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Short-Chain Fatty Acids: Microbial Metabolites That Alleviate Stress-induced Brain-Gut Axis Alterations

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Key point summary

- Chronic (psychosocial) stress changes gut microbiota composition, as well as induces behavioural and physiological deficits.

- The microbial metabolites short-chain fatty acids (SCFAs) have been implicated in gastrointestinal functional, (neuro)immune regulation and host metabolism. However, their role in stress-induced behavioural and physiological alterations is poorly understood.

- Administration of SCFAs to mice undergoing psychosocial stress alleviates enduring alterations in anhedonia and heightened stress-responsiveness, as well as stress-induced increases in intestinal permeability.

- In contrast, chronic stress-induced alterations in body weight gain, faecal SCFAs and the gene expression of the SCFA receptors FFAR2 and FFAR3 remained unaffected by SCFAs supplementation.

- These results present novel insights into mechanisms underpinning the influence of the gut microbiota on brain homeostasis, behaviour and host metabolism, informing the development of microbiota-targeted therapies for stress-related disorders.

Abstract

There is a growing recognition of the involvement of the gastrointestinal microbiota in the regulation of physiology and behaviour. Microbiota-derived metabolites play a central role in the communication between microbes and their host with short-chain fatty acids (SCFAs) being perhaps the most studied. SCFAs are primarily derived from fermentation of dietary fibres and play a pivotal role in host gut, metabolic and immune function. All these factors have previously been demonstrated to be adversely affected by stress. Therefore, we sought to assess whether SCFA supplementation could counteract the enduring effects of chronic psychosocial stress. C57BL/6J male mice received oral supplementation of a mixture of the three principle SCFAs (acetate, propionate and butyrate). One week later, mice underwent 3 weeks of repeated psychosocial stress, followed by a comprehensive behavioural analysis. Finally, plasma corticosterone, faecal SCFAs and caecal microbiota composition were assessed.

SCFA treatment alleviated psychosocial stress-induced alterations in reward-seeking behaviour, increased responsiveness to an acute stressor and *in vivo* intestinal permeability. In addition, SCFAs exhibited behavioural test-specific antidepressant and anxiolytic effects, which were not present when mice had also undergone psychosocial stress. Stress-induced increases body weight gain, faecal SCFAs and the colonic gene expression of the SCFA receptors free fatty acid receptor 2 and 3 remained unaffected by SCFAs supplementation. Moreover, there were no collateral effects on caecal microbiota composition. Taken together, these data show that SCFAs supplementation alleviates selective and enduring alterations induced by repeated psychosocial stress and these data may inform future research into microbiota-targeted therapies for stress-related disorders.

1. **Introduction**

Chronic stress is a considerable health concern for society, associated with various disease states including an increased risk for neuropsychiatric disorders like depression (Yang *et al.*, 2015; Ramirez *et al.*, 2017) and functional gastrointestinal disorders such as irritable bowel syndrome (Stam *et al.*, 1997; Klooker *et al.*, 2009; Moloney *et al.*, 2016). Stress-related disorders have complex and

multifactorial aetiologies and trajectories. Recent years has seen a growing interest in the potential contribution of microbiota-gut-brain axis signalling in both the aetiology and treatment of stress-related disorders (Foster *et al.*, 2017; Rea *et al.*, 2017). For instance, preclinical studies have revealed that chronic stress results in an altered gut microbial composition (Bailey *et al.*, 2011; Bharwani *et al.*, 2017; Burokas *et al.*, 2017; Galley *et al.*, 2017; Marin *et al.*, 2017; Szyszkowicz *et al.*, 2017; Dunphy-Doherty *et al.*, 2018). In addition, various microbiota-targeted interventions are able to ameliorate chronic stress-induced deficits in mice, which include the administration of live bacterial strains (probiotics) (Bharwani *et al.*, 2017; Marin *et al.*, 2017), as well as the supplementation of host-indigestible dietary fibers that are fermented by the gut microbiota (prebiotics) (Burokas *et al.*, 2017). Importantly, predictive analysis of functional microbiota pathways reveals that chronic social defeat stress resulted in a lower representation of pathways involved in fatty acid metabolism and biosynthesis, particularly involved in the metabolism of propanoate and butanoate—conjugate bases of the short-chain fatty acids (SCFAs) propionate and butyrate (Bharwani *et al.*, 2016).

The primary source of endogenous SCFAs is the gut microbial fermentation of host-indigestible dietary fibres, with the principal SCFAs being acetate, propionate and butyrate (Koh *et al.*, 2016). These SCFAs have been shown to affect the host through multiple mechanisms including the regulation of histone acetylation and methylation (Krautkramer *et al.*, 2016; Stilling *et al.*, 2016), G-protein couple receptors (GPCR) (Milligan *et al.*, 2017), facilitating the secretion of various hormones (e.g. GLP-1 and PYY) and neurochemicals (e.g. serotonin) (Chambers *et al.*, 2015; Reigstad *et al.*, 2015), and the induction of vagus nerve signaling (De Vadder *et al.*, 2014; Li *et al.*, 2017). SCFAs are also used as a mitochondrial energy source resulting in an extremely rapid uptake of these compounds in both humans and rodents (Egorin *et al.*, 1999; Schonfeld & Wojtczak, 2016; Boets *et al.*, 2017). It is therefore essential to emphasise that physiologically relevant levels of acetate and propionate have been reported to directly influence the brain (Perry *et al.*, 2016; Hoyles *et al.*, 2018). Considering the plethora of ways in which they can affect the host, it's not surprising that SCFAs have been implicated in numerous physiological functions such as gastrointestinal functionality, host metabolism, blood-pressure regulation, circadian rhythm and (neuro)immune function (Koh *et al.*, 2016; Erny *et al.*, 2017; Pluznick, 2017; Tahara *et al.*, 2018). Gut microbial-derived SCFAs are also increasingly implicated in emotional processing and behaviour, as butyrate has been shown to ameliorate cognitive impairments in a model of vascular dementia and in mid-adult high-fat-diet-induced obese mice (Liu *et al.*, 2015; Arnoldussen *et al.*, 2017), and propionate is able to reduce anticipatory reward responses to high-energy foods in the human striatum (Byrne *et al.*, 2016). Finally, correlations between SCFA levels and depressive-, anxiety- and stress-related behaviours have been reported after prebiotic administration (Burokas *et al.*, 2017). Considering that these behavioural parameters are negatively affected by chronic (psychosocial) stress (Yang *et al.*, 2015; Ramirez *et al.*, 2017), we hypothesised that SCFA supplementation could ameliorate these psychosocial stress-induced behavioural deficits.

As such, we investigated whether oral SCFA supplementation could ameliorate psychosocial stress-induced behavioural deficits in mice, specifically in sociability, anxiety- and depressive-like behaviours, cognition and stress-responsiveness. In parallel, we assessed changes in the expression of associated genes in discrete brains regions. Finally, we investigated the role of gut permeability

and gut microbiota composition in SCFA-induced amelioration of psychosocial stress-induced behavioural deficits.

2. Materials and methods

Ethical Approval

All experiments were approved by the Animal Experimentation Ethics Committee of University College Cork and conducted in accordance with the European Directive 86/609/EEC and the Recommendation 2007/526/65/EC. The animal experiments conducted also comply with the policy and regulations of The Journal of Physiology (Grundy, 2015). All efforts were made to reduce the number of animals used and minimise animal suffering. Samples sizes were based on previous studies (Burokas *et al.*, 2017).

Animals

This study used male C57Bl/6J mice (n=40; 8 weeks of age on arrival; Harlan, UK; n=10/group). Animals were habituated for four weeks after which single housing began. Food and water were provided *ad libitum* throughout the study. The holding room was under a 12-hour light/dark cycle (lights on at 7:00 am), with a temperature of 21 ± 1 °C and humidity of 55 ± 10%. Bodyweight was monitored twice per week starting at the onset of psychosocial stress.

Experimental timeline and behavioural testing

Oral SCFA treatment commenced one week after the single housing, both of which continued throughout the entire study (**Fig. 1**). After one week of SCFA treatment, animals underwent three weeks of psychosocial stress followed by a behavioural assessment. Behavioural tests were performed in order of least stressful to most stressful to lessen the likelihood of prior behavioural tests influencing subsequent ones. In addition, there was a minimum of 36-hours in between each test. Testing was performed in the following order: 1) Social interaction test; 2) Sucrose preference; 3) 3-Chamber social interaction; 4) Female urine sniffing; 5) Open field; 6) Novel object recognition; 7) Marble burying; 8) Elevated plus maze; 9) Stress-induced hyperthermia; 10) Tail-suspension; 11) Fear conditioning; 12) Hot plate; 13) *in vivo* FITC-dextran intestinal permeability; 14) Forced swim. These tests were followed by a one-week washout period, after which mice were euthanised by decapitation.

Oral short-chain fatty acid administration

A mix of the three principal short-chain fatty acids; sodium acetate (67.5 mM, Sigma-Aldrich, S7545), sodium, sodium propionate (25 mM, Sigma-Aldrich, P1880) and sodium butyrate (40 mM, Sigma-Aldrich, 303410), was administered via drinking water (pH 7.6) as described previously (Smith *et al.*, 2013; Erny *et al.*, 2015). Drinking water was refreshed two times per week.

