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A randomised trial of the effect of omega-3 polyunsaturated fatty acid supplements on the human intestinal microbiota

Running Title: Omega-3 fatty acids and human intestinal microbiota

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Word Count: 4018

Key words: bacteria; colorectal cancer; fatty acid; nutritional supplement; omega-3

Abbreviations: AA, arachidonic acid; AE, adverse event; CRC, colorectal cancer; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; *F/B*, *Firmicutes*/*Bacteriodetes*; HARU, Human Appetite Research Unit; LC-MS/MS, liquid chromatography tandem mass spectrometry; OUT, operational taxonomic unit; PCoA, principal coordinate analysis; PUFA, polyunsaturated fatty acid; RBC, red blood cell; SCFA, short-chain fatty acid.

Abstract

Objective: Omega-3 polyunsaturated fatty acids (PUFAs) have anti-colorectal cancer (CRC) activity. The intestinal microbiota has been implicated in colorectal carcinogenesis. Dietary omega-3 PUFAs alter the mouse intestinal microbiome compatible with anti-neoplastic activity. Therefore, we investigated the effect of omega-3 PUFA supplements on the faecal microbiome in middle-aged, healthy volunteers (n=22).

Design: A randomized, open-label, crossover trial of 8 weeks' treatment with 4 g mixed eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) in two formulations (soft-gel capsules and Smartfish™ drinks), separated by a 12 week 'washout' period. Faecal samples were collected at five time-points for microbiome analysis by 16S rRNA PCR and Illumina MiSeq sequencing. Red blood cell (RBC) fatty acid analysis was performed by liquid chromatography-tandem mass spectrometry.

Results: Both omega-3 PUFA formulations induced similar changes in RBC fatty acid content, except that drinks were associated with a larger, and more prolonged, decrease in omega-6 PUFA arachidonic acid (AA) than the capsule intervention (P=0.02). There were no significant changes in α or β diversity, or phyla composition, associated with omega-3 PUFA supplementation. However, a reversible increased abundance of several genera, including *Bifidobacterium*, *Roseburia*, and *Lactobacillus*, was observed with one or both omega-3 PUFA interventions. Microbiome changes did not correlate with RBC omega-3 PUFA incorporation or development of omega-3 PUFA-induced diarrhoea. There were no treatment order effects.

Conclusion: Omega-3 PUFA supplementation induces a reversible increase in several short-chain fatty acid-producing bacteria, independently of the method of administration. There is no simple relationship between the intestinal microbiome and systemic omega-3 PUFA exposure. ISRCTN18662143.

- **What is already known on this subject?**
  - The naturally occurring omega-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have anti-colorectal cancer (CRC) activity
  - High purity EPA and DHA can be provided in soft-gel capsule form or as a 'nutrition' drink providing greater than 2 g omega-3 PUFAs daily
  - The intestinal microbiota are implicated in colorectal carcinogenesis, as well as modulation of chemo- and immuno-therapy of CRC
- **What are the new findings?**
  - Oral high-dose omega-3 PUFAs do not produce marked changes in the intestinal microbiome in healthy volunteers, even in individuals with treatment-emergent diarrhoea
  - Intake of 4 g daily mixed EPA/DHA for 8 weeks was associated with a reversible increase in *Bifidobacterium*, *Oscillospira*, *Roseburia* and *Lachnospira* species, but decreased *Coprococcus* and *Faecalibacterium*
  - Similar effects of omega-3 PUFAs on the faecal microbiome were observed for both capsule and drink formulations
  - Capsule and drink formulations provide equivalent tissue omega-3 PUFA incorporation, as measured by red blood cell levels, but only drinks were associated with prolonged suppression of pro-inflammatory arachidonic acid levels
- **How might it impact on clinical practice in the foreseeable future?**
  - An increase in short-chain fatty acid-producing bacteria may be relevant to the beneficial anti-CRC effects of EPA in both prevention and adjuvant treatment settings
  - Clinical evaluation of the anti-cancer properties of omega-3 PUFAs needs to consider the intestinal microbiota and its role in carcinogenesis and immune regulation

Introduction

The two main omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA; C20:5ω3) and docosahexaenoic acid (DHA; C22:6ω3) are widely used as nutritional supplements, as fish oil or in more concentrated 'nutraceutical' form.[1] Multiple health benefits have been claimed for these long-chain omega-3 PUFAs, including secondary prevention of ischaemic heart disease,[2] treatment of rheumatoid arthritis [3] and anti-cancer activity,[4] some of which are supported by evidence from randomised trials.[5-6]

The mechanism(s) underlying the colorectal cancer (CRC) chemopreventative activity of EPA reported by West *et al* is unclear.[5] It has been proposed that the intestinal microbiota may play a role in colorectal carcinogenesis based on the association of CRC with a specific intestinal microbiome profile, or so-called dysbiosis, characterised by low phylogenetic diversity, altered *Firmicutes/Bacteriodetes* ratio, under-representation of short-chain fatty acid (SCFA)-producing genera such as *Roseburia* and *Eubacterium*, as well as presence of putative pathobionts such as *Fusobacterium nucleatum*.[7-8] One possibility is that modulation of the intestinal microbiota may contribute to the cancer preventative properties of omega-3 PUFAs.

