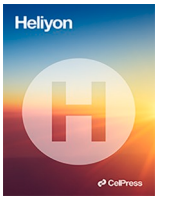
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Short-chain fatty and carboxylic acid changes associated with fecal microbiota transplant communally influence microglial inflammation

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ABSTRACT

The intestinal microbiota has been proposed to influence human mental health and cognition through the gut-brain axis. Individuals experiencing recurrent *Clostridioides difficile* infection (rCDI) frequently report depressive symptoms, which are improved after fecal microbiota transplantation (FMT); however, mechanisms underlying this association are poorly understood. Short-chain fatty acids and carboxylic acids (SCCA) produced by the intestinal microbiota cross the blood brain barrier and have been proposed to contribute to gut-brain communication. We hypothesized that changes in serum SCCA measured before and after successful FMT for rCDI influences the inflammatory response of microglia, the resident immune cells of the central nervous system. Serum SCCA were quantified using gas chromatography-mass spectroscopy from 38 patients who participated in a randomized trial comparing oral capsule-vs colonoscopy-delivered FMT for rCDI, and quality of life was assessed by SF-36 at baseline, 4, and 12 weeks after FMT treatment. Successful FMT was associated with improvements in mental and physical health, as well as significant changes in a number of circulating SCCA, including increased butyrate, 2-methylbutyrate, valerate, and isovalerate, and decreased 2-hydroxybutyrate. Primary cultured microglia were treated with SCCA and the response to a pro-inflammatory stimulus was measured. Treatment with a combination of SCCA based on the post-FMT serum profile, but not single SCCA species, resulted in significantly reduced inflammatory response including reduced cytokine release, reduced nitric oxide release, and accumulation of intracellular lipid droplets. This suggests that both levels and diversity of SCCA may be an important contributor to gut-brain communication.

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Abbreviations

SCCA	short chain fatty and carboxylic acids
LD	lipid droplets
LPS	lipopolysaccharide
IFNγ	interferon γ
TNF	tumour necrosis factor
IL	interleukin
ROS	reactive oxygen species
rCDI	recurrent *Clostridioides difficile* infection
FMT	fecal microbiota transplant(ation)
CNS	central nervous system
BBB	blood-brain barrier
CSF	cerebrospinal fluid
SF-36	36-item short form survey

1. Introduction

The intestinal microbiota plays an important role in the bidirectional interaction in the gut-brain axis [1]. This interaction involves multiple pathways and is implicated in the underlying pathophysiology linking intestinal disorders and mental health. The gut microbiota can affect the immune system directly via activation of the vagus nerve, which in turn triggers bidirectional communication with the central nervous system (CNS) [2]. In addition, indirect effects of the gut microbiota can occur through changes in bacterial metabolites and cytokines on the gut–brain axis and local and systemic immunity. In particular, short-chain fatty acids and carboxylic acids (SCCA), produced by the bacterial fermentation of dietary carbohydrates, have immunomodulatory properties and can stimulate the autonomic nervous system [3–5]. Gut microbiota-derived SCCA enter systemic circulation and can cross the blood brain barrier (BBB), modulating brain development and affecting host behavior [6–9]. Further, SCCA have been proposed to modulate microglial homeostasis and inflammatory function, necessary for proper brain development and brain tissue homeostasis, and have been implicated in the pathophysiology of depression, underscoring the importance of intestinal microbiota in mental health [10].

Microglia are classically considered the innate immune cells of the CNS, responding with inflammation to pathogens, foreign bodies, and tissue damage. However, their role is increasingly understood to be more pervasive and complex [11]. During both development and adulthood, microglia are implicated in immune surveillance, synaptic pruning, neural circuit development and refinement, plasticity, and signalling. In addition to these homeostatic functions, microglia have important inflammatory roles, such as initiation of inflammatory cascades and neuronal apoptosis, which under most conditions are adaptive, acting to preserve tissues and homeostasis during stress and illness. Microglial inflammation has also been proposed to modulate behavior [12,13]; sickness behaviors (i.e. depressed mood, anhedonia, social withdrawal, appetite changes, and fatigue) are considered an adaptive response to systemic illness, encouraging rest and recovery and limiting the spread of pathogens both within the individual and through social contacts. However, persistent pro-inflammatory microglial activation in the absence of illness is implicated in maladaptive behavioural responses and contribution to the psychopathology of psychiatric disorders [12–15].

A limited number of previous studies have shown that SCCA can differentially regulate microglial function; butyrate and acetate have been demonstrated to exert anti-inflammatory effects on microglia, while propionic acid has been shown to exacerbate inflammation, microgliosis, and behavioural deficits in rodent models of autism, but may also be implicated in anti-inflammatory roles [16–19]. The SCCA lactate can be an energy source for CNS cells, including microglia where it has been associated with resolution of inflammation, but is also produced locally by astrocytes in addition to microbial production [20]. The potential connection between microbiota-derived SCCA and microglial function is promising, but thus far there is a lack of evidence relevant to humans.

However, changes in microbial community states are associated with changes to mood and quality of life in human patients; for instance, antibiotic-induced depression is frequently observed in patients with recurrent *Clostridioides difficile* infection (rCDI), which is associated with profound dysbiosis [21,22]. Restoration of intestinal microbiome composition and functionality via fecal (or intestinal) microbiota transplant (FMT/IMT; a highly-effective therapy for rCDI [23]) often incidentally may mitigate or improve symptoms related to depression, that cannot be explained by improvement of the underlying condition alone [24,25]. Bariatric surgery, which has been associated with increased diversity of gut microbiota [26,27], is also generally shown to associate with decreased depression or anxiety symptoms [28] though some studies have noted divergent post-surgical changes in depression scores [29]. Pre-clinical studies in rodents tend to support gut-brain coupling. In mice, antibiotic-induced dysbiosis has been shown to increase depressive behaviours, which could be reversed with a probiotic supplement [30]. Middle-aged rats given pro-biotic supplements showed improved cognition that was associated with concomitant changes in the SCCA lactate in the brain [31]. It has been speculated that circulating SCCA mediate communication in the microbiota-gut-brain axis, but the specific mechanisms for the observed improvements in depression are poorly understood [32].

The aim of this study is to employ a reverse translational approach to address the role of bacterial metabolites in the gut-brain-axis. We first characterized the changes in serum SCCA in rCDI patients before and after successful treatment with FMT. Pre- and post-treatment patient SCCA profiles were then used to guide investigation of the direct effect of four SCCA on primary microglia

cultured from rat brain to model inflammation, with the hypothesis that SCCA associated with recovery from rCDI would have anti-inflammatory effects on microglia. The combined profile of recovery-associated SCCA notably decreased release of inflammatory modulators from primary microglia cultures, yet no single SCCA was able to elicit anti-inflammatory effects in isolation. These findings suggest that, much like the noted benefits of gut microbiota diversity, a diverse range of circulating SCCA may be a key component of healthy gut-brain communication.

2. Methods

2.1. Materials and reagents

Cell culture reagents including Hank's Balanced Saline Solution (HBSS), 10,000 U/ml Penicillin/10,000 μg/ml streptomycin (PS), fetal bovine serum (FBS), horse serum, Dulbecco's Modified Eagle Medium with Ham's F-12 supplement (DMEM/F12), and 0.25% (w/v) Trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were from Gibco (Thermo-Fisher Scientific, Burlington, ON, Canada). Rabbit *anti*-Iba1 antibody was from Wako (Osaka, Japan; RRID:AB_839504). Reagents for fluorescence assays including 4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a, 4a-Diaza-*s*-Indacene (BODIPY 493/503), To-Pro-3 iodide, and donkey anti-rabbit Alexa Fluor 488 antibodies (RRID:AB_2535792) were from Invitrogen (Thermo Fisher Scientific, Burlington, ON, Canada). Chemicals and reagents were from Millipore Sigma (St. Louis, MO, USA) including *Escherichia coli* O111:B4 lipopolysaccharide (LPS), poly-L-lysine (PLL), and all SCCA used in culture experiments. Interferon γ (IFNγ) was from Peprotech (Montreal, QC, Canada). Enzyme-linked immunosorbent assay (ELISA) kits were from R&D Systems (Toronto, ON, Canada). Phosphate buffered saline was prepared from tablets to pH 7.4 (Bioshop Canada, Burlington, ON, Canada).

2.2. Sample collection and chromatography

Blood samples from 38 patients who participated in a clinical trial comparing oral capsule vs colonoscopy delivered FMT [NCT02254811, approved by Health Canada (control No. 176567) and the ethics board of each participating center] [33] were quantified for short chain carboxylic acids by gas-chromatography-mass spectroscopy (GC-MS) [34]. Inclusion criteria for the study and patient characteristics are fully described in Ref. [33]. Longitudinal blood samples were collected from screening visit, 4 weeks and 12 weeks after successful FMT treatment.

