The response factor was first calculated using the **equation 6.1** in **section 6.2.13.3**.

The concentration of SCFAs within the extracted fermentation samples was then calculated by dividing the ratio of the peak area of the SCFA to the peak of the internal standard area by the response factor and then multiplying it by the internal standard concentration (**equation 6.2**)

SCFAs: isobutyrate (iC4), valerate (C5), isovalerate (iC5), caproate (C6), isocaproate (iC6), enanthate (C7) and caprylate (C8) were quantified. However, the concentrations were very small and therefore not reported.

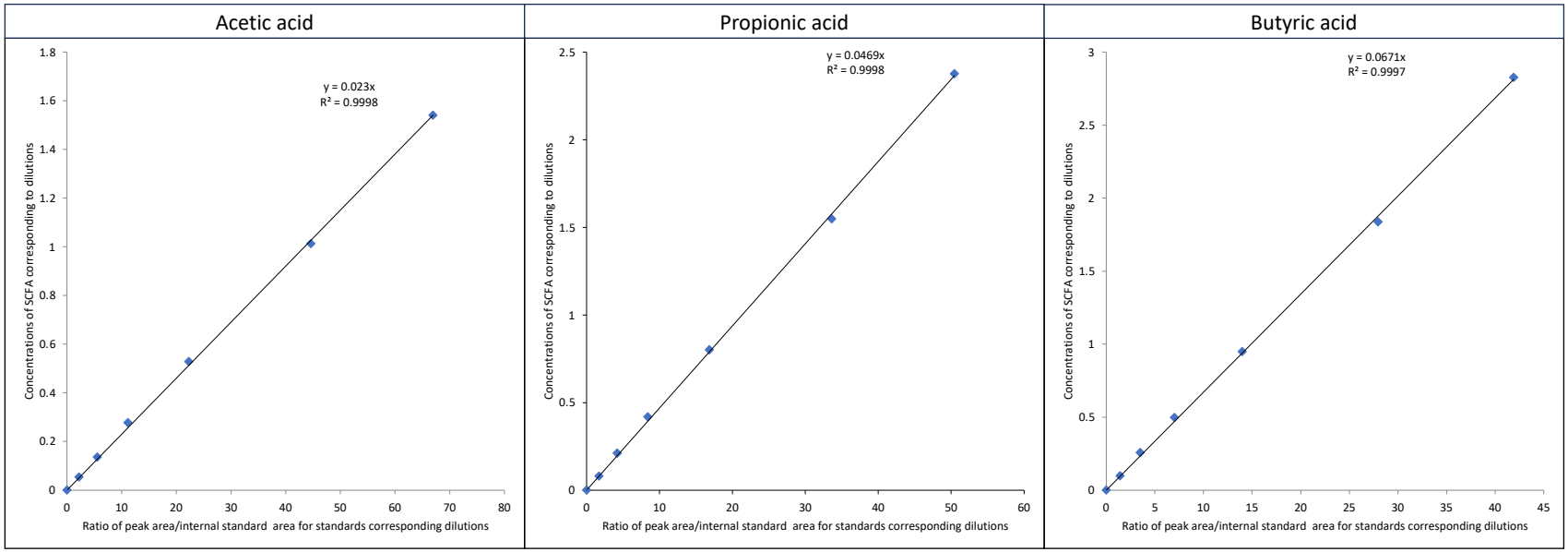


Figure 6.4 Standard curve examples for acetic, propionic, and butyric acid.

Standard curves calculated from the concentration of SCFA corresponding to the standard curve dilution on the y-axis and the ratio of the peak area to the internal standard for the corresponding dilutions on the x-axis.

6.3.2 Faecal donors

Faecal samples were obtained from ten healthy volunteers aged between 24 to 42 years (mean 30.37 [SD 5.14]) with a mean body mass index (BMI) of 22.93 (SD 3.18) kg/m^2 (**table 6.3**). Half of the donors were male, with regards to ethnicity: seven were White, two were Arab and one was Asian.

Table 6.3 Donor demographics.

Sex	
Male	5
Female	5
Age	
Mean	30 (5.1)
Median	30 (27.50, 32.50)
Range	24 to 43
BMI	
Mean	22.93 (3.2)
Median	21.80 (20.5, 25.8)
Range	19.7 to 28
Ethnicity	
White	7
Arab	2
Asian	1
Median (interquartile range) Mean (standard deviation)	

6.3.3 Omega-3 polyunsaturated fatty acids

O3FAs at a concentration of 1, 25 and 50 mcg/ml were added to the model without the addition of any dietary fibre. There was a mean pH decrease of <0.1 within the O3FA fermentations compared to the control (mean pH at 24 hours for O3FA 50mcg/ml was 7.33 [0.11] *vs*. 7.40 [0.12] for the control, P=0.02). Although a mean difference in pH of 0.07 is unlikely to be of biological significance, this decrease may reflect the slight increase in SCFA levels with the O3FA reactions (**table 6.4**).

There was a small but statistically significant increase in the total SCFA level at 24 hours observed for O3FA tested at 25 mcg/ml and 50 mcg/ml concentrations (**table 6.5**). The largest increase was for the O3FA 25 mcg/ml concentration, with a mean difference of 1.87 mmol/L in the total SCFA level

for the O3FA 25 mcg/ml concentration (23.57 [SD 2.88] mmol/L) above control (21.70 [3.07] mmol/L), (P=0.03). This increase equated to a percentage increase of 9.26% (10.61) above the control (**table 6.5**). A statistically significant percentage increase in total SCFA level at 24 hours was also observed for the 50mcg/ml concentration (7.55% [SD 8.15], P=0.02). The data suggest that the optimal O3FA concentration (without the presence of dietary fibre) to increase total SCFA was 25 mcg/ml, as there was a bell-shaped type of distribution of the total SCFA level, with the 1 mcg/ml and 50 mcg/ml concentrations being lower than the 25 mcg/ml concentration (**figure 6.5**).

With regards to the increase of individual SCFAs at 24 hours, there was a slight (non-statistically significant) increase in acetate and butyrate level for all the O3FA concentrations, with the largest increase observed for the O3FA 25 mcg/ml concentration (**table 6.5**). However, the largest increase in individual SCFAs was observed for propionate, with a mean increase of 0.67 mmol/L for the O3FA 50 mcg/ml concentration compared to control (P=0.003), the increase in propionate was also statistically significant for the O3FA 25 mcg/ml concentration (P=0.002), (**table 6.5 & figure 6.5**). There were similar patterns and levels of individual and total SCFA production observed for each of the different volunteers (**figure 6.6**). The biological significance of the percentage change in the total SCFA level is discussed later in this chapter.

Table 6.4 Changes in pH for O3FA only fermentations.

Experimental condition	Timepoint[1]			8 hours P value[3]	24 hours P value[3]
	0	8	24		
Control[2]	7.47 (0.07)	7.40 (0.09)	7.40 (0.12)		
O3FA 1mcg/ml	7.48 (0.06)	7.39 (0.13)	7.32 (0.10)	0.76	0.01
O3FA 25mcg/ml	7.47 (0.07)	7.43 (0.12)	7.33 (0.08)	0.34	0.04
O3FA 50mcg/ml	7.46 (0.07)	7.41 (0.12)	7.33 (0.11)	0.76	0.02

[1] Timepoint in hours
[2] Mean (standard deviation)
[3] Paired t-test comparing 0 with 8 and 24 hours respectively
n=10 volunteers

Table 6.5 SCFA levels of O3FA only fermentations at 8 hours and 24 hours.

Timepoint	Experimental condition	C2[1,2]	C3	C4	Total[3]	P value[4]	Percentage change from control[5]	P value[6]
8 hours	**Control**	10.72 (2.34)	2.69 (0.61)	2.26 (0.86)	15.67 (3.55)			
	O3FA 1mcg/ml	11.31 (2.76)	2.87 (0.79)	2.34 (0.90)	16.53 (4.19)	0.17	4.94 (12.58)	0.25
	O3FA 25mcg/ml	11.43 (2.35)	2.60 (0.68)	2.28 (0.86)	16.66 (3.64)	0.13	6.96 (13.05)	0.13
	O3FA 50mcg/ml	10.60 (2.56)	2.78 (0.71)	2.18 (0.88)	15.57 (3.92)	0.87	-0.91 (11.80)	0.81
24 hours	**Control**	13.73 (2.67)	4.76 (0.94)	3.20 (0.63)	21.70 (3.07)			
	O3FA 1mcg/ml	14.37 (3.00)	5.25 (1.36)	3.39 (0.75)	23.01 (4.41)	0.24	6.16 (15.72)	0.25
	O3FA 25mcg/ml	14.80 (2.42)	5.32 (1.02)	3.46 (0.85)	23.57 (2.88)	0.03	9.26 (10.61)	0.02
	O3FA 50mcg/ml	14.39 (2.09)	5.42 (1.06)	3.37 (0.46)	23.18 (2.31)	0.03	7.55 (8.15)	0.02

[1] Mean (standard deviation).
[2] SCFA levels measured in mmol/L.
[3] Total SCFA level is the sum of acetate (C2) + propionate (C3) + butyrate (C4).
[4] Paired t-test comparing total SCFA for each experimental condition (concentration of O3FAs) with control at either 8 or 24 hours.
[5] Percentage change (%) is the total SCFA level divided by the total SCFA level for the control, (standard deviation).
[6] Paired t-test comparing percentage change for each experimental condition (concentration of O3FAs) with control at either 8 or 24 hours.
n=10 volunteers

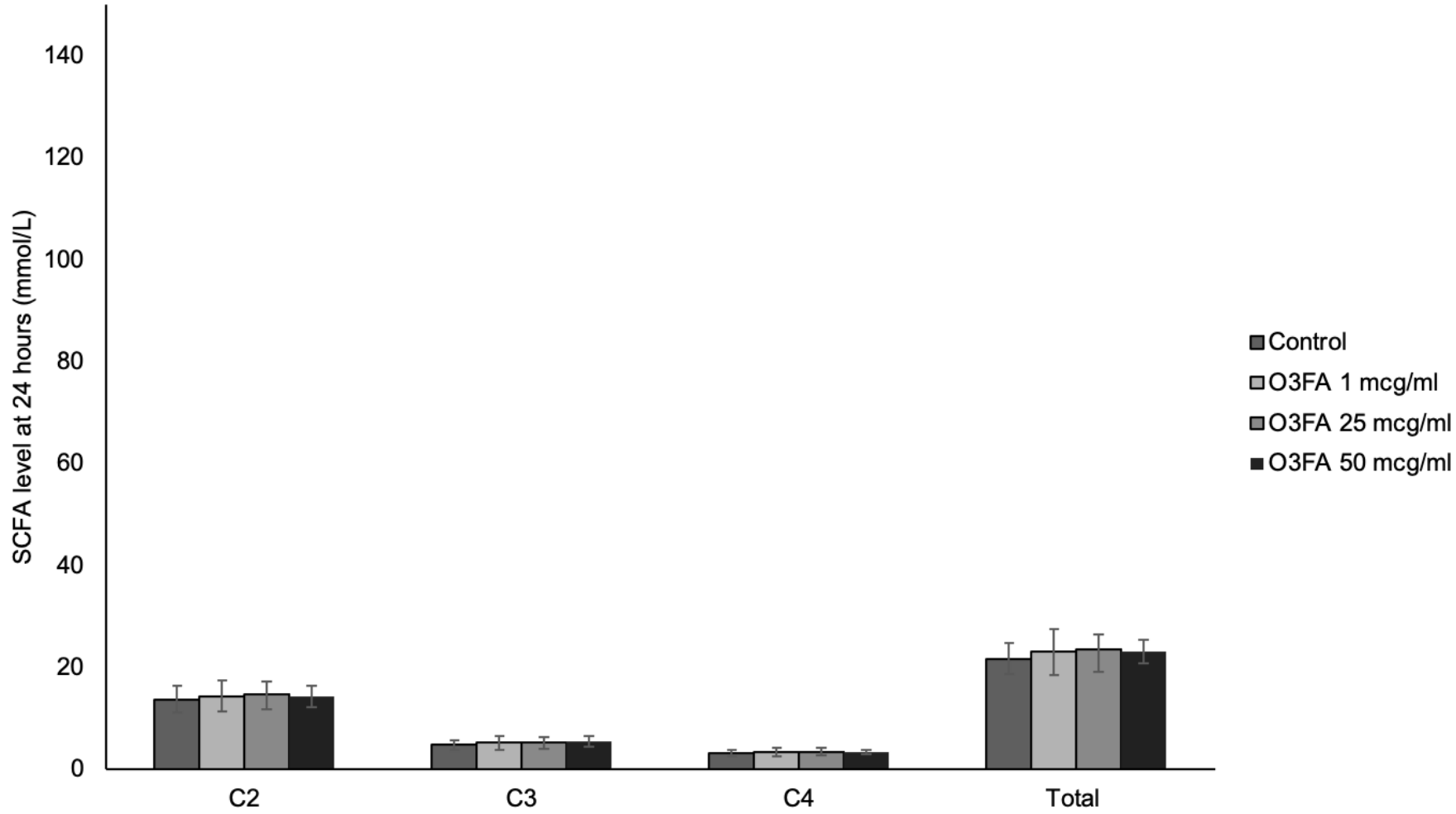


Figure 6.5 SCFA levels of O3FA-only (no added fibre) fermentations at 24 hours.

Mean level shown, error bars show standard deviation. Acetate (C2) + propionate (C3) + butyrate (C4), total SCFA is the sum of C2+C3+C4. The maximum Y axis value of 140 mmol/L is used to allow comparisons between the different O3FA

concentrations and fibre additives within the fermentation reactions.

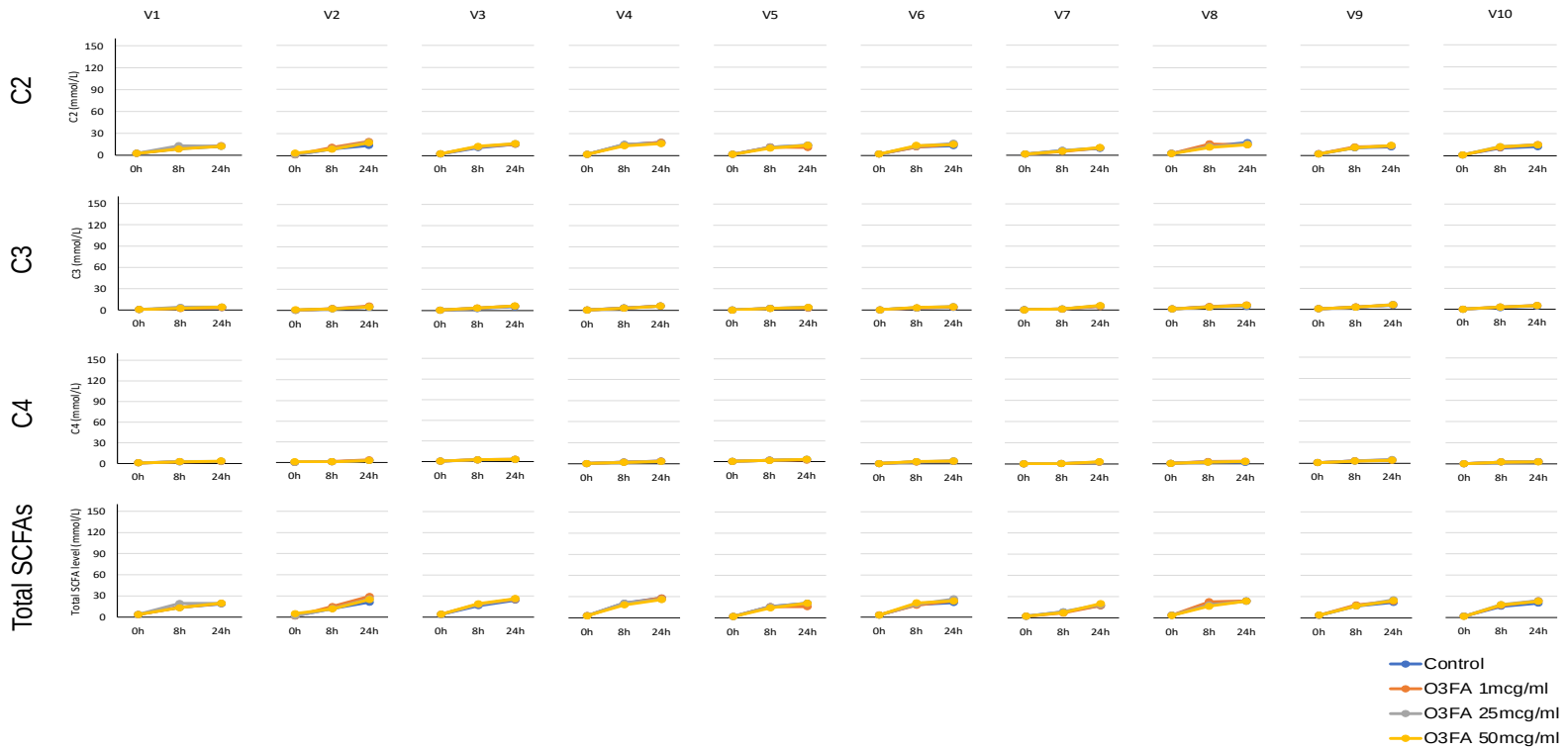


Figure 6.6 Individual and total SCFA levels measured at 0, 8 and 24 hours in O3FA-only fermentations (no added fibre) for individual volunteers.

Individual SCFAs C2, C3 & C4, in addition to the total SCFA Level at each timepoint shown for each volunteer V1 to V10.

6.3.4 Inulin

Inulin was tested at two different concentrations, 0.01 mg/ml (500 mg in 50 ml reaction volume) and 0.02 mg/ml (1 g in 50 ml reaction volume).

6.3.4.1 Inulin 0.01 mg/ml

There was a decrease in the pH of all fermentations containing inulin 0.01 mg/ml with a mean pH of 7.47 (0.06) at 0 hours, decreasing to 5.48 (0.38) at 24 hours. Of the individual experimental conditions tested, the largest mean decrease in pH at 24 hours was observed for the reaction containing O3FA 25 mcg/ml & inulin (pH 5.41 [0.34]) compared to inulin only (pH 5.54 [0.44]), however this was not statistically significant (**table 6.6**).

Increasing concentrations of O3FAs & inulin were associated with a dose response related increase in the total SCFA level at 24 hours (**table 6.7 and figure 6.7**). The O3FA concentrations of 1, 25 and 50 mcg/ml & inulin were associated with a respective mean increase of 5.26, 9.79 and 12.89 mmol/L in total SCFA level at 24 hours. These levels amounted to a respective percentage increase of 8.39 [SD 21.09], 15.28 [17.58] and 19.26 [20.31]. When compared to the inulin only reaction, the total SCFA level, and percentage change were statistically significantly greater for the O3FA 25 mcg/ml and 50 mcg/ml concentrations & inulin (P=0.02), but not the O3FA 1 mcg/ml concentration & inulin (P=0.28).

There was an increase in the individual levels of acetate, propionate, and butyrate within each of the O3FA & inulin fermentations. However, the greatest increase was observed for butyrate at 24 hours, for the O3FA 50 mcg/ml concentration & inulin. The mean of the differences for butyrate at 24 hours between the inulin control (11.77 [3.08] mmol/L) and the O3FA 50

mcg/ml concentration & inulin (15.82 [5.30] mmol/L) was 4.05 mmol/L, (P=0.02). A statistically significant increase was also observed for the O3FA 25 mg/ml concentration & inulin when compared to the inulin only reaction at 24 hours (14.83 [4.78] mmol/L) (P=0.02). Although there were observed dose related increases in acetate and propionate levels associated with increasing O3FA concentrations & inulin within the fermentations, these did not reach statistical significance (**table 6.7, figures 6.7**).

Levels of acetate, propionate, butyrate and total SCFA levels at 0, 8 and 24 hours are shown for individual volunteers (**figure 6.8**). Inter-individual variability was observed for the total level of SCFA production between individual volunteers. Within the O3FA 50 mcg/ml & inulin fermentation, the total SCFA level at 24 hours ranged from 66.41 in volunteer 7 (V7) to 119.88 mmol/L in V5. These differences were seen for individual SCFAs at 24 hours within the O3FA 50 mcg/ml & inulin fermentation, with levels of acetate ranging from 29.67 (V2) to 88.37 mmol/L (V5), propionate 12.49 (V5) to 37.02 mmol/L (V2) and for butyrate from 7.85 (V2) to 23.82 mmol/L (V4).

There was an increase in the percentage change in SCFA level at 24 hours within the O3FA 50 mcg/ml & inulin fermentation in all volunteers, except for volunteers 6 and 10 (**figure 6.8**).

The overall mean percentage increase in total SCFA level for the O3FA 50 mcg/ml concentration & inulin was 19.26% (20.31%) and ranged from -14.75% to 48.14%. This percentage increase was statistically significant for the O3FA 25mcg/ml (P=0.02) and the O3FA 50mcg/ml & inulin fermentation reactions (P=0.01) (**table 6.7**). Given the sample size (n=10) and aims of the study as hypothesis generating with regards to examining the interaction

between O3FAs and different dietary fibres, I did not test for synergy within the study, this would be difficult given the small sample size. However, testing for synergy between O3FAs and inulin interventions would be a crucial aspect of any future study leading on from this work, and an important consideration within any future study protocol.

Table 6.6 Changes in pH within fermentations containing O3FAs and inulin.

Experimental condition	Timepoint[1]			8 hours P value[3]	24 hours P value[3]
	0	8	24		
Inulin only (0.01mg/ml)[2]	7.47 (0.07)	6.01 (0.38)	5.54 (0.44)		
O3FA 1mcg/ml & inulin (0.01mg/ml)	7.47 (0.07)	5.96 (0.47)	5.55 (0.39)	0.78	0.93
O3FA 25mcg/ml & inulin (0.01mg/ml)	7.47 (0.07)	5.85 (0.33)	5.41 (0.34)	0.30	0.21
O3FA 50mcg/ml & inulin (0.01mg/ml)	7.46 (0.05)	5.90 (0.41)	5.43 (0.39)	0.53	0.28
Inulin (0.02mg/ml)	7.47 (0.07)	5.08 (0.33)	4.74 (0.27)		
O3FA 1mcg/ml & inulin (0.02mg/ml)	7.46 (0.07)	5.11 (0.26)	4.70 (0.19)	0.82	0.42
O3FA 25mcg/ml & inulin (0.02mg/ml)	7.47 (0.07)	5.12 (0.30)	4.86 (0.39)	0.64	0.26
O3FA 50mcg/ml & inulin (0.02mg/ml)	7.46 (0.05)	5.12 (0.32)	4.75 (0.20)	0.74	0.89

[1] Timepoint in hours
[2] Mean (standard deviation)
[3] Paired t-test comparing 0 with 8 and 24 hours respectively
n=10 volunteers

Table 6.7 SCFA levels of O3FA & inulin fermentations at 8 hours and 24 hours.

