A randomized dietary intervention to increase colonic and peripheral blood short-chain fatty acids modulates the blood B- and T-cell compartments in healthy humans

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Sources of support

PAG was supported by a scholarship from the Central Clinical School, Monash University, MCvZ by a NHMRC Senior Research Fellowship GNT1117687, JGM by NHMRC Senior Research Fellowship GNT1136988.

Running title: Effects of SCFA intervention in healthy humans

List of abbreviations:

BCFA: Branched-chain fatty acids

BL: Baseline

BSS: Bristol stool scale

GPR: G-protein coupled receptor

HAMS: High-amylose maize starch

H-SCFA: High-SCFA diet

IBD: Inflammatory bowel disease

L-SCFA: Low-SCFA diet

MAIT: Mucosal-associated invariant T cell

PBMC: Peripheral blood mononuclear cells

RS: Resistant starch

SCFA: Short-chain fatty acids

TCR: T cell receptor

Tfh: T follicular helper

Th: T-helper

Treg: Regulatory T cell

t-SNE: t-distributed stochastic neighbor embedding

Clinical trial registry:

Australian New Zealand Clinical trials registry: ACTRN12618001054202.

<https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=375342&isReview=true>

ABSTRACT (298 WORDS)

Background: Short-chain fatty acids (SCFA) have immune-modulating effects in animal models of disease. However, there is limited evidence that this may occur in humans. **Objective:** This study aimed to determine the effects of increased exposure to SCFA via dietary manipulation on colonic fermentation and adaptive immune cells.

Methods: Twenty healthy, young adults (18-45 years of age) underwent a blinded, randomized, cross-over dietary intervention, consuming a high-SCFA producing diet and matched low-SCFA diet for 21 days with 21-day wash-out in between. SCFA were provided through resistant starch, inulin and apple cider vinegar. Blood and 3-day total fecal output were collected at baseline and at the end of each diet. Gas chromatography was used to measure fecal and plasma SCFA. Flow cytometry was used for peripheral blood immuno-phenotyping. **Results:** High-SCFA diet was associated with significantly (paired samples Wilcoxon test) higher median [IQR] fecal SCFA concentrations (86.6 [59.0] vs 75.4 [56.2] µmol/g, *P*=0.02) and significantly lower median fecal ammonia concentrations (26.2 [14.7] vs 33.4 [18.5] µmol/g, *P*=0.04) than the low-SCFA diet. Plasma propionate (9.87 [12.3] vs 4.72 [7.6] µmol/L, *P*=0.049) and butyrate (2.85 [1.35] vs 2.02 [1.29] µmol/L, *P*=0.03) were significantly higher after high-SCFA diet than after low-SCFA diet. Blood total B cells (184 [112] vs 199 [143] cells/µL, *P*=0.04), naive B cells (83 [66] vs 95 [89] cells/µL, *P*=0.02), Th1 cells (22 [19] vs 29 [16] cells/µL, *P*=0.03) and mucosal-associated invariant T (MAIT) cells (62 [83] vs 69 [114] cells/µL, *P*=0.02) were significantly lower after high-SCFA diet than low-SCFA diet.

Conclusion: Increasing colonic and peripheral blood SCFA has discrete effects on circulating immune cells in healthy humans following 3-week intervention. Further studies, e.g. in patients with inflammatory disease, are necessary to determine if these changes have immunomodulatory effects, whether these are therapeutically beneficial, and whether prolonged intake might be required.

Key words: dietary fiber, short-chain fatty acids, colonic fermentation, adaptive immunity, T cells, B cells, resistant starch

INTRODUCTION (539 WORDS)

Short-chain fatty acids (SCFA), predominantly acetate, propionate, and butyrate are 2-4 carbon chain molecules that are primarily generated in the colon through fermentation of nondigestible dietary carbohydrates by gut microbiota(1). In addition, SCFA are present in some foods and beverages via generation in fermentation processes that occur during their production(2). Increased generation of SCFA in the colon and subsequent systemic delivery have been have been associated with improved colonic health, reduced metabolic disease and systemic inflammation, primarily in small animals(3). However, there have been difficulties translating these observations to therapeutic effects in humans, due to challenges with delivering adequate amounts of SCFA to the colon and inherent differences in SCFA metabolism and gut physiology between animals and humans(4).

Although SCFA have potent effects on the immune system within animal models, evidence for the purported effects of SCFA on the human immune system are primarily limited to studies *in-vitro*, in which blood immune cells are exposed to SCFA, often at concentrations well above physiological levels(5). The few studies performed in vivo have been limited to using dietary fiber supplements, have lacked dietary controls and have not observed changes to peripheral blood SCFA concentrations(6-8). On the other hand, dietary intervention studies that successfully used fiber supplementation to increase both fecal and plasma SCFA have not addressed effects on immune parameters(9, 10). Exposure to SCFA may modulate the activity of both the innate and adaptive immune responses through directly engaging SCFA-specific G protein-coupled receptors (GPR43,41,109a) on the cell surface, or by entering the cell to alter cellular metabolism and histone deacetylase activity(11). Therefore, effects on a wide variety of immune cell lineages and subsets might be anticipated. We have previously shown that a 5-day high-fiber diet that successfully

increases plasma SCFA levels did not change proportions of circulating regulatory T(reg) cells or concentrations of a panel of plasma cytokines(12). The increased exposure to SCFA in terms of concentrations achieved and/or its duration may have been insufficient to induce changes in healthy individuals. Altogether, there is a clear need for well-designed intervention studies to investigate if SCFA can indeed modulate the human immune system.

Fermentable fiber supplements, such as high-amylose maize starch (HAMS) and inulin, are an effective way to increase colonic SCFA production in human subjects, and are amenable to use in a blinded study design since they have little taste themselves and take up flavors well(13). In addition, oral intake of fermented foods such as apple cider vinegar can be used to acutely deliver SCFA to the systemic circulation(2). We hypothesized that supplementing diet with a combination of dietary fermentable fiber (inulin and resistant starch) and oral SCFA (apple cider vinegar) would be a feasible strategy to increase colonic and plasma SCFA sufficiently to modulate aspects of the immune system in humans. Hence, the current study aimed to address this hypothesis with three major goals: first, to define the pharmacokinetics of blood SCFA concentrations during 3-weeks' consumption of a high-fiber diet together with oral SCFA-rich foods; secondly, to assess changes to colonic fermentation resulting from increased fiber and oral SCFA intake; and thirdly, to assess if this dietary intervention affects circulating immune cells in blood, particularly subsets of B and T lymphocytes shown to be modulated by SCFA in pre-clinical models.

METHODS (1936 WORDS)

Participant recruitment

Healthy adults who were aged 18-45 y inclusive and believed themselves to be in good health were recruited for this study. Exclusion criteria included a history of gastrointestinal or other chronic inflammatory disease; recent acute illness or infection; antibiotic use within one month of study; consumption of probiotics or prebiotics within one month of study; use of medication that could alter gastrointestinal transit (e.g. laxatives or hypomotility agents);

pregnancy or planning pregnancy; suffering from an eating disorder; have special dietary requirements (vegetarian/vegan) or currently taking medication for hypertension. Volunteers who expressed interest in the study were screened with an eligibility questionnaire, followed by a face-to-face meeting with the study coordinator to obtain written, informed consent.

Study design

The study was designed to be a dietary intervention composed of a randomized, single-blinded crossover design as outlined in **Figure 1**. Each study participant undertook a 1-week baseline period, before being randomized using a random number generator (http://www.randomizer.org/, Version 4) to either a low-SCFA or high-SCFA diet for 3 weeks. Afterwards, participants underwent a minimum 3-week wash-out period where they were free to consume their habitual diet, prior to completing the other 3-week interventional diet. Subjects, but not the investigators were blinded to the nature of the diet. On the morning of day 1 of the baseline week, participants had 30 mL of peripheral venous blood collected. They also undertook 3-day total fecal collection during the baseline week and completed a food and gastrointestinal symptom diary. This diary was also completed throughout the 3-week dietary intervention period. At the end of the dietary intervention period participants again had blood collected on the morning of day 20 or 21. They also completed 3-day total fecal collection on days 18-20 of the intervention period. Weight measurements were recorded using a digital physician scale (Tanita, Illinois, USA) during each study visit at the Alfred Hospital, Melbourne, Australia. The study protocol was approved by the Monash University Human Research Committee (project no: 11190, Clayton, Melbourne, Australia) and prospectively listed on the Australian New Zealand Clinical trials registry (ACTRN12618001054202).