Psychosocial stress – Social defeat and overcrowding procedures

The social defeat and overcrowding procedure schedule was performed for three weeks as previously described, see **figure 1** (Finger *et al.*, 2011; Burokas *et al.*, 2017). For the social defeat procedure, age-matched male CD1 mice (n=30; Harlan, UK) were tested for aggression on three

individual days. Briefly, CD1 mice were exposed to another CD1 intruder mouse in their homecage until the first attack commenced. The 25 CD1 mice with the shortest attack latencies were used for the social defeat procedure, while the rest was used in the social interaction test. Test mice undergoing psychosocial stress were pseudo-randomly assigned to a different CD1 mouse on each day, counterbalanced by the SCFA group. During the social defeat procedure, stress mice were gently placed in the home cage of the CD1 aggressor and allowed to interact until the first attack of the CD1 mouse took place, which was followed by a defeat posture of the stress mouse. Mice were then separated for two hours by a perforated plexiglass divider allowing for auditory, olfactory and visual, but not physical contact. The divider was subsequently removed, after which another social defeat took place, and test mice were placed in their original home cage. For the overcrowding procedure, stress mice of one group (n = 10) were housed in a standard holding cage for 24 hours. During this period, control mice were left undisturbed.

Social interaction test

The social interaction test was used to assess avoidance of the CD1 aggressors one day following the final psychosocial stressor and was conducted as previously described (Burokas *et al.*, 2017). The test was performed in an open arena (40 × 32 × 24 cm, L × W × H) containing an empty wire mesh cage (9.5 × 7.5 × 7.0 cm) opposed to one side. The test consisted of two 2.5-min trials, with a 1-min intertrial interval, wherein mice were allowed to explore the arena freely. In the first trial ('no target'), the wire mesh cage was left empty, whereas an unfamiliar CD1 aggressor was placed in the wire mesh cage in the second trial. Both mice were returned to their homecage after the test, and the arena was cleaned with 70% ethanol. To reduce potential anxiogenic factors, all mice were habituated 45 minutes before testing, and testing was conducted in red light (5 lux). All trials were videotaped using a ceiling camera and analysed for time spent in the interaction zone using Ethovision version 13 software (Noldus). Social avoidance was calculated by dividing the time spent in the interaction zone in trial 2 by the time spent in the interaction zone in trial 1.

Sucrose preference test

Mice were assessed for hedonic-like behaviour using the sucrose preference test, which was conducted as previously described (Kelly *et al.*, 2016). Mice were presented with two drinking water bottles, one containing regular drinking water and the other one a sucrose solution (2% w/v), for 48 hours. The side on which each drinking water bottle was, was randomised and alternated every 12 hours. Mice were kept in their home cages throughout the test, and the same type of drinking water bottles were used as they had gotten their drinking water in previously. Sucrose preference was calculated using the following formula: (Total Sucrose Intake / Total fluid intake) * 100%.

Three-chamber sociability test

The three-chamber sociability test was used to assess social preference and recognition and was conducted as previously described (Desbonnet *et al.*, 2014). The testing apparatus was a three-chambered, rectangular box. The dividing walls between each chamber (20 × 40 × 22 cm, L × W × H) had small circular openings (5 cm diameter), allowing for access to all chambers. The two outer chambers contained wire cup-like cages (10 cm bottom diameter, 13 cm H), allowing for auditory, olfactory and visual, but not physical contact. The test consisted of 10-minute three phases: 1)

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Habituation, 2) Social preference, 3) Social recognition. In the first phase (Habituation), mice were allowed to explore the entire box with both wire cup-like cages left empty to allow for habituation to the novel environment. In the second phase (Social preference), one wire cup-like cage contained a novel, age-matched, conspecific, male mouse, whereas the other cage contained an object (rubber duckie). In the third phase (Social recognition), the mouse of the previous trial was left in the wire cup-like cage (Familiar mouse), while the object was replaced with a conspecific mouse (Novel mouse). The test mouse was held in the middle chamber while the conspecific mouse and object were placed in the cup wire-like cages. The location of the conspecific mice and object were systemically altered between test mice. The three-chamber test apparatus and wire cup-like cages were cleaned with 10% ethanol after each test mouse and left to dry for a few minutes. To reduce potential anxiogenic factors, all mice were habituated to the testing room 40 minutes before the test, the floor of the testing arena was covered with sawdust and testing was performed under dim light (60 lux). All experiments were videotaped using a ceiling camera and were scored blinded for the time interacted with the wire cup-like cages.

Female urine sniffing test

Mice were assessed for hedonic and reward-seeking behaviour in the female urine sniffing test, which was performed as previously described (Finger *et al.*, 2011). Before this experiment, vaginal smears from age-matched female C57Bl/6 mice (n=12; Harlan, UK) were taken and assessed for the estrous cycle. Urine from female mice in the estrous stage was collected and pooled. Male mice were habituated 45 min before the start of the test to the test room, with a cotton bulb attached to the lid of their housing cage. The test mice were subsequently introduced to a new cotton bulb containing 30 µl sterile water. After a 45 min intertrial-interval, mice were introduced to a new cotton bulb containing 30 µl urine from a female mouse in estrous for 3 min. The experiment was conducted in red light (5 lux). All tests were videotaped using a ceiling camera for 3 minutes and interaction time with the cotton bulbs was scored blinded.

Open field test

Mice were assessed for their locomotor activity and response to a novel environment in the open field test, which was conducted as previously described (Burokas *et al.*, 2017). Mice were placed in an open arena (40 × 32 × 24 cm, L × W × H) and were allowed to explore the arena for 10 minutes. Animals were habituated to the room 1 hour before the test. Testing was performed under dim light (60 lux). Experiments were videotaped using a ceiling camera and were analysed for time spend in the virtual centre zone (defined as 50% away from the edges) and total distance travelled using Ethovision version 13 software (Noldus).

Novel object recognition test

The day after the open field test, mice were allowed to explore two identical objects (acquisition phase), after which one object was replaced on the third day (retention phase). Like the open field test, testing was performed under dim light (60 lux) and lasted 10 minutes. Mice were habituated to the room 1 hour before the test. Experiments were videotaped using a ceiling camera and time of directed contact of the mouse with the objects on day three (retention phase) was scored blinded by

an independent researcher. Object preference was calculated using the following formula: (Novel object exploration time – Familiar object exploration time) / Total exploration time * 100%.

Marble burying test

Mice were tested for repetitive and anxious behaviour with the marble burying test, which was conducted as previously described (Burokas *et al.*, 2017). Animals were individually placed in a novel Plexiglas cage (35 × 28 × 18.5 cm, L × W × H), which was filled with sawdust (5 cm) and had 20 marbles placed on top (5 x 4 rows, equally spread between the walls and individual marbles). After mice had spent 30 minutes in the cage, the number of buried marbles was counted by two researchers and averaged, which was defined as 2/3 of the marble not anymore being visible.

Elevated plus maze

The elevated plus maze test was used to assess anxiety-like behaviour, which was conducted as previously described (Burokas *et al.*, 2017). The elevated plus maze apparatus consisted of a grey cross-shaped maze with two open arms and two closed arms (50 × 5 × 15 cm walls or 1 cm no wall), elevated 1 m from the floor. Mice were allowed to explore the maze for 5 min. Experiments were conducted in red light (5 lux). Mice were habituated to the room 1 hour before the test. Experiments were videotaped using a ceiling camera and videos were scored blinded for time spent in the open arms, which was defined as all paws in the open arm.

Stress-induced hyperthermia test

The stress-induced hyperthermia test was used to assess stress-responsiveness, which was conducted as previously described (Burokas *et al.*, 2017). Body temperature was taken at baseline (T1) and 15 minutes later (T2) by gently inserting a sterile, Vaseline-covered thermometer 2.0 cm into the rectum. The temperature was noted to the nearest 0.1 °C after it stabilised (~10 s). Mice were restrained by scruffing during this procedure which was the stressor. Animals were habituated to the testing room 30 min before the test. The difference between T1 and T2 reflected the stress-induced hyperthermia.

Tail-suspension test

The tail-suspension test was used to assess depressive-like behaviour, which was conducted as previously described (Burokas *et al.*, 2017). Mice were hung by their tail using adhesive tape (2 cm from the tip of the tail) to a 30 cm-elevated grid bar for 6 min. Experiments were videotaped using a numeric tripod-fixed camera and videos were scored blinded by an independent researcher for the time spent immobile.

Fear conditioning

Mice were assessed for fear-associated contextual and cued learning in the fear conditioning test, which was conducted as previously described (Burokas *et al.*, 2017). This test consisted of three phases: 1) Acquisition, 2) Retention, 3) Extinction, each of which was carried out on successive days with a 24-hour interval. The acquisition phase consisted of a 3-minute baseline recording, followed by 6 tone-conditioned stimuli (70 dB, 20 s) and shock pairings (0.6 mA, 2 s) with a 1-min interval. At

the Retention and Acquisition phases, the same experimental procedure was carried out in the absence of the shocks. The fear conditioning apparatus was cleaned with 70% ethanol and left to dry for a few minutes between runs.

Hot plate test

The hot plate test was used to assess somatic pain response, which was conducted as previously described (Tramullas *et al.*, 2012). Animals were gently placed on a hot plate (Stoelting) set at 52 °C. After mice licked their front paw, they were immediately removed from the hot plate and returned to their homecage. Animals were habituated 60 minutes to the room before the test. Experiments were videotaped using a numeric tripod-fixed camera and videos were scored blinded for the time it took the mice to lick either of their front paws.