Data from mouse models suggest that dietary omega-3 PUFA intake or high tissue levels of omega-3 PUFAs are associated with differences in intestinal microbiota, including increased quantities of certain genera, including *Bifidobacterium* and *Lactobacillus*.[9-10] There has been only one case report of the effect of an omega-3 PUFA-rich diet on human intestinal microbiota.[11] In this case, there was a notable increase in several SCFA (butyrate)-producing genera including *Blautia*, *Bacterioides*, *Roseburia* and *Coprococcus*.[11]

Therefore, a plausible hypothesis is that omega-3 PUFA intake alters the composition of human intestinal microbiota, thereby attenuating the intestinal dysbiosis associated with colorectal carcinogenesis.

Nutritional supplementation with omega-3 PUFAs can occur in several ways, either as unrefined fish oil, in 'nutraceutical' form, usually as the triglyceride or ethyl ester conjugate in soft-gel capsules, taken with food, or more recently as an emulsion in drink form.[1]

In order to address the above hypothesis and, at the same time, compare two different formulations of omega-3 PUFAs, we performed a randomised, open-label, crossover study of the effect of omega-3 PUFAs on the intestinal microbiome of healthy volunteers aged over 50

years (a population relevant to CRC screening and chemoprevention), thus comparing equivalent doses of equimolar amounts of EPA and DHA in capsule or drink form, with an integrated 'washout' period, with which to determine reversibility.

Methods

Study design and interventions

The randomized, crossover trial was carried out in the Human Appetite Research Unit (HARU), University of Leeds. Approval was obtained from the South Yorkshire Research Ethics Committee (15/YH/0142). Interventions were not classified as Investigational Medicinal Product by the Medicines and Healthcare Products Regulatory Agency. The study was registered with ISRCTN (18662143).

Healthy volunteers aged ≥ 50 years of both sexes were sought using a HARU volunteer database and advertising across the University. Participants received £20 per study visit. At a screening visit, the following exclusion criteria were considered: ongoing or planned regular use of other omega-3 PUFA or cod-liver oil supplements; seafood allergy; concomitant use of non-steroidal anti-inflammatory medications, including aspirin; current treatment for any chronic inflammatory condition or malignancy; previous colonic or small bowel resection; current smoker (minimum 6 months smoking cessation); and pregnancy. If an individual was eligible, he/she underwent a 'lead-in' taste-test of the peach-flavoured drink and also swallowed two study capsules with water, after providing written informed consent, in order to confirm likely compliance and minimise drop-out.

Visit 1 occurred within 2 weeks of the screening visit, at which participants were randomised to take either 1) two 200 ml Smartfish® Remune drinks (see Supplementary Methods for content) per day (providing approximately 2000 mg EPA and 2000 mg DHA, as the triglyceride) at any suitable time of day, or 2) Four soft-gel capsules (each containing 250 mg EPA and 250 mg DHA as the ethyl ester) twice daily with meals (providing 2000 mg EPA and 2000 mg DHA per day), both for eight weeks (Intervention A; Figure 1). After a 12-week 'washout' period, participants took the second intervention for 8 weeks (Intervention B; Figure 1). We also included a final study visit after a second 12-week 'washout' period (V5; Figure 1). Randomisation was performed by Leeds Teaching Hospitals Pharmacy using random permuted block allocation in concealed envelopes. Neither participants nor researchers were blinded to the interventions and hence allocation order.

At each visit, adverse event (AE) monitoring was undertaken by a brief interview based on questioning for recognised AEs of omega-3 PUFA supplements, including loss of appetite, eructation ('fishy' burping), nausea, vomiting, dyspepsia, abdominal pain and diarrhoea, as well as bleeding events. Review of AEs was performed by an Independent Data Monitoring

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Committee every three months. Tolerability of both drinks and capsules was assessed with a Palatability Questionnaire and capsule acceptability questionnaire at the end of each eight-week intervention period (either visit 2 & 4). Participant height and weight were measured at the start of the study.

Blood and urine were collected at each visit and a faecal sample (obtained with a Fe-Col™ faecal collection device) was returned by hand or by Royal Mail Safebox® within 2 days of each visit. Participants did not start either intervention until the baseline (visit 1 or 3) faecal sample had been collected. Faecal samples were stored at -20°C in RNAlater® (Thermofisher Scientific) until DNA extraction, which occurred within 2 weeks of collection, in the majority of cases.