Intervention diets

High- and low-SCFA diets were developed to meet the standards of the Australian Dietary guidelines(14) and provided equivalent levels of carbohydrates, proteins, fats and other

micronutrients. Nutritional composition was assessed using Foodworks Professional software (Version 7.01, Xyris, 124 Queensland, Australia) with data from the NUTTAB (Nutrient Tables for use in Australia) (Food standards Australia New Zealand, 125 ACT, Australia) and Monash University FODMAP database. The meal plans for both diets are shown in **Table 1**. The low-SCFA diet provided 18.6 g/day of fiber, of which 1 g/day was resistant starch and 1 g/day was non-digestible oligosaccharide. The high-SCFA diet contained an additional 20 g/day of high-amylose maize starch (HAMS-1043) (Ingredion, Lane Cove, NSW, Australia) that was added to 2 food items, as indicated in Table 1. This was equivalent to approximately 10 g/day of resistant starch(15). In addition, 8 g of inulin (average degree of polymerization ≥10) (OraftiGR, Beneo, Mannheim, Germany) was also included in a daily meal, as indicated in **Table 1.** Taken together, the high-SCFA diet provided an additional 18 g of fermentable fiber each day. The macronutrient and fiber breakdown of the high-SCFA and low-SCFA diet is contained in **Table 2.** To further boost SCFA-delivery, an apple cider vinegar drink consisting of apple cider vinegar (Melrose, Notting Hill, Victoria, Australia) mixed with an equal volume of flavored syrup (Cottee's blackcurrant cordial, Melbourne, Victoria, Australia) was also provided to participants to consume after each main meal (breakfast, lunch, dinner). Each drink contained approximately 25.8 mmol of acetate, 0.05 mmol of propionate, and 0.04 mmol of butyrate as previously described(2). A placebo vinegar-drink was provided on the low-SCFA diet that contained flavored syrup and approximately 0.01% citric acid (McKenzie's, Altona, Victoria, Australia). This was pH matched to the high-SCFA vinegar drink. Participants consumed 40 mL of this vinegar drink with 250 mL of water to prevent irritation of the esophagus. All meals were prepared, cooked, packed and frozen under supervision of qualified chefs in a licensed commercial kitchen with storage facilities (Be Active Sleep Eat (BASE) facility, Monash University, Notting Hill, Victoria, Australia). Study food was packed in freezer bags and delivered to participants who recorded their intake into a food diary as a measure of adherence to the diet. The study researcher provided a list of additional snacks

(**Supplementary Table 1**) that could be consumed to improve adherence to each diet. Furthermore, the study coordinator contacted study participants either in person or by phone each week to further maintain adherence to the study diet.

Dietary and symptom analysis

Participants completed 7-day food diaries at baseline, whereby they recorded all food and drinks consumed. Scored measuring cups and spoons were provided to participants to assist with this process. In addition, participants recorded information on brands of food items consumed and documented recipes of meals cooked to improve the accuracy of data collection. Information contained in these diaries was analyzed using Foodworks Professional V9.0 in order to calculate energy, macronutrient and micronutrient intake, and then analyzed using additional data from the Monash University FODMAP database (includes oligosaccharide information) and a resistant starch composition report(16) to generate detailed information on fiber intake.

During the baseline week and intervention diets participants also scored daily overall abdominal symptoms, abdominal pain, bloating, passage of wind (gas), satisfaction with stool consistency, tiredness and nausea using 100-mm visual-analogue scales, as previously applied(17). Participants also recorded the number of bowel movements they had each day and their stool form based on the Bristol Stool Chart.

Compliance to the intervention diet was assessed by examining the fold differences in resistant starch and inulin intake from the low-SCFA to high-SCFA diet relative to the 18-fold change designed. Participants who achieved 80% of this expected fold-change (equivalent to x15.2 fold-change) were considered compliant to the intervention diets.

Fecal collection and analysis

Participants collected all fecal output for 3 days by placing freshly passed stools into provided plastic containers. These were sealed with a lid, sealed in another bag and immediately placed into a portable freezer set at -20 ºC. After the collection period, stool samples were transported to the lab at -20 ºC for storage and processing. All fecal material collected was weighed to obtain 3-day and daily fecal output. The samples were thawed, pooled and homogenized. Aliquots were subsequently stored at -80 ºC. Fecal water content was estimated as previously described(18), and expressed as a percentage of the initial wet weight of sample.

Fecal pH was measured using a calibrated pH probe (Mettler Toledo, Schwerzenbach, Switzerland) inserted into thawed fecal material warmed to 25 ºC. Fecal ammonia was measured in duplicate using an enzymatic assay (Megazyme rapid ammonia assay) after protein precipitation and filtration as previously described(17). Fecal SCFA were measured in triplicate via gas-chromatography as previously described(19). Thawed fecal material was spiked with 3 times the volume of internal standard (1.68 mM heptanoic acid), homogenized and centrifuged (2000 *g,* 10 mins, 4 °C), after which 300 µL of supernatant was added to a 0.2 µm filter vial containing 10 µL of 1 M phosphoric acid. The vials were then analyzed for SCFA content via gas chromatography using an Agilent GC6890 coupled to a flame-ionization detector (FID). Coefficient of variation (CV) <10% taken as a valid result for fecal ammonia and SCFA concentrations (µmol/g). Daily fecal output was used to calculate daily excretion of SCFA (mmol/day).

Blood collection

Peripheral blood samples were collected in EDTA-coated vacutainers and, within 4 h of collection, plasma was collected and peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque gradient. PMBC were cryopreserved in liquid nitrogen, whilst

aliquots of plasma were stored at -80 ºC for later use. A 50 µL volume of peripheral blood was used to perform immune cell counts (see flow cytometry),

Plasma SCFA analysis

Blood plasma was analyzed in triplicate for SCFA content using gas-chromatography as previously described(12). Briefly, 300 µL of plasma was spiked with 200 µM heptanoic acid and acidified using 10% sulfosalicylic acid before the addition of diethyl ether solvent. The mixture was vortexed and centrifuged so that the organic layer could be clarified and transferred to 0.2 M NaOH. The alkaline solution containing SCFA was concentrated by evaporation using nitrogen, dissolved in 1 M phosphoric acid and transferred into a cold GC glass vial for analysis using an Agilent GC6890 coupled to FID. Concentrations for acetate, propionate and butyrate were determined by the average of the triplicate results, where the CV was <20%. Total SCFA was calculated by the sum of the individual SCFA. Results were expressed as µmol/L. Data points below limit of detection were excluded from relative change calculations.

Flow cytometry

Absolute numbers of major leukocyte subsets (granulocytes, monocytes, B cells, T cells and natural killer cells) were determined using a lyse-no-wash method within 24 h of blood sampling as previously described(20). In brief, 50 µL of whole blood was added to a TruCount tube (BD Biosciences, San Jose, CA, USA) together with 20 µL of antibody cocktail (CD3FITC, CD45-PerCP-Cy5.5, CD16-PE, CD56-PE, CD4-PE-Cy7, CD19-APC, APC-H7) for 15 minutes in darkness (gating strategy in **Supplementary Figure 1**). Red blood cells were lysed with the addition of 500 µL ammonium chloride solution (0.155 M) prior to analysis on an LSRII flow cytometer (BD Biosciences). A small aliquot of whole blood was also analyzed using a Cell-Dyn Emerald (Abbott, Illinois, USA) instrument to cross-reference leukocyte numbers enumerated using flow cytometry.