Timepoint	Experimental condition	C2[1,2]	C3	C4	Total[3]	P value[4]	Percentage change from control[5]	P value[6]
8 hours	**Inulin only (0.01 mg/ml)**	32.48 (10.21)	14.27 (7.74)	7.22 (3.01)	53.96 (14.08)			
	O3FA 1mcg/ml & inulin (0.01mg/ml)	33.11 (10.77)	13.93 (7.86)	7.47 (5.10)	54.50 (16.38)	0.85	0.10 (15.65)	0.98
	O3FA 25mcg/ml & inulin (0.01mg/ml)	36.72 (12.32)	15.72 (8.05)	8.88 (4.20)	61.33 (15.55)	0.03	14.47 (17.83)	0.03
	O3FA 50mcg/ml & inulin (0.01mg/ml)	35.30 (12.17)	14.43 (7.16)	9.09 (4.60)	58.81 (15.47)	0.16	9.88 (15.47)	0.01
24 hours	**Inulin only (0.01 mg/ml)**	39.92 (10.45)	20.88 (7.33)	11.77 (3.08)	72.57 (12.43)			
	O3FA 1mcg/ml & inulin (0.01mg/ml)	41.70 (10.70)	22.03 (6.14)	14.10 (6.74)	77.83 (15.51)	0.28	8.39 (21.09)	0.24
	O3FA 25mcg/ml & inulin (0.01mg/ml)	43.83 (12.62)	23.70 (7.14)	14.83 (4.78)	82.36 (11.18)	0.02	15.28 (17.58)	0.02
	O3FA 50mcg/ml & inulin (0.01mg/ml)	46.26 (17.41)	23.38 (8.10)	15.82 (5.30)	85.46 (15.87)	0.02	19.26 (20.31)	0.01

[1] Mean (standard deviation).
[2] SCFA levels measured in mmol/L.
[3] Total SCFA level is the sum of acetate (C2) + propionate (C3) + butyrate (C4).
[4] Paired t-test comparing total SCFA for each experimental condition (concentration of O3FAs) with inulin only (0.01mg/ml) at either 8 or 24 hours.
[5] Percentage change (%) is the total SCFA level divided by the total SCFA level for the inulin only reaction, (standard deviation).
[6] Paired t-test comparing percentage change for each experimental condition (concentration of O3FAs) with inulin only (0.01mg/ml) at either 8 or 24 hours.
n=10 volunteers

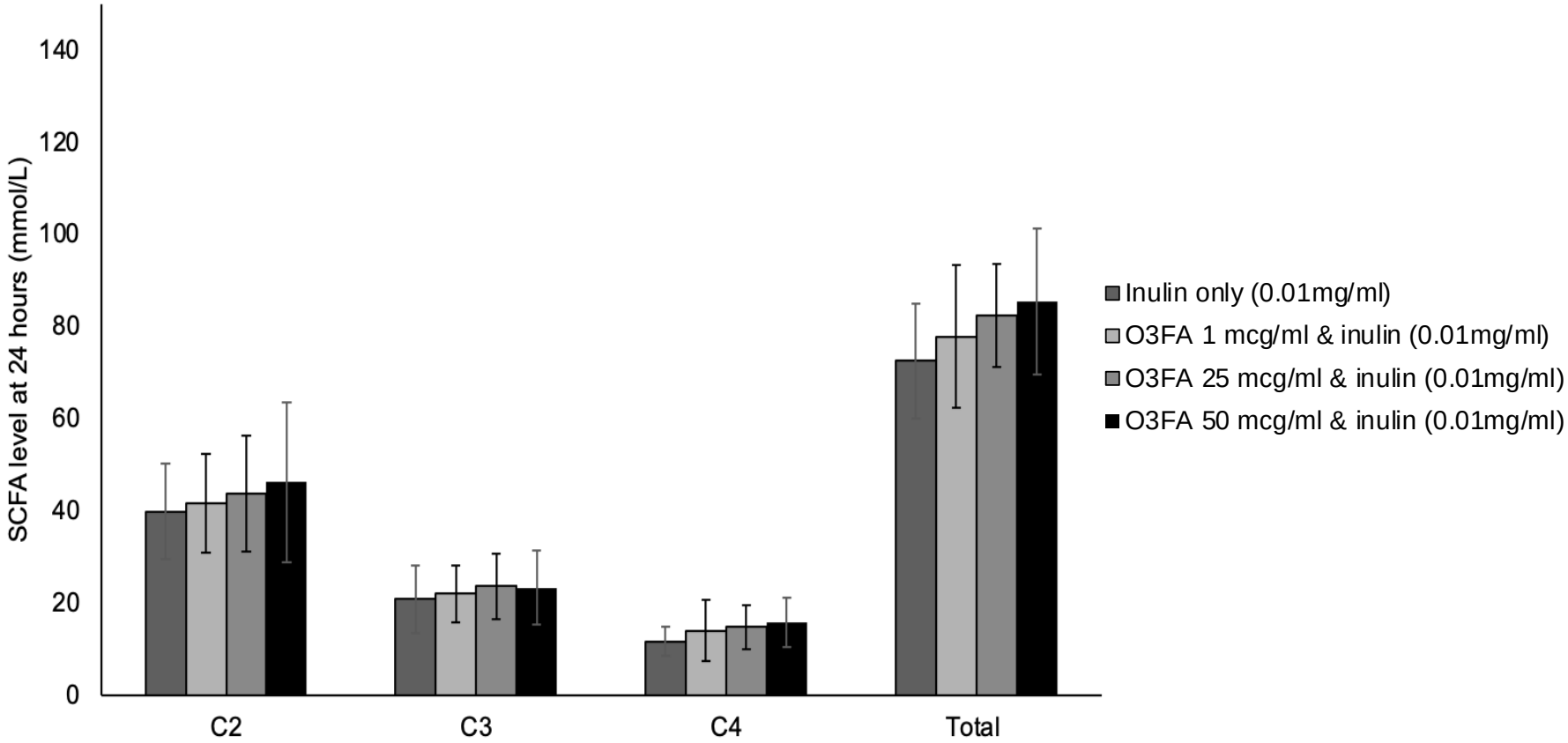


Figure 6.7 SCFA levels in O3FA & inulin (0.01 mg/ml) fermentations at 24 hours.

Mean level show, error bars show standard deviation. Acetate (C2) + propionate (C3) + butyrate (C4), total SCFA is the sum of C2+C3+C4.

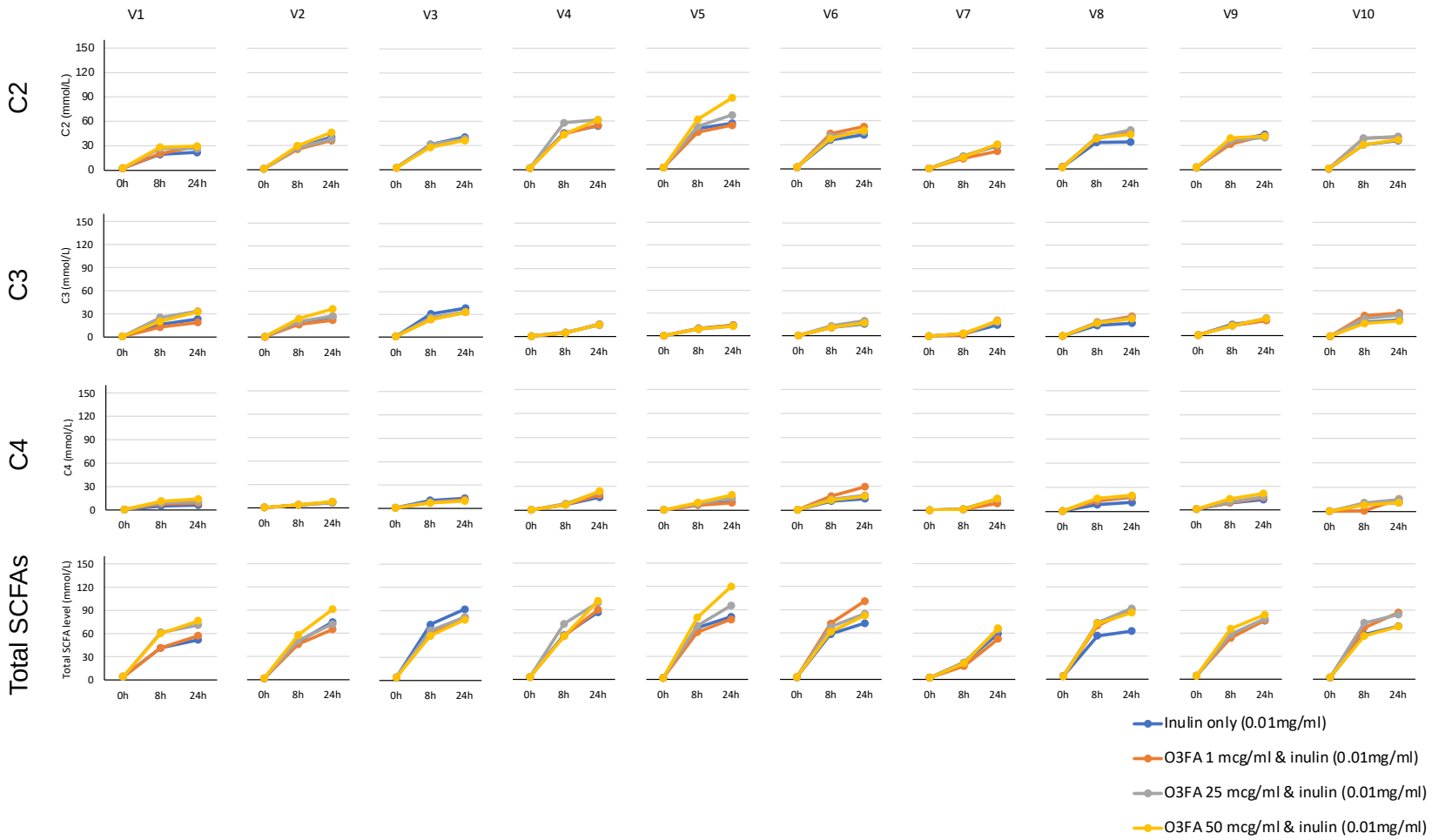


Figure 6.8 Individual and total SCFA levels measured at 0, 8 and 24 hours in O3FA & inulin (0.01 mg/ml) fermentations for individual volunteers.

Individual SCFAs C2, C3 & C4, in addition to the total SCFA Level at each timepoint shown for each volunteer V1 to V10.

6.3.4.2 Inulin 0.02 mg/ml

Inulin was tested at double the concentration, 0.02 mg/ml. The increased inulin concentration was associated with a larger decrease in pH within all the fermentations containing the higher inulin concentration, with a mean pH of 7.47 at 0 hours to 4.76 at 24 hours. The pH results at 24 hours were more variable across the O3FA concentrations tested, with the mean pH at 24 hours for the O3FA 25 mcg/ml concentration & inulin being 4.86 (0.39) which was 0.12 higher than the mean pH of the inulin only reaction at 24 hours (4.74 [0.27]), P=0.26. Whereas there was a decrease in the mean pH at 24 hours for the O3FA 1 mcg/ml concentration & inulin (4.70 [0.19]) and a similar pH to the inulin only reaction observed for the O3FA 50 mcg/ml concentration & inulin (4.75 [0.20]) (**table 6.6**).

The heterogeneity observed within the pH results was reflected in the total SCFA level at 24 hours. The higher concentration of inulin was associated with an increase in overall SCFA levels, with a mean total SCFA level of 114.88 (19.19) mmol/L across all experimental conditions tested. The highest increase in total SCFA level at both 8 and 24 hours was observed for the O3FA 1 mcg/ml concentration & inulin (77.98 [24.30] & 117.56 [25.72] mmol/L) compared to the inulin only reaction (67.80 [18.24] & 113.06 [13.73] mmol/L, respectively), however this did not reach statistical significance (P=0.17 at 8 hours, P=0.60 at 24 hours) (**table 6.8 & figure 6.9**).

There was greater variability and lack of discernible differences in SCFA levels within the fermentations with the increased inulin concentration. This possibly reflects that the reaction reached a steady state within the fermentation prior to the 24-hour timepoint. However, there were no significant differences in either the total SCFA level or percentage change

from control at 8 hours for any of the O3FA concentrations & inulin. As inulin is highly fermentable, there could have potentially been a difference observed between the reactions at 4 or 6 hours of fermentation.

The higher inulin concentration (0.02 mg/ml) was associated with a similar mean increase in propionate (30.37 [20.20] mmol/L) and butyrate (30.03 [17.42] mmol/L) levels at 24 hours across all fermentations. Whereas the lower inulin concentration (0.01 mg/ml) which was associated with a larger increase in mean propionate levels (22.50 [7.02] mmol/L) but lower levels of butyrate (14.13 [5.17] mmol/L), across all fermentations.

Inter-individual variability observed for individual and total SCFA levels at 8 and 24 hours is shown in **figure 6.10**. For V6 and V10 there was an increase in SCFA level in the fermentations including the higher inulin concentration (0.02mg/ml) whereas there was no increase observed at the lower inulin concentration (0.01 mg/ml) for those individual volunteers (**figure 6.10**), suggesting that there is an individual response.

Table 6.8 SCFA levels of O3FA & inulin (0.02 mg/ml) fermentations at 8 hours and 24 hours.

Timepoint	Experimental condition	C2[1,2]	C3	C4	Total[3]	P value[4]	Percentage change from control[5]	P value[6]
8 hours	**Inulin only (0.02mg/ml)**	38.74 (19.08)	16.29 (10.85)	12.77 (7.36)	67.80 (18.24)			
	O3FA 1 mcg/ml & Inulin (0.02mg/ml)	47.56 (16.80)	17.32 (14.91)	13.10 (6.15)	77.98 (24.30)	0.17	16.03 (33.93)	0.23
	O3FA 25 mcg/ml & Inulin (0.02mg/ml)	44.28 (13.46)	17.78 (12.47)	12.77 (6.66)	74.83 (21.66)	0.28	10.52 (26.07)	0.48
	O3FA 50 mcg/ml & Inulin (0.02mg/ml)	45.21 (13.86)	17.47 (12.38)	13.05 (7.23)	75.72 (22.09)	0.37	11.92 (25.78)	0.43
24 hours	**Inulin only (0.02mg/ml)**	54.48 (18.86)	30.38 (20.20)	30.03 (17.42)	113.06 (13.73)			
	O3FA 1 mcg/ml & Inulin (0.02mg/ml)	58.60 (21.70)	29.76 (21.72)	29.20 (16.45)	117.56 (25.72)	0.60	4.55 (22.96)	0.55
	O3FA 25 mcg/ml & Inulin (0.02mg/ml)	51.28 (21.52)	30.20 (20.70)	30.20 (20.70)	113.01 (20.51)	0.99	-0.32 (13.29)	0.94
	O3FA 50 mcg/ml & Inulin (0.02mg/ml)	54.99 (17.74)	31.11 (21.97)	29.78 (21.37)	115.89 (17.55)	0.33	2.28 (8.35)	0.41

[1] Mean (standard deviation).
[2] SCFA levels measured in mmol/L.
[3] Total SCFA level is the sum of acetate (C2) + propionate (C3) + butyrate (C4).
[4] Paired t-test comparing total SCFA for each experimental condition (concentration of O3FAs) with inulin only (0.02mg/ml) at either 8 or 24 hours.
[5] Percentage change (%) is the total SCFA level divided by the total SCFA level for the inulin only reaction, (standard deviation).
[6] Paired t-test comparing percentage change for each experimental condition (concentration of O3FAs) with inulin only (0.02mg/ml) at either 8 or 24 hours.
n=10 volunteers

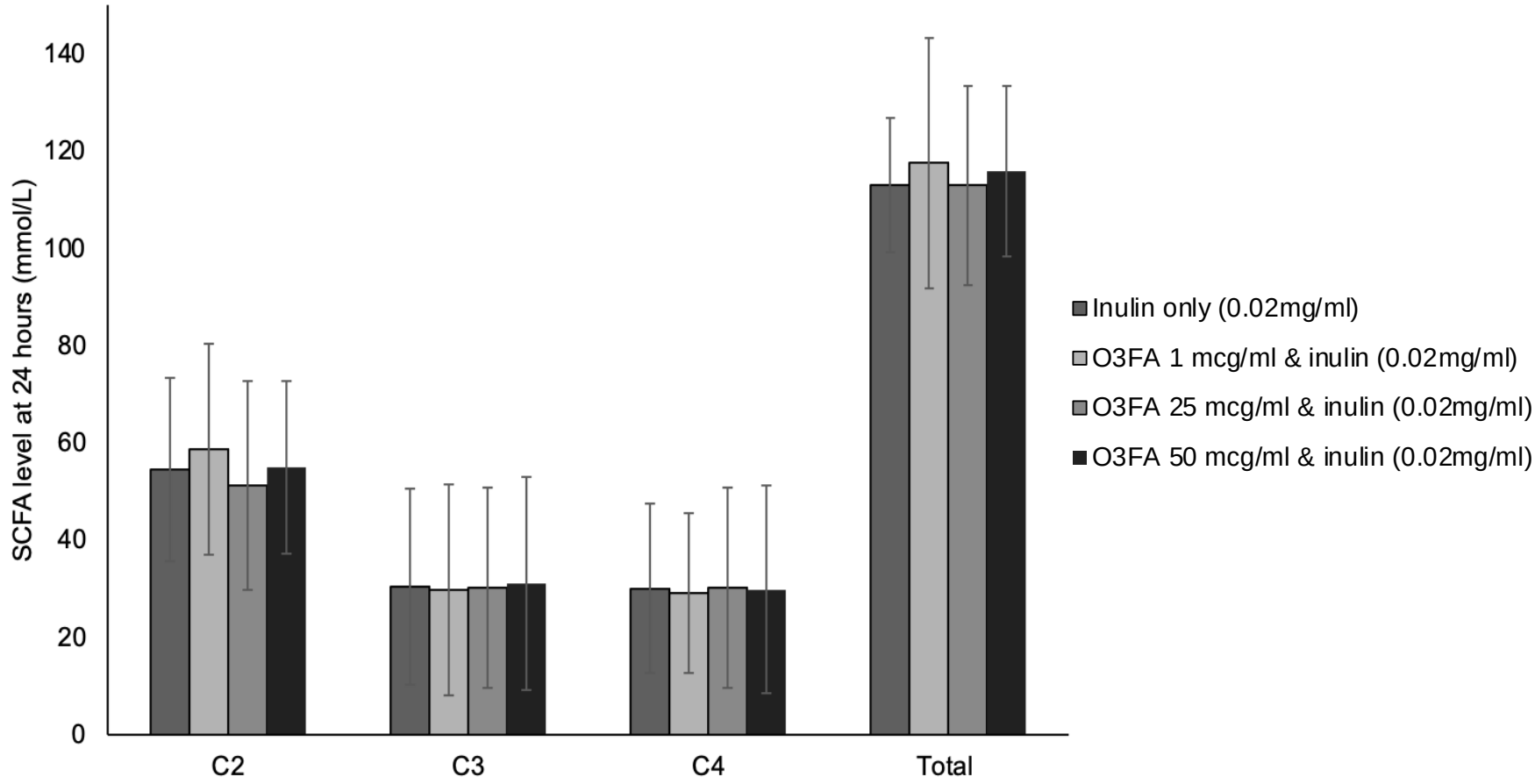


Figure 6.9 SCFA levels of O3FA & inulin (0.02 mg/ml) fermentations at 24 hours.

Mean level show, error bars show standard deviation. Acetate (C2) + propionate (C3) + butyrate (C4), total SCFA is the sum of C2+C3+C4.

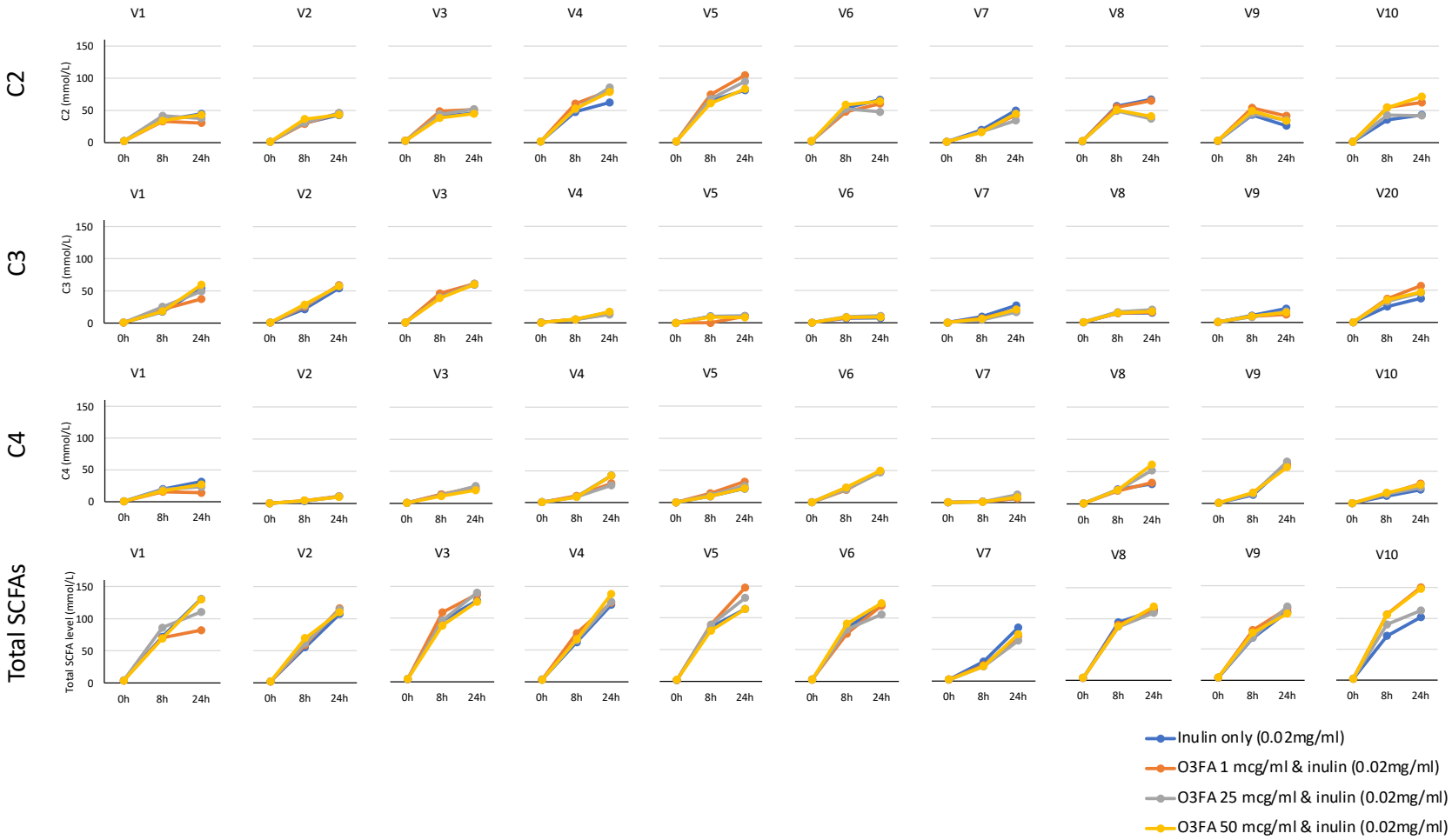


Figure 6.10 Individual and total SCFA levels measured at 0, 8 and 24 hours in O3FA & inulin (0.02 mg/ml) fermentations according to individual volunteers.

Individual SCFAs C2, C3 & C4, in addition to the total SCFA Level at each timepoint shown for each volunteer V1 to V10.

6.3.5 Wheat bran

In comparison to inulin, wheat bran was fermented less within the model, as reflected in the pH results. The mean pH of all fermentations containing wheat bran was 7.46 (0.06) at 0 hours and decreased to 6.38 (0.26) at 24 hours. A similar pH was observed in the O3FA 25, and 50 mcg/ml concentrations & wheat bran compared to the wheat bran only reaction at 24 hours. However, the O3FA 1 mcg/ml concentration & wheat bran was associated with a higher mean pH at 24 hours (6.40 [0.25]) compared to the wheat bran only reaction (6.36 [0.28]), (mean difference 0.04). Although this finding was statistically significant (P=0.04), it could be due to multiple testing, however it could also suggest a lower rate an anaerobic activity within the fermentation, reflected in lower SCFA levels observed for the O3FA 1 mcg/ml concentration (**table 6.9**).

The higher pH level was reflected in levels of SCFAs production following the addition of wheat bran within the model. The mean total SCFA level at 24 hours for all the wheat bran fermentations was 63.57 (9.75) mmol/L. The addition of O3FAs & wheat bran was not associated with an increase in total SCFA level at 8 or 24 hours for any of the O3FA concentrations. There was a slight decrease in the SCFA levels for the O3FA 1mcg/ml & wheat bran (63.31 [11.36] mmol/L) and the 25 mcg/ml & wheat bran (62.58 [9.63] mmol/L) concentrations compared to control (64.14 [9.85] mmol/L), with a similar level to the wheat bran only reaction observed for the O3FA 50 mcg/ml concentration & wheat bran (64.22 [9.55] mmol/L) (**table 6.10 & figure 6.11**). These findings suggest a potential negative association of O3FAs & wheat bran within the model.

Table 6.9 Changes in pH for wheat bran fermentations.

Experimental condition	Timepoint[1]			8 hours P value[3]	24 hours P value[3]
	0	8	24		
Wheat bran only[2]	7.45 (0.07)	6.61 (0.23)	6.36 (0.28)		
O3FA 1 mcg/ml & wheat bran	7.46 (0.07)	6.66 (0.22)	6.40 (0.25)	0.32	0.04
O3FA 25 mcg/ml & wheat bran	7.46 (0.07)	6.64 (0.23)	6.37 (0.27)	0.54	0.76
O3FA 50 mcg/ml & wheat bran	7.45 (0.05)	6.59 (0.21)	6.37 (0.28)	0.59	0.78

[1] Timepoint in hours
[2] Mean (standard deviation)
[3] Paired t-test comparing 0 with 8 and 24 hours respectively
n=10 volunteers

Table 6.10 SCFA levels for wheat bran fermentations as 8 and 24 hours.