***In vivo* FITC-dextran intestinal permeability test**

Gastrointestinal permeability was assessed using the FITC-dextran intestinal permeability test, which was conducted as previously described (Scott *et al.*, 2017). Mice were fastened overnight and received an oral gavage of FITC-dextran dissolved in sterile PBS (Sigma, FD4) at 9 a.m. The administered dosage was 600 mg/kg body weight, and the approximate volume administered per mouse was 0.21 ml. Two hours after the FITC-dextran was administered, approximately 90 µl of whole blood was taken by tail-tip. For this procedure, the end of the tail was held with two fingers without restraining the mouse. A 2-4 mm long diagonal incision was made at the end of the tail using a single edge razor blade, and blood was collected in an EDTA-containing capillary. Blood was then transferred to a tube, centrifuged for 15 min at 3,500 g at 4°C, and plasma was collected and stored at −80 °C for later analysis. Plasma FITC-dextran concentrations were assessed with a multi-mode plate reader (Synergy HT, BioTek Instruments) with an excitation of 490 nm and emission of 520 nm.

Forced-swim test

The forced swim test was used to assess depressive-like behaviour, which was conducted as previously described (Cryan & Mombereau, 2004). Mice were individually placed in a transparent glass cylinder (24 × 21 cm diameter) containing 15-cm-depth water (23-25 °C) for 6 minutes. Mice were gently dried after the test, and water was discarded and renewed after each animal. Experiments were videotaped using a ceiling camera and videos were scored blinded by an independent researcher for immobility time in the last 4 min of the test.

Plasma sampling for Corticosterone

Plasma from each animal was sampled by tail-tip five minutes before the forced swim test and repeatedly after the test in 30-min intervals up to 120 minutes. For the tail-tip, the end of the tail was held with two fingers without restraining the mouse. A 2-4 mm long diagonal incision was made at the end of the tail using a single edge razor blade. Approximately 40 µl of whole blood was taken per time point using an EDTA-containing capillary, deposited in an Eppendorf and centrifuged for 15 min at 10,000 g at 4°C. Plasma was collected and stored at −80 °C for later analysis.

Tissue collection

Animals were sacrificed by decapitation in a random fashion regarding testing groups between 9.00 a.m. and 3.00 p.m. Trunk blood was collected in EDTA-containing tubes and centrifuged for 15 min at 10,000 g at 4°C. Plasma was collected and stored at −80 °C for later analysis. The caecum, spleen, adrenals and thymus were removed, weighed, snap-frozen on dry ice and stored at −80 °C. Proximal colon tissue was either immediately frozen on dry ice and stored at −80 °C for later noradrenalin quantification by HPLC, or stored in RNAlater (Sigma, R0901) for 48 hours at 4°C, after which the RNAlater was removed, and tissues were stored at −80 °C for later gene expression analysis. The brain was excised and gross-dissected as described previously (Schellekens *et al.*, 2012). Brain tissues were then stored in RNAlater and processed in the same manner as colon tissues.

Plasma corticosterone and lipopolysaccharide-binding protein quantification

Corticosterone quantification of plasma samples (15 µl) obtained in the forced swim test was performed using a corticosterone ELISA (Enzo Life Sciences, ADI-901-097), and was carried out according to the manufacturer's guidelines. Lipopolysaccharide-binding protein (LBP) was quantified in plasma (10 µl) obtained when animals were sacrificed using an ELISA (Enzo Life Sciences, ALX-850-305), and was also performed according to the manufacturers' instructions. A multi-mode plate reader (Synergy HT, BioTek Instruments) was used to measure light absorbance in both assays.

Gene expression analysis

Total mRNA was isolated using the mirVana™ miRNA Isolation Kit (ThermoFisher Scientific, AM1560), after which samples were DNase treated (ThermoFisher Scientific, AM1907). RNA was determined using the NanoDrop™ spectrophotometer (ThermoFisher Scientific) and synthesised into cDNA using the High Capacity Reverse Transcription cDNA kit (ThermoFisher Scientific, 4368814). Gene expression analysis was finally carried out by quantitative real-time polymerase chain reaction (qRT-PCR) using various probes designed by Applied Biosystems and Integrated DNA Technologies (**Table 1**). The TaqMan™ Universal Master Mix II, no UNG (ThermoFisher Scientific, 4440040) was used. Singleplex amplification reactions were performed in duplicates in 384-well plates on the LightCycler®480 system. Data were normalised using Actb as endogenous control and transformed using the $2^{-\Delta\Delta CT}$ method. All procedures were carried out according to the manufacturers' instructions.

Table 1. qRT-PCR probes used in this study

Gene symbol	Common gene name	Probe ID	Supplier
Actb	β-actin	4352341E	Applied Biosystems
BDNF IV	Brain-derived neurotrophic factor, Exon IV	Mm.pt.58.13575048	Integrated DNA Technologies
Cldn1	Claudin-1	Mm.PT.58.6163880	Integrated DNA Technologies
Crh	Corticotropin-releasing hormone	Mm01293920_s1	Applied Biosystems
Crhr1	Corticotropin-releasing hormone receptor 1	Mm.PT.58.13604366	Integrated DNA Technologies
Crhr2	Corticotropin-releasing hormone receptor 2	Mm.PT.58.12499462	Integrated DNA Technologies
Drd1a	Dopamine receptor D1a	Mm01353211_m1	Applied Biosystems
Drd2	Dopamine receptor D2	Mm00438545_m1	Applied Biosystems
FFAR2/GPR43	Free fatty acid receptor 2	Mm.02620654_s1	Applied Biosystems
FFAR3/GPR41	Free fatty acid receptor 3	Mm.02621638_s1	Applied Biosystems
GR/Nr3c1	Glucocorticoid receptor	Mm.PT.58.42952901	Integrated DNA Technologies
Tjp1	Tight junction protein ZO-1	Mm.PT.58.12952721	Integrated DNA Technologies
MR/Nr3c2	Mineralocorticoid receptor	Mm.PT.58.30752774	Integrated DNA Technologies
Muc2	Mucin 2	Mm.PT.58.29496069	Integrated DNA Technologies
Ocln	Occludin	Mm.PT.58.30118962	Integrated DNA Technologies
TrkB/NTRK2	Tropomyosin receptor kinase B	Mm.PT.58.11070732	Integrated DNA Technologies

DNA extraction from caecal contents and Illumina MiSeq sequencing

Total DNA was extracted from thawed caecal contents using the QIAamp Fast DNA Stool Mini Kit (Qiagen, 51604), coupled with an initial bead-beating step (Fouhy *et al.*, 2015). Briefly, a 0.2 g aliquot of caecal sample was added to 0.4 g of zirconia beads together with 1 ml InhibitEx buffer supplied in the kit. Samples were subsequently homogenised using the Biospec Minibeadbeater for 3 minutes.

Removal of RNA and protein, as well as purification of the DNA, was completed according to the manufacturer's instructions. Finally, DNA was stored at -20 °C until further analysis.

The 16S metagenomic sequencing library protocol (Illumina) was used to amplify the V3-V4 variable region of the 16S rRNA gene from the DNA extracts. Briefly, DNA was amplified with V3-V4 region-specific to the 16S rRNA gene which also incorporates the Illumina overhang adaptor (Forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Each PCR reaction contained DNA template, 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM), 12.5 µl 2X Kapa HiFi Hotstart ready mix (Anachem) and PCR grade water to a final volume of 25 µl. PCR amplification was carried out as follows: initial denaturation at 95 ºC for 3 min; 25 cycles of 95 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 30 s; 72 ºC for 5 min and held at 4 °C. PCR products were then visualised using gel electrophoresis (1X TAE buffer, 1.5% agarose, 100V) and cleaned using AMPure XP magnetic bead-based purification (Labplan). A second PCR reaction was performed on the purified DNA (5 µl) to index each of the samples, allowing samples to be pooled for sequencing on the one flow cell and finally demultiplexed for analysis. Two indexing primers (Illumina Nextera XT) were used per sample. Each PCR reaction contained 5 µl index 1 primer (N7xx), 5 µl index 2 primer (S5xx), 25 µl 2x Kapa HiFi Hot Start Ready mix and 10 µl PCR grade water. PCR was mostly carried out as described above, although only 8 amplification cycles were completed instead of 25. PCR products were visualised using gel electrophoresis and subsequently cleaned (as described above). Samples were quantified using the Qubit (Bio-Sciences) along with the high sensitivity DNA quantification assay kit (Bio-Sciences), and samples were pooled in an equimolar fashion. The pooled samples were run on the Agilent Bioanalyser for quality analysis. The sample pool was prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform (Clinical Microbiomics, Denmark), using a 2 x 250 cycle kit, following standard Illumina sequencing protocols.

Bioinformatic sequence analysis was performed as described previously (Murphy et al., 2017). Briefly, paired-end sequences were assembled using FLASH (Magoc & Salzberg, 2011), and analyzed using QIIME v1.8.0 (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010). Sequences were then quality checked, and the remaining sequences were clustered into operational taxonomic units using USEARCH (v7-64bit) (Edgar, 2010). Taxonomic ranks were assigned with a BLAST search against the SILVA SSURef database release 116 (Quast et al., 2013). Alpha and beta diversities were generated in QIIME and calculated based on weighted Unifrac distance matrices (Lozupone et al., 2011). Principal coordinate analysis (PCoA) plots were made using EMPeror v0.9.3-dev (Vazquez-Baeza et al., 2013). The relative abundance of bacterial taxa was expressed as a percentage of identified sequences.