PBMC collected during the study were thawed and subjected to two staining panels to evaluate B cell(21) and T cell lineages and subsets therein. Stored PBMC were thawed from liquid nitrogen storage in a water bath at 37 ºC and re-suspended in RPMI containing 10% fetal calf serum (FCS). Viable PBMC were enumerated using a cellometer K2 instrument (Nexcelom Bioscience, Massachusetts, USA) and resuspended in FACS wash buffer at a concentration of 4.0 x 10^7 cells/mL prior to staining with markers outlined in **Supplementary Table 2**. For the B cell tube, 2.0 x10^6 cells were stained and 1.0 x 10^6 cells were included in the T cell tube. Stained cells were run on a 5-laser LSR Fortessa X-20 (BD Biosciences) flow cytometer. Instrument setup and calibration were performed as previously described(20), with additional optimization for the BUV395, BUV496, BUV737 and BV785 channels. All data were analyzed using Flow-Jo software (V10.6; BD Bioscience).

Statistical analysis

A sample size calculation was initially performed based on preliminary data (Treg %$CD4^+$ T cells) collected during a pilot study(12), to estimate the number of participants needed to observe a statistically significant difference in Treg number (20% change, effect size = 0.55) between the high and low-SCFA dietary interventions ((G*Power 3.1, Dusseldorf, Germany). The minimum sample size required was 30 participants for a statistical power equal to 0.8 and α level of 0.05 (two-tailed). With a 10% dropout rate, the sample size required has been calculated as 33 participants.

Statistical analysis was performed using GraphPad prism (V8.02, San Diego, CA, USA) and R (V3.6.1) on a per-protocol basis. The primary endpoint examined changes to Treg number. Assessment of fecal metabolites and characteristics, plasma SCFA concentration and other blood immune cell subsets were secondary endpoints. Endpoints were compared between the low-SCFA and high-SCFA diet using the paired samplesWilcoxon test. Correlation analysis between variables was calculated using Pearson correlation test, with a correlation matrix assembled for all parameters recorded. False discovery rate corrections were applied

to correlation data using Benjamini & Hochberg method to correct for multiple comparisons. Immune network analysis was conducted using the R package Rtnse (V0.15, https://github.com/jkrijthe/Rtsne), with perplexity set to 10. The significance level for this study was determined at 0.05.

RESULTS (1480 WORDS)

Study cohort

Of the 126 individuals who expressed interest in the study, 22 met the study criteria. Major reasons for ineligibility were logistical restrictions (n=43) being outside the age range (n=12), dietary requirements (n=9) and pre-existing disease (n=8), thus the original sample size of 30 participants was not reached. Of the 22 participants who were randomized, 2 participants withdrew after completing one arm, both due to additional dietary requirements that arose during the wash-out period. A total of 20 participants completed the study between August 2018 and September 2019 as illustrated in Figure 1.

Table 3 contains baseline demographic, anthropometric and habitual dietary characteristics of the 20 participants who completed the protocol according to sex. They were predominantly young, 12 were female, most were of normal weight and all were normotensive. Median [IQR] energy intake of the study cohort was 9345 [2396] kJ/d and total dietary fiber intake 25.2 [12.3] g/d, this being in the range recommended by the Australian dietary guidelines (25-30 g/d).

Dietary intake and adherence

Dietary consumption of the study diets is shown in **Figure 2**. Median [IQR] overall fiber intake was 29.5 [3.3] g/d on high-SCFA diet, which was greater than 18.7 [3.8] g/d on low-SCFA diet. (P<0.001). This was primarily due to a greater intake of resistant starch (10.5 [1.1] vs 1.0 [0.3] g/d, P<0.0001) and inulin (7.4 [1.0] vs 0 [0] g/day, P<0.0001) on the high-SCFA diet. Daily macronutrient intake (g/d) was similar between the high-SCFA and low-

SCFA diet (**Supplementary Table 3**). Overall median dietary fiber and resistant starch intake was also significantly greater during high-SCFA diet and significantly lower during the low-SCFA diet, when compared to habitual diet as consumed during the baseline period (**Figure 2B**). The weight of the participants was stable across the study periods (data not shown).

Of the 20 participants, 18 were adherent and consumed >80% of the expected fold-change increase in resistant starch and inulin intake between the intervention diets **(Supplementary Figure 2).** Two participants achieved less than the minimum 80% cut-off with a fold-change increase of 14.3 fold and 14.6 fold change, respectively. Similarly, adherence to the prescribed 3 vinegar drinks per day was high. Thus, during the low-SCFA diet 18/20 participants reported consuming the allocated 3 drinks each day, with the remaining 2 participants consuming on average 2 drinks each day. During the high-SCFA diet 16/20 participants consumed 3 drinks each day, with the remaining 4 consuming 2 drinks each day. When blinding was formally assessed upon completion of the study, 75% (15/20) of participants correctly recognized each diet. The vinegar drink containing apple cider vinegar was also correctly chosen by 80% (16/20) of the study cohort.

Effects of intervention on gastrointestinal symptoms and fecal characteristics

The study diets were well tolerated, with overall symptom scores generally low throughout the intervention periods. There were no significant changes in overall or individual self-reported symptoms on the VAS from baseline to intervention diets except for a greater, but small magnitude, difference in passage of wind/gas between the diets (median [IQR], High-SCFA 11 [12] vs low-SCFA 7 [12] mm, $P<0.05$, **Supplementary Figure 3**). Upon formal assessment at the conclusion of the study, 74% of the cohort identified the high-SCFA diet as causing more abdominal symptoms.

The fecal characteristics are shown in (**Table 4**). The frequency of bowel movements and self-reported BSS scores were similar for each diet, although the proportion of stools with a 3-5 BSS score during the high-SCFA diet was marginally higher than during the low-SCFA diet (P<0.05). No differences in the 3-day or daily fecal output, fecal water content and fecal pH were observed between the diets.

Effect of interventions on fecal metabolites

Fecal concentrations of SCFA are shown in **Supplementary Table 4**. Total SCFA concentrations were 19% [7.3%] higher (mean [SEM] % change) with the high *vs* low-SCFA diet as reflected in the acetate and butyrate concentrations (**Figure 3A**). This was in part due to significantly higher median [IQR] acetate concentration (48.8 [39.9] vs 41.4 [34.9] µmol/g, P<0.05, Supplementary Table 4) and a trend toward higher butyrate concentration (Figure 3A). Overall total SCFA concentration was significantly higher after high-SCFA diet (86.6 [59.0] µmol/g) than after the low-SCFA diet (75.4 [56.2] µmol/g)(**Figure 3B**). However, the median [IQR] ratio of butyrate to total SCFA was similar between low-SCFA (14.9% [4.3%]) and high-SCFA diet (15.2% [6.4%])(data not shown). Furthermore, the ratio of the total amount of branched chain fatty acid (BCFA) to SCFA (**Figure 3C**) produced tended to be lower (P=0.07) after the high-SCFA diet (5.4% [5.1%]) than after the low-SCFA diet (6.8% [4.5%]) The median [IQR] fecal ammonia concentration was 33.4 [18.5] µmol/g after the low-SCFA diet, which was significantly higher than the 26.2 [14.7] µmol/g after the high-SCFA diet (P<0.05) (**Figure 3D**).

Effect of interventions on plasma SCFA

There were no significant differences in total plasma SCFA (P=0.11) and plasma acetate (P=0.22) between the intervention diets (**Figure 4A,B**). However, median [IQR] plasma propionate (9.87 [12.3] vs 4.72 [7.6] µmol/L) and butyrate (2.85 [1.35] vs 2.02 [1.29] µmol/L) concentrations were significantly higher after high-SCFA diet than after the low-SCFA diet (**Figure 4C,D**). Furthermore, percentage change in plasma propionate and butyrate was a

mean [SEM] increase of 21% [19%] and 19% [9%] respectively with the high *vs* low-SCFA diet (**Figure 4E**). The proportions of plasma acetate, propionate and butyrate within total SCFA were similar after the intervention diets (data not shown).

Effect of interventions on circulating immune cells

Following 3-week high-SCFA and low-SCFA diet interventions, no differences were observed in total Treg number (median [IQR], 44 [29] vs 45 [22] cells/μL, *P*=0.25)(**Supplementary Figure 4**) or the frequency of Treg within T cells (3.5% [1.2%], 3.8% [1.5%], *P*=0.76)(data not shown). Total leukocyte (*P*=0.90), monocyte (*P*=0.87) and granulocyte (*P*=0.84) numbers were unchanged after consumption of the L-SCFA and H-SCFA diets (**Supplementary Figure 5**). Although median [IQR] total lymphocyte numbers were not statistically different (*P*=0.14) after the high-SCFA diet (1788 [519] cells/μL) than after the low-SCFA diet (1853 [863] cells/μL), they were investigated further with detailed analysis of B and T cell subsets.