Timepoint	Experimental condition	C2[1,2]	C3	C4	Total[3]	P value[4]	Percentage change from control[5]	P value[6]
8 hours	**Wheat bran only[2]**	30.33 (6.16)	8.56 (2.68)	6.01 (1.88)	44.95 (9.94)			
	O3FA 1 mcg/ml & wheat bran	30.82 (6.23)	8.75 (2.27)	6.05 (1.95)	45.61 (8.09)	0.69	2.81 (10.51)	0.42
	O3FA 25 mcg/ml & wheat bran	30.90 (6.20)	9.03 (2.40)	6.05 (1.93)	45.98 (8.21)	0.54	3.68 (11.94)	0.36
	O3FA 50 mcg/ml & wheat bran	29.27 (5.97)	7.90 (3.56)	5.90 (1.89)	43.08 (8.95)	0.23	-3.63 (9.45)	0.26
24 hours	**Wheat bran only[2]**	39.37 (7.87)	13.43 (2.99)	10.76 (2.65)	64.15 (9.85)			
	O3FA 1 mcg/ml & wheat bran	39.53 (8.47)	13.13 (3.69)	10.64 (2.87)	63.31 (11.36)	0.83	-0.29 (15.96)	0.95
	O3FA 25 mcg/ml & wheat bran	38.66 (8.03)	13.27 (3.16)	10.65 (2.48)	62.58 (9.63)	0.61	-1.74 (12.23)	0.66
	O3FA 50 mcg/ml & wheat bran	39.10 (8.03)	14.42 (2.53)	10.71 (2.69)	64.22 (9.55)	0.98	0.70 (10.58)	0.84

[1] Mean (standard deviation).
[2] SCFA levels measured in mmol/L.
[3] Total SCFA level is the sum of acetate (C2) + propionate (C3) + butyrate (C4).
[4] Paired t-test comparing total SCFA for each experimental condition (concentration of O3FAs) with wheat bran only at either 8 or 24 hours.
[5] Percentage change (%) is the total SCFA level divided by the total SCFA level for the wheat bran only reaction, (standard deviation).
[6] Paired t-test comparing percentage change for each experimental condition (concentration of O3FAs) with wheat bran only at either 8 or 24 hours.
n=10 volunteers

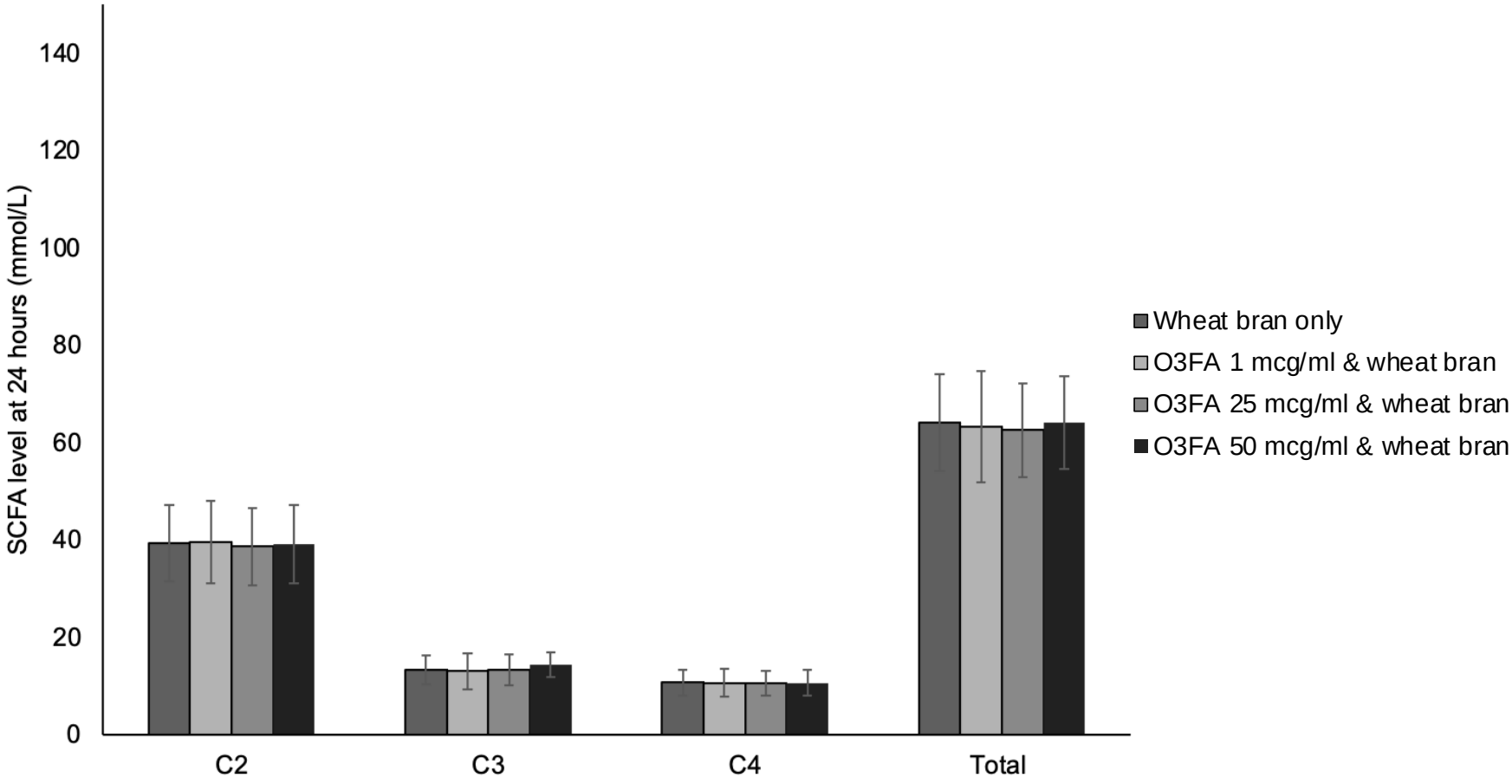


Figure 6.11 SCFA levels of O3FA & wheat bran fermentations at 24 hours.

Mean level show, error bars show standard deviation. Acetate (C2) + propionate (C3) + butyrate (C4), total SCFA is the sum of C2+C3+C4

Figure 6.12 Individual and total SCFA levels measured at 0, 8 and 24 hours in O3FA & wheat bran fermentations according to individual volunteers.

Individual SCFAs C2, C3 & C4, in addition to the total SCFA Level at each timepoint shown for each volunteer V1 to V10.

6.3.6 Pectin

Due to limitations of the size of the faecal sample provided by one volunteer (V10), it was not possible to test the O3FA concentrations of 1 mcg/ml and 25 mcg/ml with the addition of pectin, therefore the data for those concentrations is based on n=9.

The addition of pectin within the *in vitro* fermentation model was associated with a mean decrease in pH from 7.36 (0.09) at 0 hours to 5.44 (0.21) at 24 hours. There was no statistically significant decrease in pH observed in any of the O3FA concentrations & pectin (**table 6.11**), however the O3FA 1 mcg/ml concentration & pectin was associated with the largest decrease in pH at 24 hours (5.40 [0.19]) compared to the pectin only reaction (5.44 [0.18]) (P=0.07). The decrease in pH observed for the O3FA 1 mcg/ml concentration & pectin was reflected in an increase in total SCFA level at 24 hours (88.17 [11.77] mmol/L) compared to pectin only reaction (83.37 [12.01] mmol/L), a mean 5.05 mmol/L increase (P=0.004). No increase in total SCFA level was observed for the 25 and 50 mcg/ml O3FA concentrations & pectin (**table 6.12, figure 6.13**). The findings observed within the 1 mcg/ml concentration & pectin could be due to multiple testing or alternatively they may suggest that there may be an interaction between specific O3FA concentrations and dietary fibres (O3FA 1mcg/ml & pectin in this case).

Table 6.11 Changes in pH for pectin fermentations.

Experimental condition	Timepoint[1]			8 hours P value[3]	24 hours P value[3]
	0	8	24		
Pectin only	7.37 (0.13)	6.24 (0.40)	5.44 (0.18)		
O3FA 1 mcg/ml & pectin[4]	7.37 (0.09)	6.33 (0.28)	5.40 (0.19)	0.92	0.07
O3FA 25 mcg/ml & pectin[4]	7.36 (0.09)	6.24 (0.34)	5.48 (0.19)	0.59	1.00
O3FA 50 mcg/ml & pectin	7.35 (0.08)	6.23 (0.29)	5.45 (0.27)	0.95	0.91

[1] Timepoint in hours
[2] Mean (standard deviation)
[3] Paired t-test comparing 0 with 8 and 24 hours respectively
[4] n=9 volunteers due to faecal sample size available for an individual volunteer.

Table 6.12 Changes in SCFA levels for pectin fermentations at 8 and 24 hours.

Timepoint	Experimental condition	C2[1,2]	C3	C4	Total[3]	P value[4]	Percentage change from control[5]	P value[6]
8 hours	**Pectin only**	39.12 (12.47)	9.52 (4.55)	5.27 (2.19)	53.75 (17.00)			
	O3FA 1 mcg/ml & pectin[7]	37.25 (12.61)	8.94 (4.54)	4.99 (2.08)	51.19 (17.71)	0.87	0.43 (22.65)	0.96
	O3FA 25 mcg/ml & pectin[7]	40.08 (15.17)	9.11 (3.97)	5.54 (2.52)	54.62 (19.01)	0.60	6.97 (27.63)	0.47
	O3FA 50 mcg/ml & pectin	40.41 (12.23)	9.97 (5.18)	5.52 (2.35)	55.90 (17.20)	0.60	7.30 (22.57)	0.09
24 hours	**Pectin only**	58.77 (10.49)	16.03 (4.51)	10.42 (2.37)	83.37 (12.01)			
	O3FA 1 mcg/ml & pectin[7]	61.27 (10.53)	16.42 (4.13)	10.48 (2.47)	88.17 (11.77)	0.004	6.43 (4.99)	0.004
	O3FA 25 mcg/ml & pectin[7]	57.75 (12.57)	16.46 (5.60)	10.56 (2.56)	84.77 (13.71)	0.66	2.67 (12.86)	0.55
	O3FA 50 mcg/ml & pectin	58.63 (9.81)	15.41 (4.81)	10.74 (2.27)	84.78 (11.58)	0.31	1.92 (5.26)	0.15

[1] Mean (standard deviation).
[2] SCFA levels measured in mmol/L.
[3] Total SCFA level is the sum of acetate (C2) + propionate (C3) + butyrate (C4).
[4] Paired t-test comparing total SCFA for each experimental condition (concentration of O3FAs) with pectin only at either 8 or 24 hours.
[5] Percentage change (%) is the total SCFA level divided by the total SCFA level for the pectin only reaction, (standard deviation).
[6] Paired t-test comparing percentage change for each experimental condition (concentration of O3FAs) with wheat bran at either 8 or 24 hours.
[7] n=9 volunteers due to faecal sample size available for an individual volunteer.

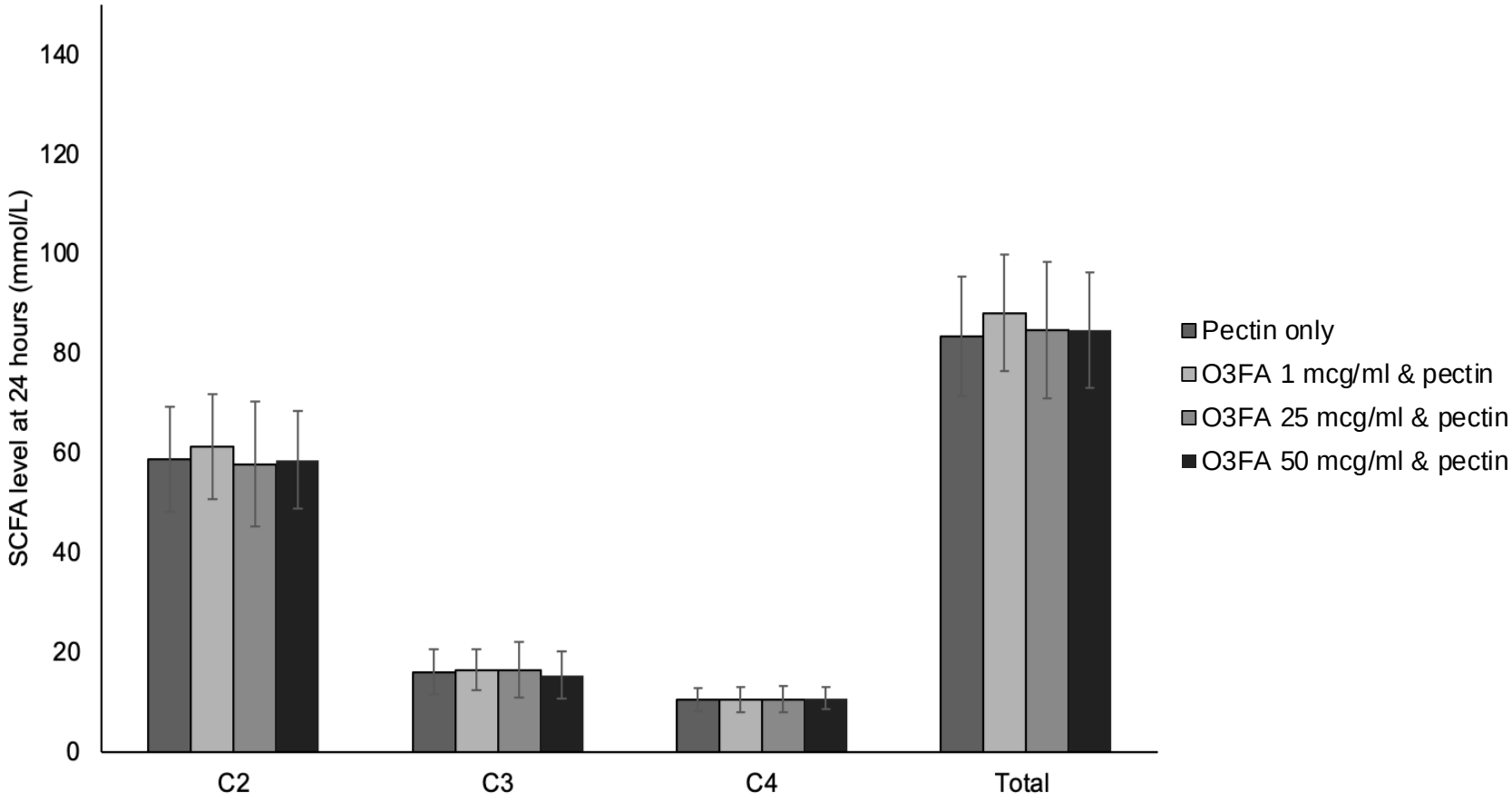


Figure 6.13 SCFA levels of O3FA & pectin fermentations at 24 hours.

Mean level show, error bars show standard deviation. Acetate (C2) + propionate (C3) + butyrate (C4), total SCFA is the sum of C2+C3+C4.

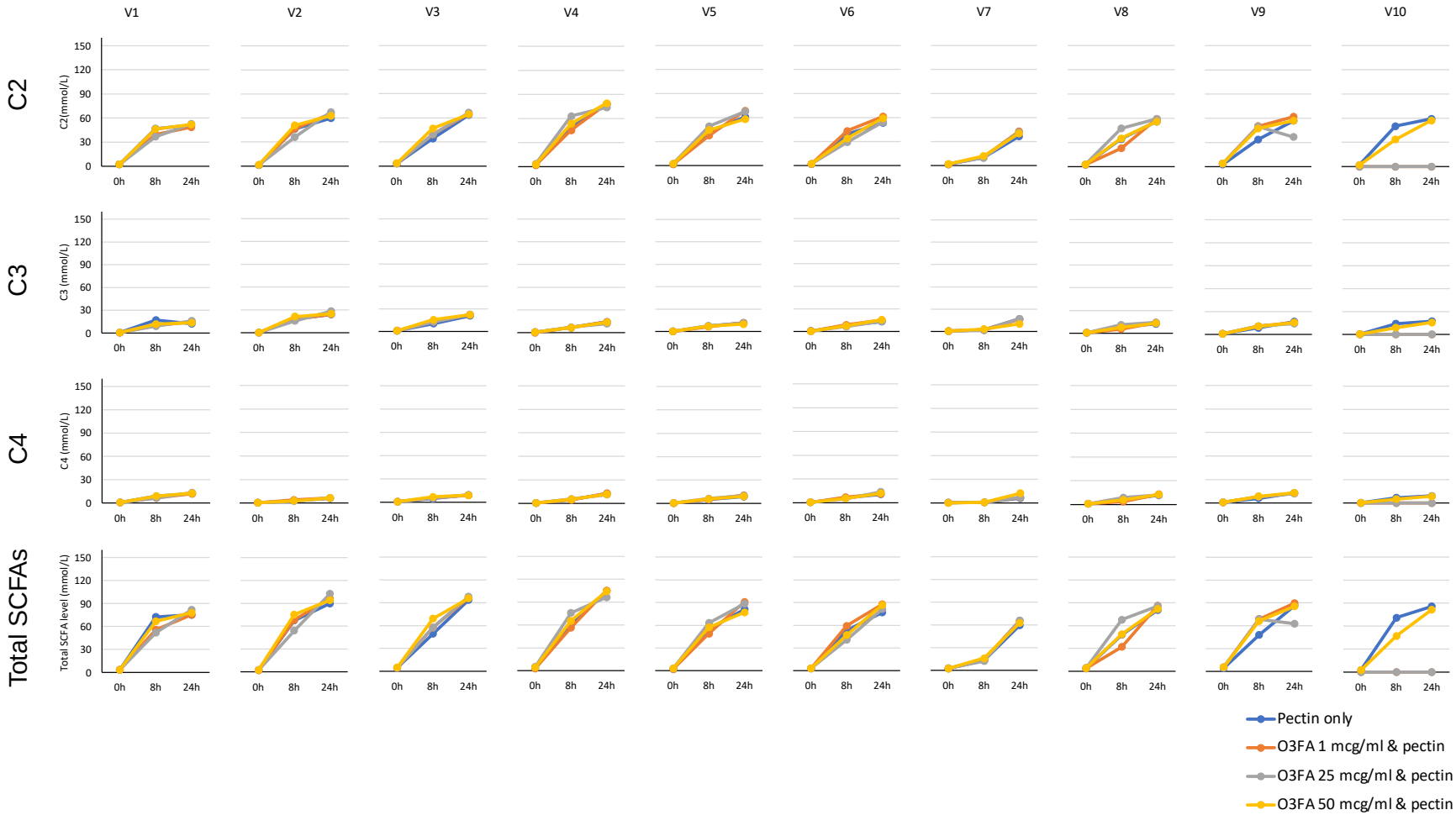


Figure 6.14 Individual and total SCFA levels measured at 0, 8 and 24 hours in O3FA & pectin fermentations according to individual volunteers.

Individual SCFAs C2, C3 & C4, in addition to the total SCFA Level at each timepoint shown for each volunteer V1 to V10.

6.3.7 Comparisons between different dietary fibres

The fermentability of different dietary fibres was reflected in the pH results (**figure 6.15**). The most significant decrease in pH was observed in the fermentations containing inulin, a soluble non-viscous fibre, followed by pectin a soluble viscous fibre (mean pH at 24 hours 5.44 [0.21]) and wheat bran an insoluble fibre, which was much less fermentable (mean pH at 24 hours 6.38 [0.26]). These findings were also reflected in the colour changes of the fermentation reactions (**figure 6.16**).

The pH findings reflected the total SCFA levels at 24 hours. The fermentations containing inulin at the 0.02 mg/ml concentration had the highest SCFA level, followed by pectin, then the inulin 0.01 mg/ml concentration and wheat bran (**figure 6.17**).

The only dietary fibre to display a concentration response increase in total SCFA level at 24 hours was inulin 0.01 mg/ml concentration, which was also reflected in the percentage change from the inulin 0.01 mg/ml only reaction (**figures 6.17 & 6.18**). There was a negative association between wheat bran and O3FAs on total SCFA production. This negative association was also observed when pectin was added to higher O3FA concentrations (25 and 50 mcg/ml), but not observed for the 1 mcg/ml concentration & pectin (**figures 6.17 & 6.18**).

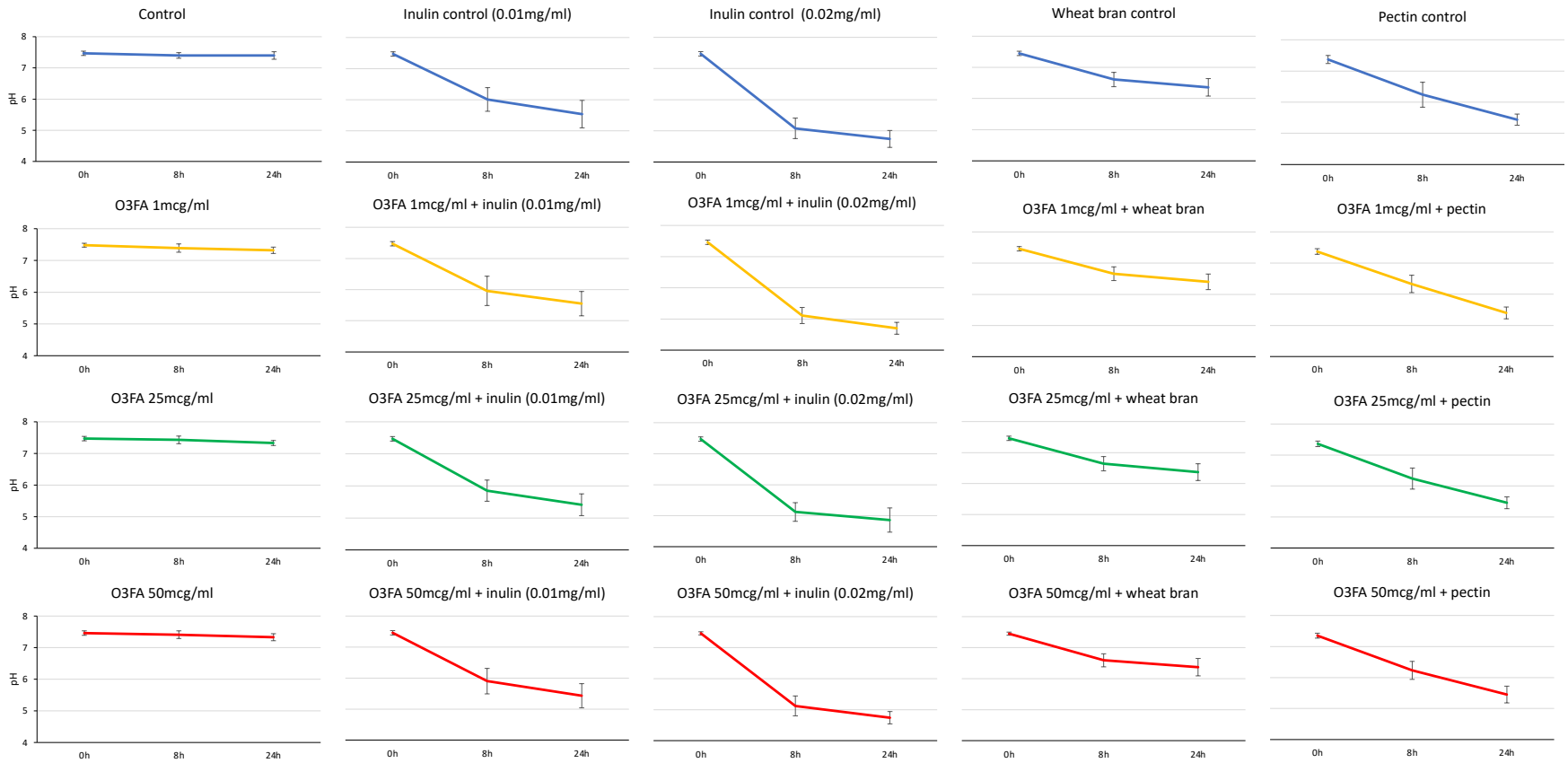


Figure 6.15 pH changes according to experimental conditions and timepoints.

Mean pH shown, error bars show standard deviation. n=10 for all experimental conditions except for n=9 for O3FA 1 mcg/ml & pectin and O3FA 50 mcg/ml & pectin

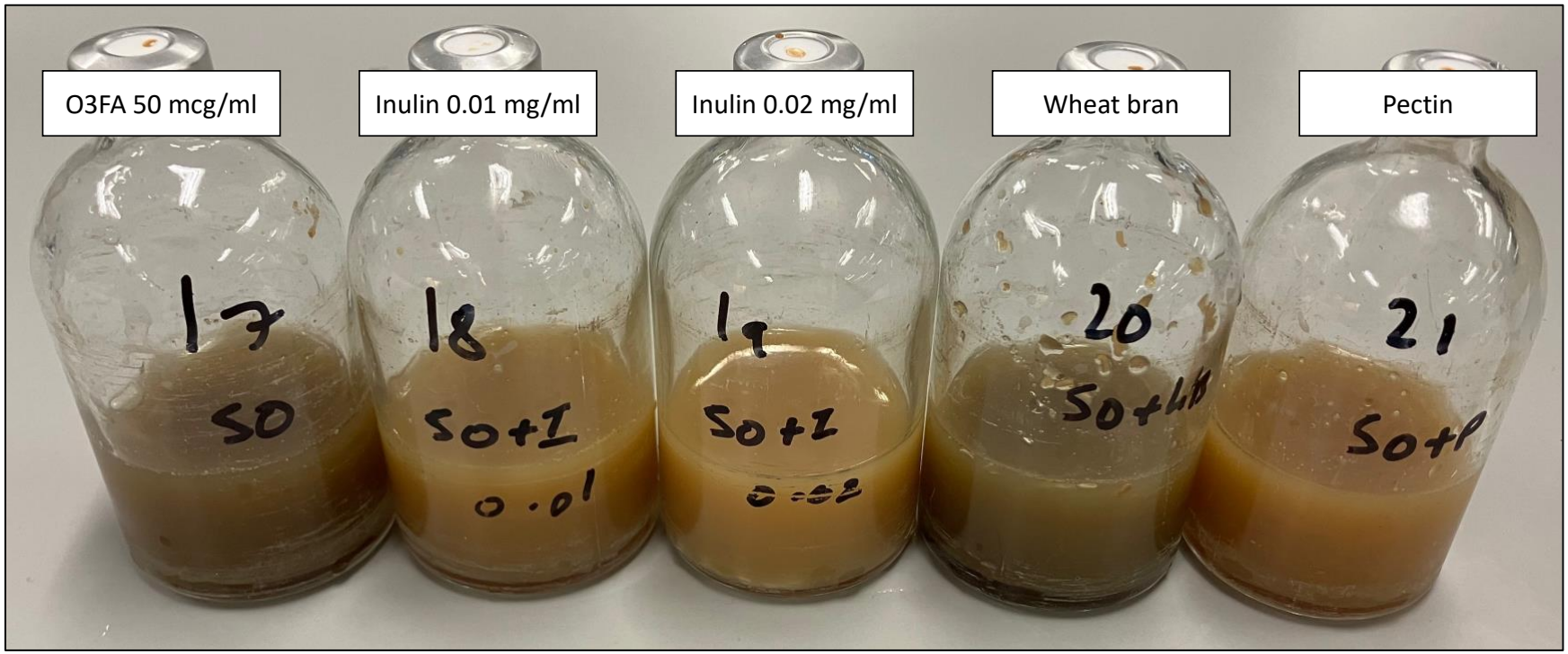


Figure 6.16 Fermentation reactions containing O3FA 50 mcg/ml and different dietary substrates.

The fermentability of the dietary fibre within the fermentation is reflected in colour changes seen within the reactions. The fermentation reaction containing O3FA (50 mcg/ml) without any dietary fibre is the darkest in colour (far left) with the fermentation containing O3FA & wheat bran being slightly lighter, followed by the O3FA & pectin and the O3FA & inulin fermentation reactions being the lightest in colour.