Omega-3 PUFA measurement

Red blood cells (RBCs) were obtained from whole blood EDTA samples as described.[12] Samples were stored at -80°C until lipid extraction and measurement of fatty acids by liquid-chromatography tandem mass-spectrometry (LC-MS/MS) as described.[13] Data are presented as the % (w/w) content of the total fatty acid pool measured.[13]

Intestinal microbiome analysis

Microbial DNA extraction, PCR and Illumina sequencing were performed as described (see Supplementary Methods).[14]

De-multiplexed FASTQ files were trimmed of adapter sequences using cutadapt.[15] Paired reads were merged using fastq-join [16] under default settings and then converted to FASTA format. Consensus sequences were removed if they contained any ambiguous base calls, two contiguous bases with a PHRED quality score lower than 33 or a length more than 2 bp different from the expected length of 240 bp. Further analysis was performed using QIIME.[17] Operational taxonomy units (OTUs) were picked using Usearch,[18] and aligned to the Greengenes reference database using PyNAST.[19] Taxonomy was assigned using the RDP 2.2 classifier.[20] The resulting OTU biom files from the above analyses were imported in MEGAN for detailed group specific analyses, annotations and plots.[21]

As well as comparisons of observed taxa, rarefication was performed to various levels to compare α diversity. All groups were rarefied to the lowest read number, and β diversity calculated using weighted and unweighted UniFrac as well as the non-phylogenetic Bray-Curtis dissimilarity measure.[14] β diversity was compared using Principal Coordinate Analysis

(PCoA) on all samples. Correlations between the microbiome and RBC fatty acid data were computed using package 'Hmisc',[22] in R.[23]

<u>Endpoints and sample size calculation</u>

In the absence of any data on the effect of omega-3 PUFAs on human intestinal microbiota, we determined the sample size based on testing non-inferiority of the drink formulation compared with capsules for the RBC level attained after 8 weeks' dosing. There are no prior RBC omega-3 PUFA data available in subjects consuming Smartfish® Remune. However, based on data from healthy individuals consuming omega-3 PUFA capsules that reported intra-individual variability of 4%,[24] we set a non-inferiority margin of 3%. Assuming participant drop-out of 20%, we estimated that a sample size of 20 participants would be required in order to exclude a difference in combined levels of RBC EPA and DHA between the intervention groups of greater or equal to 0.3 absolute % points, with 90% power at a significance level of 0.05.

Data were analysed on an intention-to-treat basis. The paired Student's t-test was used to evaluate the difference between RBC omega-3 PUFA levels for participants who completed both intervention periods. Unpaired data from participants, who did not complete both intervention periods, were included in unpaired data analyses. Comparison of absolute pre- and post-supplementation levels of RBC omega-3 PUFA levels within intervention groups was by paired Student's t-test. Adverse events in each group were compared by Chi-squared analysis.

For intestinal microbiota analysis, OTU read numbers at different time-points for the two interventions were compared by unpaired and paired analysis. Having excluded a significant treatment order effect on both RBC omega-3 PUFA incorporation and the intestinal microbiome profile, data for the capsule intervention (termed Int1) and the drinks intervention (termed Int2) were combined regardless of the intervention order (either A or B).

Results

Twenty-two participants were randomised between July 2015 and April 2016 (Figure 1). There were 12 females and 10 males with a median age of 57 years (range 51-65 years) and a median body mass index of 27.1 Kg/m^2 (range 22.0-33.8 Kg/m^2). Both interventions were well tolerated with good acceptability scores (Supplementary Results). Two individuals withdrew from the study during intervention period A (both capsules) because of an AE. One individual did not take drinks because of concern about calorific intake, but remained in the study. Of those individuals completing intervention periods for drinks (n=16) and capsules (n=20), the median duration of the intervention period was 57 (range 54-59) and 57 (55-63) days respectively.

Symptoms reported during all capsule interventions (n=22) and all drink interventions (n=19) are detailed in Table 1. In general, there was an excess of treatment-emergent AEs during the capsule intervention, particularly for dyspeptic symptoms (heartburn, acid regurgitation). All AEs were defined as minor (any symptom experienced during intervention, but did not require cessation of intervention or dose reduction) except for three AEs in each group that were classified as moderate (any symptom experienced that required subsequent dose reduction or cessation of intervention) and led to cessation of the intervention. All the AEs during the capsule intervention and the AEs in 9/10 individuals during the drink intervention had resolution of the AE during washout, suggesting that these were definite adverse reactions.

Changes in RBC omega-3 PUFA content

Fatty acid analysis was performed on those samples from participants who completed one or both intervention periods and washout (n=20). Only three individuals did not complete a second intervention-washout cycle with full blood sampling. Overall, 98 RBC samples underwent lipid extraction for omega-3 PUFA analysis. Data were available for 97 samples as one sample did not extract well and was uninterpretable.

Individual PUFA data are represented as the absolute % level, change between baseline and the post-treatment level, and difference between baseline and 'washout' value in Figure 2. Data for the order of intervention (drinks then capsules or capsules then drinks) are shown only for EPA and DHA (Figure 2A and B). The 'washout' period (12 weeks) was effective with return to baseline RBC levels of EPA and DHA for both drinks and capsules, for either order of intervention. There was a lower post-treatment % RBC content of EPA or DHA (Figure 2A and

B) if drinks were consumed after capsules compared with the opposite intervention order, but this difference was not statistically significant. There were no intervention order effects on content of the other fatty acids measured (data not shown).