B cells and subsets therein were defined as per **Supplementary Table 5** and gated using the strategy outlined in **Supplementary Figure 6**(20). Total B cell numbers were lower after the high-SCFA diet than after the low-SCFA diet (median [IQR], 184 [112] vs 199 [143] cells/μL, *P*<0.05) **(Figure 5A).** This was equivalent to median relative change of -20.6% [60.9%] from the low-SCFA to high-SCFA diet (**Figure 5G**). The difference (delta) in B-cell number between the high- and low-SCFA diets was positively correlated with the difference (delta) in fecal iso-valeric concentrations (*P*<0.01, q= 0.1, r= 0.58, 95% CI: 0.18, 0.82) (**Figure 6A**). Detailed B-cell sub setting (**Supplementary Figure 7**) showed lower naive mature B cell numbers after the high-SCFA diet than after the low-SCFA diet (82.5 [65.8] vs 94.7 [88.9] cells/μL, *P*<0.05)(**Figure 5B**) whereas absolute numbers of transitional B cells, memory B cell subsets, plasma cells and $CD21^{lo}$ B cells were not different between the intervention diets (Supplementary Figure 6). However, positive correlations were found between changes in plasma cell (*P*<0.01, q= 0.08, r= 0.59, 95% CI: 0.19, 0.83) and

transitional B cell numbers (*P*<0.01, q= 0.049, r= 0.65, 95% CI: 0.27, 0.85) with changes in fecal SCFA concentrations between the high-SCFA and low-SCFA diets (data not shown). Furthermore, changes to the $CD27^{+}IgA^{+}$ (*P*<0.01, q= 0.07, r= 0.62, 95% CI: 0.23, 0.84)(**Figure 6B**) and $CD27^{+}IgG^{+}$ memory B cell subsets (*P*<0.01, q= 0.07, r= 0.62, 95% CI: 0.22, 0.84)(data not shown) positively correlated with changes to fecal iso-valeric concentrations between the intervention diets.

T cell subsets were defined as before(20), listed in **Supplementary Table 6** and gated according to **Supplementary Figure 7**. Total T cell numbers were not statistically different after the high-SCFA diet than after the low-SCFA diet (median [IQR], 1233 [543] vs 1253 [619] cells/µL; *P*=0.12; **Supplementary Figure 4**), although 12/20 participants had lower T cell numbers. This was examined further with detailed analysis of T cell subsets (Supplementary Figure 8). Following the high-SCFA diet, significantly lower numbers were found of MAIT cells (median [IQR], 62 [83] vs 69 [114] cells/µL), $CD8^{+}$ Tfh cells (4.9 [3.4] vs 5.5 [4.5] cells/µL), Th1 cells (22 [19] vs 29 [16] cells/µL) and Tfh1 cells (5.2 [4.3] vs 5.6 [4.7] cells/µL) than after the low-SCFA diet (**Figure 5C-F**). Changes to Th2 cell numbers between the high- and low-SCFA diets (**Figure 6C**) were negatively correlated (*P*<0.01, q= 0.08, r= -0.61, 95% CI: -0.83, -0.22) to changes in blood total SCFA concentrations. Furthermore, changes to $CD8^{+}$ Tfh cells were positively correlated (*P*<0.01, q= 0.09, r= 0.59, 95% CI: 0.18, 0.82) with changes in fecal iso-butyric concentration (**Figure 6D**).

Overall changes to the phenotype of the immune system were visualized by plotting all immune cell subsets using a t-distributed stochastic neighbor embedding (t-SNE) approach. Changes to the overall immune phenotype were found to cluster to each individual, as opposed to clustering by intervention diet (**Figure 6E**).

DISCUSSION (1359 words)

We here demonstrated with a controlled dietary intervention that high dietary intake of SCFA and fibers can increase total fecal SCFA levels and reduce fecal ammonia, consistent with increasing in colonic fermentation of fiber and reducing that of protein. Furthermore, increases in plasma propionate and butyrate were observed after the high-SCFA diet, whereas acetate and total SCFA levels were unchanged. The high-SCFA diet was associated with changes in the circulating B- and T-cell compartments, with significant reductions in B cell, Th1 cell, Tfh1 cell and MAIT cell numbers. However, the magnitudes of these differences were small, and overall modulation of immune parameters tended to be unique to each individual.

Building on our previous work(12), we had hypothesized that dietary fiber and oral SCFA supplementation would increase delivery of SCFA to the systemic circulation. Indeed, colonic fermentation of carbohydrates was highly likely to be increased in the high-SCFA relative to the low-SCFA dietary period. First, the delivery of fiber to the colon was successful reflected in good compliance to the diets, the greater colonic gas production experienced by the participants as anticipated from other studies using, for example, RS and inulin(22, 23), and the higher fecal output and proportion of stools that could be classified within 3-5 on the BSS. Secondly, fecal acetate and butyrate concentrations were greater relative to those associated with the low-SCFA diet. Thirdly, protein fermentation was suppressed as shown by a higher SCFA:BCFA ratio and reduced ammonia concentrations(24). However, we did not achieve a significant increase to overall plasma SCFA concentrations. This most likely relates to the low bioavailability of SCFA to the systemic circulation given that, for following oral ingestion of SCFA or production in the colonic lumen, the vast majority of SCFA will be metabolized by colonocytes or hepatocytes on their first pass. Indeed, systemically availability of colonic acetate, propionate and butyrate has been estimated to be 36%, 9% and 2%, respectively(25). After consumption of a vinegar drink containing 1.5 g of acetate,

plasma acetate levels may transiently increase within 60 minutes, indicative of rapid absorption in the upper gastrointestinal tract(2). In contrast, colonic fermentation of inulin primarily in the cecum produces a plasma SCFA peak approximately 4-6 hours after consumption(13). Consumption of RS may also increase plasma SCFA for >6 hours as it is slowly fermented, although previous studies have examined different forms of RS (e.g. pearl barley kernels, Nutriose) than the current study(13, 26). Such observations indicate that timing of blood sampling and participant meal consumption are critical if plasma SCFA concentrations are to be used a marker of increased delivery of SCFA to the systemic circulation. These may not have been adequately controlled in this study to capture the transient increases to rapidly metabolized plasma SCFA (e.g. acetate) with a single measurement in all participants. The limited ability of plasma SCFA to reflect marked increases in delivery of SCFA during fermentable fiber supplementation also manifested in a feeding study of patients with irritable bowel syndrome in whom fecal, symptomatic and local fermentation characteristics were evaluated using a novel telemetric gas-sensing capsule(27, 28) Furthermore, variation in fecal SCFA concentration may occur due to the heterogenous nature of the sample, which was overcome by pooling fecal samples, despite the caveat of requiring an additional freeze-thaw step during processing(29).

A novel aim of this dietary intervention study was to use detailed immuno-phenotyping to examine changes to the immune system that may have occurred as result of increased delivery of SCFA to the systemic circulation. We observed that naive B cells, Th1 cells, Tfh1 cells and MAIT cells were significantly lower after the high-SCFA when compared to after the low-SCFA diet. This may be suggestive of an anti-inflammatory effect of increased systemic SCFA delivery, in line with decreased peripheral blood inflammatory markers observed to occur in healthy people provided with prebiotic fiber supplements(30). Indeed, healthy people who followed a high-fiber diet for 14 weeks had increased fecal butyrate concentrations that correlated with decreases to overall B cell frequency, a similar result to

what we have observed after 3 weeks of controlled interventional diets(31). Increased naive B cell numbers have been observed in the peripheral blood of children with inflammatory bowel disease (IBD), which may be due to impaired conversion to memory B cells(32, 33). Indeed, SCFA are potent at inducing Ig class-switched B cell subsets in animal models(34, 35). Although no changes to memory B cell subsets were observed in our study, further intervention studies should examine whether increasing systemic SCFA delivery affects B cell maturation during an immune challenge such as vaccination.