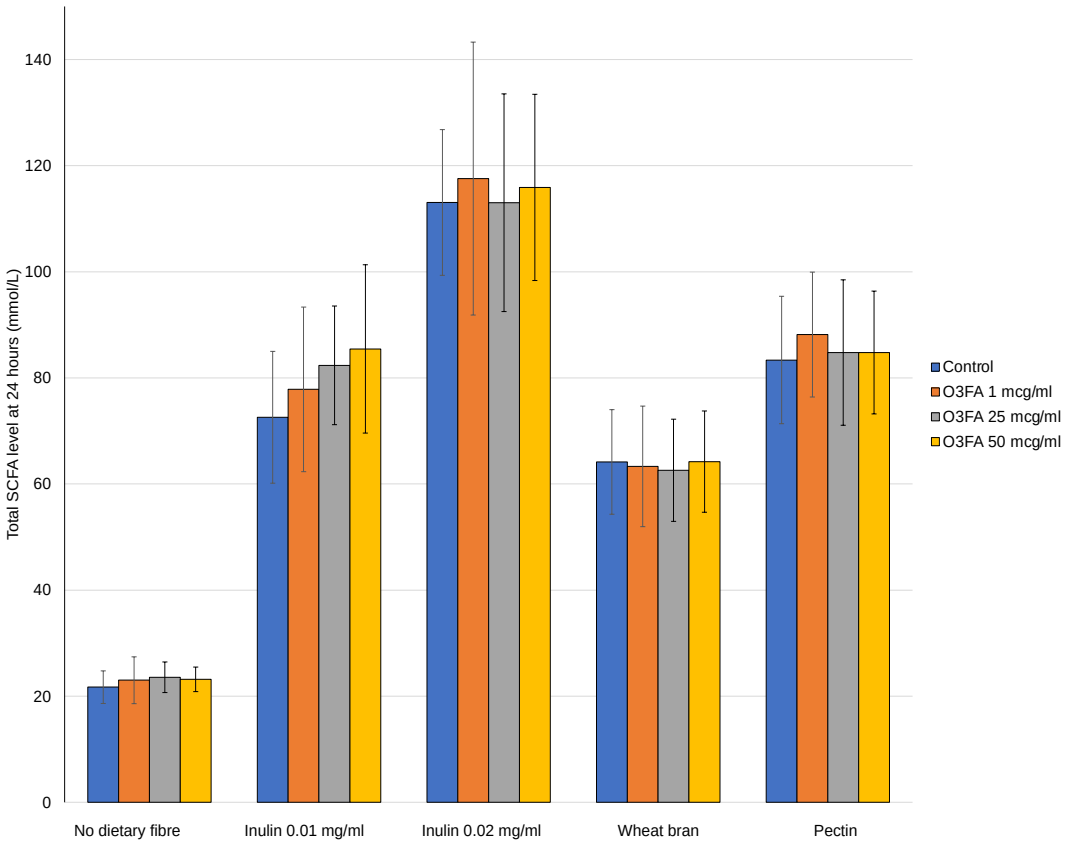


Figure 6.17 Total SCFA level at 24 hours according to increasing O3FA concentrations and different dietary fibres tested.

Mean total SCFA level shown, error bars show standard deviation. Control is described as either the fermentation model on its own inoculated with the faecal slurry, or the dietary fibre described on its own without the addition of O3FAs.

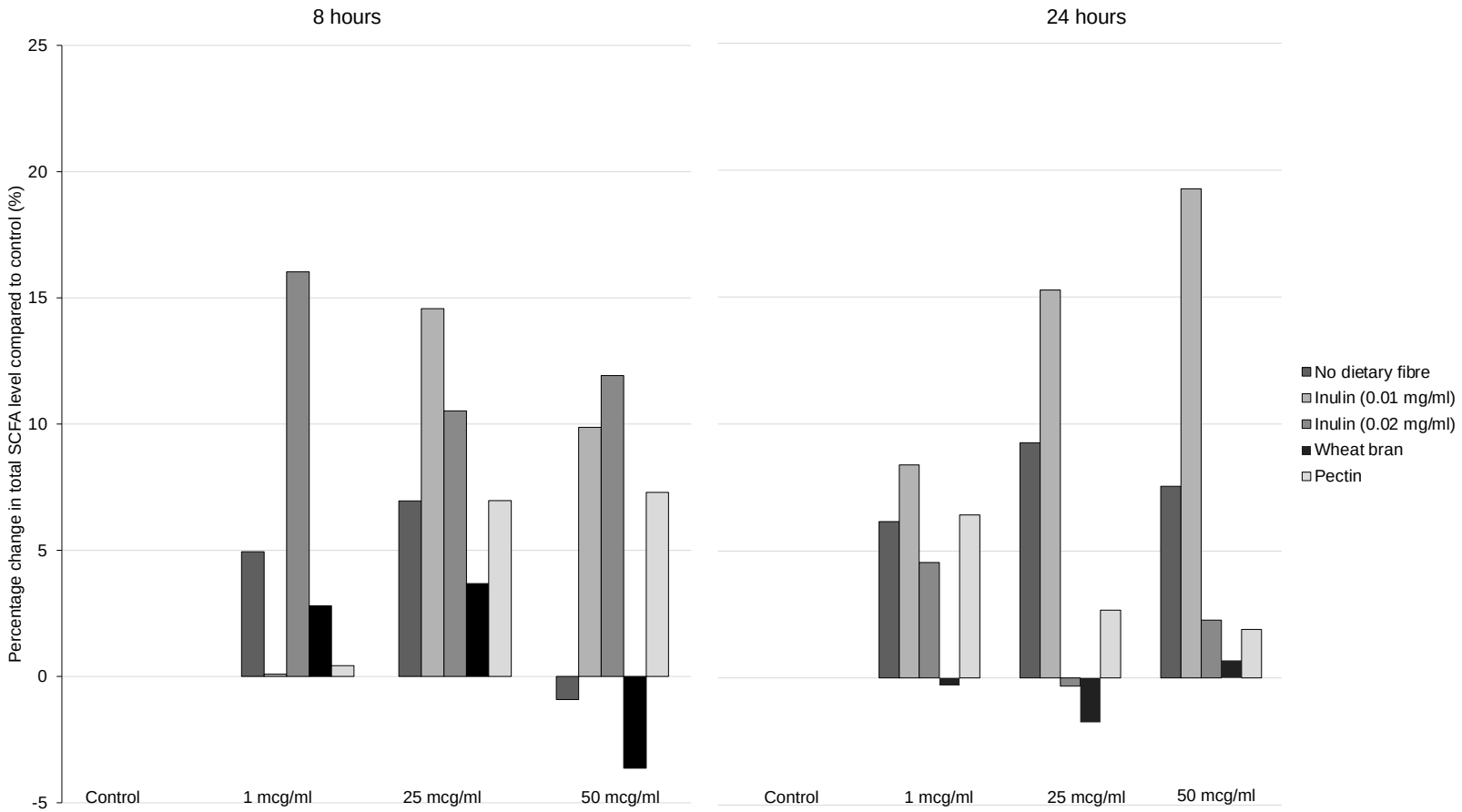


Figure 6.18 Percentage change in total SCFA level at 8 and 24 hours according to increasing O3FA concentrations and different dietary fibres.

Mean percentage change shown compared to control. Control is described as either the fermentation model on its own inoculated with the faecal slurry, or the dietary fibre described on its own without the addition of O3FAs.

The different dietary fibres were associated with the increased production of specific SCFAs, this is important as specific SCFAs are associated with anti-CRC activity, mainly butyrate (**chapter 1, section 1.5.2**).

The total SCFA level at 8 hours (**table 6.13 and figure 6.19)** and 24 hours (**table 6.14 and figure 6.20**) and the ratios of acetate, propionate and butyrate are shown. Conversion of acetate to butyrate was observed from 8 to 24 hours for the reactions containing inulin and wheat bran.

Pectin was associated with the largest proportional increase in acetate at 24 hours (mean ratio 69%), and the lowest ratio of butyrate (12%). Although the SCFA levels produced from the fermentation of wheat bran were lowest compared to the other dietary fibres, wheat bran was associated with an increase in the ratio of butyrate, comparable to inulin at the lower concentration (0.01 mg/ml), 17% for wheat bran *Vs.* 18% for inulin 0.01mg/ml. Inulin at the lower concentration (0.01 mg/ml) was associated with a larger ratio of propionate at 24 hours (mean ratio 30%) (**table 6.14**). With regards to the differences in O3FA concentrations tested with the different dietary fibres. There was a dose response increase in the proportion of butyrate produced when inulin (0.01 mg/ml) was added to the reactions (**figure 6.20**). Overall, the greatest increase in the proportion of butyrate was observed when inulin was tested at a higher concentration (0.02 mg/ml), mean ratio 28%.

Table 6.13 Ratios of individual SCFAs making up total SCFA level at 8 hours.

Fibre tested	Experimental conditions	Total SCFA level[1]	% Ratios of individual SCFAs[2]		
			C2	C3	C4
No dietary fibre	Control	15.67 (3.55)	69	17	14
	O3FA 1 mcg/ml	16.53 (4.19)	69	17	14
	O3FA 25 mcg/ml	16.66 (3.64)	69	18	13
	O3FA 50 mcg/ml	15.57 (3.92)	69	18	14
Inulin 0.01 mg/ml	Inulin only (0.01 mg/ml)	52.96 (14.08)	61	26	13
	O3FA 1 mcg/ml & inulin (0.01 mg/ml)	54.50 (16.38)	62	25	13
	O3FA 25 mcg/ml & inulin (0.01 mg/ml)	61.33 (15.55)	61	26	14
	O3FA 50 mcg/ml & inulin (0.01 mg/ml)	58.81 (15.47)	61	25	15
Inulin 0.02 mg/ml	Inulin only (0.02 mg/ml)	71.80 (19.63)	60	23	17
	O3FA 1 mcg/ml & inulin (0.02 mg/ml)	77.98 (24.30)	62	22	16
	O3FA 25 mcg/ml & inulin (0.02 mg/ml)	74.83 (21.66)	61	23	16
	O3FA 50 mcg/ml& inulin (0.02 mg/ml)	75.72 (22.09)	61	23	16
Wheat bran	Wheat bran only	44.95 (9.94)	67	19	13
	O3FA 1 mcg/ml & wheat bran	45.61 (8.09)	67	20	13
	O3FA 25 mcg/ml & wheat bran	45.98 (8.21)	67	20	13
	O3FA 50 mcg/ml & wheat bran	43.08 (8.95)	68	18	14
Pectin	Pectin only	51.84 (16.86)	73	18	9
	O3FA 1 mcg/ml & pectin	51.19 (17.71)	73	17	10
	O3FA 25 mcg/ml & pectin	54.62 (19.01)	73	17	10
	O3FA 50 mcg/ml & pectin	56.88 (17.95)	73	18	10

[1] Mean (standard deviation)
[2] Ratios of C2, C3 & C4 expressed as percentage (%) of total SCFA level at 24 hours

Table 6.14 Ratios of individual SCFAs making up total SCFA level at 24 hours.

Fibre tested	Experimental conditions	Total SCFA level[1]	% Ratios of individual SCFAs[2]		
			C2	C3	C4
No dietary fibre	Control	21.70 (3.07)	63	22	15
	O3FA 1 mcg/ml	23.01 (4.41)	63	23	15
	O3FA 25 mcg/ml	23.57 (2.88)	63	23	15
	O3FA 50 mcg/ml	23.18 (2.31)	62	23	15
Inulin 0.01 mg/ml	Inulin only (0.01 mg/ml)	72.57 (12.43)	55	29	16
	O3FA 1 mcg/ml & inulin (0.01 mg/ml)	77.83 (15.51)	53	29	18
	O3FA 25 mcg/ml & inulin (0.01 mg/ml)	82.36 (11.18)	52	30	18
	O3FA 50 mcg/ml & inulin (0.01 mg/ml)	85.46 (15.87)	53	29	19
Inulin 0.02 mg/ml	Inulin only (0.02 mg/ml)	113.06 (13.73)	47	27	26
	O3FA 1 mcg/ml & inulin (0.02 mg/ml)	117.56 (25.72)	50	26	24
	O3FA 25 mcg/ml & inulin (0.02 mg/ml)	113.01 (20.51)	45	27	28
	O3FA 50 mcg/ml& inulin (0.02 mg/ml)	115.89 (17.55)	48	27	25
Wheat bran	Wheat bran only	64.15 (9.85)	62	21	17
	O3FA 1 mcg/ml & wheat bran	63.31 (11.36)	62	21	17
	O3FA 25 mcg/ml & wheat bran	62.58 (9.63)	61	22	17
	O3FA 50 mcg/ml & wheat bran	64.22 (9.55)	60	23	17
Pectin	Pectin only	83.37 (12.01)	69	19	12
	O3FA 1 mcg/ml & pectin	88.17 (11.77)	69	19	12
	O3FA 25 mcg/ml & pectin	84.77 (13.71)	68	20	13
	O3FA 50 mcg/ml & pectin	84.78 (11.58)	69	18	13

[1] Mean (standard deviation)
[2] Ratios of C2, C3 & C4 expressed as percentage (%) of total SCFA level at 24 hours

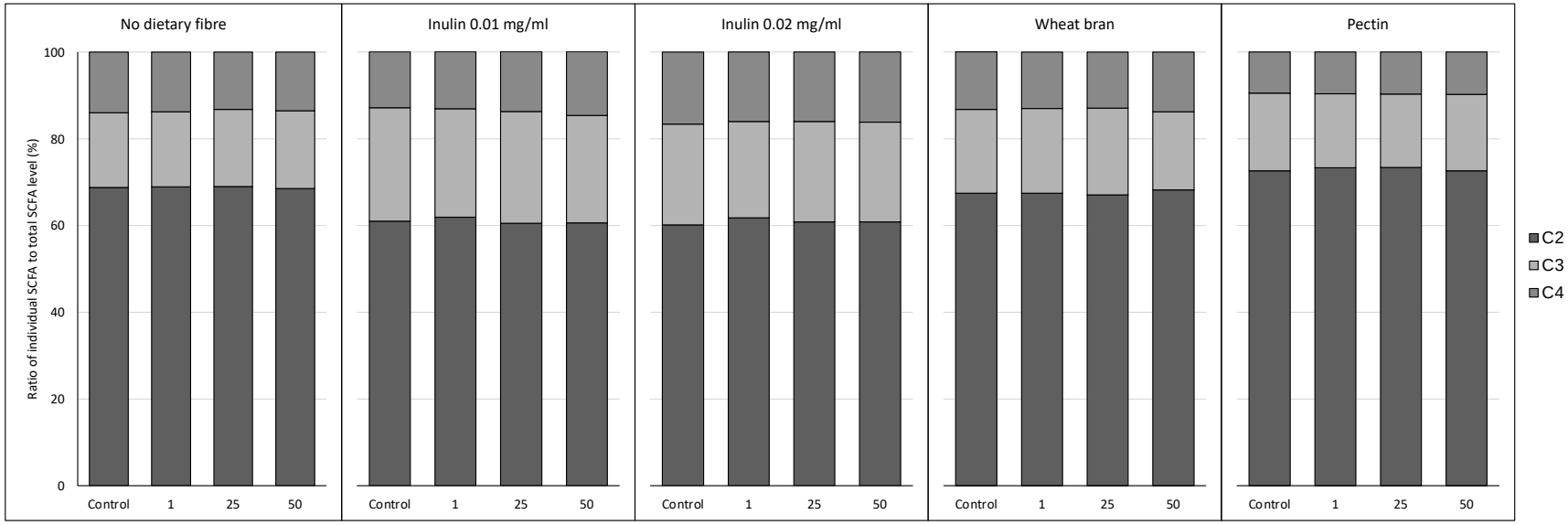


Figure 6.19 Ratios of individual SCFAs constituting the total SCFA level at 8 hours for different dietary fibres and increasing O3FA concentrations.

Acetic (C2), propionic (C3) and butyric (C4) acid.

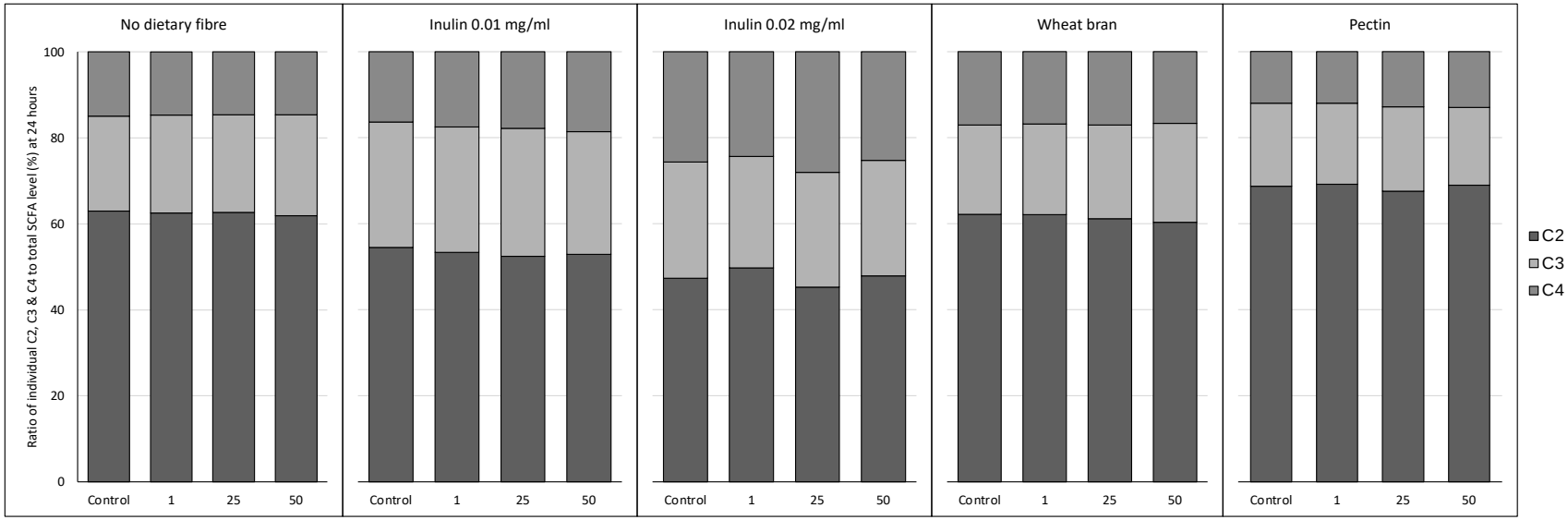


Figure 6.20 Ratios of individual SCFAs constituting the total SCFA level at 24 hours for different dietary fibres and increasing O3FA concentrations.

Acetic (C2), propionic (C3) and butyric (C4) acid.

6.4 Discussion

This study demonstrated that O3FAs are associated with an increase in total SCFA production within an *in vitro* colonic fermentation model. In addition, I discovered a specific interaction between O3FAs (50 mcg/ml) & inulin (0.01 mg/ml) on increasing total SCFA production within the model at 24 hours.

The fermentation model used within the study was based on the batch static *in vitro* fermentation model developed by Edwards et al. 1996 (299). It is an established model with which to examine SCFA production (300, 303, 304). Batch static fermentation models create anaerobic conditions ranging from closed bottles to controlled reactors, these models are inoculated using a faecal sample from a single donor or mixed samples from several donors and are run for up to 24 hours. The advantages of batch static *in vitro* models are the ability to measure metabolites including SCFAs, control the temperature within a water bath and that they are relatively inexpensive to run. However, as these models are static, they are limited by substrate depletion and the accumulation of microbial metabolites.

Overall, the batch static *in vitro* fermentation model reflects the proximal colon and therefore the effects observed are limited to this specific region of the colon. It is therefore possible that dietary fibres such as pectin and wheat bran, which are fermented more slowly, may increase SCFA and have more effects in the distal colon. In addition, there are limitations in relation to the type of inoculum used within the model. Despite the model being representative of the proximal colon and using concentrations of O3FAs which I knew were able to reach the proximal colon, I used a faceal sample as the inoculum, this is most representative of the rectal microbiome and compositionally different to the microbiota of the proximal colon as it is likely

to contain less SCFA producing bacteria and have lower microbial diversity (305). A faecal inoculum was chosen as a pragmatic solution with regards to the practicalities and feasibility of the experiments, as using an inoculum from the proximal colon would require invasive procedures which are likely to require bowel preparation. Alternatives such as ileostomy fluid could have been used, however there are potential difficulties in sourcing volunteers and/or they are likely to have other health conditions which may effect their microbial composition. Therefore the use of fecal inoculum may limit my findings.

In comparison to batch *in vitro* fermentation models, alternative, more sophisticated models exist, such as dynamic continuous fermentation models which aim to create a stable microbial ecosystem by controlling conditions such as pH and allow for evaluation over a longer duration. Such models can be single chamber (single stage, stimulating the proximal colon) or made of multiple chambers (multistage) reflecting the anatomical regions of the colon (proximal, transverse and distal colon) (306). This type of model is based on the work of Gibson et al. 1988 who initially described a three-stage continuous culture system which consisted of three chambers each reflecting the pH and substrate availability within the different anatomical regions of the colon (307). The Gibson model was validated against measurements of the colonic content of sudden death victims (307, 308). Overall, these models are more time intensive, taking longer to set up and may take over 7 days to achieve a steady state. They therefore do not allow for the high throughput of experiments and are much more costly, requiring specific expertise (309). Commercial models are also available such as: the TNO (Netherlands Organisation for Applied Scientific Research) *in vitro*

model of the colon (TIM-2) which is a computer controlled model and the Simulator of Human Intestinal Microbial Ecosystem (SHIME®) (309).

O3FAs interventions have been examined within the dynamic SHIME® model. One study examined the effects of EPA and DHA over a 48-hour period using a faecal sample from a healthy volunteer. The dose of EPA and DHA (dissolved in ethanol) were tested at different concentrations within the fermentation vessels (estimated dose delivered to the colon for DHA was 60, 300 and 1500 mg [concentrations of 0.11, 0.54 and 2.68 mg/ml respectively] and for EPA 360, 1800 and 9000 mg [concentrations of 1.29, 6.43 and 32.14 mg/ml respectively]) (310). An increase in total SCFA production at both 24 and 48 hours above control was observed, however there were differences regarding increasing EPA concentrations with the largest increase in total SCFA concentration observed for the 360 mg EPA dose (1.29 mg/ml in the fermentation vessel). The total SCFA level (acetate, propionate, and butyrate) at 24 hours was 60.6, 60.4 and 57 mmol/L for the increasing DHA concentrations and 61.7, 57.6 and 41 mmol/L for increasing EPA doses, respectively (310). These data display total SCFA levels much higher than those within my study (mean total SCFA level ranged from 21.7 to 23.2 mmol/L) and may reflect the dynamic model used or could be due to the much higher DHA and EPA concentrations tested within their study. They similarly found a decrease in total SCFA level at higher EPA concentrations, potentially reflecting non effective toxicity of high O3FA concentrations within the model. With regards to the individual SCFAs produced there was, a proportional increase in propionate followed by butyrate; I also found higher levels of propionate compared to butyrate at 24 hours (310).

Another study used a similar model (Mucosal Simulator of the Human Intestinal Microbial Ecosystem [M-SHIME®]) to examine the effects of a 7-day intervention of EPA and DHA enriched fish oil (990mg EPA and 990mg DHA esterified in triacylglycerols) which were added to the model, using faecal samples from a single healthy volunteer. They reported an increase in the production of propionate following 6 days of fermentation compared to control (311). Despite the levels of total SCFAs within the SHIME® model studies being much higher than those observed within my study, I also observed an increase in total SCFA above control at a specific O3FA concentration (25 mg/ml) and a decrease at much higher concentrations (100 & 200 mcg/ml), however my intervention was of mixed EPA and DHA. It would be interesting to repeat my study examining the effects of EPA and DHA alone. I also reported a specific increase in the proportion of propionate production in the O3FA only fermentation reactions, in keeping with the findings using the SHIME® models (310, 311).

I found a specific interaction between O3FA and inulin when added to the model. Although the mean difference in total SCFA concentration above the inulin only reaction for O3FA 50 mcg/ml & inulin 0.01 mg/ml was 12.9 mmol/L, some individuals had levels above the inulin only reaction as high as 40.0 mmol/L and another volunteer had a negative relationship with -13.6 mmol/L below control. These findings suggest a host and/or microbiome factor determining SCFA production. These SCFA findings have been examined in parallel with the changes in the microbiome within the model (**chapter 7**). The inter-individual variability within the study could be explained by multiple factors. The participants reflected those of different sex, age, BMI, and ethnicities. Diet and fibre intake were not controlled prior to faecal sample collection, this was in order to maximise availability of

volunteers and to ensure that the study was not too onerous for participants. However, this could have influenced the variability seen and should be controlled for in future studies.

The mean increase in total SCFA level that I observed is in keeping with other studies from the Edwards group that used the same static batch *in vitro* fermentation model. The catabolism of rutin (a form of quercetin) in the presence of inulin, pectin, and wheat bran at three different concentrations (0.8, 1.7 and 3.3 g / 50ml), was examined using a similar methodology (300). Although the concentrations of inulin within my study differed, the most comparable was the 0.8 g / 50ml (0.016 mg/ml) concentration which was in-between the 0.01 and 0.02 mg/ml concentrations I tested. Although they did not report the raw data, when inulin (0.016 mg/ml) was added to the model on its own, they observed a similar mean reduction in pH, that I observed for both inulin concentrations tested at 0.01 and 0.02 mg/ml. I also observed similar levels of C2, C3, C4 and total SCFA production when inulin, pectin and wheat bran were tested at 0.8 g / 50ml within their study (300). These findings reflect the reliability of the model.

Although I observed an increase in mean total SCFA production associated with O3FAs & inulin, it is difficult to interpret the biological significance of these findings and how they may translate clinically. This is due to limitations of the model including substrate depletion, the build of metabolites within the model and the use of a faecal sample as a proxy for the colonic microbiome; therefore an *in vivo* study is required to further examine the relationship.

Several animal models have examined dietary O3FA interventions in rats and mice, demonstrating an increase in faecal SCFA production above the

control diet (312, 313). The effects of O3FAs on SCFA production may be related to the composition and type of background diet consumed. In a study of male Wistar rats fed either a control or cafeteria diet, consisting of high-energy ultra-processed foods representative of the modern western diet for 20 weeks, both supplemented with O3FAs (500 mg/kg/day, 10% EPA, 50% DHA) between weeks 16 and 20, plasma levels of SCFA were measured, in which there were no effects of O3FA supplementation in the control diet (314). However those supplemented with O3FAs in the cafeteria diet had a significant decrease in butyrate and isobutyrate concentrations (314). I observed similar rates of butyrate production according to increasing O3FA concentrations, with no significant difference between O3FAs and the control.