Faecal SCFA quantification

Faecal pellets were collected six weeks post psychosocial stress. Faecal water was prepared by homogenising the faecal samples (approx. 200mg) with 800 µl of 0.003M HCl and 3 zirconia beads for 10 minutes using a vortex mixer. The use of HCl results in the acidification of samples ensuring protonation of SCFAs before analysis which prevents the occurrence of split peaks in chromatograms (Zhao *et al.*, 2006). Samples were centrifuged at 15,000 g for 5 minutes, after which supernatants were syringe filtered with 0.22uM filters (Corning) and duplicate aliquots of 270 µL of filtrate were

mixed with 30 μL of 10 mM internal standard. Samples were subsequently homogenised briefly and were centrifuged at 15,000 g for 3 minutes. The supernatant was transferred to 250 μL inserts (Agilent) placed in amber glass 2mL GC vials (Agilent) sealed with silicone/PTFE screw caps (Agilent).

Samples were analysed by gas chromatography flame ionisation detection (GC-FID) using a Varian 3800 GC system, fitted with a Zebron ZB-FFAP column (30 mL x 0.3 2mm ID x 0.25 μm df; Phenomenex) and a flame ionisation detector with a CP-8400 auto-sampler. Helium was employed as the carrier gas at an initial flow rate of 1.3 mL/min. The initial oven temperature was 50 °C and was maintained for 30 seconds, then raised to 140 °C at 10 °C/min and held for 30 seconds. The temperature was finally increased to 240 °C at 20 °C/min and held for 5 minutes. The temperatures of the detector and the injection port were 300 °C and 240 °C respectively. A split-less injection of 0.2 μL was carried out for each sample or standard using a 10 μL syringe (Agilent) installed to a CP-8400 auto-sampler (Varian). Peak integration was carried out using Varian Star Chromatography Workstation version 6.0 software. Vials containing 1800 μL of water were run between each sample duplicates as blanks to check for any potential carryover. Standards were included in each run to maintain the calibration.

Statistical analysis

All data were assessed for normality using the Shapiro-Wilk test and Levene's test for equality of variances. Normally distributed data were analysed using a two-way ANOVA, followed by Fishers' least significant difference (LSD) post hoc test. Non-parametric datasets were analysed using the Kruskal-Wallis test, followed by the Mann-Whitney U test. Statistical analysis was performed using SPSS software version 24 (IBM Corp). Data are expressed as mean ± SEM. A p-value < 0.05 was deemed significant.

3. Results

Social Behaviour

Three weeks of psychosocial stress induced a trend towards decreased social interaction with a CD1 mouse used in the social defeat sessions (**Fig. 2A**), independently of SCFA treatment ($X^2(1) = 2.875$, $p = 0.090$). Mice were later assessed for their preference between a conspecific mouse or an object in the 3-chamber sociability test. All groups exhibited a significant preference towards the conspecific mouse compared to the object (**Fig. 2B**). Changing the object to a novel conspecific mouse to test for social recognition, resulted in a significant preference towards interacting with the novel conspecific mouse for all test groups (**Fig. 2C**).

Anxiety- and Depressive-like Behaviour

Assessment of anxiety-like behaviour using the open field test showed that SCFA supplementation increased the time spent in the centre of the open field ($X^2(3) = 15.622$, $p = 0.001$) (**Fig. 3A**). This effect was abolished when mice underwent psychosocial stress. These effects were independent of locomotor activity, as no differences in the total distance travelled were found in the open field test (**Fig. 3B**). Nonetheless, no effects were found in other tests assessing anxiety-like behaviours like the marble burying test (**Table 2**) and the elevated plus maze (**Table 2**). Assessment of depressive-like behaviour using the tail-suspension test did not reveal any significant differences (**Table 2**), even

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though mice supplemented with SCFAs exhibited a decreased immobility time in the forced swim test ($F(1, 38) = 5.548$, $p = 0.024$) (**Fig. 3C**). Surprisingly, no significant stress effects, independently of SCFAs, were found in any of the tests assessing anxiety- and depressive-like behaviour.

Table 2. Behavioural Measurements. Tests for anxiety-like behaviour were the marble burying test, which was performed two weeks post-stress and the elevated plus maze which was done three weeks post-stress. Depression was assessed using the tail-suspension test three weeks post-stress. Reward-seeking behaviour was tested using the sucrose preference test four days post stress. Cognition was determined using the novel object recognition test two weeks post stress. Somatic pain response was assessed with the hot plate test four weeks post stress. Data from the sucrose preference test were non-parametrically distributed and analysed using the Kruskal-Wallis test, followed by the Mann-Whitney test. Data from the hot plate test were normally distributed and analysed using a two-way ANOVA. Significant differences are depicted as: *$p < 0.05$; Control/Control compared to Control/Stress, ##$p < 0.01$; Control/Stress compared to SCFA/Stress. All data are expressed as mean ± SEM (n = 8-10).

Factor	Control/Control	SCFA/Control	Control/Stress	SCFA/Stress
Marble Burying Test				
Marbles buried (#)	13.7 ± 1.5	12.6 ± 1.8	11.0 ± 1.6	16.1 ± 0.5
Elevated Plus Maze				
Time spend in open arm (s)	7.6 ± 2.8	5.9 ± 2.3	9.1 ± 2.6	12.6 ± 3.3
Entries into open arm (#)	1.6 ± 0.5	1.6 ± 0.5	2.3 ± 0.4	2.2 ± 0.5
Tail Suspension Test				
Immobility time (s)	127.0 ± 18.5	169.6 ± 4.0	144.8 ± 8.5	131.4 ± 12.2
Sucrose Preference Test				
Sucrose preference (%)	81.5 ± 1.6	73.4 ± 3.8	86.4 ± 0.6*	80.6 ± 2.1##
Water consumption (mL)	3.53 ± 0.14	4.10 ± 0.39	3.28 ± 0.07	3.90 ± 0.37
Sucrose consumption (mL)	16.6 ± 1.6	12.4 ± 1.4	21.4 ± 1.5	17.0 ± 1.6
Novel Object Recognition				
Object preference (%)	65.2 ± 3.5	58.6 ± 4.8	63.3 ± 5.6	65.7 ± 3.7
Hot Plate Test				
Time until frontpaw lick (s)	17.6 ± 1.2	17.7 ± 0.7	16.0 ± 0.6	15.6 ± 0.9

Hedonic and Reward-seeking Behaviour

Assessment of hedonic and reward-seeking behaviour using the sucrose preference test revealed that stressed mice had an increased sucrose preference (**Table 2**), which was not seen following SCFA treatment ($X^2(3) = 12.839$, $p = 0.005$). Conversely, in the female urine sniffing test, stressed mice spent less time interacting with the cotton bulb containing the urine of a female mouse in estrus stage (**Fig. 4A**), which was not present when mice additionally received SCFAs ($X^2(3) = 12.785$, $p = 0.005$). We subsequently pursued potentially underlying mechanisms by investigating the expression of genes associated with reward-signalling in the striatum, part of the mesolimbic reward pathway (**Fig. 4B**). SCFA treatment increased dopamine receptor D1a expression (DRD1a) independent of psychosocial stress ($F(1, 36) = 13.416$, $p = 0.024$), whereas dopamine receptor D2 (DRD2) gene expression remained unaffected. As brain-derived neurotrophic factor (BDNF) has been shown to essential for dopaminergic signalling during psychosocial stress (Berton *et al.*, 2006), we subsequently analysed the expression of BDNF (exon IV) and its receptor tropomyosin receptor kinase B (TrkB). Although no significant alterations were found in BDNF expression, the combination of SCFA treatment and stress decreased TrkB expression ($F(1, 37) = 4.264$, $p = 0.047$).

Memory and Learning

Neither stress nor SCFA administration affected the discrimination index for memory in the novel object recognition test (**Table 2**). In addition, no effect of SCFAs or stress on the acquisition, retention and extinction in the fear conditioning test were found (**Fig. 5**).

Stress Responsiveness and HPA-axis reactivity

Assessment of stress responsiveness and HPA-axis reactivity in the stress-induced hyperthermia test revealed a potentiated increase in body temperature after an acute stressor in psychosocially stressed mice (**Fig. 6A**), which was restored by SCFAs ($X^2(3) = 8.676$, $p = 0.034$). Additionally, SCFAs ameliorated the stress-induced corticosterone potentiation after an acute stressor observed in the psychosocially stressed group ($X^2(3) = 11.058$, $p = 0.011$) (**Fig. 6B, C**). To further understand how SCFAs affect HPA-axis reactivity, we investigated the expression of hypothalamic genes involved in stress signalling (**Fig. 6D**). SCFAs induced a trend towards decreased corticotropin-releasing hormone (Crh) expression ($p = 0.122$), which was significantly upregulated by psychosocial stress ($X^2(3) = 9.530$, $p = 0.023$). In addition, SCFAs downregulated mineralocorticoid receptor (MR) expression, which was reversed by stress ($X^2(3) = 14.805$, $p = 0.002$). Finally, no differences were found in glucocorticoid receptor (GR) expression. As glucocorticoid signalling in the hippocampus has previously been shown to affect behaviour (Kim *et al.*, 2015), we investigated the gene expression of various glucocorticoid receptors in the hippocampus (**Fig. 6E**). SCFAs induced a trend towards decreased corticotropin-releasing hormone receptor 1 (Crhr1) expression, which was upregulated when mice also underwent stress ($X^2(3) = 7.426$, $p = 0.059$). In accordance with the hypothalamic gene expression analysis, SCFAs downregulated MR expression, whereas stress increased MR expression ($X^2(3) = 19.185$, $p < 0.001$). Finally, no differences were found in hippocampal GR expression.