As there was no significant difference in RBC PUFA levels related to the order of intervention, data for individual PUFAs were pooled, independently of the intervention order, for the further comparison of drinks and capsules (Figure 2C-F). Baseline and post-treatment levels of EPA, DPA and DHA were well matched with no significant difference between the post-treatment level of EPA and DHA attained following consumption of drinks or capsules (Figure 2C and E). The mean individual difference between the combined RBC EPA and DHA level attained after the drink intervention and that attained after capsule use was -0.12 (95% confidence interval -1.84 to 1.61; n=16 paired data), thereby confirming non-inferiority of the drink formulation compared with capsules for omega-3 PUFA incorporation in RBCs. There was little conversion to $C22:5\omega-3$ docosapentaenoic acid (DPA) from EPA (Figure 3D). Incomplete washout of DHA from RBC membranes after the capsule intervention was observed unlike that after the drink intervention (Figure 2E). Drink intake was associated with a larger decrease in relative $C20:4\omega-6$ arachidonic acid (AA) content compared with capsules, which remained evident at the end of washout (Figure 2F). However, there was no statistical post-treatment difference in the % AA content between drink and capsule use (Figure 2F). The omega-3/omega-6 ratio is commonly used as a biomarker of omega-3 PUFA bioactivity.[12] Therefore, we examined the effect of drink consumption and capsule intake on the EPA+DHA/AA ratio (Figure 2G). The larger decrease in AA content contributed to the higher EPA+DHA/AA ratio gained after use of drinks compared with capsules (Figure 2G), a difference which was statistically significant (P=0.02).

Supplementary Figures 1 and 2 demonstrate individual EPA and DHA profiles based on intervention order. As expected, there was wide variability in omega-3 PUFA incorporation between individuals. In general, EPA profiles were less variable, for both capsule and drink interventions (Supplementary Figure 1), than for RBC DHA levels, both within and between individuals (Supplementary Figure 2). Incorporation of either EPA or DHA with one intervention did not predict the individual response to the other intervention.

Changes in the intestinal microbiome

A faecal sample was collected at all five time-points from all 20 volunteers. Three samples were excluded from PCR amplification because of repeated poor quality DNA extraction,

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leaving 97 samples that were analysed (median 46603 reads; min 26387, max 114130). Bray-Curtis PCoA of all samples considering all taxonomic levels is shown in Figure 3A. Inter-individual differences in the intestinal microbiome exceeded any treatment effect of omega-3 PUFA in either capsule or drink form. In particular, volunteers #8 and #13 were markedly different from the others related to a reduced proportion of *Clostridia* and a larger proportion of the class *Gammaproteobacteria* with high abundance of *Succinivibrionaceae* (Supplementary Figure 3). For unpaired analysis of microbiome profiles across time points, data from these volunteers were excluded. Volunteer #16 also had a different microbiome with very high abundance of *Succinivibrionaceae*, but the rest of the taxonomic groups in this case were consistent with the other volunteers (Supplementary Figure 4). Therefore, we did not exclude this (#16) individual's data from further analysis. For the subsequent analyses, all capsule (termed Int1) and drink (termed Int2) intervention data were combined, regardless of the order of the interventions, on the basis of complete tissue (RBC) omega-3 PUFA washout and similarity of the microbiome profile at the two baseline assessments at visit 1 and visit 3 for either order of interventions (Supplementary Figure 5). The cladogram highlights that the omega-3 PUFA intervention, either as capsules or as drinks, was not associated with any significant overall taxonomic shift (Figure 3B). There was no significant change in α (Shannon diversity index) or β (unweighted and weighted unifrac distance) diversity at the end of each intervention period (Int1.2 and Int2.2) compared with baseline (Int1.1 and Int2.1) or the final washout time-point (V5) (Figure 3C).

The *Firmicutes/Bacteriodetes* (*F/B*) ratio is frequently reported in cohort studies investigating the relationship between intestinal dysbiosis and CRC.[7] However, neither capsule nor drink intake were associated with statistically significant changes in the *F/B* ratio (supplementary Figure 6).

However, presentation of data at individual family- and genus-level revealed consistent differences associated with both capsule omega-3 PUFA and drink omega-3 PUFA interventions that returned towards baseline upon cessation (Figure 3D). For example, some relatively low abundance families including *Clostridiaceae*, *Sutterellaceae* and *Akkermansiaceae* were each increased at the end of both interventions with reversibility after the washout period (Figure 3D). A similar phenomenon was observed at genus level, for example *Oscillospira* and *Lachnospira* (Figure 3D). Individual histograms are shown for the top five abundant genera in Figure 4A-E, which highlight changes associated with 8 weeks'

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intervention with capsules or drinks, each time with reversibility after 12 weeks. Supplementation with omega-3 PUFAs was associated with increased abundance of *Bifidobacterium*, *Oscillospira*, *Lachnospira* and a reduction in abundance of *Coprococcus* and *Faecalibacterium* (Figure 4A-E). We also performed a paired analysis, in which OTU read numbers for the top 20 genera were compared before and after capsule or drink interventions within individuals. This confirmed that *Bifidobacterium* and *Oscillospira* (increase), as well as *Coprococcus* (decrease), changed most for both interventions, whereas an increase in *Roseburia* and *Lachnospira* abundance was prominent only after the drink omega-3 PUFA intervention (Figure 5). A paired t test comparing the treatment effect (in OTU reads) between the capsule and drink intervention for the 17 individuals who had complete paired (before and after intervention) data revealed that the drink intervention was associated with increased abundance compared with capsules for *Roseburia* only (P<0.05). Mouse studies have consistently demonstrated an increase in *Lactobacillus* abundance after dietary fish oil supplementation.[9-10,25-26] Therefore, we analysed changes in this lower abundance genus following both omega-3 PUFA interventions. There was an increase in *Lactobacillus* OTU number after both interventions that returned towards baseline values after washout (Figure 4F). However, these differences were not statistically significant between baseline and post-intervention time-points (P=0.11 capsules; P=0.9 drinks) or comparing drinks *versus* capsules in a pair-wise manner (P=0.59). *Fusobacterium* spp. (including *nucleatum*) were not detected in faeces from this cohort of non-cancer individuals.