Two studies examined the effects of plant derived O3FAs, one looked at the effects of ALA-rich flaxseed oil on the development of atherosclerosis in ApoE-/- mice and measured faecal SCFAs following 12 weeks of intervention with a high fat diet with and without flaxseed oil, in addition to a control group who were fed a normal diet (315). They found that compared to the high fat diet without flaxseed oil group, those supplemented with ALA-rich flaxseed oil had statistically significant higher levels of acetate, propionate, valerate, isovalerate and isobutyrate. However, when compared to mice fed the non-high fat control diet, an increase in isovaleric and isobutyric acid only was observed (315). Another study examined flaxseed oil in rats to reduce the severity of type 2 diabetes in which there was a significant increase in SCFAs which were negatively correlated with inflammatory cytokines (316).

It is difficult to compare the SCFA levels I observed with *in vivo* intervention studies as measurement of faecal SCFAs within such studies may not be

representative of SCFA production, as they are absorbed and metabolically used (317). One study measured plasma SCFAs in healthy human volunteers taking part in a RCT exploring either O3FA (500 mg/day, 165 mg EPA and 110mg DHA) (n=34) or inulin (20 g/day) (n=30) interventions for 6 weeks. It demonstrated a statistically significant increase in plasma iso-butyrate and iso-valerate levels following the O3FA intervention, however the total SCFA data were not shown (121). The inulin intervention was also associated with an increase in butyrate. The differences in SCFA levels pre- and post- treatment for the O3FA intervention were low, 2.69 mmol/L for iso-butyric acid and 1.08 mmol/L for isovaleric acid and were 3.23 mmol/L for butyrate in the inulin intervention group (121). It is important to consider the biological samples (faces, plasma) used to quantify SCFA in any future studies examining O3FA intervention *in vivo*.

The novel aspect of my study was the investigation of the interaction of O3FAs with different types of dietary fibre. I demonstrated that SCFA production was dependent on the concentration of O3FAs and the type and concentration of the fibre with which it was fermented. I found a positive association with inulin at a concentration of 0.01 mg/ml. The interaction between inulin and O3FA should be further explored.

A recent study found that mice fed a high soluble fibre diet of 20% inulin were found to have an increase in tumour number and load in azoxymethane treated and $APC^{min/+}$ mice (318). They reported an increase in faecal butyrate and a specific dose response for the increasing concentration of inulin diet (5, 10, 15, 20%) and colorectal tumorigenesis (318). The authors suggested that their findings could be due to the reduction in microbial diversity associated with inulin which may contribute to CRC pathogenesis.

An alternative hypothesis is that the findings related to '*butyrate paradox*', (**chapter 1, section 1.5.2**) meaning that the ability for butyrate to induce cell proliferation or apoptosis is dependent on cell type and butyrate concentration and therefore butyrate may promote CRC in some circumstances (318, 319). It is important that the interaction I observed between inulin and O3FAs within the *in vitro* model is examined *in vivo*, this is discussed in greater detail in **Chapter 8**.

I reported a negative association between O3FAs and wheat bran within the model. This relationship has also been seen in animal studies exploring O3FA interventions and other insoluble fibres. An animal study in Sprague Dawley rats examined the effects of resistant starch (high amylose maize starch) or alpha cellulose with fish oil (100 g/kg of tuna fish oil) on SCFA production compared to a control of sunflower oil. Fish oil was associated with a decrease in total SCFA level compared to sunflower oil for both fibres tested (320). Another animal study examined O3FAs (mixed EPA/DHA, 0.8 ml oil per kg body weight) with and without the insoluble fibre buckwheat, in the form of D-fagomine (mean daily dose 4.6 mg per 100 g body weight) in male Sprague-Dawley rats. It reported statistically significant lower levels of faecal total SCFAs, in addition to acetate and isobutyrate, in both the O3FA and O3FA & D-fagomine groups compared to control (321, 322). The introduction of the fibre appeared to have no effect on total SCFA levels compared to O3FA alone. The low levels of acetate observed may have reflected absorption of acetate by the colon rather than production (317).

The differences observed between the different fibres may be related to a number of different factors. This could be due to the model itself which is representative of the proximal colon and therefore may reflect the findings

observed for inulin, which is a soluble non-viscous fibre which is highly fermentable, whereas the insoluble fibre such as wheat bran may display effects in the distal colon where it has had greater time exposure to fermentation within the colon or it may not contribute to SCFA production at all as it is more likely to have mechanical effects within the colon by contributing stool bulk and therefore may not interact with O3FAs or have a negative effect by reducing their bioavailability. I observed higher concentrations of total SCFA production when pectin was added to the model compared to inulin, in addition to an interaction with O3FAs at a lower concentration (1mcg/ml), the viscous nature of pectin may account for these findings. Overall, the data suggests that there is an interaction between O3FAs and inulin which requires further investigation.

6.5 Conclusion

This study demonstrated that O3FAs (50 mcg/ml) & inulin (0.01 mg/ml) within an *in vitro* colonic fermentation model increased SCFA production above the inulin only reaction. The effects of the O3FA & inulin intervention on the microbiome are described and discussed in **chapter 7**.

This study suggests that O3FAs have the potential to modulate the gut microbiota in favour of SCFA production as a potential anti-CRC mechanism, through a probiotic-prebiotic relationship between O3FA & inulin. This should prompt a future *in vivo* human study examining this relationship.

Chapter 7 Investigating the effect of omega-3 polyunsaturated fatty acids on the gut microbiome within an *in vitro* colonic fermentation model

7.1 Introduction

This chapter describes the secondary analysis of the study examining the interaction between O3FAs and SCFA production described in **chapter 6**. It tests the hypothesis, is O3FA exposure within an *in vitro* colonic fermentation model associated with changes in the bacterial content within the model? The changes in bacterial content within the model were measured using shotgun metagenomics. The aims of the study are outlined in **chapter 2, section 2.2**.

7.2 Methods

7.2.1.1 Volunteers included in the microbiome analysis

Due to financial constraints, it was not possible to analyse the microbiome within all samples, for each experimental condition and volunteer. Therefore, volunteers were selected based on the increase in SCFA production compared to control or the fibre only reaction. Samples were chosen to reflect the O3FA 50 mcg/ml concentration with and without inulin (0.01 mg/ml), as this was the experimental condition for which the study was powered to detect a change in SCFA production. The samples chosen for sequencing were: the control at 0 and 24 hours, the inulin only (0.01mg/ml) reaction at 24 hours, O3FA 50 mcg/ml at 24 hours, and O3FA 50 mcg/ml & inulin 0.01 mg/ml at 24 hours. The volunteers chosen for microbiome analysis were V1, V2, V5, V6, V8 and V9 (**figure 6.8**).

7.2.2 Microbiome analysis

The two commonly used methodologies for microbial sequencing are 16S ribosomal RNA (rRNA) and shotgun metagenomics. 16S rRNA is a form of gene amplicon sequencing that uses the 16S rRNA gene as a marker gene. The 16S rRNA gene encodes the 30S subunit of prokaryotic ribosomes in most bacteria. 16S rRNA contains nine variable regions that can be targeted for amplification. In contrast to marker gene analysis (16S rRNA), metagenomics attempts to sequence all or most of the genes within a sample. The term '*shotgun*' refers to the technique by which all DNA is randomly sheered into different fragment sizes suitable for high-throughput sequencing and then assembled into longer continuous sequences (48).

Shotgun metagenomics has been shown to be more powerful at identifying less abundant taxa than 16S rRNA sequencing. It can identify microorganisms at the species (sometimes strain) level and identifies the functional potential of sequenced DNA. In comparison, 16S rRNA identifies microorganisms at the genus (sometimes species) level (323, 324). The microbiome analysis described in this chapter was undertaken using shot gun metagenomics.

7.2.2.1 DNA extraction

The samples collected from *the in vitro* colonic fermentation model underwent DNA extraction. I performed the DNA extractions in Professor Quirke's Laboratory in the Leeds Institute of Medical Research, using a Qiagen QIAamp® PowerFecal® Pro DNA kit.

Fermentation samples were removed from the -80°C freezer. Prior to sample preparation, the control samples collected at 0 hours, required dilution to reflect the concentration of the fermentation samples at 24 hours. The samples collected at 0 hours were of the neat faecal slurry, of which 5 ml was placed into a 43 ml of fermentation media and 2 mls reducing solution to create an overall reaction volume of 50 ml. 500 mcl of the neat faecal slurry at 0 hours was diluted with 4.5 ml of Nuclease-Free water and vortexed to reflect the concentrations of the fermentation samples collected at 24 hours.

The samples were prepared for DNA extraction by adding 200 mcl of faecal slurry to 800 mcl of Solution CD1 within a PowerBead Pro Tube. This was then placed in a TissueLyser II for 10 minutes to allow for bacterial cell lysis. The PowerBead Pro Tubes were then centrifuged at 14,000 rpm for 1 minute. 700 mcl of the supernatant was transferred into a clean 2 ml Microcentrifuge tube and 200 mcl of Solution CD2 was added. This was then vortexed for 5 seconds and centrifuged at 14,000 rpm for 1 minute, 600 mcl of the supernatant was then transferred into a clean microcentrifuge tube and 600 mcl of Solution CD3 was added and then vortexed for 5 seconds. This allowed for the binding of DNA to the MB Spin Column filter membrane as 650 mcl of the lysate was loaded onto a MB Spin Column and centrifuged at 14,000 rpm for 1 minute. The follow through was discarded, the MB Spin Column was placed in a clean Collection Tube and the rest of the lysate was loaded onto the MB Spin Column which was centrifuged at 14,000 rpm for l minute. The follow through was then discarded and the MB Spin Column was placed in a clean Collection Tube and 500 mcl of Solution EA was added to the MB Spin Column which was centrifuged at 14,000 rpm for 1 minute. This step removed the protein and other non-aqueous contaminant from the MB Spin Column filter membrane. The follow through was then discarded and

the MB Spin Column was placed in a clean Collection Tube, 500 mcl of Solution C5 was added to the MB Spin Column and centrifuged at 14,000 for 1 minute. This step allowed for the removal of residual salt, inhibitors and other contaminants while allowing the DNA to stay bound to the silica membrane. The follow through was then discarded, the MB Spin Column placed in a clean Collection Tube and centrifuged at 14,000 rpm for 3 minutes to remove any residual Solution C5, which could interfere with downstream DNA applications. The MB Spin Column was then placed into a new 1.5 ml Elution Tube and eluted with 50 mcl of Solution C6 which was added to the MB Spin Column. This was then centrifuged at 14,000 rpm for 1 minute, the MB Spin Column were then discarded, and the DNA quantified prior to storage at -80°C.

7.2.2.2 Quantification and quality control of faecal DNA

Quantification of faecal DNA was performed using a Nanodrop ND-1000 Spectrophotometer (Thermofisher Scientific). The ratio of absorbance at 260 and 280 nm (260/280) was used to assess the purity of DNA, with a ratio of ~1.8 accepted for good purity DNA, with a lower ratio indicating the presence or protein, phenol and or other contaminants. The ratio of absorbance of 260 and 230 nm (260/230) was used as a secondary measure of nucleic acid purity, with a ratio of 2.0 to 2.2 accepted for DNA, and a lower ratio indicating the presence of contaminants which absorb at 230 nm.

7.2.2.3 Library preparation and sequencing

The library preparation and sequencing were performed by Ms Morag Raynor, Ms Carolina Lascelles and Dr Ian Carr at the Next Generation Sequencing Facility in the Leeds Institute of Medical Research. Library

preparation was performed using the New England Biolabs NEXT® Ultra™ DNA Library Prep Kit for Illumina®.

7.2.2.3.1 End prep of PCR product

First, the 3'-5' and 5'-3' cohesive overhangs of each DNA fragment were enzymatically removed to facilitate ligation of an adaptor. This was performed in a 96 well plate, to each well: 50 mcl of fragmented DNA from each individual sample, 3 mcl of NEBNext End Prep Enzyme Mix and 7 mcl of NEBNext End Repair Reaction Buffer was added (total volume 60 mcl). Each individual well was then pipetted 10 times to ensure it was mixed thoroughly. The 96 well plate was then placed on to the Biorad C1000 thermocycler at 20˚C for 30 minutes followed by period of 30 minutes at 65˚C to denature the enzymes.

7.2.2.3.2 Adaptor Ligation

Oligonucleotide adapters were then attached to the blunt ended DNA fragments to facilitate binding to the flow cell of the Illumina next generation sequencer. Each end prep reaction mixture (60 mcl) was added to an individual well of a clean 96 well plate. 2.5 mcl of NEBNext Adaptor for Illumina was then added to each well along with 30 mcl of NEBNext Ultra II Ligation Master Mix and 1 mcl of NEBNext Ligation enhancer (total volume 93.5 mcl). Each individual well was then pipetted 10 times to ensure it was mixed thoroughly. The plate was then incubated at 20°C for 15 minutes on a thermal cycler. 3 mcl of USER® enzyme was added to each well. The plate was then incubated for 15 minutes at 37°C.

7.2.2.3.3 Clean-up of Adaptor-ligated DNA

Following ligation of the adaptor to the blunt ended DNA, a purification process was required to remove various contaminants and ligation enzymes. Solid Phase Reversible Immobilisation (SPRI) beads are para-magnetic beads activated by a magnetic field and to which DNA reversibly binds in the presence of certain chemicals. 87 cl of resuspended Appleton Woods PCR purification Beads were added to the Adaptor Ligation reaction within each well and then pipetted 10 times to ensure it was mixed thoroughly. The plate was then incubated at room temperature for five minutes. The 96 well plate was placed on a magnetic rack to activate the magnetic beads and separate the beads from the supernatant. After 5 minutes the supernatant was removed using a pipette and disposed of. The beads were then washed with 200 mcl of 80% ethanol, incubated at room temperature for 30 seconds and the supernatant then discarded. The process of washing the beads with ethanol was then repeated twice. The beads were then left to air dry for 5 minutes. In order to remove the ligated DNA from the SPRI beads, they were eluted in 17 mcl of 10 mM Tris-HCL which was added to each well and mixed by pipetting 10 times. To separate the SPRI beads from the purified ligated DNA product the 96 well plate was placed on the magnetic rack for five minutes. 15 mcl of purified ligated DNA product was aliquoted from each well into a clean 96 well plate ensuring the SPRI beads were not disturbed.

7.2.2.3.4 PCR Enrichment of Adaptor-ligated DNA

PCR amplification of the ligated PCR product was then performed to facilitate successful sequencing. To each well of the 96 well plate containing 15 mcl of purified ligated DNA product, 25 mcl of NEBNext Ultra II Q5 Master Mix was added in addition to 10 mcl of Index Primer Mix (total volume 50 mcl). Each well was then pipetted 10 times to ensure it was thoroughly mixed. The

PCR reaction was undertaken in a Biorad C1000 thermocycler. Initial denaturation was performed at 98°C for a period of 30 seconds. This was followed by 12 cycles programmed to undergo denaturation at 99°C for 10 seconds followed by a 75 second annealing step at 65°C. A final elongation step was carried out at a temperature of 65°C for five minutes and then held at 4°C.

7.2.2.3.5 Clean-up of PCR Reaction

The enriched PCR library preparation then underwent purification using SPRI beads. 45 mcl of resuspended beads were added to the PCR reaction within each well and then pipetted 10 times to ensure it was mixed thoroughly. The plate was then incubated at room temperature for five minutes. The 96 well plate was placed on a magnetic rack to activate the magnetic beads and separate the beads from the supernatant. The supernatant was removed using a pipette and disposed of. The beads were then washed with 200 mcl of 80% ethanol, incubated at room temperature for 30 seconds and then the supernatant was discarded. The process of washing the beads with ethanol was then repeated twice. The beads were then left to air dry for 5 minutes. To remove the ligated DNA from the SPRI beads they were eluted in 33µl of 0.1X TE and pipetted 10 times to mix thoroughly. It was then incubated for 2 minutes at room temperature. To separate the SPRI beads out of solution from the purified ligated DNA product, the 96 well plate was placed on the magnetic rack for five minutes. 30 mcl of the purified ligated DNA product was aliquoted from each well into a clean 96 well plate ensuring the SPRI beads were not disturbed.

7.2.2.3.6 Quantification of the library preparation product

The samples then underwent quantification using the Quant-iT dsDNA and D1000 TapeStation using the Quant-iT values to inform the pool equimolar. The pool then underwent QC using the Qubit BR dsDNA, D1000 Tapestation and qPCR in order to know the DNA concentration in molarity (nmol/L) prior to sequencing.

7.2.2.3.7 Library sequencing

Sequencing was performed on an Illumina NextSq 2000. The prepared libraries were loaded onto a flow cell and cluster generation took place, this involved fragments within the samples being captured on a lawn of surface-bound oligonucleotides which were complementary to the library adapters. Each fragment was then amplified to generate a series of clusters, each with numerous copies of the same sequence within each cluster. Each cluster then underwent simultaneous sequencing, by which a complementary fluorescently labelled base was added to the sequence of interest. The flow cell was then excited by light to generate a specific signal corresponding to the fluorescently labelled base in each sequence cluster. This cycle was repeated to sequence the entirety of each cluster.

7.2.2.4 Bioinformatics microbiome analysis

Analysis of the fermentation DNA sequences was performed by Dr Suparna Mitra and Mr Alexander Davie at the University of Leeds. FASTQ data files were generated by the Illumina sequencing platform and demultiplexed using the unique index primer associated with each individual sample. Downstream analysis was then undertaken.

Quality control and low-quality reads were found and removed using the Cutadapt tool. The total read counts were then mapped to the National Centre for Biotechnology Information (NCBI) Taxonomy using the NCBI nr database to map reads to known taxa within the database. Assignment to the NCBI taxonomy was performed according to the taxonomic tree (Family, Genus, Species). Samples were then normalised to the lowest sample size by dividing each level of the taxonomic tree by the total number of NCBI assigned values multiplied by the NCBI assigned values of the lowest sample size.

MetaPhlAn4, a metagenomic analysis tool was used to profile the composition of microbial communities from shotgun metagenomic sequencing data. MetaPhlAn and its necessary reference databases (ChocoPhlAn) were downloaded and installed onto the high-performance computer to carry out the analysis. The databases contained the marker genes and taxonomic information for microbial species found within the colonic microbiome. The taxonomic profile and abundance of the microbial community of the samples were produced at Family, and Species level, from which heatmaps were produced.

MEGAN (MEtaGenome ANalyzer) was used to create a principle coordinate analysis (PCoA) using Bray-Curtis dissimilarity to evaluate the degree of inter-individual and intra-individual variation between volunteer samples.

Results

7.2.3 DNA extraction

DNA was extracted from a total of 30 samples, this included five samples per volunteer, for a total of six volunteers (V1, V2, V5, V6, V8 and V9). The

samples chosen for DNA extraction included the control at 0 and 24 hours, and the following fermentation reactions at 24 hours: inulin only (0.01mg/ml), O3FA 50 mcg/ml, and O3FA 50 mcg/ml & inulin (0.01mg/ml).

The DNA extractions were then quantified using a Nanodrop ND-1000 Spectrophotometer. The concentration of DNA appeared to reflect the experimental conditions within the fermentation reactions. The presence of inulin was associated with an increase in the concentration of DNA, with a mean concentration of 47.85 (SD 17.59) ng/mcl, compared to the fermentation reactions without inulin, which had a mean concentration of 12.35 (2.12) ng/mcl. The mean 260/280 ratios for all samples were 1.95 (0.27) and ranged from 1.67 to 2.15, the mean 260/230 ratios were 0.22 (0.29) and ranged from 0.03 to 1.08 ng/mcl.

7.2.4 Bray-Curtis principal co-ordinate analysis (PCoA)

All samples were analysed to measure the degree of inter-individual and intra-individual variation in the bacterial composition between volunteers and experimental conditions using Bray-Curtis dissimilarity. Each volunteer is depicted with a different symbol (n=6), the experimental conditions are shown in different colours (**figure 7.1**). There were no significant differences between the control and O3FA reactions when examined at 24h. However, there was a large effect for the inulin only reaction at 24h compared to the control at 0h. This same inulin effect was observed in the reactions containing O3FA and inulin. I observed differences between the inulin only and the O3FA & inulin reactions at 24h for two of the volunteers (V1 & V2). In the presence of inulin, those volunteers had a different microbial composition compared to the other volunteers and from their own control reaction at 0h (**figure 7.1**).

7.2.5 Diversity of samples

The diversity between individual volunteers and experimental conditions was also examined using the Shannon-Weaver diversity index, which measures the differences in microbial diversity within a specific environment (**figure 7.2**). Overall, there was a similar amount of diversity between the control and O3FAs reactions at 24 hours within the model. However, the presence of inulin appeared to reduce the diversity, with a similar, yet slightly lower reduction in diversity for the O3FA & inulin reactions.

7.2.6 Heatmaps

In order to evaluate the relative differences in microbiota, heatmaps were created at Family level for each individual volunteer, with the top 26 most abundant taxa shown (**figures 7.3 & 7.4**).

I first examined the effects on the microbial composition within the model for the control reactions at 0 and 24 hours (**figure 7.3**). Within the model there was a decrease in abundance of known SCFA-producing taxa *Lachnospiraceae* and *Ruminococcaceae* within the control at 24 hours for all volunteers. However, the overall microbial composition of the model remained stable after 24 hours.

I then examined the microbial composition when O3FAs were added to the model. In the presence of O3FA (50mcg/ml) there was a very small increase in the abundance of *Ruminococcaceae* and *Lachnospiraceae* in some of the volunteers compared to the control at 24 hours. There were no differences in the abundance of *Bacteroidaceae* and Bifidobacteriaceae between the control and O3FA reactions at 24 hours. For V1, there was a higher abundance of *Prevotellaceae* in the control at 0h, however this was no longer present at 24h or when O3FAs were added to the model.

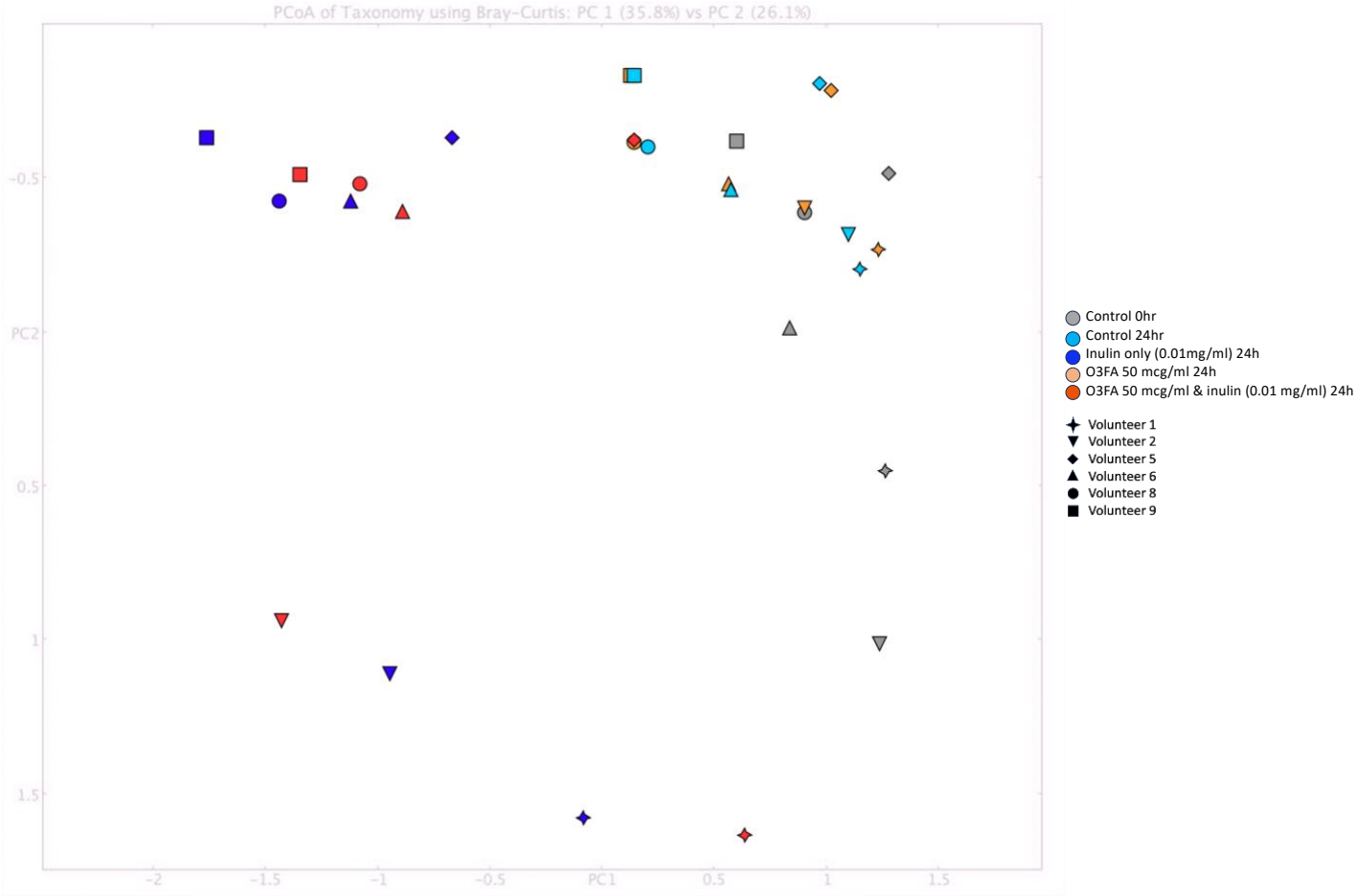


Figure 7.1 Principle component analysis plot of all volunteers and experimental conditions analysed as measured by Bray-Curtis Dissimilarity calculation.

The different volunteers are depicted with a different symbol (n=6) and the experimental conditions are shown in different colours.