Observations from mouse models suggested that delivery of SCFA to the systemic circulation may induce protective Treg cells, particularly in the gut(36, 37). We did not observe any significant changes to subsets of Treg between the intervention diets. Indeed, SCFA may directly upregaulte the Treg transcription factor FoxP3, which was not included in our phenotyping panel(38). However, we did observe significantly lower MAIT cell numbers, a recently identified T cell subset associated with gut-mucosal tissue that respond to gut microbiota-derived vitamin B metabolites(39). These cells would be exposed to high concentrations of SCFA in this environment, although it is unclear if human MAIT cells directly respond to SCFA as other gut-associated subsets such as TCR$\gamma\delta^{+}$ cells do (40). Modulation of MAIT cell numbers in the peripheral blood may have been induced by altered microbial composition and function in the gut resulting from the intervention diets. This may have led to migration of MAIT cells out of the peripheral blood to the gut, as has been shown to occur in response to enteric bacterial or viral infection(41). Similarly, Th1 cells were significantly lower after the high-SCFA diet than after the low-SCFA diet. A recent study that performed similar immuno-phenotyping in healthy people found reduced numbers of Th1 cells in peripheral blood of individuals that had increased species of SCFA-producing bacteria *Ruminococcus,* which may be further indicative of an indirect effect of the intervention diets(42).

It is important to note that the magnitude of changes observed in this study were small, with network analysis using t-SNE highlighting that overall changes to the immune cell populations clustered within each individual. Furthermore, the small size of the study cohort limited the statistical power of changes observed. Up to 80% of immune variation in humans may be related to non-heritable lifestyle factors, that cannot be completely controlled through a 10-week intervention study(43). Previous studies that have performed detailed immuno-phenotyping in patients with IBD or primary immune deficiency generally observe larger (>20%) differences in T and B numbers when compared to healthy controls(20, 33). Although, we did find that median relative changes in overall B cells, naive B cells and some T cell subsets between the diets were in the range of 15-30%(**Figure 5G**). Increasing systemic delivery of SCFA using a dietary approach may have added benefit for those with IBD, as shifts in colonic fermentation observed through our dietary approach have been associated with anti-inflammatory effects in Crohn's disease and Ulcerative colitis(44). Our diet was well tolerated by the healthy cohort of study participants, although further studies will need to assess tolerability in patients with IBD , who may already have low habitual dietary fiber intake than a healthy cohort and have reduced compliance to dietary intervention many weeks in duration(45, 46). A pilot study in small group of adults with Multiple sclerosis found that 12 months of following a high fiber vegetable predominant diet significantly altered peripheral blood $CD4^+$ T cells and improved clinical disease indices, highlighting that long-term dietary therapy to increase systemic SCFA may have more efficacy in other-immune mediated disorders(47).

In conclusion, dietary intervention with fermentable fiber and oral SCFA to increase colonic and peripheral blood SCFA is a well-tolerated intervention that is associated with favorable changes to colonic fermentation, reducing fecal ammonia. Increased SCFA also associated with changes to the distribution of lymphocyte subsets, with lower naive B cells, MAIT cells and Th1 cells in the peripheral blood. Further studies are needed in larger cohorts to assess

if phenotypical changes are also associated with changes to immune function to determine if dietary therapy may have therapeutic benefit in patients with inflammatory disease.

Author Contributions

PAG, PRG, JGM and MCvZ designed the study. PAG conducted the study, collected and analyzed the data and drafted the manuscript. JGM, MCvZ and PRG critically reviewed and edited the manuscript and supervised the entire study. All authors read and approved the final manuscript.

Acknowledgements

The authors thanks Dr Daniel So, Ms Lyndal Collins and Ms Patricia Veitch for their assistance in developing the intervention diets

Data described in the manuscript, codebook, and analytic code will be made available upon request to the corresponding author.

Conflict of interest:

MCvZ, no conflicts of interest.

PAG, PRG, and JGM work in a department that financially benefits from the sales of a digital application and booklets on the low FODMAP diet. They have published an educational/recipe book on diet. Funds raised contribute to research of the Department of Gastroenterology and to the University. The authors receive no personal remuneration.

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Table 1. Meal plan of dietary intervention study.

Meals	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Breakfast[1]	**Breakfast smoothie w/oats (RS)** & berries Kiwi fruit	**Pancake (RS)** w/berries & yoghurt	White bread toast w/spread Tub of yoghurt	**Breakfast muffin (RS)** w/eggs & tomato	**Pancake (RS)** w/ berries & yoghurt	**Breakfast smoothie w/oats (RS+I)** & berries Kiwi fruit	**Breakfast muffin (RS)** w/eggs & tomato
Morning tea	Sweet biscuits	1 medium kiwi fruit 2 sweet biscuits	**Savory muffin (RS)**	Peach & Peanuts	Sweet biscuits	Almonds	Sweet biscuits
Lunch[1]	Corn tortilla wrap (**meatballs**, spread, cheese, salad)	**Arancini (RS)** & salad	**Pumpkin soup (I)** w/white bread	White bread sandwich w/cold meat, cheese, salad	**Beef curry (I)** w/ white rice	**Tuna sweet potato patties (RS)** & salad	White bread sandwich w/cold meat, cheese, salad
Afternoon tea	**Sweet muffin (RS)**	Peanuts	Almonds	**Sweet muffin (RS)**	**Savory muffin (RS)**	Mandarin & sweet biscuits	**Smoothie w/oats (RS)** & berries
Dinner[1]	**Chicken cacciatore (I)** w/white rice	**Bolognaise (I) w/pasta** & salad	**Frittata (RS)** w/grilled meat & salad	**Chicken risotto (I)**	**Vegetable stir-fry** w/rice noodles	**Rice pilaf** w/grilled meat	**Pasta w/tomato & bacon sauce (I)**

RS: resistant starch-containing meals, I: Inulin-containing meals, Bold text indicates provided meal. w/ = with

[1]Vinegar or placebo drink was consumed after each main meal

Table 2. Nutrient breakdown of intervention diets

Component	Daily intake	
	Low-SCFA	**High-SCFA**
Energy (kj)	8687	8660
Carbohydrate (g)	212.7	216.0
Sugars (g)	68.4	69.0
Protein (g)	98.2	98.0
Fat (g)	84.2	84.4
Saturated fat (g)	29.0	29.0
Sodium (mg)	3391	3397
Total Dietary Fiber[1] (g)	18.6	39.2
Resistant starch (g)	1.0	11.0
Inulin (g)	0	8.0
Oligosaccharides[2] (g)	1.0	1.0
Energy Intake		
Carbohydrate (% total energy intake)	41.6	42.4
Fat (% total energy intake)	35.9	36.0
Protein (% total energy intake)	19.2	19.2

[1]*Total Dietary Fiber includes RS, inulin and oligosaccharides in the supplied background diet.*

[2]*Oligosaccharides: fructans + galacto-oligosaccharides*

Table 3. Baseline characteristics of study participants who completed the protocol according to sex

		Male	Female
Number of participants		8	12
Age, median (range), years		30 (25-36)	25 (18-43)
Body mass index, median (range), kg/m^2		24 (20.2-26.2)	22.0 (17.3-31.9)
Habitual dietary intake per day	Energy (kJ)	9949 (9143-12343)	8252 (6377-11831)
	Carbohydrate (g)	269 (233-377)	230 (52-327)
	Sugars (g)	111.3 (56.2-173.0)	67.5 (26.6-131.5)
	Protein (g)	114.1 (103.1-128.0)	87.7 (59.6-111.3)
	Fat (g)	89.5 (65.8-100.7)	72.1 (45.1-140.0)
	Saturated fat (g)	31.3 (20.0-47.2)	30.4 (13.6-64.6)
	Sodium (mg)	2063 (1176-3058)	2141 (1230-3639)
	Total dietary fiber[1] (g)	28.5 (17.1-45.1)	23.6 (9.2-31.8)
	Resistant Starch (g)	3.5 (3.2-7.9)	2.1 (1.2-4.4)
	Oligosaccharides (g)	4.0 (2.3-7.8)	3.2 (1.4-4.7)
	Proportion of total energy intake		
	Carbohydrate (%)	46.4 (41.4-53.0)	43.3 (37.6-55.3)
	Fat (%)	30.0 (26.6-39.0)	34.4 (20.4-43.8)
	Protein (%)	18.8 (15.9-22.1)	17.2 (13.5-22.5)

[1]*Total dietary fiber includes RS and oligosaccharides. Data shown as median (range)*

Table 4. Participant bowel habit and fecal characteristics during study intervention periods.