Intestinal Permeability

In the intestinal permeability test, we found increased levels of plasma FITC-dextran in psychosocially stressed mice (**Fig. 7A**), which wasn't present when mice were also supplemented with SCFAs ($X^2(3) = 10.024$, $p = 0.018$). Surprisingly, further analysis of intestinal permeability by plasma lipopolysaccharide-binding protein (LBP) quantification, a marker for systemic exposure to the intestinal bacterial product lipopolysaccharide, didn't reveal any difference between groups (**Table 3**). As intestinal permeability is largely dependent on proper functioning of tight junctions (Zihni *et al.*, 2016), we investigated gene expression of tight junction proteins in the colon (**Fig. 7B**). Here we found increased expression of claudin-1 (Cldn1) and tight junction protein 1 (Tjp1) in stressed mice, which was ameliorated by SCFAs ($X^2(3) = 15.311$, $p = 0.002$ and $X^2(3) = 10.927$, $p = 0.012$, respectively). No significant differences were found in occludin (Ocln) expression. We further hypothesised that mucus layer thickness could explain some of the observed findings, as this layer is impermeable to luminal bacteria (Johansson *et al.*, 2011). And similar to the observed plasma LBP levels, no changes were found in mucin 2 (Muc2) expression, the primary marker for colonic mucus thickness. Finally, as stress signalling has been shown to induce intestinal permeability (Rodino-Janeiro *et al.*, 2015), we investigated colonic gene expression markers involved in glucocorticoid signalling (**Fig. 7C**). Similar to the hippocampus, SCFAs decreased the expression of Crhr1, which was upregulated by stress ($X^2(3) = 11.003$, $p = 0.012$). And surprisingly, both stress and SCFAs decreased Crhr2 expression ($X^2(3) = 10.280$, $p = 0.016$). Stress also increased MR expression, whereas SCFAs administration resulted in a downregulation ($X^2(3) = 20.076$, $p < 0.001$). No differences were found in GR expression.

Table 3. Physiological Measurements. Lipopolysaccharide-binding protein (LBP) was quantified from plasma obtained when animals were sacrificed. Body weights and drinking water consumed were assessed throughout the study and normalised to the starting body weight to calculate body weight change (Δ Bodyweight). Organs were collected at the end of the study and weighted. Thymus, adrenals, spleen and caecum weights were normalised to body weight, whereas the colon is expressed in total colon length. Faecal pellets were collected six weeks post psychosocial stress and analysed for SCFA and BCFA levels. Colonic gene expression analysis for SCFA GPCRs was carried out for free fatty acid receptor 2 and 3 (FFAR2 and FFAR3 respectively). Body weight data and gene expression data were non-parametrically distributed and analysed using the Kruskal-Wallis test, followed by the Mann-Whitney test. Data from SCFA levels were normally distributed and analysed using a two-way ANOVA, followed by an LSD post hoc test. Significant differences are depicted as: *$p < 0.05$ and **$p < 0.01$; Control/Control compared to Control/Stress, $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$; SCFA/Control compared to SCFA/Stress. Data are expressed as mean ± SEM (n = 8-10).

Factor	Control/Control	SCFA/Control	Control/Stress	SCFA/Stress
Plasma Lipopolysaccharide-binding protein				
LBP (µg/ml)	7.6 ± 0.3	8.0 ± 0.2	8.5 ± 0.2	7.8 ± 0.3
Body Weight Change				
Day 3 of stress (Δg)	0.24 ± 0.13	0.15 ± 0.05	-0.54 ± 0.20**	-0.48 ± 0.21$^{\$}$
Day 9 of stress (Δg)	0.41 ± 0.16	0.39 ± 0.11	-0.25 ± 0.17*	0.18 ± 0.25
Day 16 of stress (Δg)	0.21 ± 0.25	0.28 ± 0.16	1.82 ± 0.28**	1.85 ± 0.31$^{\$\$}$
6 weeks post-stress (Δg)	0.39 ± 0.42	0.90 ± 0.17	1.63 ± 0.30*	2.26 ± 0.27$^{\$\$}$
End of study (Δg)	1.48 ± 0.29	1.44 ± 0.25	1.91 ± 0.40	2.61 ± 0.29
Daily water intake at end of the study				
Drinking water consumed (mL)	5.4 ± 0.2	4.8 ± 0.4	5.0 ± 0.2	4.7 ± 0.3
Organ Measurements				
Left adrenal (mg/g BW)	0.052 ± 0.006	0.060 ± 0.006	0.067 ± 0.006	0.066 ± 0.005
Right adrenal (mg/g BW)	0.037 ± 0.006	0.047 ± 0.006	0.041 ± 0.006	0.043 ± 0.007
Spleen (mg/g BW)	2.48 ± 0.09	2.44 ± 0.05	2.60 ± 0.08	2.60 ± 0.07
Thymus (mg/g BW)	1.52 ± 0.08	1.64 ± 0.05	1.61 ± 0.08	1.67 ± 0.06
Caecum (mg/g BW)	17.2 ± 0.5	16.7 ± 0.7	17.2 ± 1.0	15.9 ± 0.4
Colon (cm)	7.3 ± 0.2	7.5 ± 0.2	7.3 ± 0.18	7.7 ± 0.3
Faecal SCFAs and BCFAs				

Acetate (µmol/g wet mass)	32.2 ± 5.6	35.0 ± 5.7	64.8 ± 11.4**	54.1 ± 9.0
Propionate (µmol/g wet mass)	2.5 ± 0.3	3.2 ± 0.4	4.4 ± 0.8	4.0 ± 0.6
Butyrate (µmol/g wet mass)	2.0 ± 0.4	1.7 ± 0.2	2.3 ± 0.6	1.7 ± 0.2
Valerate (µmol/g wet mass)	0.85 ± 0.17	0.91 ± 0.21	1.00 ± 0.18	0.84 ± 0.14
Total SCFAs (µmol/g wet mass)	38.3 ± 5.7	42.0 ± 5.8	74.0 ± 12.6**	62.2 ± 8.8
Isobutyrate (µmol/g wet mass)	0.53 ± 0.14	0.49 ± 0.10	0.85 ± 0.26	0.95 ± 0.19
Isovalerate (µmol/g wet mass)	0.52 ± 0.09	0.52 ± 0.07	0.58 ± 0.07	0.62 ± 0.08
Total BCFAs (µmol/g wet mass)	1.05 ± 0.18	0.99 ± 0.09	1.43 ± 0.29	1.57 ± 0.24
SCFA GPCR expression				
FFAR2 (fold change)	1.00 ± 0.17	0.89 ± 0.10	1.79 ± 0.23*	1.89 ± 0.30[$]
FFAR3 (fold change)	1.00 ± 0.09	0.92 ± 0.04	1.14 ± 0.14	1.36 ± 0.12[$]

Somatic Pain Response

Mice were assessed for their response to a noxious thermal stimulus using the hot plate test 4-weeks post-stress (**Table 2**). Animals that previously underwent psychosocial stress showed a slight, but significant, increase in somatic response ($F_{(1, 36)}$ = 4.677, p = 0.038) as measured by the time until mice licked their front paw. Further post hoc test did not reveal any differences between groups.

Body Weight Change

We sought to further understand the underlying causes of the behavioural phenotypes seen in the previous analyses. Considering that chronic psychosocial stress has previously been shown to affect host metabolism and body weight (Finger *et al.*, 2011; Tramullas *et al.*, 2012), and the crucial role of gut microbial-derived SCFAs in host energy metabolism (Byrne *et al.*, 2015), we hypothesized that SCFAs might ameliorate stress-induced alterations in body weight, which was monitored throughout the study (**Table 3**). Interestingly, psychosocial stress significantly decreased body weight in the first two weeks of stress (day 3; $X^2(3)$ = 13.601, p = 0.004, to day 9; $X^2(3)$ = 8.854, p = 0.031). This decrease in body weight subsequently reversed, and a significant increase in body weight was observed in week 3 (day 16; $X^2(3)$ = 22.537, p < 0.001). The increase in body weight persisted throughout the rest study (6 weeks post stress; $X^2(3)$ = 14.991, p = 0.002), even though the effect size seemed to lessen over time and no significant differences were found at the endpoint of the study. In accordance with this, no differences were observed in organ measurements like the thymus, adrenal, spleen and caecum weight, as well as colon length (**Table 3**) Overall, SCFA treatment did not affect any stress-induced changes in body weight, nor organ weights.