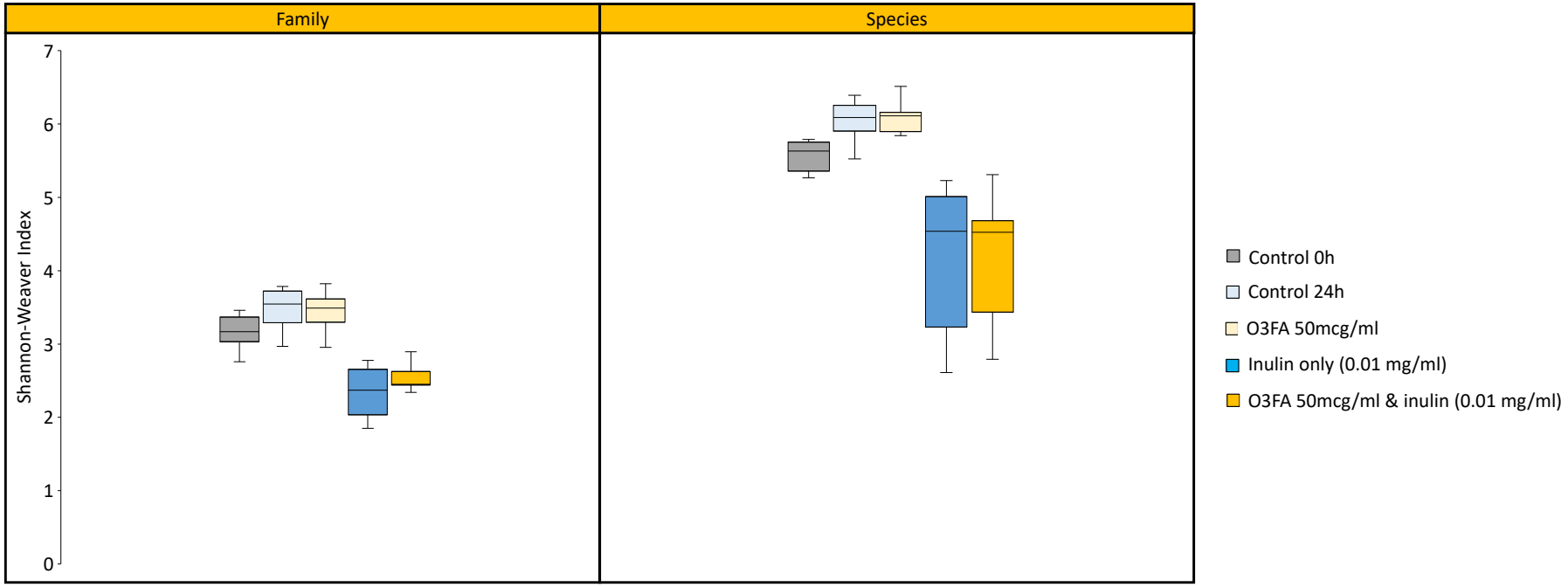


Figure 7.2 Box and whisker plots showing the Shannon Weaver Index of diversity at family and species level for all volunteers according to each experimental condition.

The median is shown as the middle line, with boxes extending to the 25th and 75th percentile. The minimum and maximum data points are denoted by the whiskers.

I then examined the effects of inulin within the model (**figure 7.4**). Despite the presence of inulin decreasing bacterial diversity (**figure 7.2**), there was a greater abundance of specific taxa observed when inulin was added to the model, compared to the control reactions, as reflected in the scale of the heat maps. This is likely to also reflect the increase in DNA concentration that I also observed in the presence of inulin (**section 7.3.2**). Inulin appeared to increase the abundance of specific families, there was an increase in *Prevotellacae* observed in V1 and V2 compared to control at 24 hours whereas there was an increase in the abundance of *Bacteroidaceae* compared to control at 24 hours in V5, 6, 8 & 9.

When O3FAs & inulin were added to the model, the increase in the abundance of *Bacteroidaceae* was slightly lower than for the inulin only reactions within some of the volunteers (V6, V7, V9). Whereas there was an increase in *Bifidobacteriaceae* in the O3FA & inulin reactions compared to the inulin only reaction in others (V5, V6, V8, V9).

The microbial composition of V1 and V2 differed from the others, as reflected in the PCoA plot (**section 7.3.3**). Those two volunteers had a lower abundance of *Bifidobacteriaceae* compared to the other volunteers and a higher abundance of *Prevotellaceae*. Despite the difference in microbial composition of these two volunteers (V1 & V2), they still exhibited an increase in total SCFA level above the inulin control in the presence of O3FAs (**figure 6.8**).

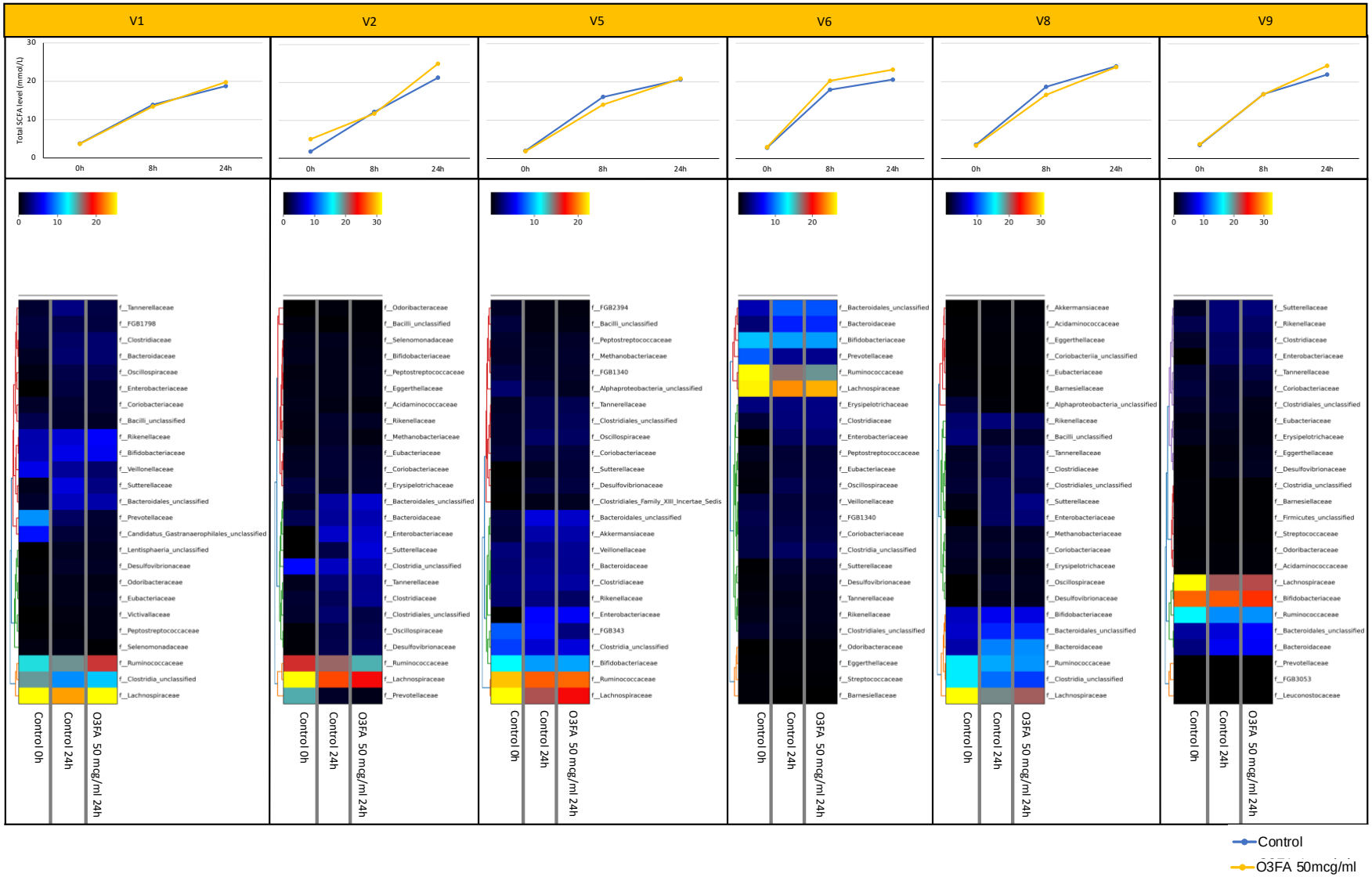

V1
V2
V5
V6
V8
V9
Total SCFA level (mmol/L)
0h
8h
24h
f__Tannerellaceae
f__FGB1798
f__Clostridiaceae
f__Bacteroidaceae
f__Oscillospiraceae
f__Enterobacteriaceae
f__Coriobacteriaceae
f__Bacilli_unclassified
f__Rikenellaceae
f__Bifidobacteriaceae
f__Veillonellaceae
f__Sutterellaceae
f__Bacteroidales_unclassified
f__Prevotellaceae
f__Candidatus_Gastranaerophilales_unclassified
f__Lentisphaeria_unclassified
f__Desulfovibrionaceae
f__Odoribacteraceae
f__Eubacteriaceae
f__Victivallaceae
f__Peptostreptococcaceae
f__Selenomonadaceae
f__Ruminococcaceae
f__Clostridia_unclassified
f__Lachnospiraceae
f__Odoribacteraceae
f__Bacilli_unclassified
f__Selenomonadaceae
f__Bifidobacteriaceae
f__Peptostreptococcaceae
f__Eggerthellaceae
f__Acidaminococcaceae
f__Rikenellaceae
f__Methanobacteriaceae
f__Eubacteriaceae
f__Coriobacteriaceae
f__Erysipelotrichaceae
f__Bacteroidales_unclassified
f__Bacteroidaceae
f__Enterobacteriaceae
f__Sutterellaceae
f__Clostridia_unclassified
f__Tannerellaceae
f__Clostridiaceae
f__Clostridiales_unclassified
f__Oscillospiraceae
f__Desulfovibrionaceae
f__Ruminococcaceae
f__Lachnospiraceae
f__Prevotellaceae
f__FGB2394
f__Bacilli_unclassified
f__Peptostreptococcaceae
f__Methanobacteriaceae
f__FGB1340
f__Alphaproteobacteria_unclassified
f__Tannerellaceae
f__Clostridiales_unclassified
f__Oscillospiraceae
f__Coriobacteriaceae
f__Sutterellaceae
f__Desulfovibrionaceae
f__Clostridiales_Family_XIII_Incertae_Sedis
f__Bacteroidales_unclassified
f__Akkermansiaceae
f__Veillonellaceae
f__Bacteroidaceae
f__Clostridiaceae
f__Rikenellaceae
f__Enterobacteriaceae
f__FGB343
f__Clostridia_unclassified
f__Bifidobacteriaceae
f__Ruminococcaceae
f__Lachnospiraceae
f__Bacteroidales_unclassified
f__Bacteroidaceae
f__Prevotellaceae
f__Ruminococcaceae
f__Lachnospiraceae
f__Eubacteriaceae
f__Oscillospiraceae
f__Veillonellaceae
f__FGB1340
f__Coriobacteriaceae
f__Clostridia_unclassified
f__Sutterellaceae
f__Desulfovibrionaceae
f__Tannerellaceae
f__Rikenellaceae
f__Clostridiales_unclassified
f__Odoribacteraceae
f__Eggerthellaceae
f__Streptococcaceae
f__Barnesiellaceae
f__Akkermansiaceae
f__Acidaminococcaceae
f__Eggerthellaceae
f__Coriobacteriia_unclassified
f__Eubacteriaceae
f__Barnesiellaceae
f__Alphaproteobacteria_unclassified
f__Rikenellaceae
f__Bacilli_unclassified
f__Tannerellaceae
f__Clostridiaceae
f__Clostridiales_unclassified
f__Sutterellaceae
f__Enterobacteriaceae
f__Methanobacteriaceae
f__Coriobacteriaceae
f__Erysipelotrichaceae
f__Oscillospiraceae
f__Desulfovibrionaceae
f__Bifidobacteriaceae
f__Bacteroidales_unclassified
f__Bacteroidaceae
f__Ruminococcaceae
f__Clostridia_unclassified
f__Lachnospiraceae
f__Sutterellaceae
f__Rikenellaceae
f__Clostridiaceae
f__Enterobacteriaceae
f__Coriobacteriaceae
f__Eubacteriaceae
f__Erysipelotrichaceae
f__Eggerthellaceae
f__Desulfovibrionaceae
f__Clostridia_unclassified
f__Barnesiellaceae
f__Firmicutes_unclassified
f__Streptococcaceae
f__Odoribacteraceae
f__Acidaminococcaceae
f__Lachnospiraceae
f__Bifidobacteriaceae
f__Ruminococcaceae
f__Bacteroidales_unclassified
f__Bacteroidaceae
f__Prevotellaceae
f__FGB3053
f__Leuconostocaceae
Control 0h
Control 24h
O3FA 50 mcg/ml 24h
Control
O3FA 50mcg/ml

Figure 7.3 Heatmaps showing the abundance of the top 26 taxa at Family level for each individual volunteer for samples collected from the control reaction at 0 and 24 hours and the O3FA (50 mg/ml) fermentation reaction at 24 hours.

The total SCFA level at 0,8 and 24 hours is also shown for each individual volunteer above the respective heatmap.

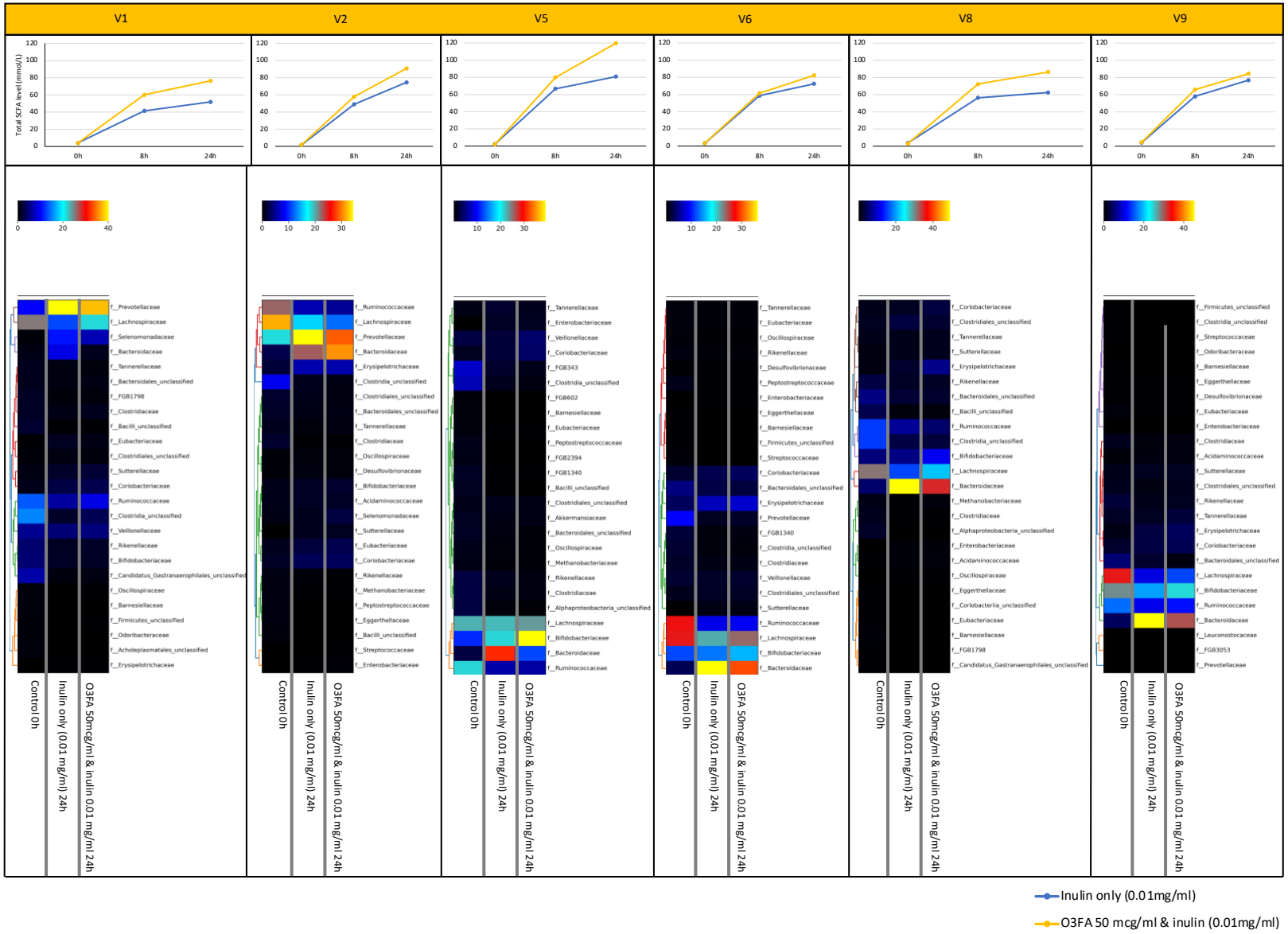

V1
V2
V5
V6
V8
V9
Total #SCFA level (mmol/L)
0h
8h
24h
Control 0h
Inulin only (0.01 mg/ml) 24h
O3FA 50mcg/ml & inulin 0.01 mg/ml 24h
Inulin only (0.01mg/ml)
O3FA 50 mcg/ml & inulin (0.01mg/ml)

Figure 7.4 Heatmaps showing the abundance of top 26 taxa at Family level for each individual volunteer for samples collected from the control reaction at 0, inulin only (0.01 mg/ml), O3FA (50 mg/ml) & inulin (0.01 mg/ml) fermentation reactions at 24 hours.

The total SCFA level at 0,8 and 24 hours is also shown for each individual volunteer above the respective heatmap.

Heatmaps were then created showing the top 25 most abundant species in which all volunteer samples were compared within the same heatmap rather than separately; allowing comparison using the same scale.

Within the control reaction, there was a reduction in some specific species according to individual volunteers after 24 hours of fermentation, such as *Clostridum bacterium* and *Prevotella corpi clade* in V1, *Prevotella corpi clade* in V2 and V6, and *Candidatus Cibiobacter qucibialis* in V5. There was an increase in a range of different species within the control after 24 hours of fermentation that included *Escherichia coli* and *Bacteroides Uniformis* **(figure 7.5)**.

When O3FAs 50 mcg/ml only was added to the model, there were no differences between the abundance of taxa in the control and the O3FA reactions at 24 hours, apart from a higher abundance of *Phocaeicola vulgatus* in V9 which is a SCFA-producer (**figure 7.5**).

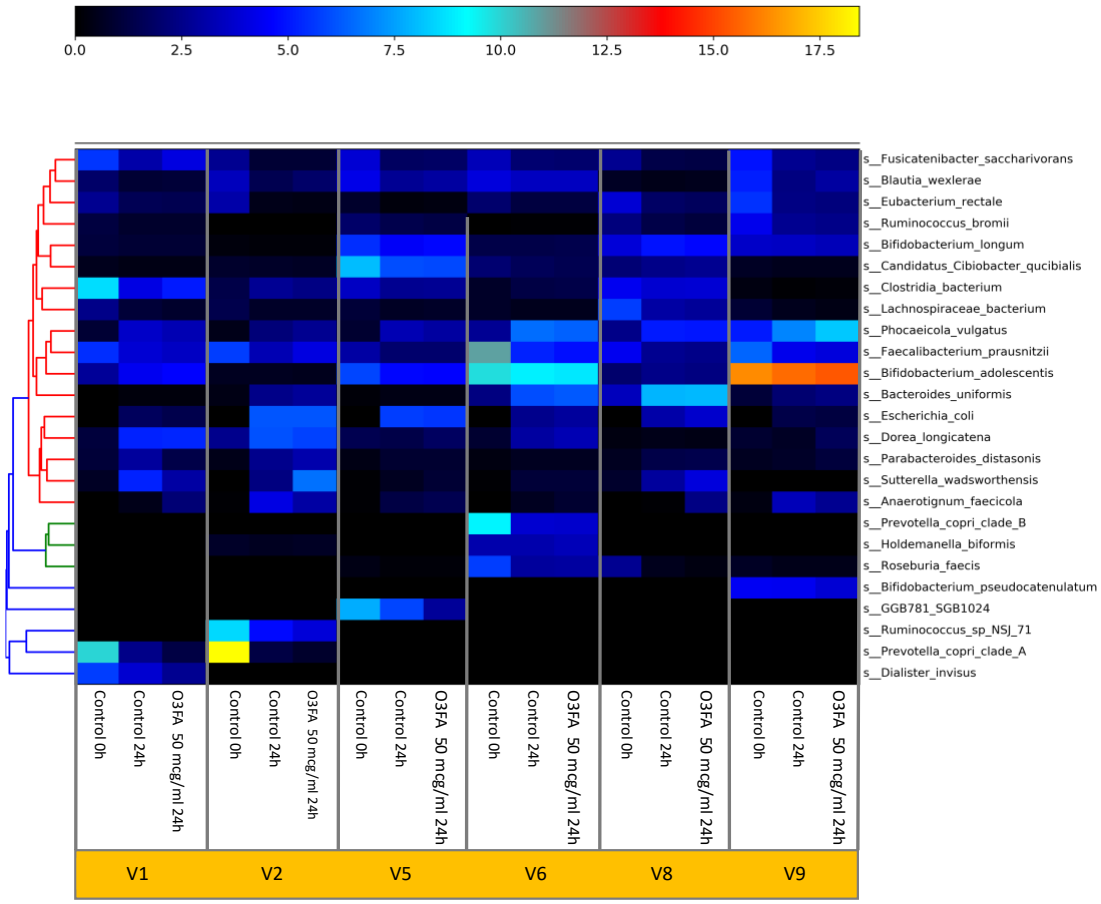


Figure 7.5 Heatmaps showing the abundance of Species level for each individual volunteer for samples collected from the control at 0 and 24 hours and O3FA 50 mg/ml only reactions at 24 hours.

All samples are normalised to each other.

I then examined the differences in the abundance of species between the reactions containing inulin compared to the control at 24 hours (**figure 7.6**). In the presence of inulin, there was an increase in the abundance of *Prevotella corpi clade* in volunteers 1 and 2. There was an increase in the acetate-producing species *Bifidobacetrium Adolescentis* in V5, 6, 8 and 9. In addition to a consistent increase in the SCFA-producer *Bacteroides Uniformis* in all volunteers. An increase in Bacteroides stercoris was observed in V5.

When O3FA 50 mcg/ml & inulin was added to the model, compared to the inulin only reactions there was an increase in *Bifidobacetrium Adolescentis* in three of the volunteers (V5, V6, V9). This was particularly striking for V5 and may correlate with the large production of acetate seen for this volunteer in the presence of O3FAs and inulin **(figure 6.8**). There was a slight decrease in the abundance of *Bacteroides Uniformis*, within and O3FA & inulin reactions compared to the inulin only reactions at 24 hours in some volunteers (V2, V6, V8, V9), suggesting a dampening effect of O3FAs.

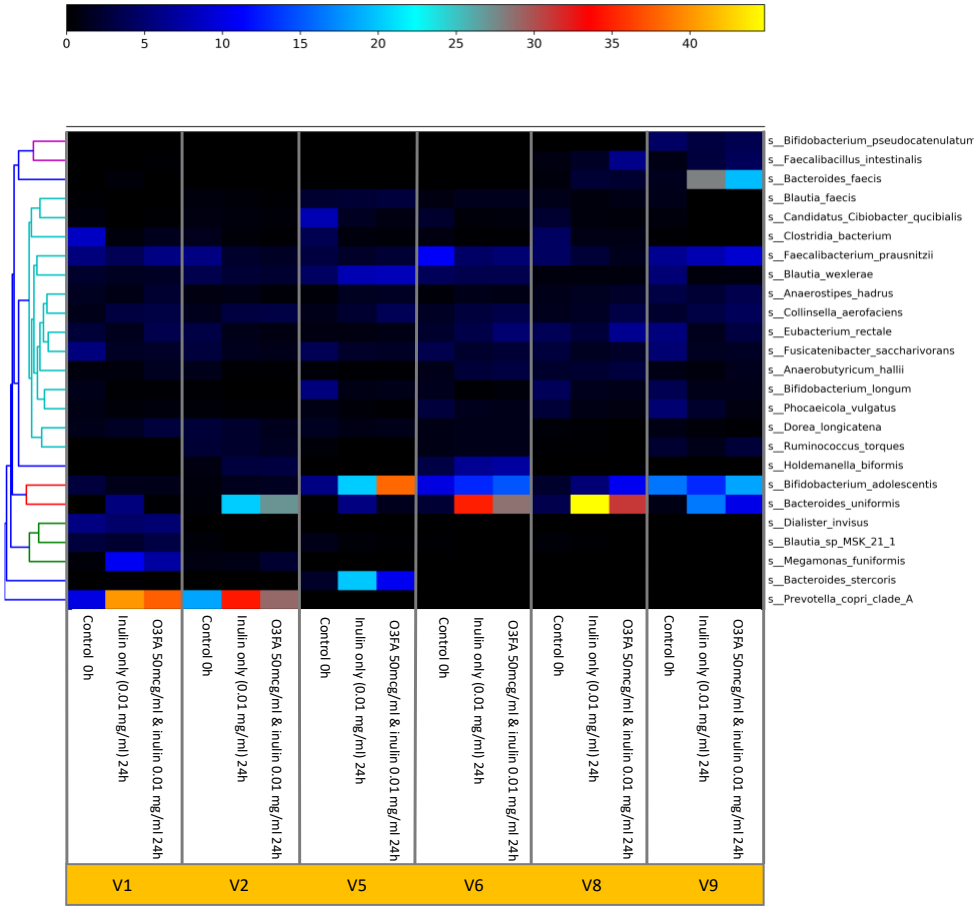


Figure 7.6 Heatmaps showing the abundance at Species level for each individual volunteer for samples collected from the control at 0h, inulin (0.01 mg/ml) only and O3FA (50 mcg/ml) & inulin 0.01 mg/ml fermentation reactions at 24 hours.