A targeted GC-MS protocol was used for the analysis of short chain carboxylic acids, via adaptation of an established method [35]. The full protocol used for the GC-MS experiments was as described previously [34]. Pooled study reference samples were included within the run as a performance monitor for analysis. SCCA standards (Sigma) of known concentration were used to obtain calibration curves; MTBE with 100 parts per million (ppm) methyl stearate (Sigma) was used as internal standard. Samples were randomised, and analysis performed on an Agilent 7890B GC system coupled to an Agilent 5977A mass selective detector (Agilent, Santa Clara, California). Identification and quantification of SCCA from spectral data was performed using MassHunter software (Agilent), using retention times as stated in Supplementary Table 1, and by applying a previously-described analytical approach [34].

2.3. Cell culture treatments

All animal work was carried out under an Animal Use Protocol approved by the Animal Care and Use Committee at the University of Alberta in accordance with Canadian Council for Animal Care guidelines. Microglia were isolated from the brain of postnatal day 1 Sprague-Dawley rat pups (RRID:RGD_70508) as previously described [36]. A total of 37 animals were used in this study. In brief, pups were killed by acute decapitation and brains were dissected in warm HBSS +1% PS (37 °C) to remove meninges and superficial blood vessels. Single-cell suspension was produced by digestion with trypsin-EDTA (37 °C, 20 min) followed by mechanical trituration with a Pasteur pipette. Cells were washed with DMEM/F12 + 10% FBS and plated in 12-well polystyrene culture plates previously coated with PLL at a density of 1 brain per plate and cultured with DMEM/F12 + 10% FBS for 14 days in a humidified 5% CO_2 incubator to reach confluence. At 14 days *in vitro* (d.i.v.) microglia were isolated from mixed cell cultures by differential adhesion [37] by briefly digesting with trypsin-EDTA diluted to 30% with DMEM/F12 (15 min, 37 °C). Mixed cells were removed by the treatment leaving isolated microglia adherent in the wells at high purity (≥98%). Cell culture purity was routinely assessed at the end of experiments using fluorescence microscopy for the specific microglial marker Iba1 compared to the general nuclear dye To-Pro-3 iodide and experiments were excluded if purity was determined to be below 98%.

Treatments were carried out on isolated microglia over a total 24 h time course (Fig. 2). Isolated microglia in wells were randomly assigned to a treatment or control condition, and were treated with SCCA from prepared aqueous stocks in DMEM/F12 to a final treatment concentration as indicated (Figs. 3–9) or aqueous vehicle. One hour after the start of SCCA treatment cells microglia were treated with the endotoxin LPS (100 ng/ml), the cytokine IFNγ (100 ng/ml), or aqueous vehicle as an inflammatory challenge. After a total of 24 h treatment cell culture media was collected for analysis of secreted inflammatory modulators and cells were fixed with 10% buffered formalin (5 min, room temperature) for fluorescence and immunofluorescence assays.

2.4. Molecular analyses and fluorescence imaging

Secreted cytokines in cell culture media including interleukins-1β (IL1β), −6 (IL6), and −10 (IL10) and tumour necrosis factor (TNF) were determined by ELISA according to the manufacturer's instructions. Secreted nitric oxide (NOx) was estimated by the stable

decay product nitrite using the Greiss reaction [38,39]. Cells were counted using automated analysis of To-Pro-3 iodide staining as described below and secreted cytokines or nitrite were normalized to the number of cells per well to account for any well-to-well variation in cell density.

Fixed cells were blocked and permeabilized using 1% horse serum and 0.5% Triton X-100 in PBS for 2 h. Lipid droplets were quantified using fluorescence microscopy and the lipophilic dye BODIPY 493/503 [40] and counterstained with To-Pro-3 iodide. BODIPY 493/503 was prepared by dissolving to saturation in ethanol (approximately 1 mg/ml). The staining solution was prepared by diluting the saturated BODIPY 493/503 stock 1:100 into pre-warmed PBS with 5% horse serum, 0.05% Triton X-100, and 0.5 μM To-Pro-3 iodide and fixed cells were stained for 15 min at room temperature and washed twice with PBS +1% horse serum. BODIPY 493/503 was prone to precipitate from aqueous solutions and as such was always prepared fresh with 5% horse serum as a lipophilic carrier, diluted into PBS pre-warmed to 37 °C, and used immediately. BODIPY 493/503 was also not compatible with most mounting reagents so images were acquired immediately after staining in wells with PBS.

Images were acquired using a MiniMax Imaging Cytometer (Molecular Devices, San Jose, CA, USA) to detect To-Pro-3 iodide using the 713 nm channel and BODIPY 493/503 using the 541 nm channel with 200 ms exposure times. For cell counting To-Pro-3 iodide was imaged across a grid 24 non-overlapping positions per well giving total coverage of 28% of the well surface, and particles were detected using the MiniMax analysis software for particle sizes of 7.5–30 μm. Particle counts were then corrected for the well surface area to give a total cell count per well. For BODIPY 493/503 analysis the same imaging and particle detection parameters and were applied to the 713 nm channel however each detected object was used to determine an ellipsoid region of interest (ROI) that was then increased in diameter by 3.5 μm to encompass the approximate area of the cell soma and the corresponding fluorescence intensity of each ROI in the 541 nm channel was determined. This value was averaged within the well to give the mean fluorescence intensity per cell in arbitrary units of fluorescence (AU).

2.5. Statistics

Power analysis was performed to determine sample size based on means, effect size, and variance determined from a preliminary dataset. All statistical analyses were carried out using GraphPad Prism 9. Serum SCCA were evaluated for normality using a D'Agostino-Pearson test, which was not successful for most samples, as such differences were assessed using a nonparametric one-way ANOVA using the Kruskal-Wallis method and Dunn's multiple comparison post-hoc. Data from cell culture assays were assessed using a two-way ANOVA (SCCA dose and inflammatory stimulus as the two independent variables) and Student's t-test post-hoc. For all comparisons differences with a P value of ≤ 0.05 were considered significant and indicated as either an * (pairwise significance vs respective control) or † (pairwise significance as indicated). Each cell culture experiment was replicated a total of 7 times from independent cultures prepared from a separate animal, however the exclusion criteria described above using cell purity resulted in some experiments being removed from analysis. The number of replicates included in the analysis for each experimental group are indicated in the figure legends.

3. Results

3.1. Serum short-chain fatty and carboxylic acid profiles changed after fecal microbiota transplant

Serum samples were collected from patients receiving FMT for treatment of RCDI [33] at pre-FMT, 4 weeks post-FMT, and 12 weeks post-FMT and SCCA were quantified by gas chromatography-mass spectroscopy (GC-MS). Of the 10 SCCA determined, 5 species were significantly different at one or more time points after FMT (Fig. 1, Supplementary Tables 1–2). Butyrate was significantly increased at 4 and 12 week timepoints compared to pre-FMT (Fig. 1A; mean increase of 3.4- and 3.7-fold and Z-score 3.5 and 4.2 vs pre-FMT at 4 and 12 weeks, respectively). 2-methylbutyrate was increased relative to pre-FMT (Fig. 1C; 1.3-fold at 4 weeks and 1.5-fold at 12 weeks), but only reached significance at the 12 week time point (Z-score vs pre-FMT 1.7 and 3.3 for 4 weeks and 12 weeks, respectively). Valerate was increased relative to pre-FMT (Fig. 1D; 1.1-fold at 4 weeks and 1.2-fold at 12 weeks), but only significantly at 4 weeks (Z-score vs pre-FMT 2.6 and 2.2 at 4 and 12 weeks, respectively). Isovalerate was increased relative to pre-FMT (Fig. 1E; 2.2-fold at 4 weeks and 2.0-fold at 12 weeks), but only reached significance at the 12 week time point (Z-score vs pre-FMT 2.3 and 2.5 at for 4 weeks and 12 weeks, respectively). 2-hydroxybutyrate was the only measured SCCA that was decreased after FMT (Fig. 1B; 0.76-fold change at 4 weeks and 0.67-fold change at 12 weeks) and this decrease was significant at 12 weeks (Z-score vs pre-FMT 2.0 and 2.4 at 4 and 12 weeks, respectively). Propionate was included in our analysis as it is often reported to increase with gut perturbations and has been suggested to have pro-inflammatory effects on immune function, but was notably unchanged in our cohort (Fig. 1F; no significant effect of treatment).