Parameter	Low-SCFA	High-SCFA	*P*-value
Bowel movements/day	1 (1-3)	1 (1-2)	0.99
Reported BSS form	3 (2-5)	4 (1-5)	0.84
% rated 3-5 BSS form	79 (0-100)	83 (5.3-94.7)	**0.01**
Total fecal output (g)[1]	274 (107-755)	335 (58-743)	0.35
Fecal output (g/day)[1]	91 (36-252)	112 (19-248)	0.35
Fecal water content (%)[1]	72 (57-80)	71 (62-84)	0.10
Fecal pH[1]	6.7 (6.3-7.5)	6.7 (6.1-7.5)	0.44

Data shown as median (range) n=20 matched pairs, except. [1]n=19 matched pairs. Statistical significance as determined by paired samples Wilcoxon test denoted by bolded P-values. BSS: Bristol stool score

Figure legends (n=6)

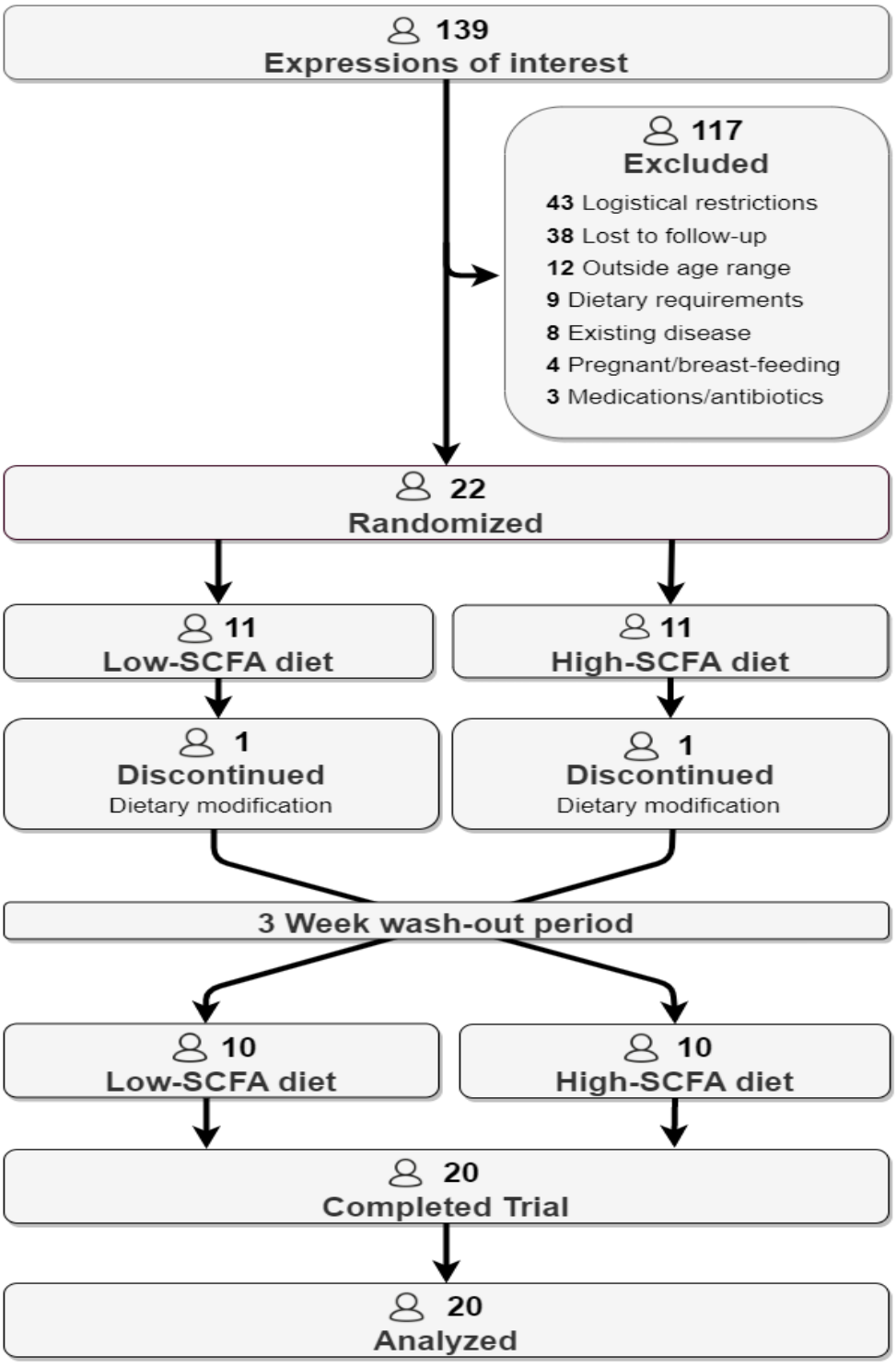

Figure 1. Participant flow diagram for intervention study

Of 139 expressions of interest, 22 healthy participants were randomized to intervention. A total of 20 participants completed the study, with 2 drop-outs after the first arm.

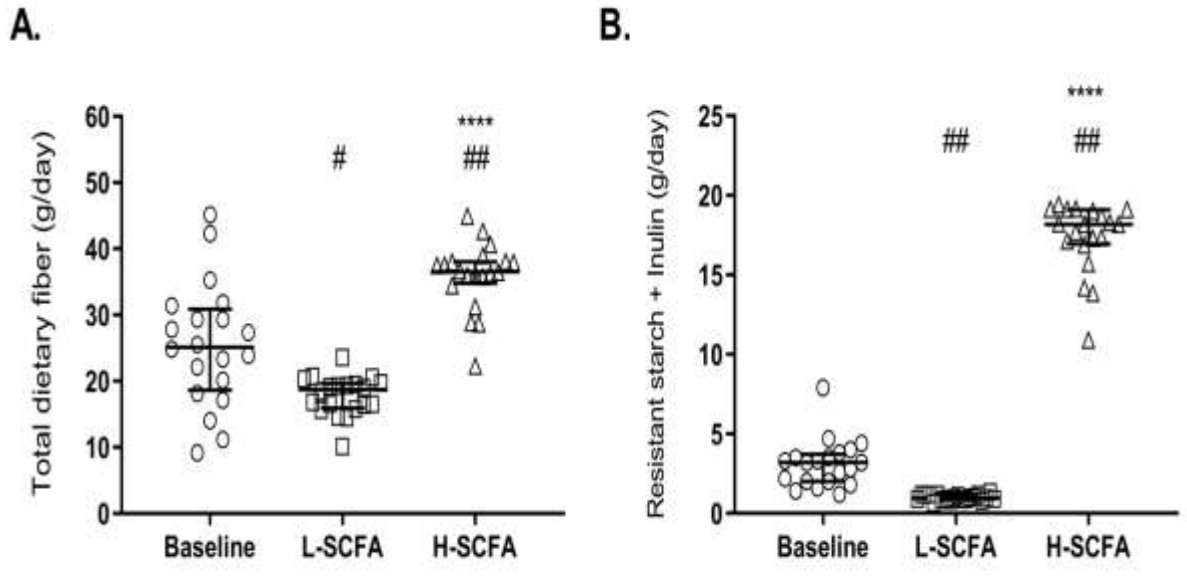

Figure 2. Dietary fiber intake during study intervention periods.

Daily intake of **A)** Total dietary fiber, **B)** resistant starch & inulin at baseline (BL) and during intervention diets (L-SCFA and H-SCFA). Data shown as median±IQR. Significance to Low-SCFA as calculated by paired samples Wilcoxon test denoted as ****P<0.001. Significance to BL as calculated by Friedman test denoted as #P<0.05, ##P<0.01. n=20 matched pairs.