All samples are normalised to each other.

7.2.7 Bar charts of the abundance of specifc SCFA producing taxa

In order to illustrate the absolute microbiota abundance data for each volunteer as a percentage of read counts, bar charts of SCFA-producing taxa at Family and Species level were plotted according to each volunteer and experimental condition (**figures 7.7 & 7.8**). Specific taxa were chosen based on the heatmap abundance and known SCFA-producing potential (**chapter 1, section 1.5.1**).

I observed inter-individual variability with regards to the abundance of the specific taxa, with some volunteers exhibiting higher percentage read counts. Within some of the volunteers there was an increase and decrease in the abundance of specific taxa in the presence of O3FAs, such as an increase in *Bifidobacteriaceae* in V5 and *Bacteroidaceae* for V2 and a decrease *Bacteroidaceae* in V9 and Prevotella *corpi* in V1 and V2 (**figures 7.7 & 7.8**). There was no overall pattern observed in the changes of microbiota when O3FAs were added to the model.

As previously observed, there was large inulin effect observed for some of the taxa, including an increase in the Family *Bacteroidaceae* and Species *Bacteroides uniformis*. When O3FA & inulin was added to the model there was a slight, but consistent increase in *Lachnospiraceae* across most of the volunteers (V1, V5 to V9), in addition to an increase in *Bifidobacteriaceae* within some of the volunteers (V5, V6, V8) (**figure 7.7**). The presence of O3FAs & inulin appeared to slightly decrease the abundance of *Bacteroides uniformis* and *Prevotella corpi* in some of the volunteers (**figure 7.8**).

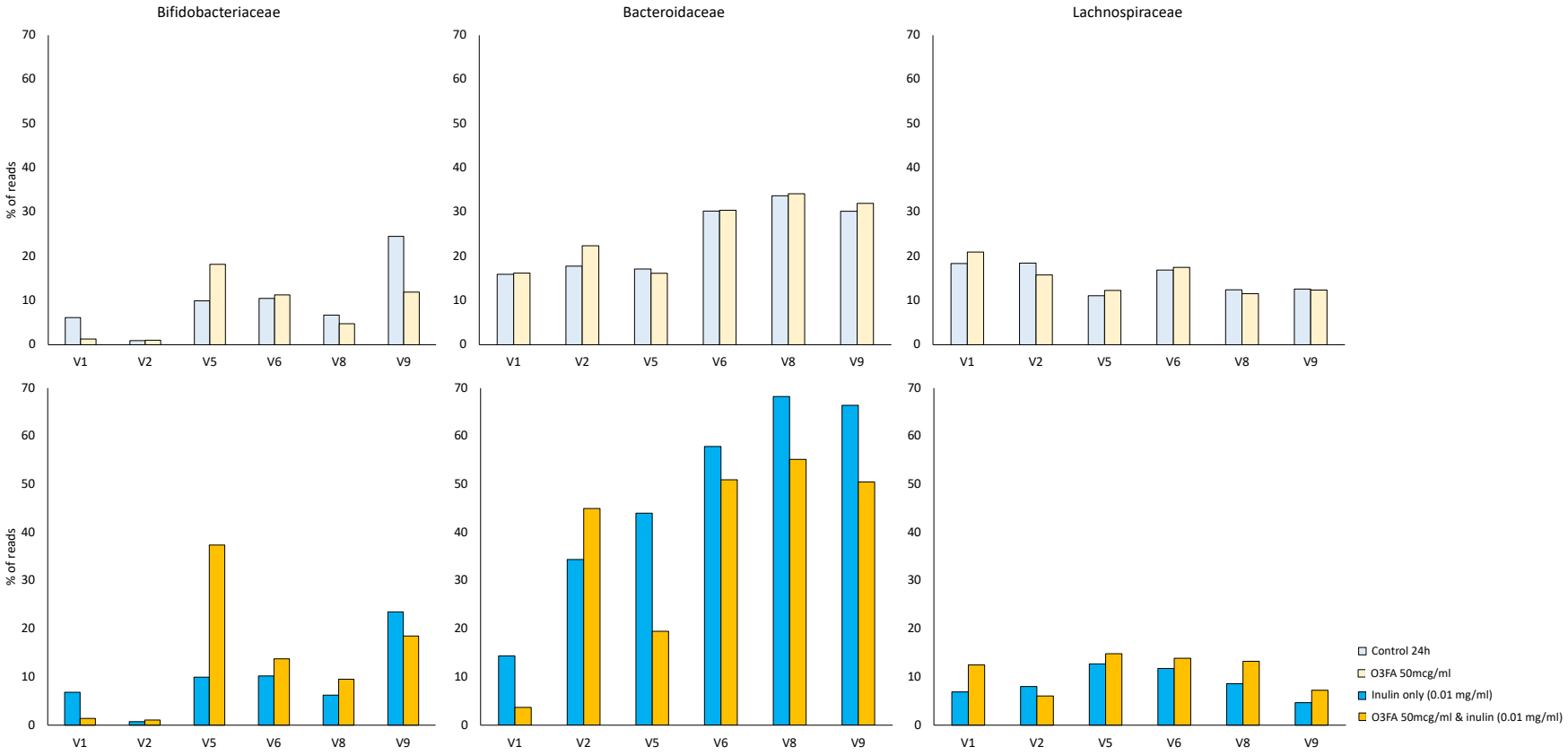


Figure 7.7 Bar charts showing the percentage of reads for *Bifidobacteriaceae, Bacteroidaceae* and *Lachnospiraceae* families for individual volunteers and according to experimental conditions.

Volunteers are shown on the x axis and the percentage number of reads on the y axis.

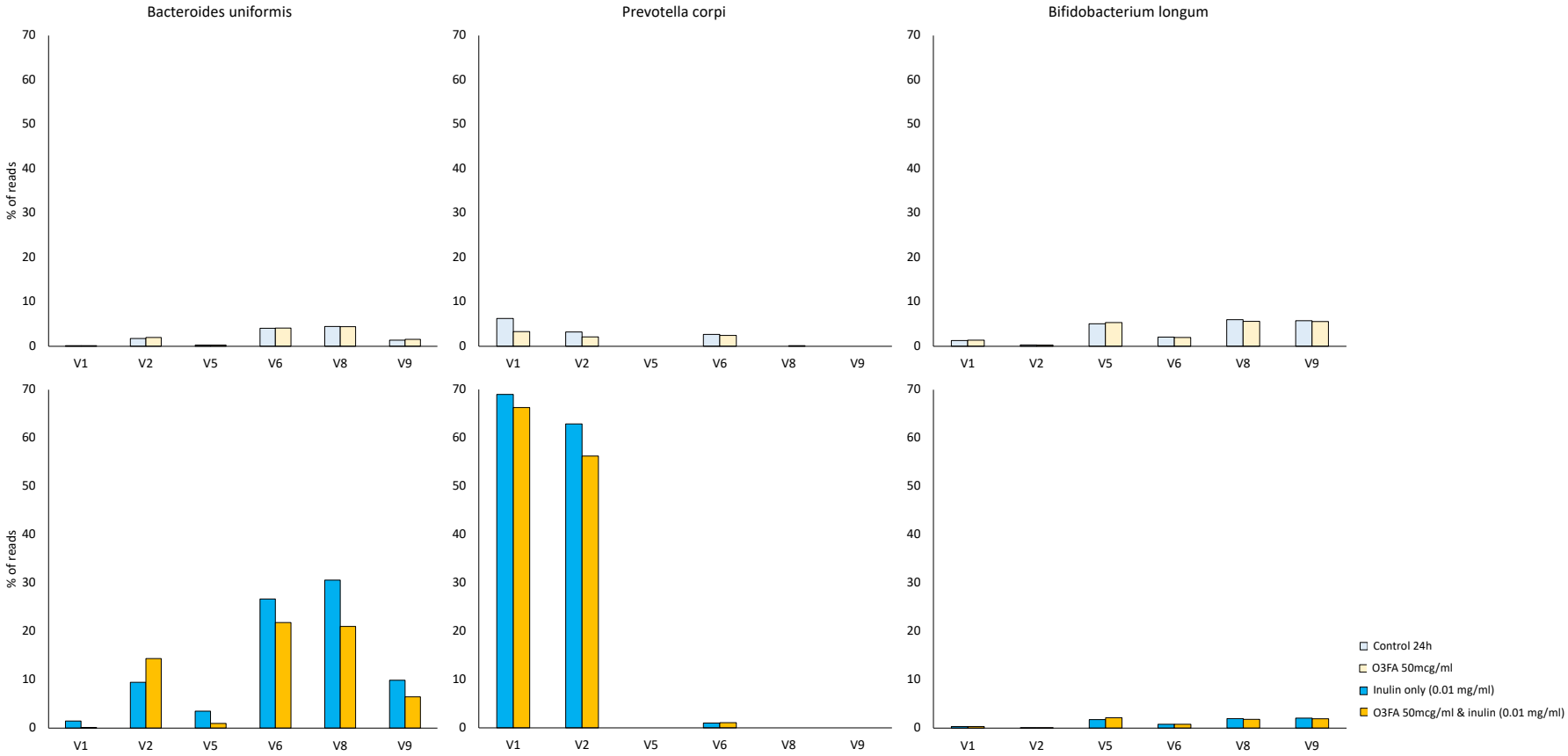


Figure 7.8 Bar charts showing the percentage of reads for *Bacteroides uniformis, Prevotella corpi* and *Bifidobacterium longum* species for individual volunteers and according to experimental conditions.

Volunteers are shown on the x axis and the percentage number of reads on the y axis.

7.3 Discussion

There were changes in the microbiome within the *in vitro* colonic fermentation model examining the effects of inulin (0.01 mg/ml), O3FAs (50 mcg/ml) and O3FAs (50mcg/ml) & inulin (0.01 mg/ml).

The analysis inlcuded samples from six volunteers who were chosen due to the increase in SCFA production observed when their faecal samples were tested within the model. There was inter-individual variablity in relation to SCFA production and the changes in the microbiome observed. This variabiity is likely to reflect the differences in demographics (sex, age, ethnicity) between the volunteers. In addition, the diets of individual volunteers were not controlled beyond the avoidance of oily fish intake for two days prior to faceal sample collection. Due to the small number of volunteers and variability it is difficult to draw definitive conclusions, however I observed some specific patterns within the data.

There was an increase in the microbial diversity within the *in vitro* colonic fermentation model following 24 hours of fermentation of the control reaction. There was a decrease in SCFA-producing taxa *Lachnospiraceae* and *Ruminococcaceae* within the control at 24 hours for all volunteers which may reflect the lack of substrate availability for those specific taxa. Overall, the *in vitro* colonic model remained stable with regards to the microbiota after 24 hours of fermentation, reflecting its ability to create a stable environment to examine the microbiome.

There was a signfiicant effect observed for inulin in altering the microbiome and increasing the abundance of specific taxa including *Bacteroidaceae* within the model. A systematic revew of human studies (n=9) examining the

effects on inulin on gut microbial composition reported a consistent increase in *Bifidobacterium* and a decrease in *Bacteroides* following inulin supplementation, overall they reported no significant change in microbial diversity (325). I observed an increase in *Bifidobacteriaceae* in only two volunteers when inulin was added to the model, which may reflect the differences in individual microbial compositions. I also found a decrease in microbial diversity associated with inulin, which may be due to the limitations of a batch static *in vitro* colonic model and/or the concentration of inulin tested within the model. It is possible that there is an optimal dose of inulin in modulating the colonic microbiome, as observed in a study of mice fed a high soluble fibre diet of 20% inulin who were found to have an increase in tumour number and load in azoxymethane treated and $APC^{min/+}$ mice, these findings were associated with a significant decrease in microbial diversity associated with the inulin intervention (318). There are also likely to be inter-individual responses to inulin, dependent on an individual's microbiota (326).

When O3FAs 50 mcg/ml only were added to the model, I observed a very slight increase in the SCFA producing families *Ruminococcaceae* and *Lachnospiraceae* within most of the volunteers. These findings are consistent with a study of 876 middle aged and elderly female twins, in whom plasma DHA levels were positively correlated with operational taxonomy units (OTUs) of the *Lachnospiraceae* family within the faceal microbiome measued using 16S sequencing (327). The authors also reported an increase in microbial diversity after adjusting for dietary fibre intake, suggesting that O3FAs improve the colonic microbial compostition (327). Unfortunately they did not include plasma NMR EPA levels within their analysis. An increase in the abundance of *Ruminococcaceae* was observed in a study of mice receiving an intervention of the biologically inactive O3FA,

ALA (0.5g per kg) for 30 days. The increase in Ruminococcaceae was thought to activate the Wnt/B-catenin signalling pathway leading to an increase in intestinal stem cells, in addition there was an increase in levels of caecal acetate, propionate and butyrate (328). I observed a decrease in microbial diversity when O3FAs were added to the model, whereas other *in vivo* studies have found an increase in microbial diversity associated with O3FA interventions suggesting this as a potential anti-CRC mechanism of O3FAs (327-330). A potential explanation for this is that longer term intervention studies may result in changes throughout the gut and have repeated impact in the colon, in contrast to a static batch *in vitro* fermentation model.

I did find that O3FAs and inulin were associated with a lower reduction in microbial diversity when compared to inulin alone. This could suggest that O3FAs may modulate the effect of inulin on microbial diversity within the model. It could also explain the increase in SCFA production I observed when inulin & O3FAs were examined together within the model, compared to inulin on its own; however this requires further evaluation.

There are limitations associated with the *in vitro* colonic fermentation model which may account for some of the findings I observed. The model included samples of the facecal microbiome as a surrogate for the colonic microbiome, and are likely to compositionally different from the microbiome within specifc regions of the colon (331). The model is static and therefore subject to substrate depletion and the accumulation of metabolites, which will effect the composition of the microbiome wthin the model and result in the relative abundance of specific taxa. The findings from this study should be evaluated further *in vivo* without the limitations of the model.

As O3FAs may be depleted within the *in vitro* colonic model due to its static nature, I undertook a PUFA analysis to measure the degredation of EPA and DHA within the model using ultra performance liquid chromatography mass spectrometry (data not shown). I measured EPA and DHA in fermetation samples from the O3FA 50 mcg/ml only and O3FA 50 mcg/ml & inulin 0.01 mg/ml reactions at 8 and 24 hours. Approximately 80% of available EPA and DHA was degraded after 24 hours of fermentation. There was less degredation of EPA and DHA in the O3FA & inulin reactions. This could be due to inulin being a more available food source for the microbiota than O3FAs on their own, therefore resulting in less degredation. Again I observed inter-individual variability in the relative degredation of EPA and DHA between volunteers which may reflect the differences in microbial composition.

7.4 Conclusion

The presence of inulin, O3FAs and O3FAs & inulin was associated with changes in the microbiome within an *in vitro* colonic model. O3FAs may potentially modulate the effects of inulin by limiting its reduction on microbial diversity within the model and increasing the abundance of some SCFA-producing taxa.

Chapter 8 Concluding remarks and further considerations

8.1 Differential effects of O3FAs according to host, tumour, and microbiota factors

This thesis explored the potential differential effects of O3FAs in the prevention and treatment of CRC according to host, tumour and microbiome factors through a biomarker-driven, epidemiological approach and a parallel translational, mechanistic study.

A comprehensive literature review of clinical data including RCTs, and observational studies published within the last decade, identified the potential benefits of O3FAs in CRC prevention and treatment. Potential host factors included individuals with low baseline, pre-treatment O3FA levels. A prespecified polyp study within the VITAL trial reported that participants with low baseline O3FA levels who were supplemented with an O3FA intervention had a lower risk of conventional adenomas (146).

Other host factors included a potential benefit of O3FAs according to ethnic background. The VITAL trial specifically sampled African American participants (20% of the study population) to investigate ethnic-specific treatment effects (332). They found that African Americans who received the O3FA intervention had a reduced risk of myocardial infarction, and colorectal adenomas (145, 146). This was despite African American participants having higher baseline plasma O3i levels and oily fish intake, compared to non-Hispanic White participants within the trial (146, 332). Within the literature, I also identified sex differences associated with O3FA dietary intake and reduced CRC risk, with males more likely to benefit from O3FAs (143, 177, 199).

These findings highlight the need for future studies to further examine these specific host factors. Dietary O3FAs have an important role in predicting plasma O3FA levels, however there are likely to be other factors such as genetics and environment that determine plasma O3FA levels (285). The VITAL trial was unique in that it specifically recruited African American participants (145). It is important that future studies aim to measure blood O3FA levels and record dietary O3FA intake including FOS use, in addition to collecting data on ethnicity.

The sex differences related to O3FAs, and CRC risk require further testing to help understand the mechanism of O3FAs in CRC (267, 268). Do these relate to dietary O3FA intake or if there is a mechanism by which males benefit for O3FA supplementation, due to hormone signalling affecting fatty acid metabolism?

Overall, these host factors potentially move the trajectory for the future of O3FAs in the prevention and treatment of CRC towards a more precision medicine approach. This may become increasingly possible in the future with the ability to perform high throughput metabolomics analysis using NMR spectroscopy, in addition to whole genome sequencing – which would allow for the identification of factors such as a specific plasma O3FA level and/or genetic polymorphisms (such as FADS2 indel rs66698963), that could be used to identify individuals with which to target O3FAs interventions.

With regards to tumour factors, I identified potential benefits of O3FAs at different stages of the CRC tumorigenesis pathway. Polyp studies included the seAFOod Trial which found a decrease in left sided adenomatous polyps associated with the O3FA intervention (103), in addition to a large

observational study demonstrating a reduced risk of conventional adenomas and serrated polyps asscoiated with dietary O3FA intake (150). Several studies have suggested that dietary O3FAs reduce the risk of proximal colon cancer (139, 142, 143). In addition to identifying an association with reducing the risk of MSI tumours, which are dMMR and have a prediliction for the proximal colon (141). These findings suggest that O3FAs may augment the host anti-tumour immune response and require further evaluation through *in vivo* mechanistic studies (140, 141).

I identified several gaps within the current literature surrounding the use of O3FAs in the prevention and treatment of CRC, these included, 1) the tolerability and safety of high dose O3FA interventions and 2) evaluation of the cost effectiveness and health economics perspective of dietary O3FA guidance and the pharmacological use of O3FAs.

8.2 O3FA dietary intake and plasma levels

A key finding from this thesis is that FOS is a significant contributor to plasma O3FA levels. FOS use will confound the assessment of dietary O3FA intake if it is not accounted for. I also found that FOS use is very common with almost one third of the UKBB population regularly using a FOS, in addition to a dose-dependent relationship between increasing oily fish intake and FOS use. Within the UKBB, FOS use increased plasma O3FA levels to a similar level observed in participants who consumed oily fish once a week and did not use a FOS.

These findings are important as the current dietary recommendations for CRC published in the WCRF 2018 report '*Diet, nutrition, physical activity and colorectal cancer*' were developed from epidemiological evidence including

observational studies, that did not account for FOS use within their analyses. Therefore, the pre-existing data is likely to be confounded by a lack of FOS use data, which may account for the equivocal WCRF recommendation that there is a '*limited, but suggestive'* relationship between fish intake and CRC risk. It is important that future observational studies evaluating dietary O3FA intake include assessment of FOS use data comprising of dose, formulation, and frequency of use, in addition to distinguishing FOS from CLO supplements.

I observed a significant overlap for plasma total O3FAs and DHA levels in oily fish intake categories with and without FOS use, which suggests that although dietary O3FA intake is a predictor of plasma O3FA levels there are likely to be other factors related to plasma levels, such as genetic variants controlling levels, environment, or the poor reporting of dietary intake. This is an important factor to consider when considering future dietary recommendations relating to oily fish intake and FOS use. It is also important to consider compliance of dietary guidance and supplements, in addition to the cost feasibility of such interventions.

Overarching population wide dietary recommendations regarding oily fish intake and/or the use of FOS may not be complied with, as they consist of blanket recommendations (333). It would perhaps be better to target individuals and tailor dietary guidance using a biomarker driven approach. There are of course practicalities and limitations with regards to how this may be best achieved which are discussed in **section 8.4**.

In addition, when considering O3FA dietary recommendations it is important to consider the sustainability of oily fish and FOS. This is particularly pertinent

considering the effects of global warming on our food chain, which has been associated with the depletion of fish and seafood stocks, in addition there are reported mass mortality events, involving the sudden death of thousands to millions of fish associated with the expansion of the fish farming industry (334, 335). It is also important to consider the variability in the O3FA content of specific oily fish species which may be accounted for by geography and/or whether fish is farmed or wild (296). However, with the increased awareness of sustainability of oily fish intake and FOS there are now commercially available EPA/DHA oils produced from algae, which are also suitable for vegetarians/vegans (87).

Future work needs to consider the implementation of dietary guidance and identify specific groups (males, those with low baseline plasma levels) who are likely to benefit from dietary O3FA interventions, so that these can be targeted. In addition to examining factors related to compliance with potential recommendations by working with those target population groups in collaboration with implementation scientists to develop dietary policy guidelines.

8.3 Host and tumour factors identified within the UKBB

I identified a reduced risk of CRC associated with plasma total O3FAs of 0.43-0.61 mmol/L in male participants within the UKBB. Males within the UKBB were less likely to consume higher frequencies of oily fish and use a FOS compared to their female counterparts. This may suggest a relationship between diet and plasma levels or other sex-related factors responsible for the findings (267, 268). These findings are important from a public health perspective, there is a male predominance of CRC and therefore there is a potential that males of a specific age can be specifically targeted with regards

to dietary guidance and/or O3FA interventions, such as through the NHS Health Check programme in England (336).

I found that plasma O3FA levels were associated with a reduced risk of proximal colon cancer risk. There was a dose-related increase observed between plasma DHA levels and proximal colon cancer risk, this finding was specific for DHA, and not observed for plasma total O3FA levels. These findings may suggest a specific relationship with DHA or that plasma O3FA levels within the UKBB will include non-bioactive O3FAs such as ALA, and therefore DHA is a better marker for bioactive O3FAs.

My findings related to reduced proximal colon cancer risk and plasma O3FA/DHA levels are supported by pre-existing data (139, 142, 143). However, existing studies only signalled a potential relationship and were unable to statistically prove their findings (139, 142, 143). This is possibly due to a lack of power as reflected in the wide confidence interval observed, and/or a lack of comprehensive dietary O3FA assessment including FOS use.

8.4 Optimal plasma O3FA levels and CRC risk

Plasma O3FA and DHA levels were associated with reduced CRC risk, however there was a strong association observed for the second tertile, suggesting that there is an optimal plasma O3FA level associated with reduced CRC risk. Other studies have suggested a non-linear relationship between both plasma and dietary O3FAs with all-cause mortality and overall cancer risk (182, 296). It would be important to examine a potential non-linear relationship between plasma O3FA and DHA levels with CRC risk within the

UKBB. It is unclear whether there is an optimal level or if there is a confounder in those individuals related to genetics and/or diet.

The idea of a threshold O3FA level in cardiovascular research has previously been described in the form of the O3i, which is a commercially available assay (168). The idea of an optimal plasma O3FA level for CRC prevention is something that would require further assessment but could ultimately leading to a biomarker driven approach to CRC risk. It would require further studies examining plasma O3FA levels and CRC risk. In addition, there are potential difficulties translating plasma levels across studies according to their method of quantification (GC-MS, LC-MS, NMR spectroscopy) and the biological substance within which they are measured (whole blood, RBC, plasma), standardised reporting would allow clear comparison between studies, building the evidence base (107).

There are potential difficulties in the clinical translation of optimal O3FA levels, for example if an individual has very high levels, it is likely that these are driven by other factors unrelated to diet for which there is no intervention and therefore a threshold O3FA level for CRC risk may present a more pragmatic approach. The cost-effectiveness of a population wide '*test and treat*' strategy for O3FA levels would not be practical, however there may be the potential in the future to adopt measurement of an O3FA blood levels as part of the NHS Bowel Cancer Screening Programme. However, this would require further evidence and a full economic evaluation.

8.5 Future work within the UK Biobank

There are limitations relating to the UKBB cohort such as '*healthy volunteer*' selection bias, a lack of plasma EPA levels and the absence of FOS use data

on dose, frequency or formulation (287). Nevertheless, the UKBB is a rich source of data for which future work characterising the potential benefits of O3FAs in CRC prevention and treatment can be further undertaken, and hypothesis tested as described below.

The UKBB is an ongoing study with future data releases planned to include updates to the cancer and mortality registries, which will increase power to any further studies through the potential additional number of CRC cases and deaths, in addition to the release of the final tranche of plasma PUFA levels for all UKBB participants by Nightingale Health, which will provide complete cohort coverage.

A limitation of the UKBB data, is that I was unable to analyse CRC beyond tumour subsite using ICD-10 codes. However, it may be possible to link the UKBB data to other national data sets such as the National Bowel Cancer Audit (NBCOA), which could provide data on the management of CRC including surgery, chemo(radio)therapy, post-operative outcomes, and complications, in addition to disease severity such as tumour stage and the presence of distant metastases. This may identify further potential benefits for O3FAs according to specific tumour factors. A potential hypothesis to test would be whether dietary O3FAs reduce the risk of post-operative complications. For example, are O3FAs more likely to be of benefit in individuals with a lower tumour stage and/or those without distant metastases, or do they help reduce CRC related cachexia associated with advanced CRC stage and the presence of metastases?

There is evidence to suggest that O3FA intervention and dietary intake is associated with increased disease free-, recurrence free- and overall survival

for CRC (222, 223). A future aim of this research would be to explore the relationship between both dietary O3FA intake and plasma levels, with CRC related mortality and overall survival within the UKBB.