3.2. Modeling serum profiles associated with improved health and wellbeing

The post-FMT conditions was associated with improved GI health and also health and wellbeing as surveyed by the SF-36 questionnaire [33]. Substantial and significant improvements were noted in metrics associated with mental health (Mental health, Role emotional, and Social functioning). Following the hypothesis that circulating SCCA participate in gut microbiome-brain communication, we defined a SCCA treatment profile based on the observed SCCA increases from patient serum: the SCCA profile consisted of butyrate, valerate, isovalerate, and 2-methylbutyrate. For simplicity we used an equimolar mix of these four SCCA as treatment concentrations *in vitro* are not well predicted by serum concentrations, and this mix was used to treat primary microglia cultured from

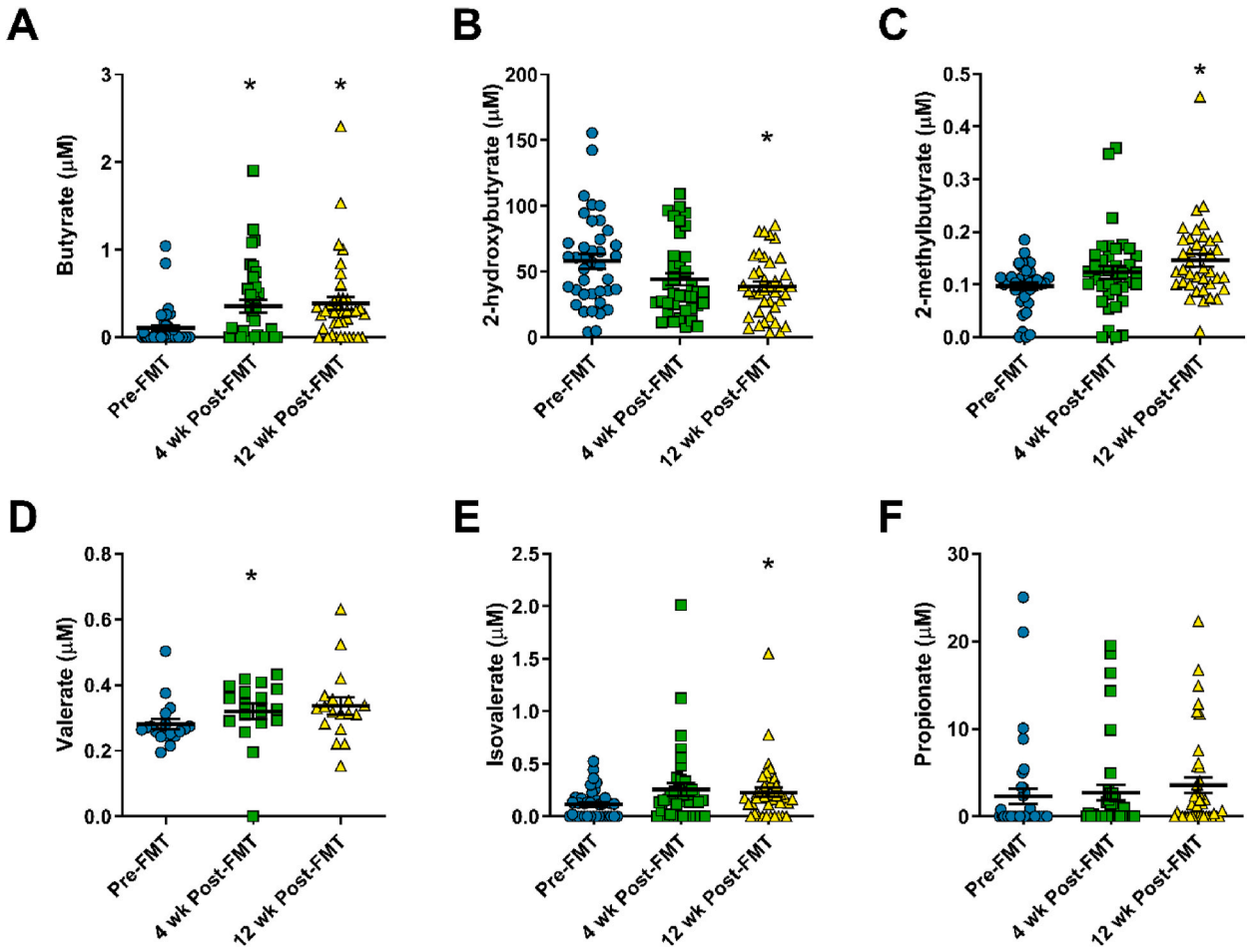


Fig. 1. Serum levels of SCCA from individuals with rCDI pre-FMT, and at 4 and 12 weeks post-FMT. (A) butyrate N = 38, (B) 2-hydroxybutyrate N = 38, (C) 2-methylbutyrate N = 38, (D) valerate N = 18, (E) isovalerate N = 38, and (F) propionate N = 38. Asterisk indicates significant difference relative to pre-FMT ($P < 0.05$) using Kruskal-Wallis ANOVA and Dunn's multiple comparison post-hoc.

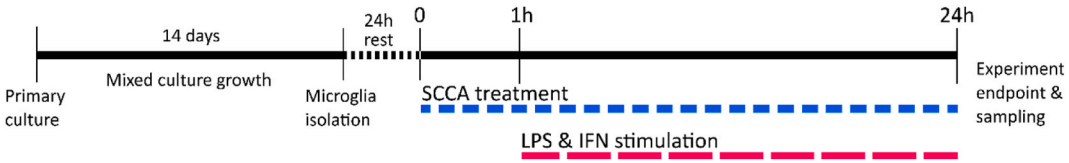


Fig. 2. Schematic representation of the *in vitro* experiment methodology and timeline. Primary cultures of mixed cells were established from neonatal rat brain, maintained for 14 days, and microglia were isolated 24 h prior to the start of experimental treatments. At t = 0 microglia were treated with SCCA as described in each subsequent figure, followed by LPS or IFN stimulation at t = 1 h. At t = 24 h cell culture media was collected to measure cumulative release of secreted factors and cells were fixed for immunofluorescence analysis.

rat brain. Accurate estimates of these SCCA concentrations in human brain tissue were not available, though butyrate has been measured in the rodent brain at ~0.4–0.7 μmol/g wet tissue weight (mmol/L) [41] which was used as a guide concentration to establish a dosing range. Further, these individual SCCA were each tested in a broad dose response (40 μM–1000 μM) for individual effects on primary cultured microglia. 2-hydroxybutyrate was additionally tested as it showed significantly higher levels in the pre-FMT serum samples associated with adverse gut and mental health.

3.3. Mixed SCCA treatment had anti-inflammatory effects on microglia

As a test of inflammation-modifying properties of SCCA primary isolated microglia cultures were treated with varied concentrations of SCCA (individually or mixed as above) and subsequently administered a pro-inflammatory stimulus of either bacterial