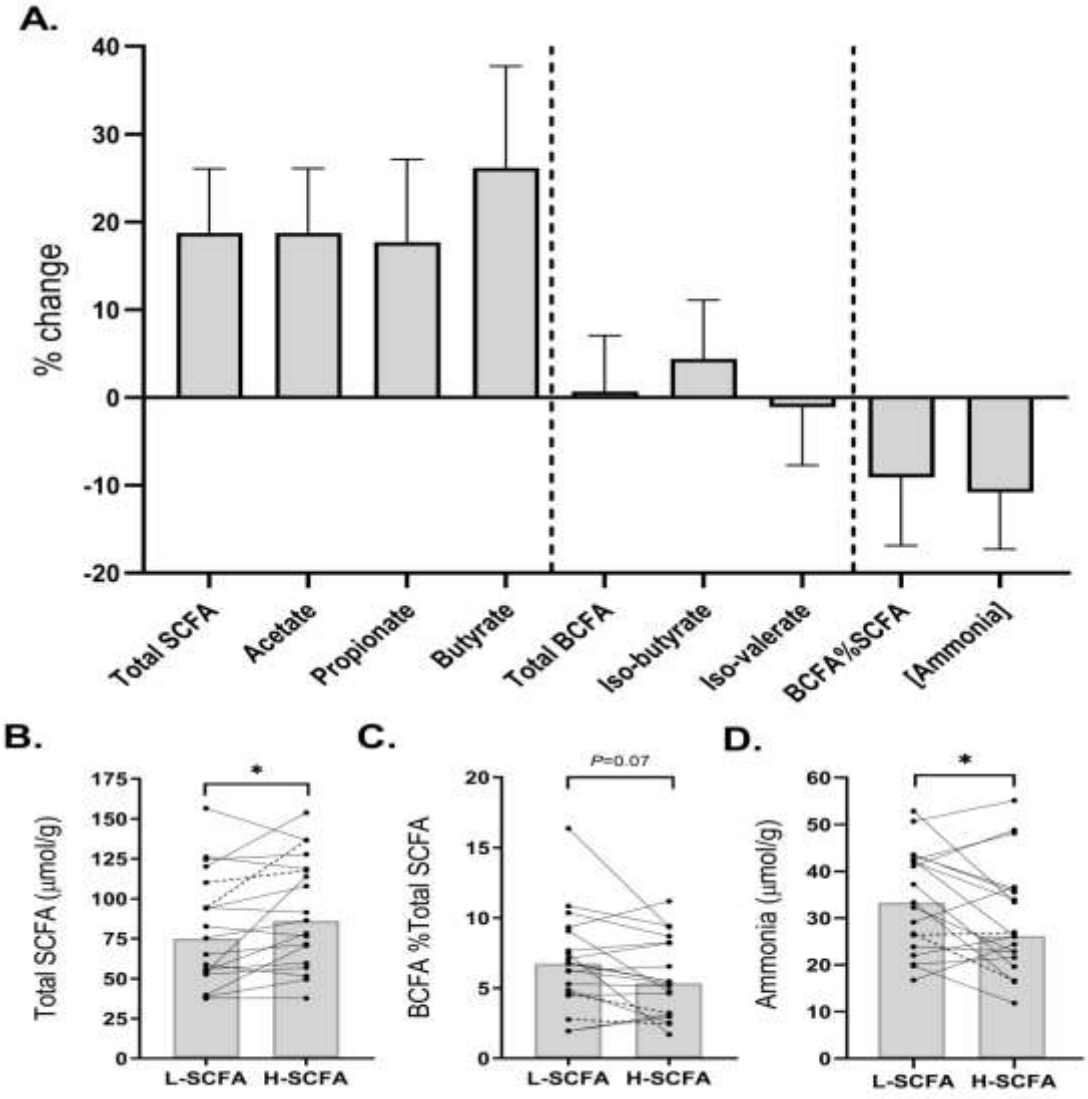

Figure 3. Fecal metabolite changes during intervention diets.

A) Percentage changes ((High-SCFA - Low-SCFA/Low SCFA) to fecal SCFA, BCFA and ammonia as measured by gas chromatography, data shown as mean±SEM. **B)** Paired total fecal SCFA concentrations, **C)** Paired ratio of amounts of BCFA to SCFA, **D)** Paired fecal ammonia concentration as measured via enzymatic assay. Dotted lines represent participants with reduced compliance to study diet. L-SCFA: Low-SCFA diet, H-SCFA: High-SCFA diet. Bar represents median. Statistical significance as calculated by paired samples Wilcoxon test. *P<0.05. n=19 matched pairs.

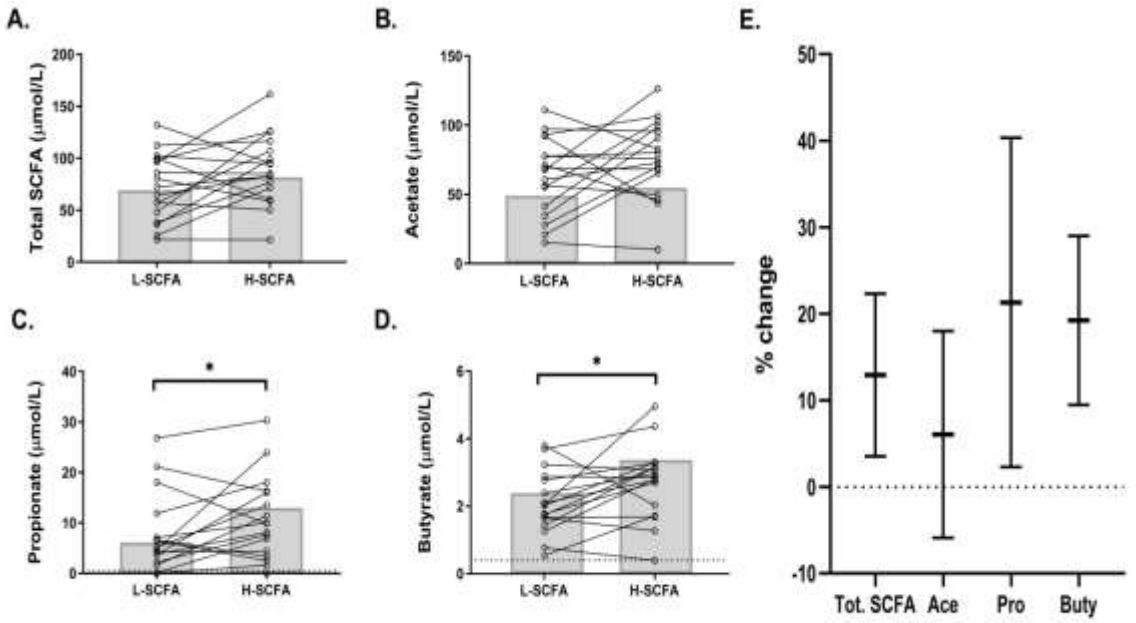

Figure 4. Plasma SCFA concentration after intervention diets

Paired concentrations of plasma **A)** total SCFA, **B)** acetate, **C)** propionate and **D)** butyrate as measured by gas chromatography after intervention diets. Dotted line represents limit of detection for propionate and butyrate. Bar represents median. **E)** Percentage changes to plasma SCFAs ((High SCFA – Low SCFA)/High SCFA), data shown as mean±SEM. Data points below limit of detection excluded from percentage change calculations. L-SCFA: Low-SCFA diet, H-SCFA: high-SCFA diet. Statistical significance as calculated by paired samples Wilcoxon test. N=17 matched pairs.