Short-Chain Fatty Acid Levels and GPCR Signaling

As an increased body weight is associated with elevated SCFA levels (Turnbaugh *et al.*, 2006; Schwiertz *et al.*, 2010; Fernandes *et al.*, 2014; Rahat-Rozenbloom *et al.*, 2014), we investigated whether the stress-induced body weight gain was also increased faecal SCFA levels (**Table 3**). Stress increased total SCFAs (F(1, 37) = 9.946, p = 0.003), which was likely due to an increase in acetate (F(1, 37) = 9.470, p = 0.004), but not propionate, butyrate and valerate levels, all of which were unaffected by SCFA administration. Notable, branched-chain fatty acids also remained unaffected. An increased bodyweight, as seen in high-fat-diet-induced obese mice, has been shown to result in an elevated colonic gene expression of the SCFA GPCRs free fatty acid receptor 2 and 3 (FFAR2 and FFAR3 respectively) (Lu *et al.*, 2016), indicating increased SCFA signalling. Similar to the faecal SCFA levels, stress increased the expression of FFAR2 (X^2(3) = 11.610, p = 0.009), whereas FFAR3 was only significantly upregulated in mice receiving both stress and SCFAs (X^2(3) = 7.986, p = 0.046) (**Table 3**).

Gut Microbiota Composition

16S sequencing revealed no apparent changes in caecal bacterial species diversity between groups (**Fig. 8A**). In addition, principal coordinate analysis did not show any clear separation of microbiota composition (**Fig. 8B**). Stress, as well as SCFA treatment, did induce some minor changes in caecal microbiota composition on the family and genus level (**Fig. 9A-B**). Most of the differences in microbiota composition detected were induced by the combination of SCFAs and stress (**Fig. 9C**).

4. **Discussion**

Microbial metabolites are receiving increased attention for their ability to modify brain and behaviour (Shultz *et al.*, 2015; Sampson *et al.*, 2016; Gronier *et al.*, 2018). In the present study, we found that psychosocial stress affected reward-seeking behaviour and increased both stress-responsiveness *in vivo* intestinal permeability, all of which were ameliorated by SCFA treatment. SCFA supplementation also induced a decrease in anxiety-like behaviour in the open field test and decreased depressive-like behaviour in the forced swim test, both of which were absent following stress. Similarly, we found that the SCFA group had a decreased gene expression of receptors involved with stress-signalling in the hypothalamus, hippocampus and colon.

The goal of these experiments was to characterise the enduring effects of chronic psychosocial stress on behavioural, physiological and microbiome parameters in the mouse and to assess if supplementation with SCFAs could counter such effects. In line with previous reports using a continuous stress exposure model, we report psychosocial stress-induced anhedonia in the female urine sniffing test, increased stress-responsiveness and increased in *in vivo* intestinal permeability (Burokas *et al.*, 2017). Interestingly, the previously reported effects of chronic stress on cognition, anxiety- and depressive-like behaviour, as well as alterations in gut microbiota composition, failed to endure in our model (Burokas *et al.*, 2017). Indeed, it is well known that many stress-induced physiological and behavioural deficits dissipate over time (Fanous *et al.*, 2010; Ver Hoeve *et al.*, 2013; Wohleb *et al.*, 2014). For instance, 24 days after the last psychosocial stressor, monocyte infiltration into the brain, microglia inflammation and anxiety-like behaviour revert back to baseline (Wohleb *et al.*, 2014). In particular, microglia inflammation has been implicated in cognition, depression- and anxiety-like behaviour (Wohleb & Delpech, 2016), which likely explains the absence

of differences in these behaviours. The absence of long-term stress-induced differences gut microbiota might also be explained by dissipation, suggesting that the caecal microbiota composition does not contribute to the enduring effects of psychosocial stress, even though more research investigating the microbiota at different timepoints post-stress, as well as gut microbiota-derived metabolites, is warranted to validate this hypothesis.

Stress induced a long-term increase in body weight, which is in line with the fact that stress exposure can lead to overeating and excessive body weight (Finger *et al.*, 2012; Vancampfort *et al.*, 2015; Razzoli & Bartolomucci, 2016; Razzoli *et al.*, 2017). Stress also increased faecal acetate and total SCFA levels, as well as colonic FFAR2 and FFAR3 gene expression. This is perhaps not surprising as increased body weight in obesity is associated with increased SCFA levels (Turnbaugh *et al.*, 2006; Schwiertz *et al.*, 2010; Fernandes *et al.*, 2014; Rahat-Rozenbloom *et al.*, 2014), as well as increased colonic FFAR2 and FFAR3 gene expression (Lu *et al.*, 2016). Surprisingly, oral SCFA supplementation did not ameliorate stress-induced changes in body weight and SCFA levels, even though SCFA supplementation has previously been shown to reduce body weight in rodent models of obesity (Lin *et al.*, 2012; Vinolo *et al.*, 2012; Lu *et al.*, 2016). Interestingly, the discrepancy between the fact that obesity is generally associated with increased SCFA levels, whereas SCFA supplementation ameliorates symptoms of obesity, has been left unexplained (Byrne *et al.*, 2015; van de Wouw *et al.*, 2017). As such, our data demonstrated a novel interaction between chronic stress, host metabolism and SCFAs, even though more research into the mechanisms involved is warranted in order to understand the exact role of chronic stress in SCFA-host metabolism interactions. Finally, oral SCFA administration itself did not affect faecal SCFA levels, which is probably explained by the rapid uptake of SCFAs (Pouteau *et al.*, 2003; van der Beek *et al.*, 2015).

We found that stress induced anhedonia in the female urine sniffing test, which was not present in combination with SCFA supplementation. However, the increased sucrose preference observed following stress is at odds with studies showing that chronic stress reduces reward-seeking behaviour in the sucrose preference test (Iniguez *et al.*, 2014; Veeraiah *et al.*, 2014), even though this is not consistently reported (Van Bokhoven *et al.*, 2011; O'Leary *et al.*, 2014). This may be explained by the fact that it is a stress-withdrawal-induced effect we are modelling. Together these data support the concept of SCFAs affecting reward-processes. In this regard, a human brain imaging study showed that colonic propionate reduces anticipatory reward responses to high-energy foods in the human striatum (Byrne *et al.*, 2016). Such findings indicate that gut microbial-derived SCFAs could provide target neural circuits underpinning hedonic food intake, in addition to being implicated in the regulation of homeostatic food intake (van de Wouw *et al.*, 2017).

As previously reported, psychosocial stress induced long-term increased responsiveness to acute stress challenges (Ver Hoeve *et al.*, 2013). Here, we show that acute stress-induced hyperthermia and corticosterone levels in chronically stressed mice are ameliorated by SCFA supplementation. Similar changes were found in colonic MR gene expression, even though the stress-induced increased gene expression in hippocampal MR remained unaffected. In addition, no differences in GR gene expression was found in any of the investigated tissues which, similar to the absence of chronic stress-induced anxiety- and depressive-like behaviour and impaired cognition, could have dissipated over time. A more robust effect was observed in the group solely receiving SCFA administration, where there was a consistent decrease in Crhr1, Crhr2 and MR gene expression

across all investigated tissues. Overall, these results reveal that SCFAs can downregulate stress-signalling and HPA-axis responsiveness, a crucial pathway in microbiota-gut-brain axis communication (Wiley *et al.*, 2017). The sympathetic nervous system could play a key role in the mechanisms involved, as FFAR2 is expressed on autonomic and somatic sensory ganglia and activation hereof results in sympathetic nervous system activation (Kimura *et al.*, 2011; Nohr *et al.*, 2015), even though more research is warranted to validate this hypothesis. This has particular implications in pathophysiology of chronic anxiety disorders (e.g. PTSD), where a recurrent stressor after the long-term cessation of chronic stress, rapidly results in behavioural and neuroinflammatory sequelae similar to the ones observed immediately after the cessation of chronic stress (McKim *et al.*, 2016), indicating that SCFA-targeted therapies might be a suitable dietary target for such disorders.

Psychosocial stress induced an enduring increase in *in vivo* intestinal permeability five weeks after the last stressor, indicating long-term stress-induced changes in gastrointestinal barrier function. This was reversed by SCFA treatment, which is not surprising as SCFAs have previously been shown to restore intestinal permeability (Kelly *et al.*, 2015; Tong *et al.*, 2016; Simeoli *et al.*, 2017). Surprisingly, stress induced an increased gene expression of tight junction proteins Cldn1 and Tjp1, even though increased intestinal permeability doesn't necessarily correlate to decreased tight junction gene expression (Golubeva *et al.*, 2017). In addition, no differences were seen in plasma LBP levels, an indirect marker of systemic exposure to the intestinal bacterial product lipopolysaccharide. This might be attributable to the fact that Muc2 gene expression remained unaffected, indicating that the mucus barrier was still functional and impermeable to bacteria (Johansson *et al.*, 2011). It is also important to note that gene expression does not necessarily correlate with protein levels. Another explanation for the discrepancy between *in vivo* intestinal permeability and LBP levels could be handling stress induced by the administration of FITC-Dextran in the *in vivo* intestinal permeability test, as stress-responsiveness was increased in the stress group, and as stress signalling has been shown to induce intestinal permeability (Rodino-Janeiro *et al.*, 2015). These findings have particular interest for chronic stress-induced gastrointestinal dysfunction, as chronic stress has previously been shown to promote colitis in mice (Gao *et al.*, 2018), and chronic stress is associated with an increased risk of functional gastrointestinal disorders in humans, as well as increased rates of relapse and surgery for inflammatory bowel diseases (Stam *et al.*, 1997; Klooker *et al.*, 2009; Bernstein, 2017). Even though it must be noted that heightened right colonic fermentation has been shown to be a potential pathophysiological factor in irritable bowel syndrome (Farmer *et al.*, 2014; Ringel-Kulka *et al.*, 2015).