Previous epidemiological studies have examined the dietary patterns and interactions between O3FAs and fibre intake with variable results (130, 131). It would be important to test the findings of a potential O3FA-fibre interaction from the *in vitro* colonic fermentation model, using a population-based approach – examining the association between dietary O3FAs and fibre on CRC risk and survival. It may be possible to examine the effects of different forms of dietary O3FA intake (FOS use with and without oily fish intake) in addition to plasma O3FA levels with different types of fibre intake (non-soluble, soluble, insoluble) on CRC risk within the UKBB (337, 338).

With the 2024 release of the whole genome sequencing data by the UKBB, future work would aim to examine diet-gene interactions for CRC risk, The Hull group recently reported a genetic polymorphism in FADS2 indel rs66698963 which predicts the colorectal polyp prevention efficacy of EPA (257). It would be important to investigate its interaction with O3FA dietary intake (oily fish and FOS interaction) for CRC risk within the UKBB.

8.6 O3FA-fibre interaction and SCFA production

I demonstrated that O3FAs within an *in vitro* colonic model were associated with subtle changes in total SCFA production and the microbiome within the model. However, when O3FAs were added to the model with inulin, at a specific concentration, I found an increase in SCFA production of almost 20%, compared to when inulin was added to the model on its own. This increase was associated with a decrease in bacterial diversity within the

model and an increase in specific taxa including *Bacteroidaceae* and *Bifidobacetrium Adolescentis*.

My findings suggest an O3FA-fibre (inulin) relationship in increasing SCFA production. The findings were specific for inulin (soluble, non-viscous fibre), as I observed a negative relationship when O3FAs added to the model with pectin (soluble, viscous) or wheat bran (insoluble) fibres. This suggests that the type of fibre intervention associated with O3FAs is very important. In addition, my findings may be related to the *in vitro* colonic model, which is most representative of the proximal colon. Inulin is a highly fermentable dietary fibre, whereas pectin and wheat bran are fermented over a longer period of time and may potentially display effects in the distal colon. The changes in SCFA production observed within the *in vitro* colonic fermentation model require further examination within *in vivo* studies to determine the clinical relevance and translation of these findings.

8.6.1 Future work

Due to limitations with regards to time and cost, I was unable to conduct all the secondary analyses I had initially planned; I collected and stored fermentation samples for both PUFA analysis and metatranscriptomics.

The samples I obtained for metatranscriptomics were collected at 0, 8 and 24 hours from the fermentations. I observed subtle changes in the microbiome within the model from samples measured after 24 hours of fermentation. It would be important to consider the changes observed in RNA at 8 and 24 hours, given the fermentation period and the production of the SCFA butyrate which is predominately converted from acetate by butyryl-

CoA : acetate CoA-transferase; to identify if there are specific taxa associated with this pathway which I would not have identified at 24 hours.

I found a decrease in microbial diversity with the addition of inulin to the model, however the decrease appeared to be lower in the presence of O3FAs. It would be important to further evaluate the effects of the higher concentration of inulin (0.02 mg/ml) tested within the model. This would help establish whether O3FAs had a modifying effect on inulin according to inulin concentration; by performing microbiome analysis on the higher inulin concentration samples collected.

I also collected samples for PUFA analysis, I measured the degradation of EPA and DHA in some of the samples using ultra performance liquid chromatography mass spectrometry. I showed that there was degradation of EPA and DHA within the model, with less observed when O3FA and inulin were tested together. Other analyses would aim to identify if there are any major anti-inflammatory lipid metabolites of EPA and DHA such as oxylipins (such as PGE_2) and resolvins within the model and their interaction with the production of SCFAs (339, 340). This would help establish further mechanistic understanding of the potential anti-CRC effects of O3FAs within the colon.

Having identified a potential O3FA-inulin interaction using an *in vitro* static batch fermentation model, the next step would be to test these findings *in vivo*. This could take the form of a human study examining an O3FA and inulin intervention. The same interventions tested within the *in vitro* colonic model could be tested *in vivo* (4 g of mixed EPA/DHA and 30 g of inulin). Inulin at a dose of 30 g/day may be associated with side effects such as

bloating, abdominal pain and/or diarrhoea and therefore may need to be reduced to 20 g/day or it could be built up to a dose of 30 g/day at 10 g/day doses over a period of time to reduce potential side effects (341, 342).

It would be potentially difficult to blind the participants from the intervention, however other aspects such as diet could be controlled during the course of such a study. Outputs would include the measurement of both plasma and faecal SCFA production, in addition to faecal microbiome analysis to evaluate changes in the microbiome related to the interventions. Another important output of such a study would be to also assess the compliance, safety, and tolerability of such an intervention.

With regards to a study population, in the first instance this would be best conducted in healthy human volunteers due to cost and time; in addition to establishing the safety and tolerability of the O3FA & inulin intervention. Once this had been established the interventions could be tested within the target population, a CRC cancer trial takes time and cost and therefore other potential strategies to target the study population could be to use a biomarker for CRC such as a polyp study.

8.7 Conclusion

In conclusion, this thesis explored the potential use of O3FAs in the prevention and treatment of CRC according to host, tumour and microbiome factors by using a biomarker-driven, epidemiological approach and a parallel translational mechanistic study. I identified host factors related to sex, with plasma O3FA levels associated with reduced CRC risk in male UKBB participants. With regards to tumour factors, I found that plasma O3FA levels were associated with reduced proximal colon cancer risk in the UKBB and

finally, I demonstrated that when O3FAs and inulin were added to an *in vitro* colonic model, they led to an increase in SCFA production and SCFA producing bacteria within the model. These findings move the trajectory for the use of O3FAs in the prevention and treatment of CRC towards a more targeted approach based on specific host, tumour and microbiome factors.

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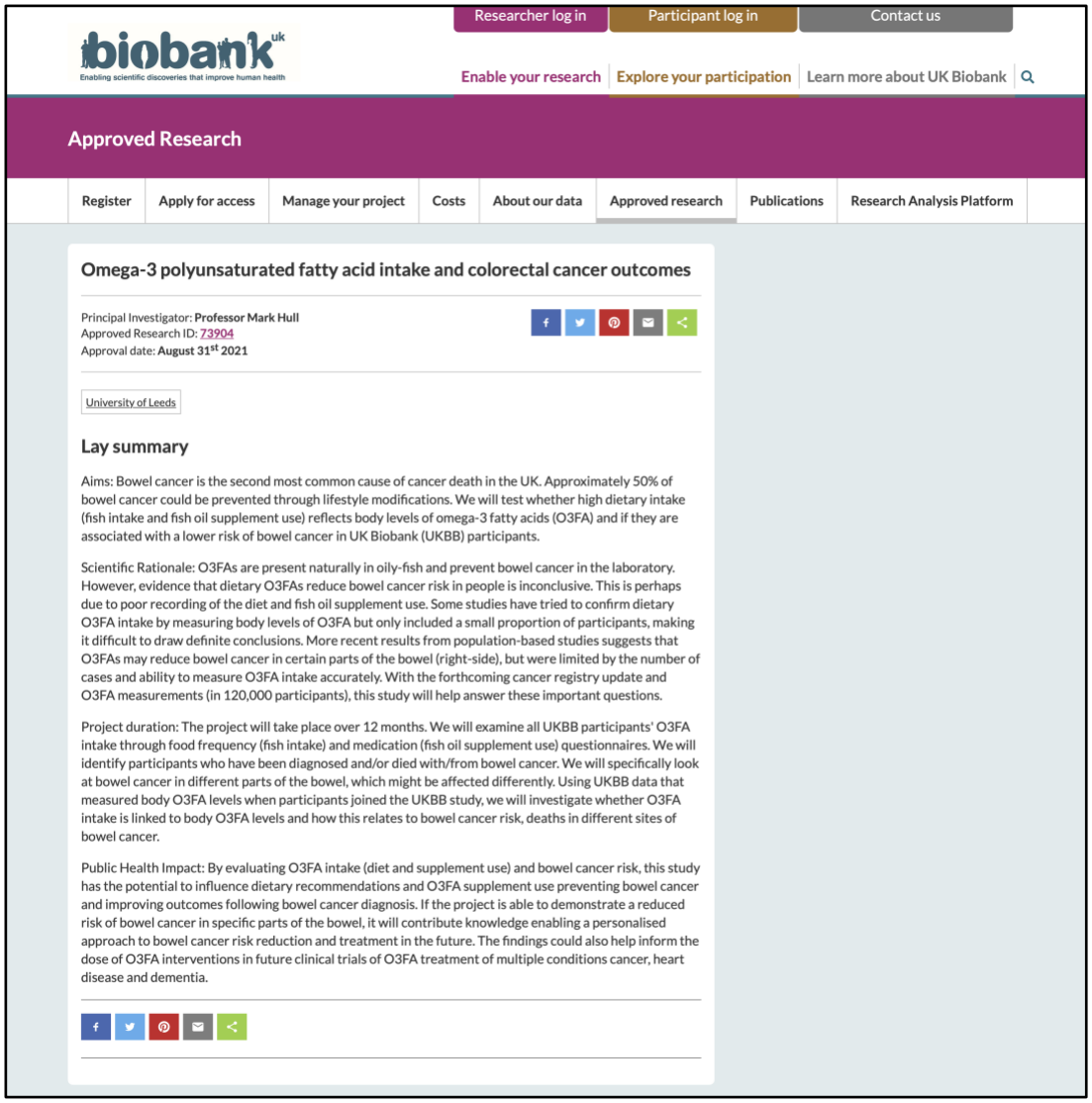
Appendix A UK Biobank Approval



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Omega-3 polyunsaturated fatty acid intake and colorectal cancer outcomes

Principal Investigator: **Professor Mark Hull**
Approved Research ID: 73904
Approval date: **August 31st 2021**

University of Leeds

Lay summary

Aims: Bowel cancer is the second most common cause of cancer death in the UK. Approximately 50% of bowel cancer could be prevented through lifestyle modifications. We will test whether high dietary intake (fish intake and fish oil supplement use) reflects body levels of omega-3 fatty acids (O3FA) and if they are associated with a lower risk of bowel cancer in UK Biobank (UKBB) participants.

Scientific Rationale: O3FAs are present naturally in oily-fish and prevent bowel cancer in the laboratory. However, evidence that dietary O3FAs reduce bowel cancer risk in people is inconclusive. This is perhaps due to poor recording of the diet and fish oil supplement use. Some studies have tried to confirm dietary O3FA intake by measuring body levels of O3FA but only included a small proportion of participants, making it difficult to draw definite conclusions. More recent results from population-based studies suggests that O3FAs may reduce bowel cancer in certain parts of the bowel (right-side), but were limited by the number of cases and ability to measure O3FA intake accurately. With the forthcoming cancer registry update and O3FA measurements (in 120,000 participants), this study will help answer these important questions.

Project duration: The project will take place over 12 months. We will examine all UKBB participants' O3FA intake through food frequency (fish intake) and medication (fish oil supplement use) questionnaires. We will identify participants who have been diagnosed and/or died with/from bowel cancer. We will specifically look at bowel cancer in different parts of the bowel, which might be affected differently. Using UKBB data that measured body O3FA levels when participants joined the UKBB study, we will investigate whether O3FA intake is linked to body O3FA levels and how this relates to bowel cancer risk, deaths in different sites of bowel cancer.

Public Health Impact: By evaluating O3FA intake (diet and supplement use) and bowel cancer risk, this study has the potential to influence dietary recommendations and O3FA supplement use preventing bowel cancer and improving outcomes following bowel cancer diagnosis. If the project is able to demonstrate a reduced risk of bowel cancer in specific parts of the bowel, it will contribute knowledge enabling a personalised approach to bowel cancer risk reduction and treatment in the future. The findings could also help inform the dose of O3FA interventions in future clinical trials of O3FA treatment of multiple conditions cancer, heart disease and dementia.

Appendix B Ethical Approval

Fw: Research Ethics Application Approved [Investigation of the effect of omega-3 polyunsaturated fatty acids on colonic short chain fatty acid production in vitro]-[200210044]

From Christine Edwards <Christine.Edwards@glasgow.ac.uk>
To joannaaldoori@doctors.org.uk <joannaaldoori@doctors.org.uk>
Date 2021-12-08 14:15

Good news

Christine

From: ResearchEthicsSystem@glasgow.ac.uk <ResearchEthicsSystem@glasgow.ac.uk>
Sent: 08 December 2021 14:14
To: Christine Edwards <Christine.Edwards@glasgow.ac.uk>
Subject: Research Ethics Application Approved [Investigation of the effect of omega-3 polyunsaturated fatty acids on colonic short chain fatty acid production in vitro]-[200210044]

Dear Professor Christine Edwards,

The following research ethics application has been approved:

Project Title	Investigation of the effect of omega-3 polyunsaturated fatty acids on colonic short chain fatty acid production in vitro
Application Number	200210044
Committee	College of Medical Veterinary and Life Sciences
Submitted By	Professor Christine Edwards

Please log in to the Research Ethics System to download the approval letter from your Application.

This is an automated message. Please do not reply to this email.
If you need additional help, please contact your ethics administrator or visit the IT Services helpdesk.

Appendix C Recruitment Poster

UNIVERSITY OF LEEDS

University of Glasgow

Would you like to be involved in our research?

Healthy volunteers (aged 18+ years) wanted for a study investigating the effects of omega-3 fatty acids (found in oily-fish & fish oil supplements) on gut bacteria in the role of bowel cancer risk

For this study we need heathy participants to collect one stool sample that we will use to carry out some simple laboratory experiments

We will ask you to avoid eating fish or taking fish oil supplements for two days before sample collection

If interested in taking part, please contact Joanna Aldoori
Email: joanna.aldoori@nhs.net

Human Nutrition, New Lister Building, Glasgow Royal Infirmary, G31 2ER

This study has been granted ethical approval by the University of Glasgow, College of MVLS Ethics Committee 2021

Appendix D Participant Information Sheet

Title of study

Investigation of the effect of omega-3 polyunsaturated fatty acids (O3FAs) on colonic short chain fatty acid production *in vitro*

Invitation to take part

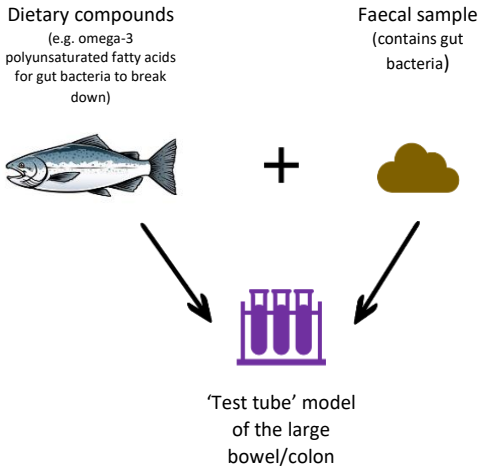
Thank you for reading this. You are being invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. If you decide to take part in this study, you will be given a copy of this Participant Information Sheet and the signed consent form to keep.

What is the purpose of the study?

Omega-3 polyunsaturated fatty acids (O3FAs) are found naturally in highest quantities in oily fish (such as mackerel, salmon, and herring). O3FAs have anti-cancer properties, including against bowel (colorectal) cancer, but it is not known how O3FAs work. Your bowel contains many millions of bacteria. Researchers at the University of Leeds have already shown that an O3FA supplement increases the number of bacteria in faecal samples that are predicted to make short-chain fatty acids (SCFAs). SCFAs are produced when bacteria digest dietary fibre. SCFAs are important for bowel health and have anti-cancer properties. This study will measure whether O3FAs lead to an increase in SCFA levels in the large bowel (colon). The results will inform future clinical studies which will improve use of O3FAs for prevention and/or treatment of bowel cancer by, for example, increasing dietary fibre intake at the same time.

We can test SCFA production in the colon by using a simple ‘test tube’ model of the large bowel where we grow bacteria from faeces in bottles mimicking conditions in the large bowel. We will also look at the type of bacteria present in the samples using bacterial (not human) DNA analysis (measured in Leeds) and measure the levels of other anti-inflammatory and anti-cancer

molecules (measured in Bradford). To carry out these studies we need fresh human faecal samples.



Why have I been invited to participate?

You are:

- A healthy individual, aged 18-65, non-smoker, in good general health, not a vegetarian or vegan and have not had a cholecystectomy (an operation to remove your gallbladder).
- You have not taken fish oil supplements (including cod liver oil), antibiotics, non-steroidal anti-inflammatory medications (such as ibuprofen, naproxen, diclofenac), laxatives and/or aspirin regularly (greater than or equal to three times a week) in the 3 months before taking part in the study.
- You do not suffer from any condition affecting bowel health e.g., inflammatory bowel disease (Crohn's disease or ulcerative colitis), irritable bowel syndrome, coeliac disease, diverticular disease etc.
- You are not pregnant or trying to get pregnant.
- You live within a half an hour radius (within the transport means available to you) of the Human Nutrition Department at Glasgow Royal Infirmary.

Do I have to take part?

No, it is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. If you decide to withdraw, any data

collected up to that point will be retained and used for the remainder of the study.

What will happen to me if I take part?

You will talk with a member of the Research Team from the University of Glasgow who will explain the study at a convenient time. All reasonable transport costs, as well as costs associated with the study, will be reimbursed.

What do I have to do?

You will have the study explained by one of the research team.
We will ask for your age, sex, and ethnicity.
We will check your suitability for the study by asking some simple questions about your usual diet, any health problems, and recent medications.

We will ask you to avoid fish or other seafood for 2 days before providing a faecal sample and ask that you record your diet during this time. You may also be asked to follow either a low- or high-fibre (most fruit, vegetables, wholegrain foods, nuts, pulses etc.) diet.
You will be given contact details in order to contact a member of the Research Team with any queries.
You will be asked to tell us if you think you may have slipped up with any of the dietary advice.

At the end of the two-day diet, we will ask you to provide a faecal sample.
You will be given full instructions on how to collect the sample and we will provide a special collection device that can be placed on the toilet seat to make collection as convenient as possible.

We will ask you to place the sample in a special container, which we will provide, that can be tightly sealed. Before the container is sealed, we will need you to add a sachet that is used to create an atmosphere to preserve the activity of the bacteria. The container then needs to be put into a plastic bag and sealed. This is then placed into a cool bag. You can then either bring the sample to our unit or we can collect it from you. You can also come to the unit to collect the sample there if that is easier for you.

We need the sample in our unit as soon as possible after passage so we need you to let us know immediately after you have produced a sample.

Samples need to be collected in the morning, so they are in the unit ready for processing before lunchtime.

We may ask if you would be willing to provide more than one sample, but this is not a requirement.

What are the possible disadvantages and risks of taking part?

There are no risks or disadvantages associated with this study other than time loss and slight inconvenience of following minor dietary restrictions.

What are the possible benefits of taking part?

There are no direct benefits to the volunteers associated with taking part. This study will provide us with a better understanding of the effects of O3FAs in the large bowel. This study will inform future clinical studies relating to the use of O3FAs in the prevention and treatment of bowel cancer.

Will my taking part in this study be confidential?

Very little information will be required from you, but all information will be kept strictly confidential. Any data in paper form will be stored in locked cabinets in rooms with restricted access at the University of Glasgow. The data will be anonymised and the only data available to researchers will be your age, sex, and ethnicity. No one will be able to find out your name, or any other information which could identify you. Some of the samples from the 'test tube' experiments will be sent to Leeds and Bradford by courier for further analysis of the bacteria and levels of anti-inflammatory and anti-cancer molecules. The samples will be anonymised so that no one will be able to identify you from it. The samples will not be used for any other purposes or other research.

What will happen to my data?

Researchers from the University of Glasgow collect, store, and process all personal information in accordance with the General Data Protection Regulation (2018). Your data will be pseudo-anonymised. A code will be assigned to identify the sample and data. The pseudo-anonymised data will

be used by the researchers. The anonymisation key will be stored separately (in paper or electronic form) from the results data. The anonymisation key will be locked and the person who has access to that will not be part of the main team. Regulators may access the data to ensure it is managed in accordance with regulations. The only data available to researchers in Glasgow, Leeds and Bradford will be your age, sex, and ethnicity.

What will happen to the results of the research study?

Results will be presented at meetings of learned societies and published in scientific journals. Results will also be included in student project reports/theses. We will arrange a meeting to discuss the generalised project results with participant volunteers, if they would like that. All results will be anonymised, and no identifiable information will be published.

Who is organising and funding the research?

This project is being organised by the Human Nutrition Group, at the University of Glasgow and the Molecular Gastroenterology Group at the University of Leeds.

Who has reviewed the study?

This project has been reviewed by the University of Glasgow, College of MVLS, Ethics Committee.

Contact for further information and/or you would like to take part.

If you require further information and/or you are interested in taking part, please contact Professor Christine Edwards.

Professor Christine Edwards: Christine.Edwards@glasgow.ac.uk
Human Nutrition, New Lister Building, Glasgow Royal Infirmary, Glasgow G31 2ER

If you wish to ask an independent contact about the research or the team, please contact Dr Alison Parrett: Alison.Parrett@glasgow.ac.uk

Thank you for reading this information sheet.

Appendix E Consent form

1

University of Glasgow | College of Medical, Veterinary & Life Sciences

Centre Number:

Project Number:

Participant Identification Number for this study:

Title of Project: Investigation of the effect of omega-3 polyunsaturated fatty acids on colonic short chain fatty acid production *in vitro*

Name of Researcher(s):

CONSENT FORM

	Please initial box
I confirm that I have read and understood the Participant Information Sheet version 1.2 dated 30/11/2021.	☐
I confirm that I have read and understood the Privacy Notice version 1.2 dated 30/11/2021.	☐
I have had the opportunity to think about the information and ask questions, and understand the answers I have been given.	☐
I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights being affected.	☐
I confirm that I agree to the way my data will be collected and processed and that data will be stored for up to 10 years in University archiving facilities in accordance with relevant Data Protection policies and regulations.	☐
I understand that all data and information I provide will be kept confidential and will be seen only by study researchers and regulators whose job it is to check the work of researchers.	☐
I agree that my name, contact details and data described in the information sheet will be kept for the purposes of this research project.	☐
I understand that if I withdraw from the study, my data collected up to that point will be retained and used for the remainder of the study.	☐
I would like to receive information about the generalised project results (if yes, please indicate how you would like to receive this at the end of the form).	☐
I agree to take part in the study.	☐

_____	_____	_____
Name of participant	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

If you would like to receive information about generalised project results, when possible, how would you like to receive this?

Email ☐ If yes, please provide your email address: _____

_____

Post ☐ If yes, please provide your postal address: _____

_____

_____

(1 copy for participant; 1 copy for researcher)

Appendix F Instructions for faecal sample collection

University of Glasgow | College of Medical, Veterinary & Life Sciences

Instructions for the collection of a faecal sample

1. Lift the toilet seat.
2. Place the bracket and the sample pot across the bowl.
3. Put the plastic bag inside the pot.
4. Lower the toilet seat.
5. Sit on the toilet seat. Check to make sure the sample pot is right below you.
6. If needed move the bracket and bucket around.

You do not have to use the bracket if you do not find it necessary.

7. Let your entire bowel movement fall into the plastic bag inside the pot.

Do NOT urinate or place toilet paper into the pot.

8. Stand up. Lift the pot out of the bracket and close (or fold over) the plastic bag in the pot. You can do this with plastic gloves on if you prefer.
9. Remove the bracket. Finish toilet activities.

Turn over the page for instructions after you have collected the sample

University of Glasgow | College of Medical, Veterinary & Life Sciences

Please follow these instructions below as carefully as possible after you have collected the sample;

1. Put on the plastic gloves provided (if they are not on already). Tear open the Anaerogen™ sachet and put into the pot above the plastic bag containing the sample. Cover the pot, containing the sample and sachet, with the lid. Seal it tight, and then place it inside the plastic bag.



2. Immediately twist the neck of the bag really tight, trying to push out as much air as you can. Then make a firm double knot. It is very important to **tie the bag firmly** to keep air out.


3. Place the bag with the sample into the cool bag.

IMPORTANT: call the researcher immediately after the sample is produced.

Please contact the researcher Joanna Aldoori on 07816180077

If there is no reply, please leave a text message with your name if possible

Appendix G Data collection proforma

University of Glasgow | College of Medical, Veterinary & Life Sciences

Investigation of the effect of omega-3 polyunsaturated fatty acids (O3FAs) on colonic short chain fatty acid production *in vitro* Study

Data collection form

Participant Number	
Age	
Sex	
Weight	
Height	
Ethnicity	

Appendix H Health and Medication Screen Questionnaire

Are you a vegetarian or vegan?

Yes •

No •

Are you pregnant or aiming to get pregnant?

Yes •

No •

Do you use fish oil supplements (including cod liver oil) three or more times a week?

Yes •

No •

Have you used any of the following in the last 3 months: non-steroidal anti-inflammatory medications, aspirin, laxatives and/or antibiotics?

Yes •

No •

Do you have a diagnosis of inflammatory bowel disease or irritable bowel syndrome?

Yes •

No •

Have you had a previous colonic or small bowel resection (bowel surgery)?

Yes •

No •

Have you had a cholecystectomy (operation to remove your gallbladder)?

Yes •

No •

Are you a current smoker?

Yes •

No •

Appendix I Dietary Guidance

For this study we will ask you to **avoid eating any fish for 2 days** prior to providing us with a sample.

We will ask you to eat a minimum of 20 grams of fibre a day.

Please see the table below for examples of foods with high fibre and their total fibre content per 100g[1]

	Total fibre per 100g
Cereals and carbohydrates	
Shredded whole wheat or bran cereals	13-24.5g
Wholemeal bread	7g
Raw porridge oats	8g
Wholemeal spaghetti	4.2g
Baked potato	2.5g
Fruit	
Apple	2.3g
Figs	6.9g
Strawberries	3.8g
Broccoli	2.8g
Nuts and seeds	
Almonds	7.4g
Peanuts	7.6g
Sunflower seeds	7.9g
Pulses	
Baked beans in tomato sauce	4.9g
Peas	5.6g
Green beans	4.1g

[1]Adapted from the British Dietetic Association Food Fact Sheet: Fibre. Accessed online 30/11/2021. https://www.bda.uk.com/resourceDetail/printPdf/?resource=fibre