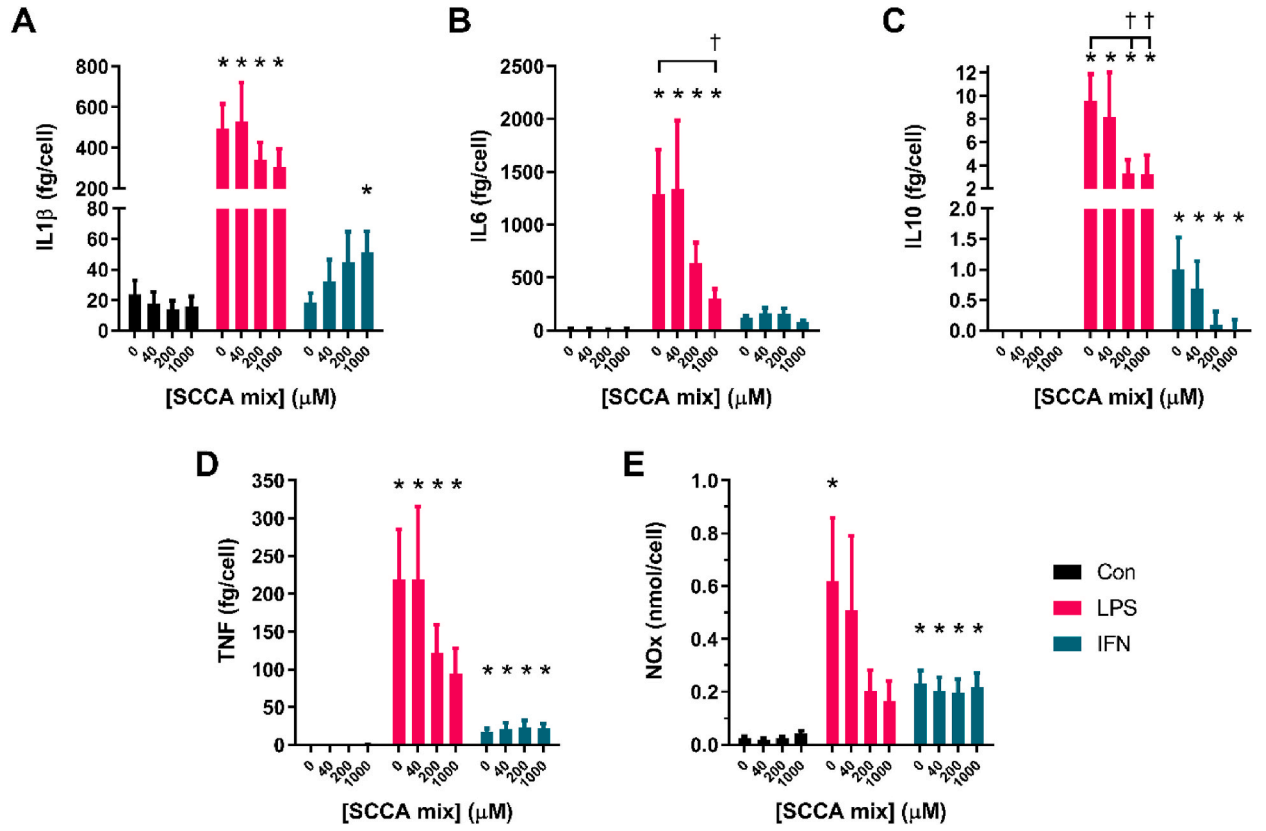


Fig. 3. Release profile of the cytokines (A) IL1β, (B) IL6, (C) IL10, (D) TNF, and (E) release of NO into culture media from microglia treated with mixed SCCA (butyrate, 2-methylbutyrate, valerate, isovalerate) and stimulated with either LPS or IFN. * indicates significant difference relative to control, † indicates pairwise difference as indicated ($P < 0.05$) using two factor ANOVA and Student's t-test post-hoc ($N = 6$).

lipopolysaccharide (LPS; a direct mimic of pathogen exposure) or interferon-γ (IFNγ; a cytokine induced by viral infection). With this experimental paradigm, an anti-inflammatory effect of SCCA would result in a decrease in the release of cytokines and inflammatory mediators after stimulus with LPS or IFNγ, while a pro-inflammatory effect would result in an increase in release of cytokines and mediators in the control condition or stimulus conditions. When treated with mixed SCCA representing the post-FMT serum profile, we observed decreased release of a number of cytokines as well as nitric oxide (Fig. 3).

For each measured cytokine and nitric oxide, LPS treatment significantly increased release relative to the control, and for most effectors mixed SCCA treatment reduced the LPS-induced release. LPS-induced interleukin-1β (IL1β) was slightly reduced after treatment with 1000 μM SCCA (0.62-fold change), though this change was not significant compared to the within group control ($P = 0.24$ vs LPS only; Fig. 3A). Interleukin 6 (IL6) was reduced after SCCA treatment (Fig. 3B; 0.50-fold at 200 μM and 0.24-fold at 1000 μM and this was significant at 1000 μM versus the LPS only condition. Interleukin-10 (IL10) release was significantly reduced after SCCA treatment (Fig. 3C; 0.35-fold at 200 μM and 0.34-fold at 1000 μM) compared to the LPS only condition. Tumour necrosis factor (TNF) was reduced (0.43-fold at 1000 μM) but this difference was not significant relative to the LPS only condition ($P = 0.12$ vs LPS only.; Fig. 3D). Nitric oxide, measured by the stable breakdown product nitrite, was also reduced (Fig. 3E; 0.33-fold at 200 μM, 0.27-fold at 1000 μM), with both SCCA treated groups no longer significantly above the respective control conditions.

IFNγ treatment resulted in a substantially smaller increase in cytokine release relative to LPS, and only significantly increased the release of IL10, TNF, and nitric oxide relative to the control condition. This lowered release profile did not show any significantly reduced release after SCCA treatment, however the relatively sensitized state induced by IFNγ treatment did reveal that SCCA treatment increased the release of IL1β (Fig. 3A; 2.78-fold change vs IFNγ) significantly above the control condition. No other significant within group effects of SCCA after IFNγ treatment were observed.

3.4. *Single-species SCCA treatments did not affect microglial inflammation*

As serum butyrate had the most substantial change after FMT, we did explore the hypothesis that butyrate would be the dominant figure explaining the observed anti-inflammatory effects of mixed SCCA; however, LPS-induced cytokine release was largely unchanged after butyrate treatment of primary microglia cultures (Fig. 4). No within group differences were measured for IL1β (Fig. 4A), IL6 (Fig. 4B), IL10 (Fig. 4C), TNF (Fig. 4D), or nitric oxide (Fig. 4E) after butyrate and LPS treatment, suggesting butyrate alone does

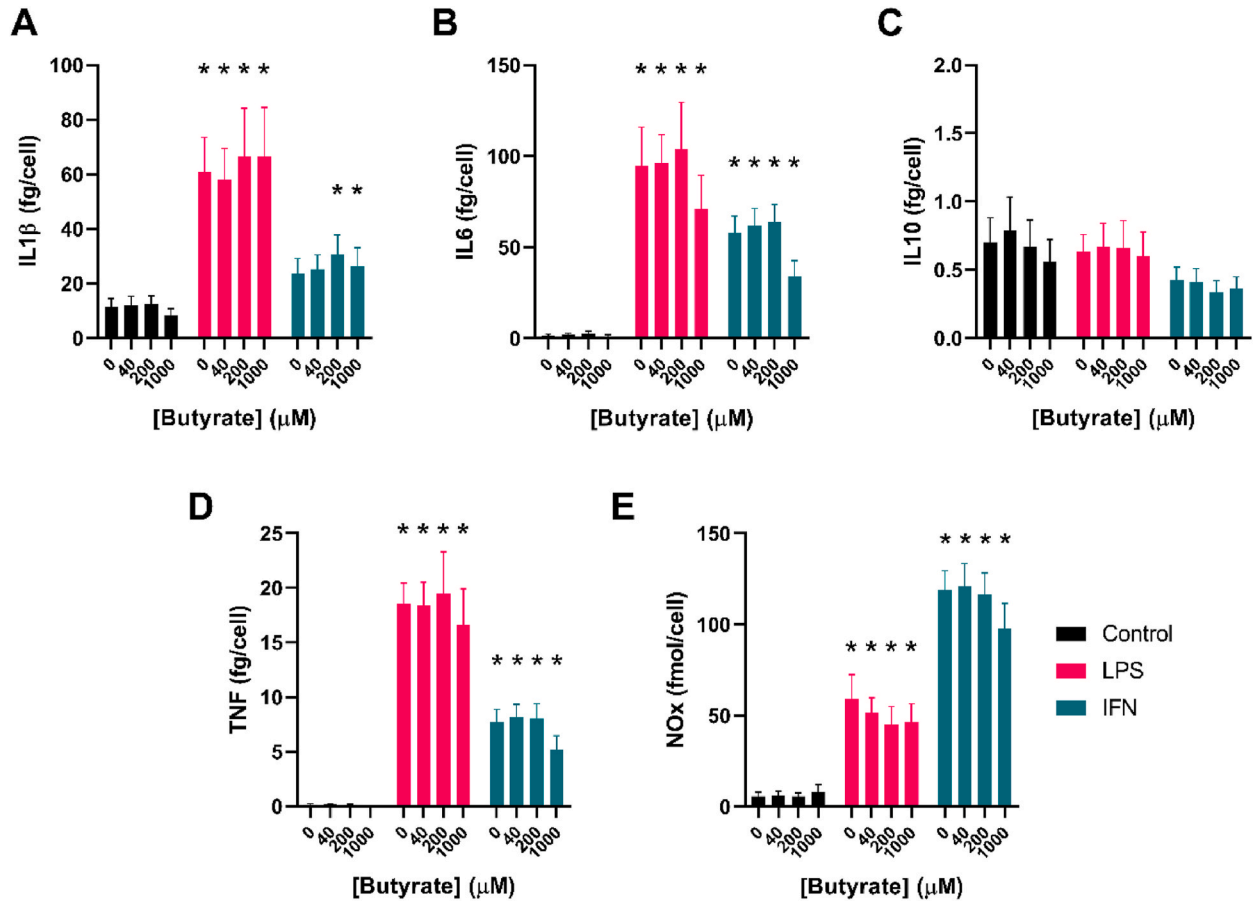


Fig. 4. Release profile of the cytokines (A) IL1β, (B) IL6, (C) IL10, (D) TNF, and (E) release of NO into culture media from microglia treated with butyrate. * indicates significant difference relative to control, † indicates pairwise difference as indicated ($P < 0.05$) using two factor ANOVA and Student's t-test post-hoc ($N = 7$).

not exert a potent anti-inflammatory effect on microglial inflammation. As with the mixed SCCA treatment we did observe a slight increase in IL1β release (Fig. 4A; 1.3-fold at 200 μM and 1.1-fold at 1000 μM) that resulted in IL1β levels significantly above control. Butyrate treatment otherwise did not alter release of any cytokine or nitric oxide after IFNγ treatment.