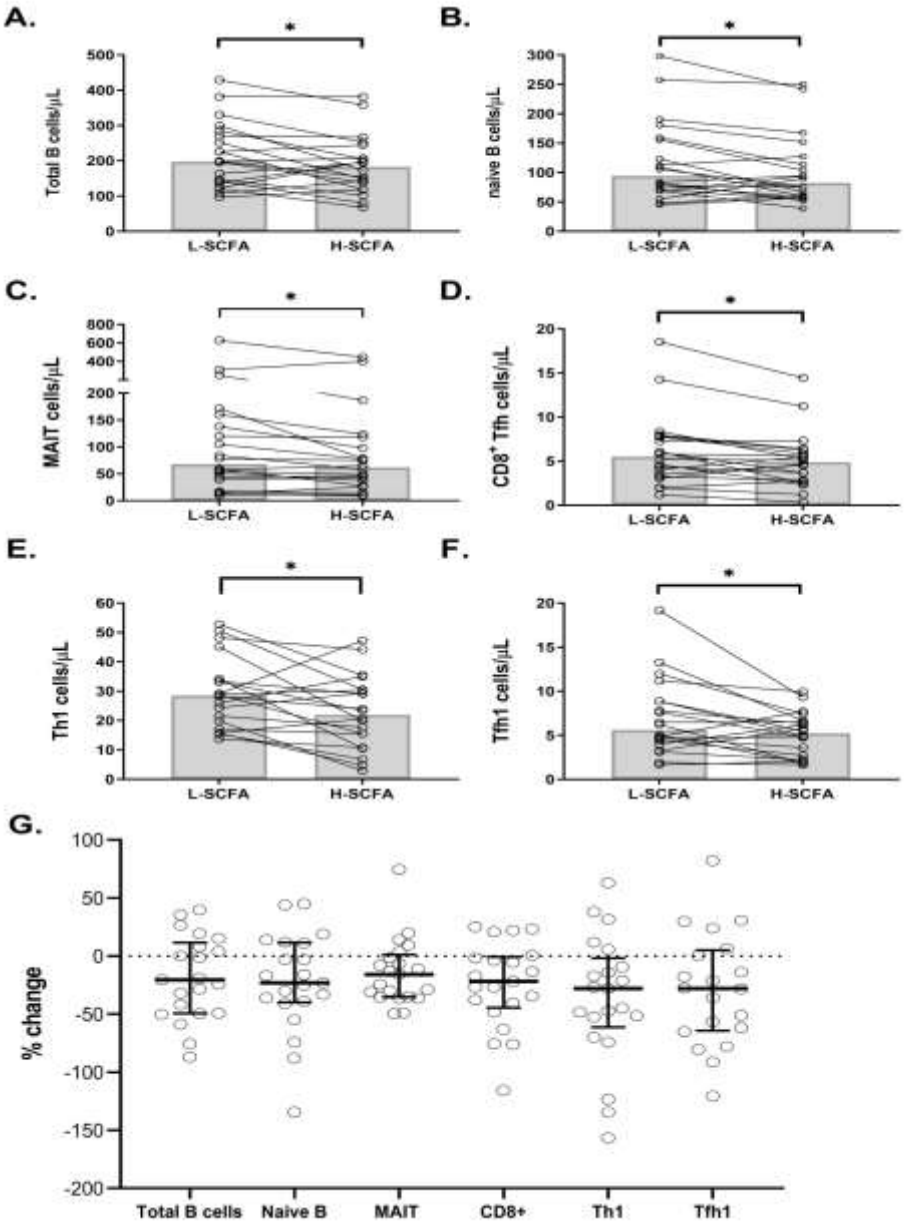

Figure 5. Significant changes to immune cell subsets associated with intervention diets

Paired changes to absolute numbers of **A)** Total B cells, **B)** naive B cells, **C)** MAIT cells, **D)** CD8+ Tfh cells, **E)** Th1 cells and **F)** Tfh1 cells as calculated from flow cytometry with BD Trucount tubes used to quantify absolute numbers. Bar represents median. **G)** Relative changes to above subsets (High SCFA – low SCFA)/High SCFA). L-SCFA: Low-SCFA diet, H-SCFA: high-SCFA diet, T_{FH}: T-follicular helper, Th: T-helper, MAIT: mucosal-associated invariant T cell. Statistical significance as determined by paired samples Wilcoxon test. *P=0.05, n=20 matched pairs.

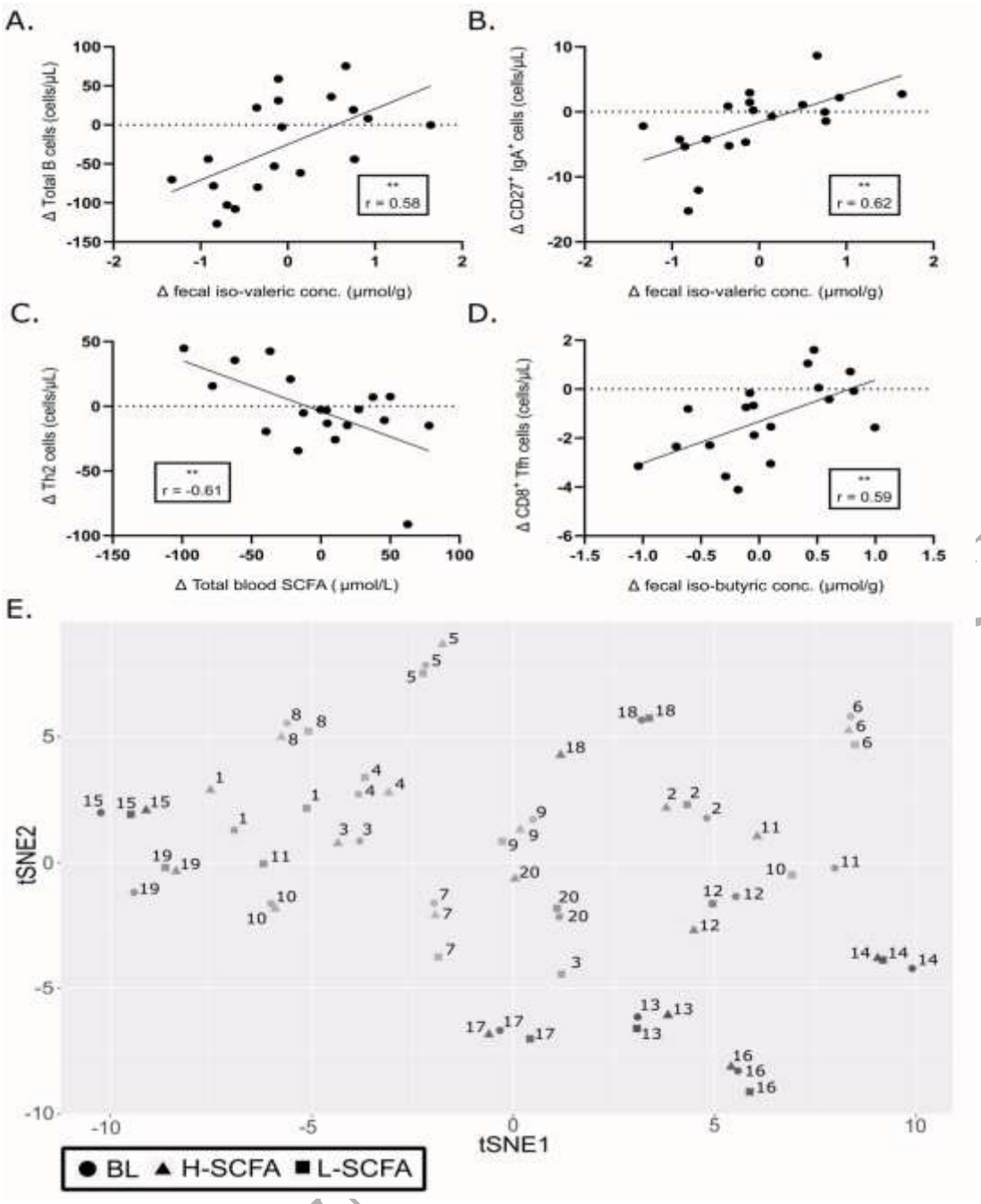

Figure 6. Correlations between individual immune cell subsets and global changes to overall immune profile.

Correlations between SCFA and immune cell subsets **(A-D)**. Δ= difference between H-SCFA and L-SCFA. Correlation as calculated by Pearson correlation test, ***P*<0.01 (n=19). **E)** t-SNE plot of global changes to immune parameters between Baseline (BL) and intervention diets. L-SCFA: Low-SCFA diet, H-SCFA: high-SCFA diet. Numbers representative of each study participant (n=20).