There was no effect of gender on baseline microbiome profile or treatment effect of either omega-3 PUFA intervention (data not shown). We also tested whether development of diarrhoea on omega-3 PUFA supplementation was associated with a shift in the intestinal microbiome. There was no consistent profile observed in stool samples collected at the visit during/after which diarrhoea was reported (Supplementary Figure 7). Moreover, we investigated whether the RBC EPA and DHA levels generated by each of the interventions correlated with changes in the intestinal microbiome. Correspondence analysis and correlation testing did not demonstrate any consistent relationship between RBC EPA/DHA levels and microbiome profile, with the relationship for EPA and DHA being weak in comparison with the other FAs tested (Supplementary Figure 8).

Discussion

We report small, consistent and reversible changes in the human intestinal microbiome associated with omega-3 PUFA supplementation for 8 weeks. The lack of significant change in microbial diversity associated with omega-3 PUFA intervention is consistent with mouse studies, in which there was either no change,[10] or a small change,[9] in α diversity. Our data are also consistent with the concept that short-term dietary interventions do not overcome the dominant inter-individual variation in the intestinal microbiome.[27]

The increased abundance of butyrate-producing, so-called 'beneficial', bacterial genera such as *Bifidobacterium*, *Lachnospira*, *Roseburia* and *Lactobacillus* during one or both types of omega-3 PUFA intervention is consistent with the mouse literature.[9-10, 25-26, 28-30] Whether the relatively small changes in intestinal microbiome that we observed at the end of the 8-week intervention period have functional consequences, including an increase in luminal SCFA levels, is a question that will require a metabolomic approach, including lipidomic profiling. The increase in *Roseburia* and *Lachnospira* was only observed during the drink intervention. Further studies are required in order to determine what aspect of the drink formulation may explain this difference.

Studies of CRC and colorectal adenoma patients have reported a reduction in OTUs of SCFA-producing bacteria compared with healthy controls.[7, 31-33] An increase in SCFA-producing bacteria such as *Bifidobacterium*, leading to enhanced mucosal SCFA exposure has been suggested to reduce mucosal inflammatory tone.[34] Therefore, our findings are compatible with a hypothesis that omega-3 PUFA intake is associated with intestinal microbiota changes driving increased luminal SCFA exposure. In future studies, it will be key to investigate the effect of omega-3 PUFAs on the colonic metabolome in concert with dietary fibre assessment. Consistent with an interaction between omega-3 PUFAs and fibre, Crim *et al* have reported that fish oil and dietary pectin have synergistic anti-neoplastic activity in rats.[35]

We did not observe any relationship between intestinal microbiome changes and RBC omega-3 PUFA levels (a measure of systemic omega-3 PUFA exposure). Therefore, we do not provide any evidence that systemic 'bioavailability' of omega-3 PUFAs was modulated by the intestinal microbiota in our study. However, mouse studies have demonstrated that supplementation with *Bifidobacterium breve* was associated with increased tissue EPA and

DHA content, suggesting an influence of intestinal microbiota on tissue fatty acid levels.[36] It remains unclear whether RBC omega-3 PUFA levels after oral omega-3 PUFA supplementation predict omega-3 PUFA exposure in the gut lumen. In a study of patients (n=6) with a permanent ileostomy and minimal terminal ileum removed, less than 1% of the total oral omega-3 PUFA dose was recovered in ileal effluent suggesting efficient proximal small intestinal absorption.[37] However, the daily dose of EPA and DHA was less than 300 mg for 4 days only.[37] It remains unclear what the small intestinal and colonic bioavailability of omega-3 PUFAs is at oral dosing ≥2000 mg daily.[5-6]

A randomized trial of control *versus* sardine diet (100 g sardines for 5 days per week for 6 months providing approximately 3 g daily of EPA and DHA combined) in type II diabetics reported a significant decrease in *F/B* ratio in the sardine diet arm compared with controls.[38] We did not observe a significant change in the *F/B* ratio in our study, which may be explained by differences in omega-3 PUFA delivery and duration of the intervention.