Interestingly, although SCFAs decreased anxiety-like behaviour in the open field test and depressive-like behaviour in the forced swim test in control mice, the presence of a stressful history prevented such effects from being manifested. In a similar vein, we have shown that dietary manipulations with n-3 polyunsaturated fatty acids, which alter the composition of the gut microbiota (Pusceddu *et al.*, 2015a), have behavioural effects in control, but not early-life stressed animals (Pusceddu *et al.*, 2015b).

It is important to note that SCFAs were administered as a sodium-salt and controls were sodium matched, which could result in yet unstudied changes in behaviour and physiology. This could have contributed to the unexpected findings in the sucrose preference test, even though it is important to

note that drinking water consumption was the same between groups. As such, it has to be pointed out that a recent study showed that a high salt diet increases the susceptibility to colitis and alters gut microbiota composition (Miranda *et al.*, 2018).

Taken together, these results present novel insights into how the gut microbial-derived metabolites SCFAs influences brain homeostasis and behaviour, as well as host metabolism. Finally, as SCFAs are one of the main products of bacterial fermentation of dietary fibres, this study supports the importance of potential novel nutrition-based therapeutic targets for stress-related disorders.

5. Additional information

Competing interests

The authors have no competing interests to declare.

Author contributions

MVDW, GC, CS, TGD and JFC have contributed to the conception and design of the work, as well as critically revising it for intellectual content. Data acquisition, analysis and interpretation were performed by MVDW, MB, JML, NW, CS and OS. Animal experiments, ELISAs and gene expression analysis were performed in APC Microbiome Institute Ireland, University College Cork, Cork, Ireland by MVDW, MB and JML. SCFA quantification and Illumina MiSeq sequencing were performed by NW, CS and OS in Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland. All authors approve the final version of the manuscript and agree to be accountable for all aspects of the work. All individuals designated as author qualify for authorship and all individuals qualified for authorship are listed.

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Tables and figure legends

**Table 1. qRT-PCR probes used in this study.**

Table 2. Behavioural Measurements. Tests for anxiety-like behaviour were the marble burying test, which was performed two weeks post-stress and the elevated plus maze which was done three weeks post-stress. Depression was assessed using the tail-suspension test three weeks post-stress. Reward-seeking behaviour was tested using the sucrose preference test four days post stress. Cognition was determined using the novel object recognition test two weeks post stress. Somatic pain response was assessed with the hot plate test four weeks post stress. Data from the sucrose preference test were non-parametrically distributed and analysed using the Kruskal-Wallis test, followed by the Mann-Whitney test. Data from the hot plate test were normally distributed and analysed using a two-way ANOVA. Significant differences are depicted as: *$p < 0.05$; Control/Control compared to Control/Stress, ##$p < 0.01$; Control/Stress compared to SCFA/Stress. All data are expressed as mean ± SEM (n = 8-10).

Table 3. Physiological Measurements. Lipopolysaccharide-binding protein (LBP) was quantified from plasma obtained when animals were sacrificed. Body weights and drinking water consumed were assessed throughout the study and normalised to the starting body weight to calculate body weight change (Δ Bodyweight). Organs were collected at the end of the study and weighted. Thymus, adrenals, spleen and caecum weights were normalised to body weight, whereas the colon is expressed in total colon length. Faecal pellets were collected six weeks post psychosocial stress and

analysed for SCFA and BCFA levels. Colonic gene expression analysis for SCFA GPCRs was carried out for free fatty acid receptor 2 and 3 (FFAR2 and FFAR3 respectively). Body weight data and gene expression data were non-parametrically distributed and analysed using the Kruskal-Wallis test, followed by the Mann-Whitney test. Data from SCFA levels were normally distributed and analysed using a two-way ANOVA, followed by an LSD post hoc test. Significant differences are depicted as: *$p < 0.05$ and **$p < 0.01$; Control/Control compared to Control/Stress, $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$; SCFA/Control compared to SCFA/Stress. Data are expressed as mean ± SEM (n = 8-10).

Figure 1. Experimental design of the short-chain fatty acid treatment, psychosocial stress and behavioural assessment. After one week of short-chain fatty acid (SCFA) treatment, animals underwent a 3-week psychosocial stress protocol containing intermittent social defeat and overcrowding procedures. Mice subsequently underwent behavioural assessment, starting with the least stressful test to the most stressful test. The order of the behavioural tests was as following; Week 4: Social interaction test (SIT) and Sucrose preference test (SPT), Week 5: 3-Chamber sociability test (3-CT) and Female urine sniffing test (FUST), Week 6: Open field (OF), Novel object recognition test (NOR) and Marble burying test (MB), Week 7: Elevated plus maze (EPM), stress-induced hyperthermia test (SIH) and tail-suspension test (TST), Week 8: Fear conditioning (FC) and Hot plate test (HPT), Week 9: FITC-dextran intestinal permeability test (FITC), Week 10: Forced swim test (FST), Week 11: Euthanasia.
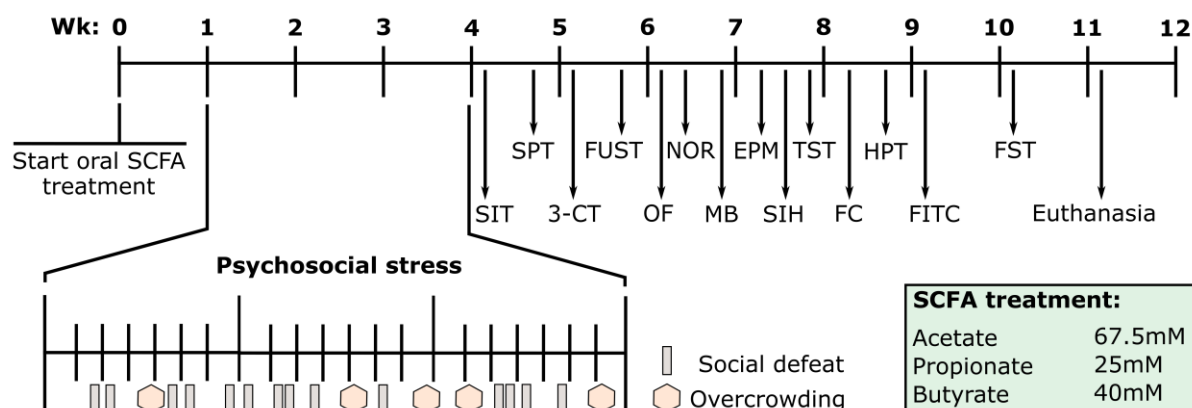
Figure 2. Chronic stress induces CD1 aggressor-specific social avoidance. Social interaction with a CD1 mouse used in the social defeat procedure was assessed in the social interaction test one day after the last stressor (**A**). Mice were additionally assessed for social preference (**B**) and recognition (**C**) with a conspecific mouse in the 3-chamber sociability test one-week post-stress. The social interaction test was non-parametrically distributed and analysed using the Kruskal-Wallis test. Significant differences are depicted as: *$p < 0.05$; Control compared to Stress. In the 3-chamber sociability test, differences between "Object" versus "Mouse" and "Familiar mouse" versus "Novel mouse" were assessed using an unpaired *t*-test. Significant differences are depicted as: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. All data are expressed as mean ± SEM (n = 9-10).
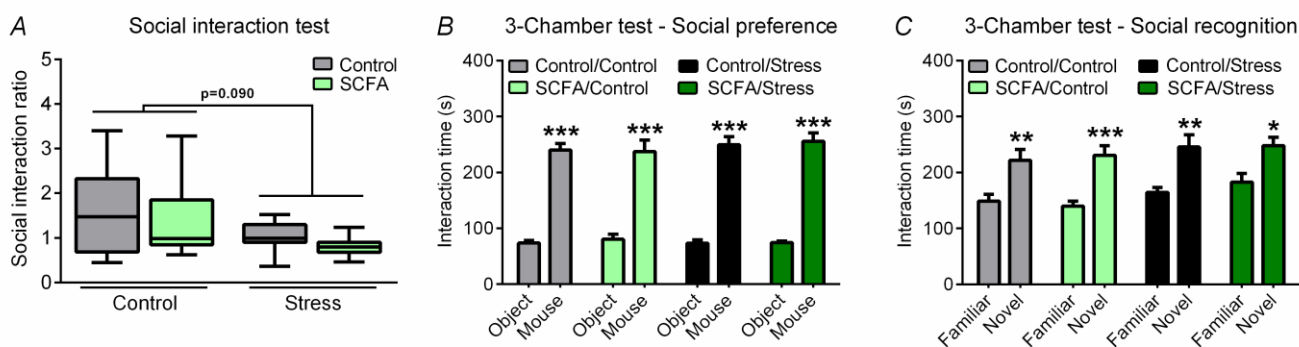