2-hydroxybutyrate was notably decreased after FMT treatment (Fig. 1B), and as the only SCCA measured to significantly decline was investigated to determine if it may have been associated with an adverse effect on microglial inflammation. Treatment with 2-hydroxybutyrate did not affect either LPS- or IFNγ-induced release of cytokines (IL1β, IL6, IL10, or TNF; Fig. 5A–D) or nitric oxide (Fig. 5E).

Each of the remaining SCCA that were observed to change following FMT were tested across a comparable dose range for effects on LPS- or IFNγ-induced inflammation from primary cultured microglia including 2-methylbutyrate (Fig. 6A–E), valerate (Fig. 7A–E), and isovalerate (Fig. 8A–E). In all cases LPS- and IFNγ-induced release of cytokines and nitric oxide were unchanged by SCCA treatment.

3.5. *Mixed SCCA treatment increased lipid storage in microglia*

Previous studies have shown that microglia store triglycerides in intracellular lipid droplets in quantities that vary with different inflammatory stimuli and environmental conditions including extracellular lipids and glucose concentrations [40,42,43]. As some SCCA may serve as metabolic precursors in addition to signalling roles we assayed lipid droplet accumulation in SCCA treated microglia using the lipophilic fluorophore BODIPY 493/503 (Fig. 9). BODIPY 493/503 fluorescence was markedly increased after treatment with mixed SCCA at 1000 μM (Fig. 9A; 1.7-fold increase in control group, 1.6-fold increase in LPS group, and 1.5-fold increase in IFNγ group vs respective 0 μM condition) though the increase was not significant in the LPS condition (LPS 0 vs 1000 μM $P = 0.15$). BODIPY 493/503 fluorescence levels were generally lower in the IFNγ group relative to the control and LPS groups, possibly due to a consistently higher rate of proliferation after IFNγ treatment which could result in dispersal of lipid droplet organelles across the daughter cells following cytokinesis.

Similar to the effects observed on cytokine and nitric oxide release, treatment with any single species of SCCA did not significantly affect accumulation of lipid droplets as indicated by BODIPY 493/503 staining. For each of butyrate (Fig. 9B), valerate (Fig. 9C), 2-

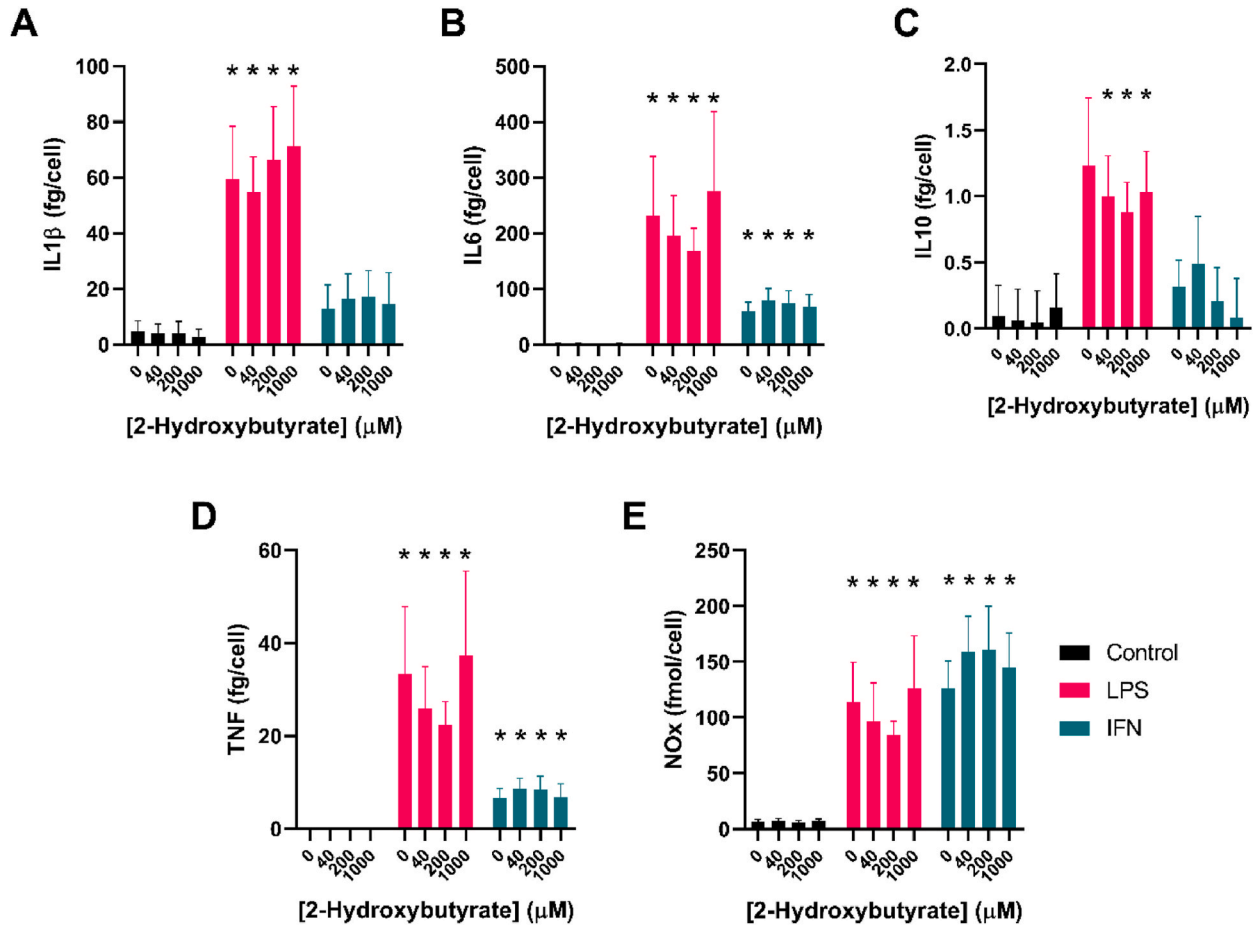


Fig. 5. Release profile of the cytokines (A) IL1β, (B) IL6, (C) IL10, (D) TNF, and (E) release of NO into culture media from microglia treated with 2-hydroxybutyrate. * indicates significant difference relative to control, † indicates pairwise difference as indicated ($P < 0.05$) using two factor ANOVA and Student's t-test post-hoc ($N = 6$).

methylbutyrate (Fig. 9D), 2-hydroxybutyrate (Fig. 9E), and isovalerate (Fig. 9F), our analysis indicated no significant effect of SCCA treatment on BODIPY 493/503 staining.

4. Discussion

In this work, we explored potential mechanisms underpinning the gut-brain axis by exploiting changes in microbiome-metabolome interactions mediated by FMT for rCDI in humans in experiments investigating the impact of SCCA on microglial inflammation *in vitro*. Based on FMT results, we tested the effects of butyrate, 2-methylbutyrate, valerate, and isovalerate separately and in combination, and 2-hydroxybutyrate on primary microglial cultures. As supported by the SCCA profiles determined pre- and post-FMT for rCDI in human clinical trial participants, each of butyrate, 2-methylbutyrate, valerate, and isovalerate were positively associated with the resolution of both gut perturbation and consequent improvements in physical and mental health status, while 2-hydroxybutyrate was negatively associated with recovery, being significantly higher in the pre-treatment condition. While we hypothesized that those SCCA with the largest magnitude of change (i.e. butyrate) would be most likely to affect induced microglial inflammation, we were surprised to see that only in a combined SCCA treatment condition was release of inflammatory modulators significantly affected. From these data we conclude that a communal influence of health-associated SCCA has the highest likelihood of anti-inflammatory effect on brain microglia and may be a partial contributor to healthy gut-brain communication. As all single-species SCCA treatments in this study did not significantly affect microglia we will emphasize discussion of the communal effects and mechanisms of SCCA on inflammation but have made available a more detailed discussion of each individual SCCA in the Supplementary Discussion.