Red blood cell fatty acid changes were similar during either the drink or capsule intervention with a significant increase in RBC EPA and DHA (and a parallel decrease in AA content) consistent with dosing at 4 g per day for 8 weeks.[39] It is not clear why the drink intervention was associated with a larger decrease in relative AA content, which led to a significantly higher EPA+DHA/AA ratio. We acknowledge that despite daily dose equivalence of EPA and DHA between drink and capsule interventions, the omega-3 PUFAs were triglyceride and ethyl ester conjugates respectively. In the longest comparative study of capsules providing 1.68 g EPA/DHA per day (6 months), the RBC omega-3 index was higher after consumption of omega-3 PUFA triglycerides compared with ethyl esters.[40] Our study suggests that, at high doses (4 g) over several weeks, possible small differences in intestinal bioavailability related to PUFA delivery are not relevant to omega-3 PUFA tissue incorporation and subsequent changes in the intestinal microbiota. Further investigation of the effect of the other macro- (eg. fibre) and micro-nutrients (eg. vitamin D) in the Smartfish® intervention on tissue PUFA incorporation and metabolism is required.

Smartfish® Remune drinks providing 4 g per day of EPA and DHA combined were well-tolerated by healthy middle-aged individuals. Treatment-emergent AEs were similar between drinks and capsules with a suggestion that upper gastrointestinal symptoms were more common during the capsule intervention. An AE occurring during one intervention did not consistently predict occurrence during the other intervention. The excellent acceptability and

tolerability, aligned with good omega-3 PUFA absorption characteristics, mirrors previous experience with an earlier version of the Smartfish® drink.[41] The aetiology of the dose-dependent diarrhoea caused by omega-3 PUFA intake in up to 5-10% of individuals remains unclear. We describe, for the first time, that diarrhoea associated with omega-3 PUFA intake is not associated with a significant change in intestinal microbiota or predicted by a particular baseline intestinal microbiome profile.

The strengths of this study include the crossover design that allowed a direct comparison between two omega-3 PUFA formulations, thus minimizing the effect of inter-individual variability in omega-3 PUFA incorporation [42] and intestinal microbiome profile,[43] as well as the middle-aged demographic of the study cohort relevant to CRC prevention. A methodological weakness was the lack of colorectal tissue in order study the effect of omega-3 PUFA treatment on the mucosa-associated microbiome. This would be particularly relevant if luminal bioavailability of oral omega-3 PUFAs is confirmed to be low and omega-3 PUFA 'exposure' is predominantly via membrane fatty acids from surface and/or shed enterocytes. We also did not obtain data on the background diet and dietary omega-3 PUFA intake.

In summary, we report that a high-dose (4 g daily) of mixed omega-3 PUFAs (EPA and DHA) given for 8 weeks is associated with small changes in the intestinal microbiota that are consistent across two different omega-3 PUFA interventions.[9-10, 25-28] The increase in density of bacteria known to be butyrate-producers concurs with the existing pre-clinical literature and is compatible with the known anti-inflammatory and anti-neoplastic properties of omega-3 PUFAs.

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Figure Legends

Figure 1. <u>Schedule of study visits and</u> **participant flow through the study.** The duration of the intervention and washout periods in weeks (w) is noted in the left column. n = number of individuals in study as per protocol. *One participant ceased the drink intervention early and completed intervention period B (capsules). **Two participants ceased drink intervention early (remained in study).

Figure 2. Red blood cell PUFA levels during the study. In each case (A-G), the left y axis is the baseline % RBC PUFA value or ratio, and the right y axis is the absolute difference between the post-treatment value or 'washout' value and the baseline % level. Columns (baseline % values) and symbols (absolute difference in % value from baseline) denote the mean. Error bars denote the standard error of the mean. A-B) Comparison of RBC EPA and DHA levels depending on whether the drink intervention was first or second. C-F) Individual RBC PUFA levels comparing pooled data from the drink *versus* capsule intervention, independently of the intervention order. G) Comparison of the EPA+DHA/AA ratio at baseline, post-treatment and after washout for the drink and capsule intervention. *$P<0.05$ for the difference between drinks and capsules; paired t test.

Figure 3. Changes in the intestinal microbiome associated with omega-3 PUFA supplementation. A) PCoA of all samples (V1-V5) for all participants. Each participant is denoted by a different colour. Clustering of data from individuals is prominent with relatively small differences between different time-points per individual. Circles highlight participants with high *Gammaproteobacteria* (blue) or *Succinivibrioacceae* alone (red). B) Cladogram. Int1.1 and Int1.2 denote visits/samples before and after the capsule intervention regardless of the intervention order (A then B, and B then A). Int 2.1 and Int2.2 are corresponding time-points for the drinks intervention regardless of intervention order. C) Shannon α diversity index (23305 sequences per sample) and weighted (open circles) and unweighted (solid squares) unifrac β diversity scores for all participant samples from each visit (Int1.1-1.2 and Int2.1-2.2 and V5). Symbols denote the mean and bars represent the standard deviation. D) Family- and genus-level profiles before and after capsule and drink interventions and at final 'washout'.

Figure 4. Abundance of top five genera and *Lactobacillus* at each visit. Columns represent the mean value for each time-point for each intervention (Int1 and Int2) irrespective of the order of the interventions (see Figure 3 legend).