4.1. Communal SCCA treatment anti-inflammatory effect on microglia

While single-species treatments of SCCA did not exert anti-inflammatory effects on LPS and IFNγ-treated microglia, the equimolar mixture of butyrate, valerate, isovalerate, and 2-metylbutyrate did. In the LPS condition, treatment with mixed SCCA produced a

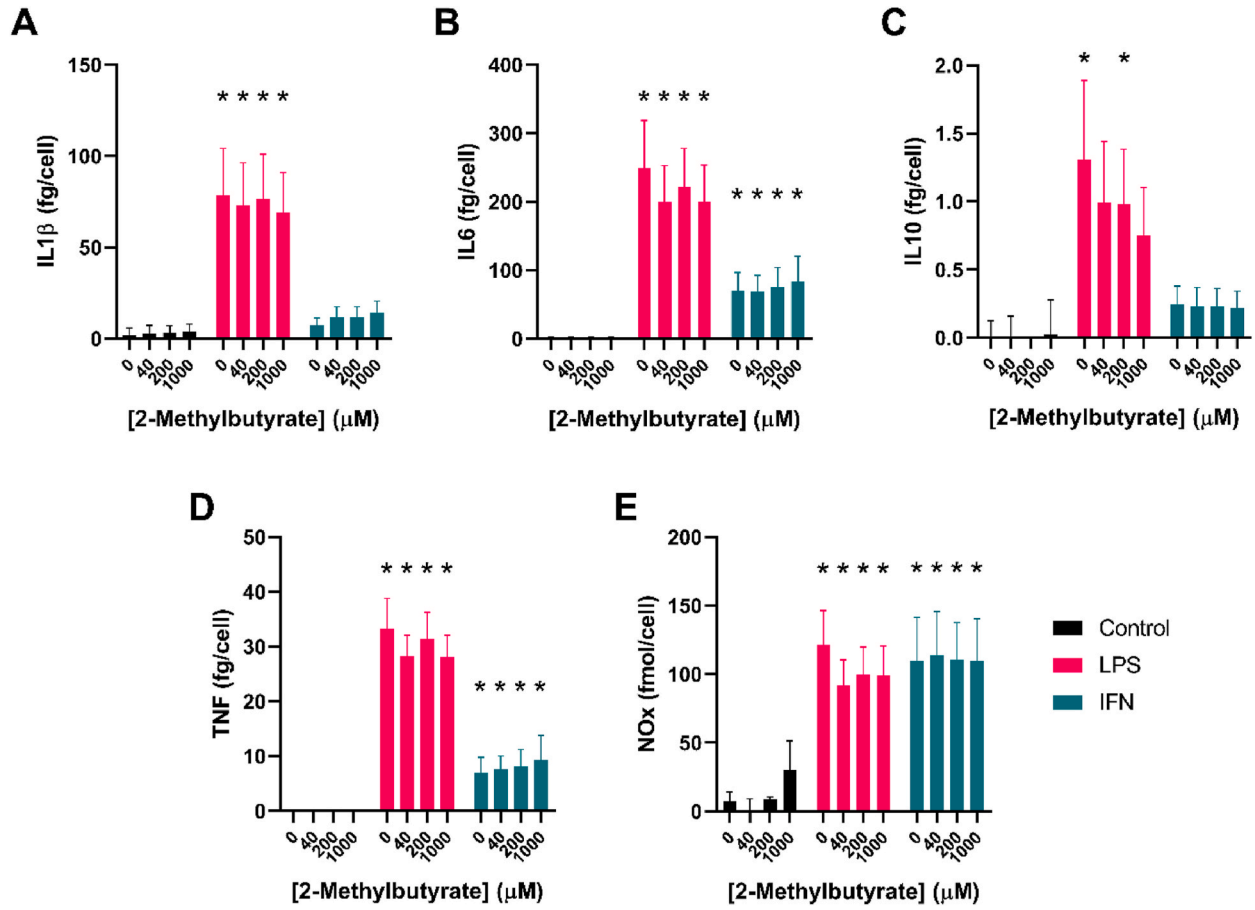


Fig. 6. Release profile of the cytokines (A) IL1β, (B) IL6, (C) IL10, (D) TNF, and (E) release of NO into culture media from microglia treated with 2-methylbutyrate. * indicates significant difference relative to control, † indicates pairwise difference as indicated ($P < 0.05$) using two factor ANOVA and Student's t-test post-hoc ($N = 7$).

significant decrease in levels of IL-6, IL-10, and nitric oxide, as well as an observable but not statistically significant decrease in IL-1B and TNF.

In the IFNγ condition, changes from SCCA treatment were more difficult to detect since the induced release of cytokines from IFNγ treatment is much lower than that of LPS. Mixed SCCA treatment produced no significant decrease in cytokine release, but the relatively sensitized state revealed a significant increase in IL-1B (Fig. 3A), similar to that detected in the IFNγ condition treated with butyrate alone.

This communal anti-inflammatory effect is supported by other related observations in models: SCCA levels were noted to be altered in the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis prompting the investigation of single-species treatment with butyrate [44]. In this model the anti-inflammatory effects of gut microbiome manipulations were associated with increased levels of butyrate, however butyrate alone was notably unable to replicate the treatment effect [44].

Conversely, recent work using an immortal human monocyte cell line (THP-1) as a model for studying microglia-like immune functions demonstrated an anti-inflammatory effect of a combined SCCA treatment, however this also noted distinct anti-inflammatory effects of individual SCCA species (notably valerate and formate) [45]. This replicates the communal effect of SCCA we observed in the present study, however, contrasts our lack of effect of valerate alone on primary microglial cells (Fig. 7). This study presents several technical contrasts to our current study: both rat primary microglia and human immortal THP-1 monocytes are imperfect models for human microglia *in vivo*; each study used a different range and concentration of SCCA; and measured outcomes differed between both studies. Our use of primary rat microglia is a well established model with known strengths and limitations. As primary brain cells, we are studying the actual target cell population, but are derived from a rodent rather than a human due to necessity and ethics, and the culturing process may induce experimental artifacts that affect the similarity to the human brain. Conversely, human THP-1 cells are an immortal peripheral macrophage, and while some studies have noted phenotypic convergence between microglia and peripheral macrophages, it is largely dependent upon the tissue microenvironment (e.g. the range of factors in the CNS milieu) and their complex interactions with cells [46]. In the absence of this influence of microenvironment, THP-1 cells may only model general immune-related functions that can be shared with microglia and should be interpreted with this limitation in mind.

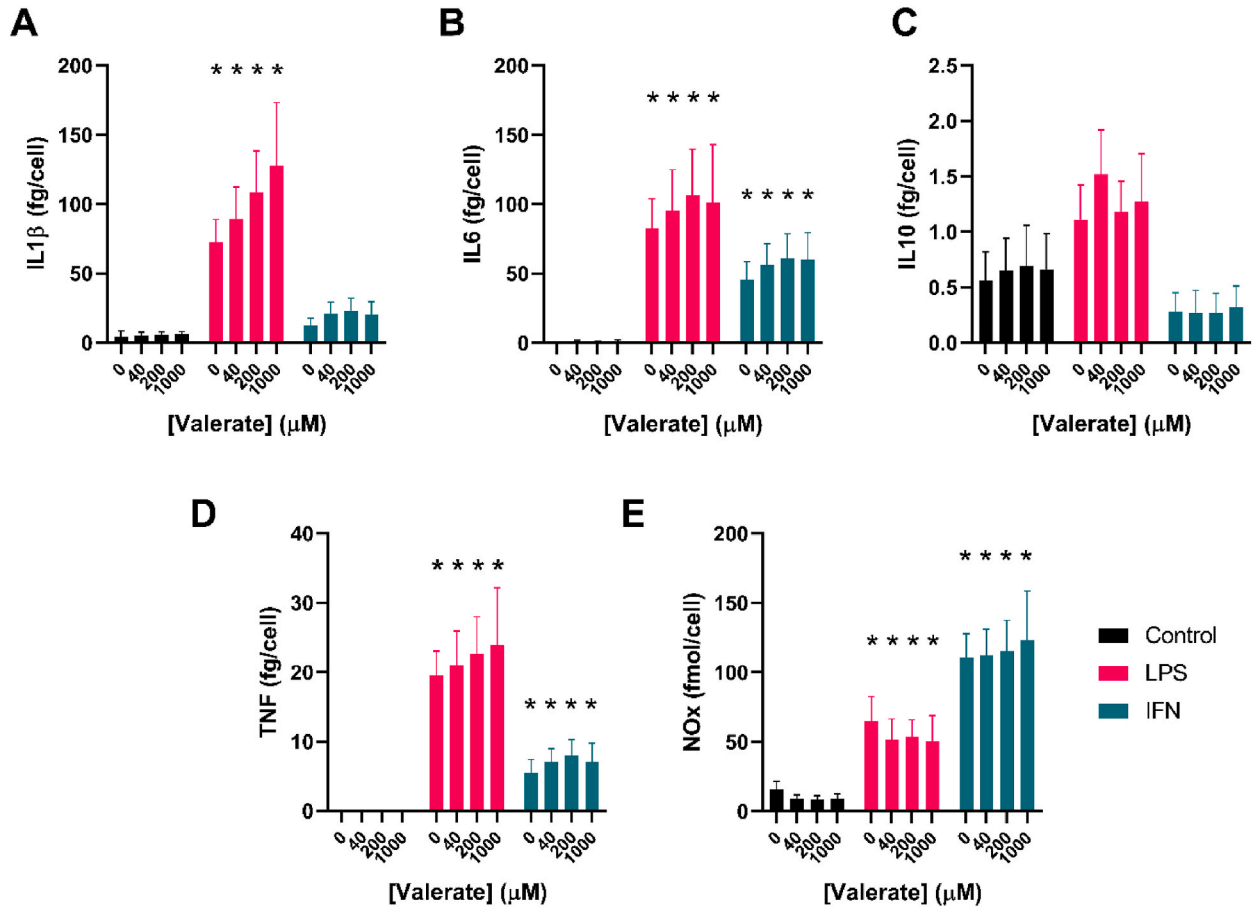


Fig. 7. Release profile of the cytokines (A) IL1β, (B) IL6, (C) IL10, (D) TNF, and (E) release of NO into culture media from microglia treated with valerate. * indicates significant difference relative to control, † indicates pairwise difference as indicated ($P < 0.05$) using two factor ANOVA and Student's t-test post-hoc ($N = 6$).

As SCCA are able to affect cells through a number of mechanisms, including direct signal transduction, further investigation into mechanisms of SCCA signalling are necessary. Critically, Wenzel and colleagues identified that the THP-1 modulating effects of SCCA treatment was likely unrelated to the free fatty acid receptor 2/3 (FFAR2/3) pathway as it was unaffected by the FFAR2/3 inhibitor GLPG 0974 [45]. Another putative mechanism is through epigenetic regulation of histone acetylation: acetate, butyrate, and propionate, as the three most common SCCA found in the gut, as well as valerate, have been shown to affect immune cells through inhibition of histone deacetylases (HDAC) [47]. Propionate and butyrate inhibited granulocyte macrophage colony stimulating factor (GM-CSF) induced dendritic cell development [48], and acetate and propionate promoted T-cell differentiation in to effector and regulatory T-cells [49] both through HDAC inhibition. Primary microglia were further noted to increase prostaglandin synthesis after valproate or butyrate treatment through a HDAC-dependent mechanism [50]. As the effects on microglial inflammation in the present study were only observed after treatment with a mixture of different SCCA, it may well be that multiple, convergent mechanisms are at play in microglia. Future studies will be required to investigate the specific signalling roles in microglia in response to mixed SCCA treatment. Taken as a whole, our present work and these previous studies indicate the potential complexity of SCCA roles both in the gut and as a component of gut-brain communication, emphasizing the need for continued investigation. In addition to SCCA there are several additional gut microbial metabolites that are affected by perturbations of gut microbiota and FMT that may contribute to gut-brain communication - including bile acids [51,52], aryl hydrocarbon receptor ligands [53–56], indole-related metabolites (tryptophan- and 5-hydroxytryptamine-related compounds) [57,58], and other neurotransmitter-related metabolites (e.g. GABA, precursors of acetylcholine) [59,60] which also merit further exploration. The present study emphasizes the importance of the communal role of SCCA on microglial inflammation. It is becoming clear that reductive investigative efforts which seek to investigate individual factors separate from the complexity of the physiological microenvironment will need further support from *in vivo* experimental studies.

4.2. *Mixed SCCA treatment increased lipid storage in microglia*

Lipid droplets play several physiological roles in microglia and other brain cells. Arising from the endoplasmic reticulum, LD are

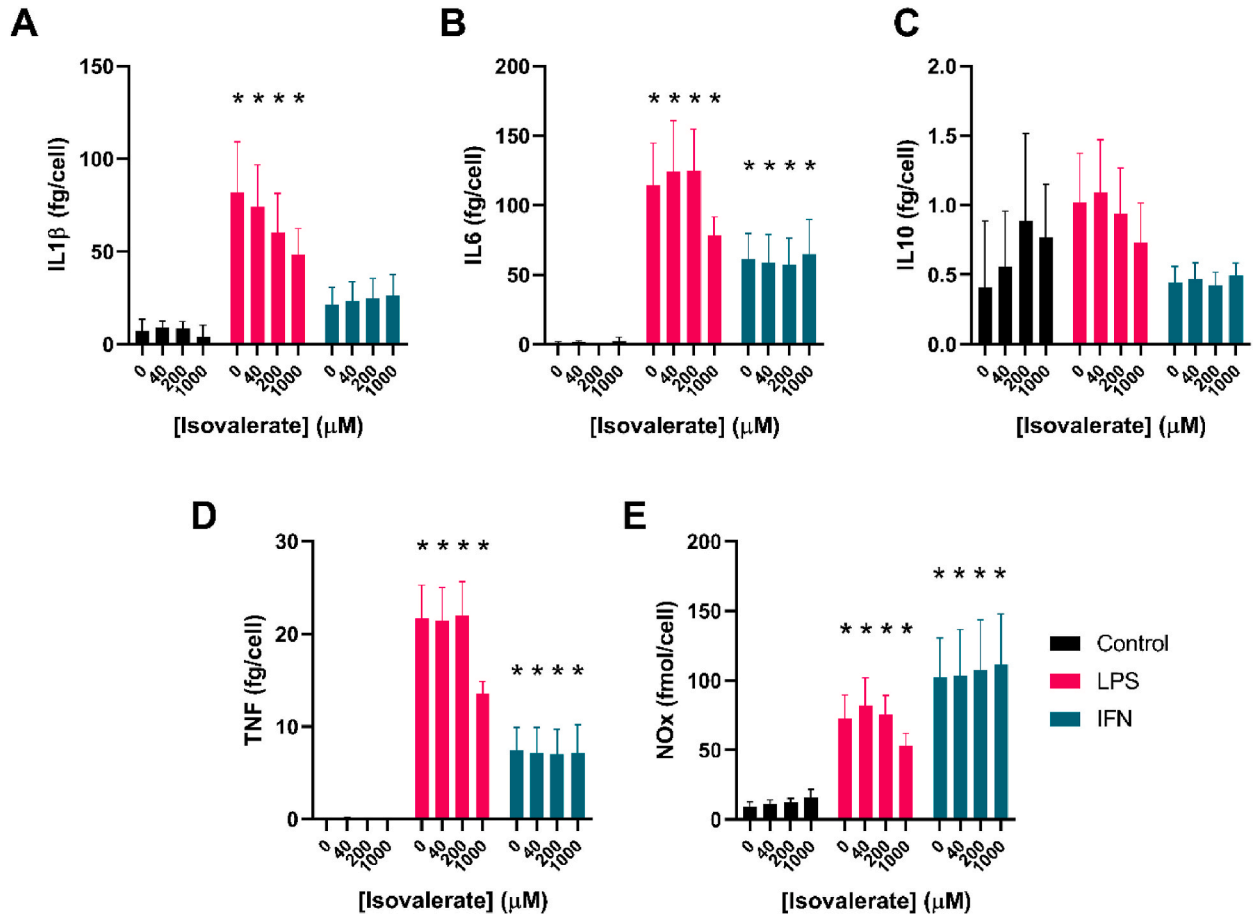


Fig. 8. Release profile of the cytokines (A) IL1β, (B) IL6, (C) IL10, (D) TNF, and (E) release of NO into culture media from microglia treated with isovalerate. * indicates significant difference relative to control, † indicates pairwise difference as indicated ($P < 0.05$) using two factor ANOVA and Student's t-test post-hoc ($N = 5$).

essential for the storage, breakdown, and metabolism of lipids, much of which is dynamically regulated by lipases and perilipins on the droplet's surface [61]. This regulation of 'lipid homeostasis' in the CNS is essential for neural function and plasticity. Stored lipids are also implicated as a key alternate energy source for microglia during metabolic stress caused by glucose deprivation [42].

The amount of lipids stored by cells can be affected by various environmental conditions and inflammatory stimulus [40,42,43]. Accumulation of LD in microglia is canonically associated with trauma, aging, and neurodegenerative disease [61,62]. Marschallinger and colleagues identified a novel Lipid Droplet Accumulating Microglia (LDAM) type in the brains of aging mice and humans, which exhibits cellular dysfunction, impaired phagocytosis, and excessive release of inflammatory factors when treated with LPS, including ROS, NO, and various pro-inflammatory cytokines, indicating a primed activation state [62]. Additionally, they implicate genes linked to neurodegenerative diseases as regulators of lipid droplet formation, solidifying the connection between disease and microglia in the aging brain. Alternately, LD in microglia have also been shown to increase in response to anti-inflammatory treatments such as polyunsaturated fats [43] suggesting changes in LD can be affected by both adverse and healthy stimuli.

LD assessment in this study was of particular interest as several carboxylic acids related to the SCCA we used herein (e.g. β-hydroxybutyrate) can act as ketone bodies as an alternate energy source to glucose, and are capable of impacting brain metabolism [63,64]. Accumulation of lipid droplets was assayed with BODIPY fluorescence. Interestingly, our results demonstrate significant accumulation of lipids in microglia after mixed SCCA treatment despite the overall anti-inflammatory effect on cytokine release and morphology. Mixed SCCA treatment yielded a significant increase in two groups: 1.7-fold in the control and 1.5-fold in the IFNγ condition. The LPS condition also had a 1.6-fold increase, but the results were not significant.