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Figure 5. Changes in abundance of genera at the end of the 8-week omega-3 PUFA intervention period. Differences in OTU read number are calculated as the value at the end of the treatment period minus the value at the start of the intervention. Bars denote the mean value and bars denote the SD. Black and red bars distinguish capsule and drink intervention data, respectively. Positive values represent an increase associated with intervention and negative values represent a decrease in abundance at the end of the intervention period. $*P<0.05$ (one sample t test).

	CAPSULES (n = 22)	DRINKS (n = 19)
AE during intervention period (no. of participants)	29 (14)	17 (10)
Diarrhoea	5	5
Abdominal discomfort	6	3
Eructation (Burping)	10	4
Dyspepsia	5	1
Nausea/vomiting	3	4
1 or more symptoms defined as moderate	3 (2 ceased intervention)	3 (3 ceased intervention early)
Resolution of symptoms after washout (no. of participants)	14	9
Symptoms experienced during both intervention periods (proportion occurring during 2nd intervention that were present during the 1st intervention)	4/10 (40%)	4/9 (44%)

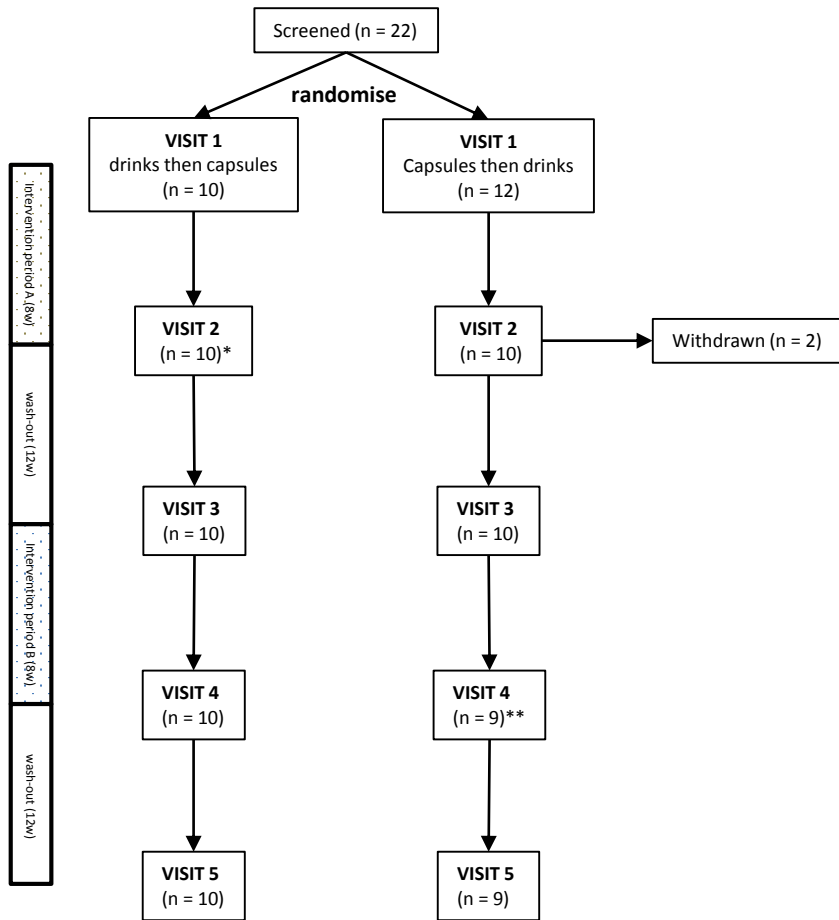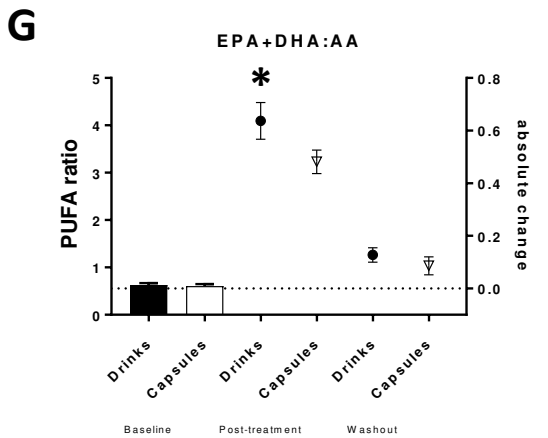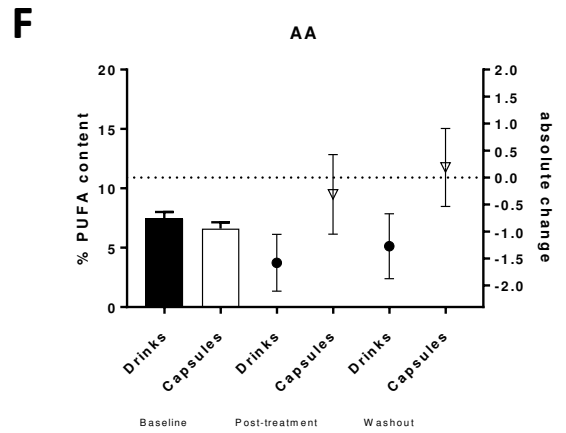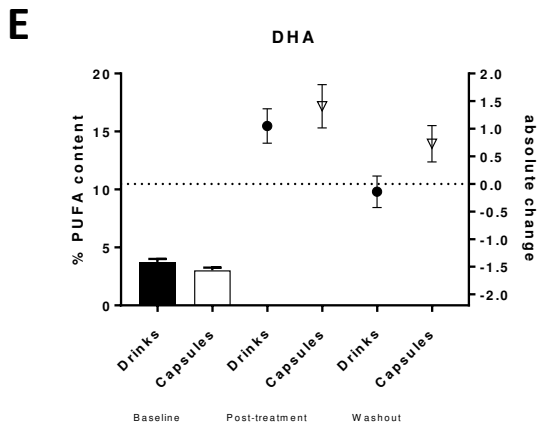**Table 1. Adverse events during capsule and drink intervention periods**

Figure 1

Figure 2

A

PCoA of Taxonomy using BrayCurtis: PC 1 (28.3%) vs PC 2 (19.0%)

B

Int1.1
Int1.2
Int2.1
Int2.2
V5

C

D

Legend (Taxa):

Ruminococcaceae | Lachnospiraceae | Bifidobacteriaceae | Erysipelotrichaceae
Succinivibrionaceae | Clostridiaceae | Sutterellaceae | Akkermansiaceae | Christensenellaceae
Acidaminococcaceae | Desulfovibrionaceae | Rikenellaceae | Bacteroidaceae
Pseudomonadaceae | Enterobacteriaceae | Pasteurellaceae | Peptostreptococcaceae
Peptococcaceae | Veillonellaceae | Victivallaceae | Porphyromonadaceae | Anaeroplasmataceae
Selenomonadaceae | Coriobacteriaceae | Lactobacillaceae | Prevotellaceae | Odoribacteraceae
Streptococcaceae | Peptoniphilaceae | Actinomycetaceae | Methanobacteriaceae
Moraxellaceae | Alcaligenaceae | Eggerthellaceae | Oxalobacteraceae | Aerococcaceae
Campylobacteraceae | Comamonadaceae | Eubacteriaceae | Gracilibacteraceae | Bacillaceae
Clostridiales Family XIII. Incertae Sedis | Micrococcaceae | Flavobacteriaceae | Leptotrichiaceae
Morganellaceae | Syntrophomonadaceae | Corynebacteriaceae | Brachyspiraceae
Carnobacteriaceae | Enterococcaceae | Leuconostocaceae | Brucellaceae | Caulobacteraceae
Erwiniaceae | Yersiniaceae | Atopobiaceae | Burkholderiaceae | Methylobacteriaceae
Microbacteriaceae | Neisseriaceae | Nocardioidaceae | Rhodobacteraceae | Synergistaceae
Bradyrhizobiaceae | Caldicoprobacteraceae | Deinococcaceae | Parachlamydiaceae
Propionibacteriaceae | Thermaceae | Vibrionaceae | Xanthomonadaceae

Legend (Taxa):

Bifidobacterium | Oscillospira | Coprococcus | Faecalibacterium | Lachnospira | Ruminococcus
Succinivibrio | Roseburia | Sutterella | Akkermansia | Blautia | Phascolarctobacterium
Desulfovibrio | Bacteroides | Pseudomonas | Haemophilus | Clostridium | Peptococcus | Dorea
Anaerostipes | Turicibacter | Dehalobacterium | Parabacteroides | Dialister | Mitsuokella
Lachnobacterium | Lactobacillus | Catenibacterium | Bilophila | Acidaminococcus | Bulleidia
Prevotella | Streptococcus | Actinomyces | Christensenella | Megasphaera | Butyricimonas
Peptoniphilus | Odoribacter | Holdemania | Veillonella | Methanobrevibacter | Parvimonas
Gemella | Acinetobacter | Coprobacillus | Aggregatibacter | Collinsella | Epulopiscium
Slackia | Scardovia | Paraprevotella | Campylobacter | Actinobacillus | Peptostreptococcus
Erysipelothrix | Lactococcus | Victivallis | Bacillus | Anaerococcus | Gracilibacter | Anaerofustis
Anaerovorax | Oxalobacter | Adlercreutzia | Citrobacter | Rothia | Myroides | Alistipes
Morganella | Syntrophomonas | Oribacterium | Porphyromonas | Desulfosporosinus | Finegoldia
Leptotrichia | Varibaculum | Facklamia | Corynebacterium | Selenomonas | Brachyspira
Leuconostoc | Desulfotomaculum | Granulicatella | Ochrobactrum | Vagococcus | Aerococcus
Butyrivibrio | Caloramator | Erwinia | Mogibacterium | Schwartzia | Serratia | Abiotrophia
Atopobium | Brevundimonas | Lautropia | Leucobacter | Methanosphaera | Methylobacterium
Oxobacter | Paracoccus | Pyramidobacter | Achromobacter | Alkaliphilus | Caldicoprobacter
Deinococcus | Eikenella | Enterococcus | Gallicola | Klebsiella | Moryella | Neisseria
Photobacterium | Propionibacterium | Stenotrophomonas | Thermus | Trichococcus

Figure 3

Figure 4

Difference in OTU reads (post-pre)

Figure 5