This discrepancy may be partially explained by observations of lipid droplet remodeling by other anti-inflammatory stimuli such as ω3 polyunsaturated fatty acids (PUFA) [43]. Given the relatively limited understanding of the role of lipid droplets in inflammatory processes it may be that the organelle is dynamic in response to changing conditions, either pro-inflammatory or anti-inflammatory and further analysis of the composition of lipid droplets within these experiments is warranted. It may be that lipid droplet modification serves as a storage location for material internalized by inflammation-associated phagocytosis [62], while remodeling of lipid droplets by upstream metabolic changes such as SCCA and PUFA is associated with an anti-inflammatory phenotype [43].

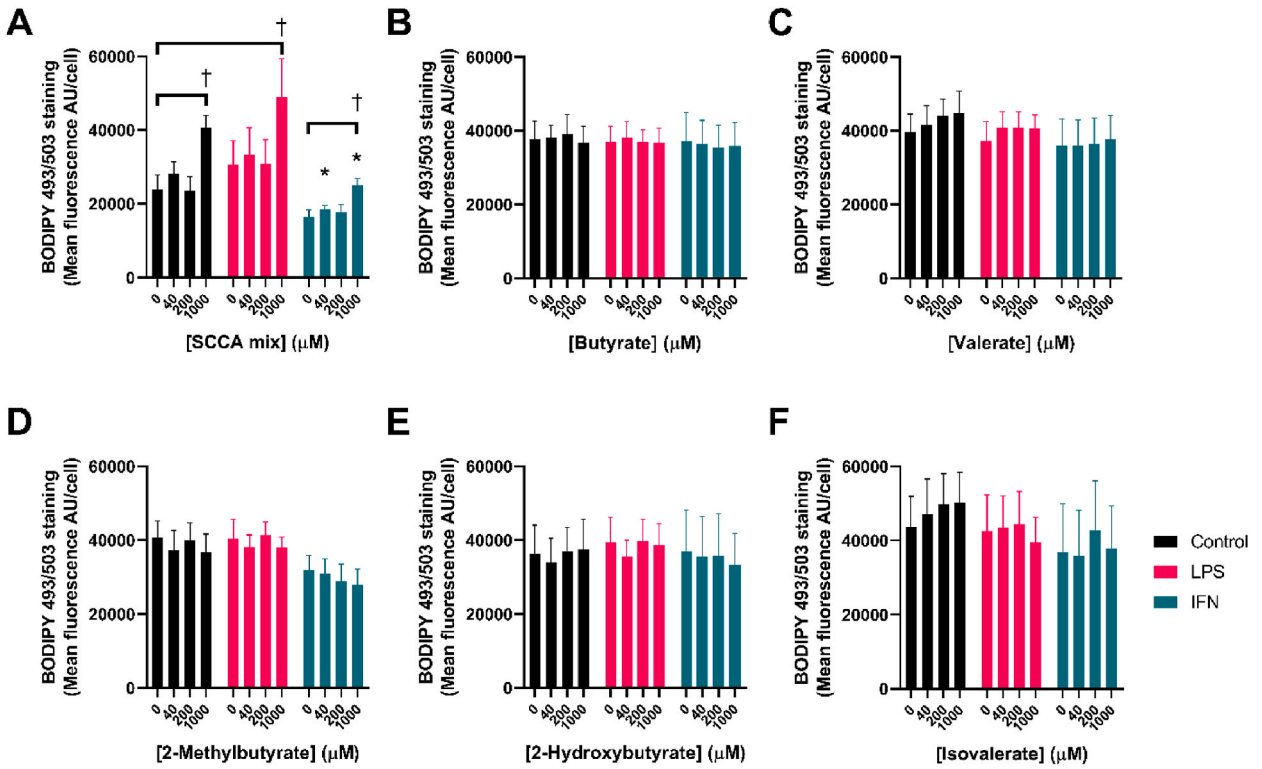


Fig. 9. Lipid droplets assayed by BODIPY 493/503 staining after treatment with (A) mixed SCCA (N = 6), (B) butyrate (N = 7), (C) valerate (N = 5), (D) 2-methylbutyrate (N = 7), (E) 2-hydroxybutyrate (N = 6), and (F) isovalerate (N = 4). * indicates significant difference relative to control, † indicates pairwise difference as indicated ($P < 0.05$) using two factor ANOVA and Student's t-test post-hoc.

Additionally, LD accumulation per cell was generally lower in the IFNγ group (significant group effect of IFNγ), which may be explained by the increased proliferation of cells and dispersal of lipid droplet organelles in the greater number of daughter cells. Treatment with any single species of SCCA had no significant effect, once again emphasizing the importance of reflecting physiological conditions in experiments due to the communal influence of factors.

4.3. Limitations

Several limitations in this work merit addressing in both the determination of serum SCCA in human trial participants and in the implementation of this paradigm in our cell culture model. The SF-36 survey captures metrics related to mental health but is not a specific survey for depression or anxiety symptoms. We have also not directly excluded the impact of changes in mental health resulting directly from recovery from a serious illness (*C. difficile* infection) which is highly disruptive to an individual's quality of life. While these themes have been broadly discussed in other literature [65], future studies will need to further address these limitations. Serum SCCA concentrations in this study are notably different from the stool concentrations measured in the same patient cohort [60], where significant increases in acetate, butyrate, and propionate were observed. Gut-derived SCCA are able to enter systemic circulation and cross the BBB through mechanisms that continue to be investigated [9], however solute carrier proteins and monocarboxylate transporters are expressed in the gut and brain and are likely catalysts of SCCA transport [7,8,66]. Differences in concentration between stool and serum can be attributed to differential transport across the gut and the utilization of some SCCA for energy in colonocytes [67]. There are likewise differences in serum concentration and brain concentration due to transport across the BBB. Butyrate, as an example, was measured in serum at low μM concentrations in the present study, but has been measured in the rodent brain at near mM concentrations [41], a difference of at least 2 orders of magnitude. These observed disparities as well as the lack of available information on levels of most SCCA in brain was the rationale for our simplified treatment paradigm using equimolar concentrations of the SCCA butyrate, 2-methylbutyrate, valerate, isovalerate for treatment *in vitro*, however accurate study of the relationship between serum and brain concentrations of SCCA would allow a more accurate treatment approach. Finally, our approach necessarily reduced the complexity of gut-brain signalling to one set of factors. As noted previously, a wide range of metabolites are derived from gut microbiota, many of which enter circulation and could contribute to gut-brain communication.

5. Conclusion

In the present work we demonstrated significant changes in serum SCCA in rCDI patients that have undergone successful FMT treatment and experienced accompanying improvements in health status. Based on previous investigations into the gut microbiome-brain communication axis, we hypothesized SCCA associated with dysbiosis or health would affect microglial inflammation *in vitro*. Testing using primary rat microglial cultures demonstrated that a mixture of SCCA upregulated post-FMT significantly decreased release of inflammatory modulators (cytokines and nitric oxide) and increased lipid droplets. No single SCCA species alone was able to significantly affect microglial release of inflammatory modulators suggesting that health associated SCCA exert a communal influence on microglial inflammation and demonstrates the need for further investigation into the complexity of SCCA signalling as a potential mechanism of gut microbiome-brain communication.

Author contribution statement

Matthew A. Churchward: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Emily R. Michaud: Performed the experiments; Wrote the paper.

Benjamin H. Mullish, Julian R. Marchesi: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jesús Miguéns Blanco, Isabel Garcia Perez: Performed the experiments.

Huiping Xu: Analyzed and interpreted the data.

Dina Kao, Kathryn G. Todd: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data associated with this study has been deposited at https://doi.org/10.6084/m9.figshare.20068817.v1.

Contributors

MAC, DK, and KGT conceived and designed the experiments; MAC, ERM, BHM, JMB, HX, IGP, and JRM performed the experiments, analyzed and interpreted the data; KGT and DK contributed reagents, materials, analysis tools or data; MAC, ERM, BHM, JRM, DK, and KGT wrote the paper; all authors had access to the study data and have read and approved the final manuscript.

Data sharing statement

The data that support the findings of this study are openly available in figshare at https://doi.org/10.6084/m9.figshare.20068817.v1.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Dina Kao reports financial support was provided by University of Alberta Hospital Foundation. Kathryn G Todd reports financial support was provided by Natural Sciences and Engineering Research Council of Canada. Julian Marchesi reports financial support was provided by National Institute for Health Research Imperial Biomedical Research Centre. Kathryn G Todd reports financial support was provided by Alberta Health Services. Benjamin H Mullish reports a relationship with Finch Therapeutics that includes: consulting or advisory. Benjamin H Mullish reports a relationship with Ferring Pharmaceuticals Inc that includes: consulting or advisory. Benjamin H Mullish reports a relationship with Summit Therapeutics plc that includes: consulting or advisory. Julian R Marchesi reports a relationship with Cultech that includes: consulting or advisory. Julian R Marchesi reports a relationship with EnteroBiotix Ltd that includes: consulting or advisory.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16908.

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