Figure 3. SCFAs decrease specific anxiety- and depressive-like behaviours in control but not stressed animals. Anxiety-like behaviour was assessed in the open field test two weeks post-stress (A, B), whereas depressive-like behaviour was determined using the forced swim test six weeks post-stress (C). The open field test was non-parametrically distributed and analysed using the Kruskal-Wallis test, followed by the Mann-Whitney test. The forced swim test was analysed using a two-way ANOVA, followed by an LSD post hoc test. Significant differences are depicted as: *$p < 0.05$ and **$p < 0.01$; Control/Control compared to SCFA/Control, $^{\$\$}p < 0.01$; SCFA/Control compared to SCFA/Stress. All data are expressed as mean ± SEM (n = 9-10). Dots on each graph represent individual animals.
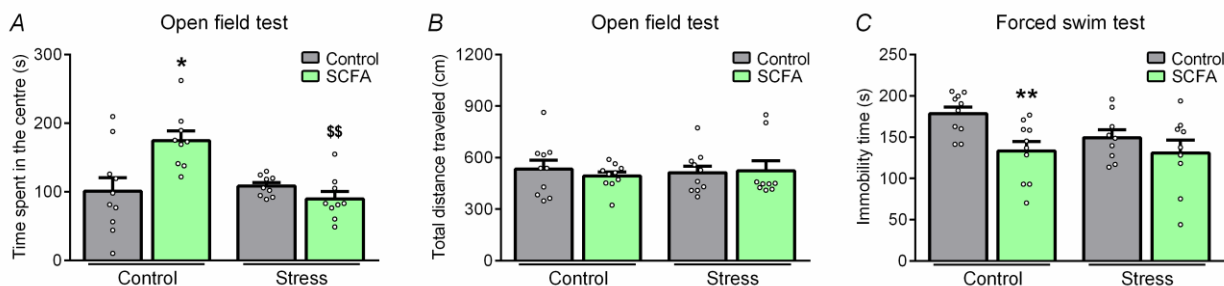
Figure 4. Psychosocial stress induces long-term anhedonia, which is absent after SCFA supplementation. Hedonic and reward-seeking behaviour was assessed using the female urine sniffing test two weeks post stress (A). The expression of genes involved in reward signalling was investigated in the striatum (B), which were the dopamine receptor D1a (DRD1a), dopamine receptor D2 (DRD2), brain-derived neurotrophic factor (BDNF Exon IV) and tropomyosin receptor kinase B (TrkB). Data from the female urine sniffing test and BDNF gene expression was non-parametrically distributed and analysed using the Kruskal-Wallis test, followed by the Mann-Whitney test. Gene expression data from all other genes were normally distributed and analysed using a two-way ANOVA, followed by an LSD post hoc test. Significant differences are depicted as: **$p < 0.01$ and ***$p < 0.001$; Control/Control compared to SCFA/Control or Control/Stress, $^\$p < 0.05$; SCFA/Control compared to SCFA/Stress, $^\#p < 0.05$; Control/Stress compared to SCFA/Stress. All data are expressed as mean ± SEM (n = 8-10).
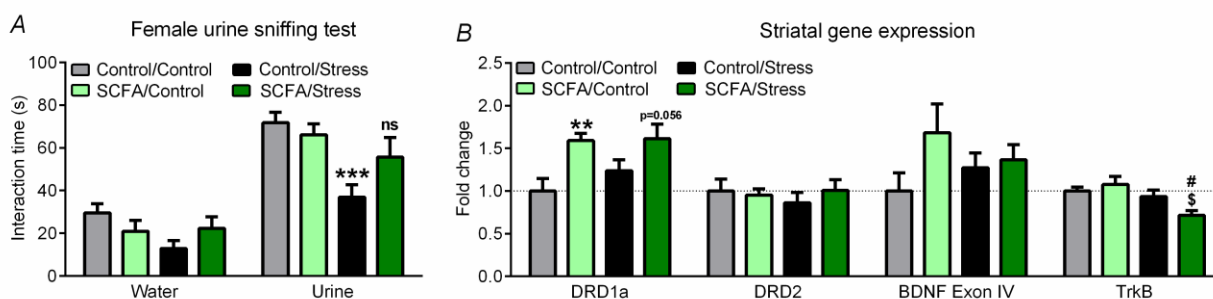

**Figure 5. Psychosocial stress induces increased responsiveness to acute stress, which was ameliorated by SCFAs.** Stress responsiveness and HPA-axis reactivity were assessed using the stress-induced hyperthermia test three weeks after psychosocial stress (A), and by assessing the corticosterone levels in response to acute stress six weeks after psychosocial stress (B, C). The stressor used for the latter was the forced swim test. Hypothalamic genes involved in HPA-axis signalling of which expression was investigated were corticotropin-releasing hormone (Crh), mineralocorticoid receptor (Mr) and glucocorticoid receptor (Gr) (D). For the hippocampus, these were corticotropin-releasing hormone receptor 1 (Crhr1), Mr and Gr (E). All data were non-parametrically distributed and analysed using the Kruskal-Wallis test, followed by the Mann-Whitney test. Significant differences are depicted as: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$; Control/Control compared to SCFA/Control or Control/Stress, $^\$p < 0.05$, $^{\$\$}p < 0.01$; SCFA/Control compared to SCFA/Stress, $^\#p < 0.05$ and $^{\#\#}p < 0.01$; Control/Stress compared to SCFA/Stress. All data are expressed as mean ± SEM (n = 8-10).
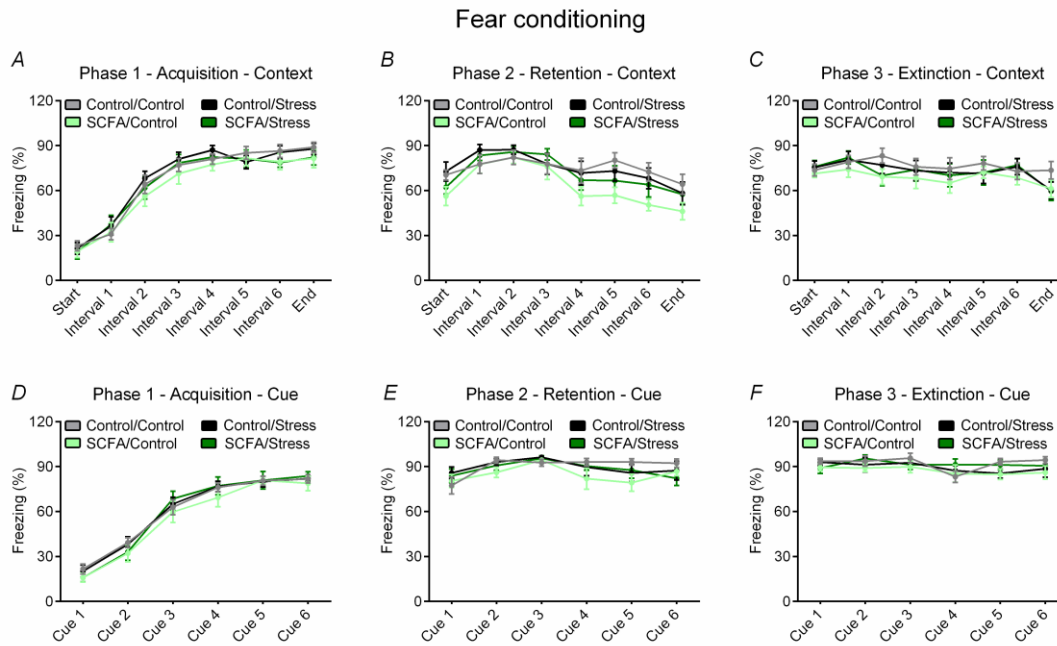
Fear conditioning

Figure 6. Neither stress, nor SCFA treatment affected the acquisition, retention and extinction phase data from the fear conditioning test. Cue- and context-associative learning induced by foot shock was evaluated using fear conditioning four weeks post stress. At Phase 1 - Acquisition, mice were presented with a tone, followed by a foot chock. The context-associative learning was assessed by measuring freezing behaviour in between tones (A), whereas cue-associative learning was determined during the presentation of the tone (D). At Phase 2 - Retention, mice underwent the same protocol without the shocks to assess context- and cue-associative retention (B and E respectively). At Phase 3 – Extinction, mice were assessed for context- and cue-associative extinction (C and F respectively). All data are expressed as mean ± SEM (n = 9-10). Dots on each graph represent individual animals.
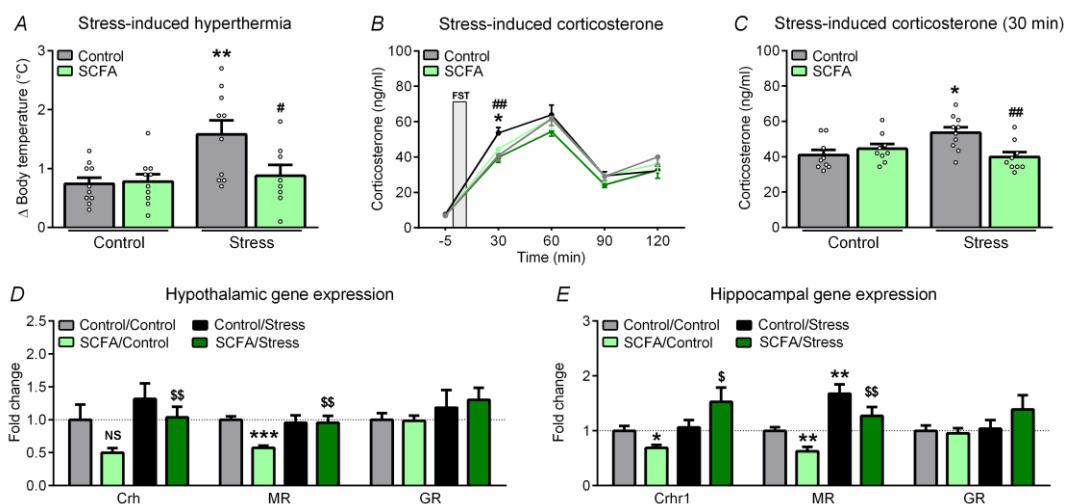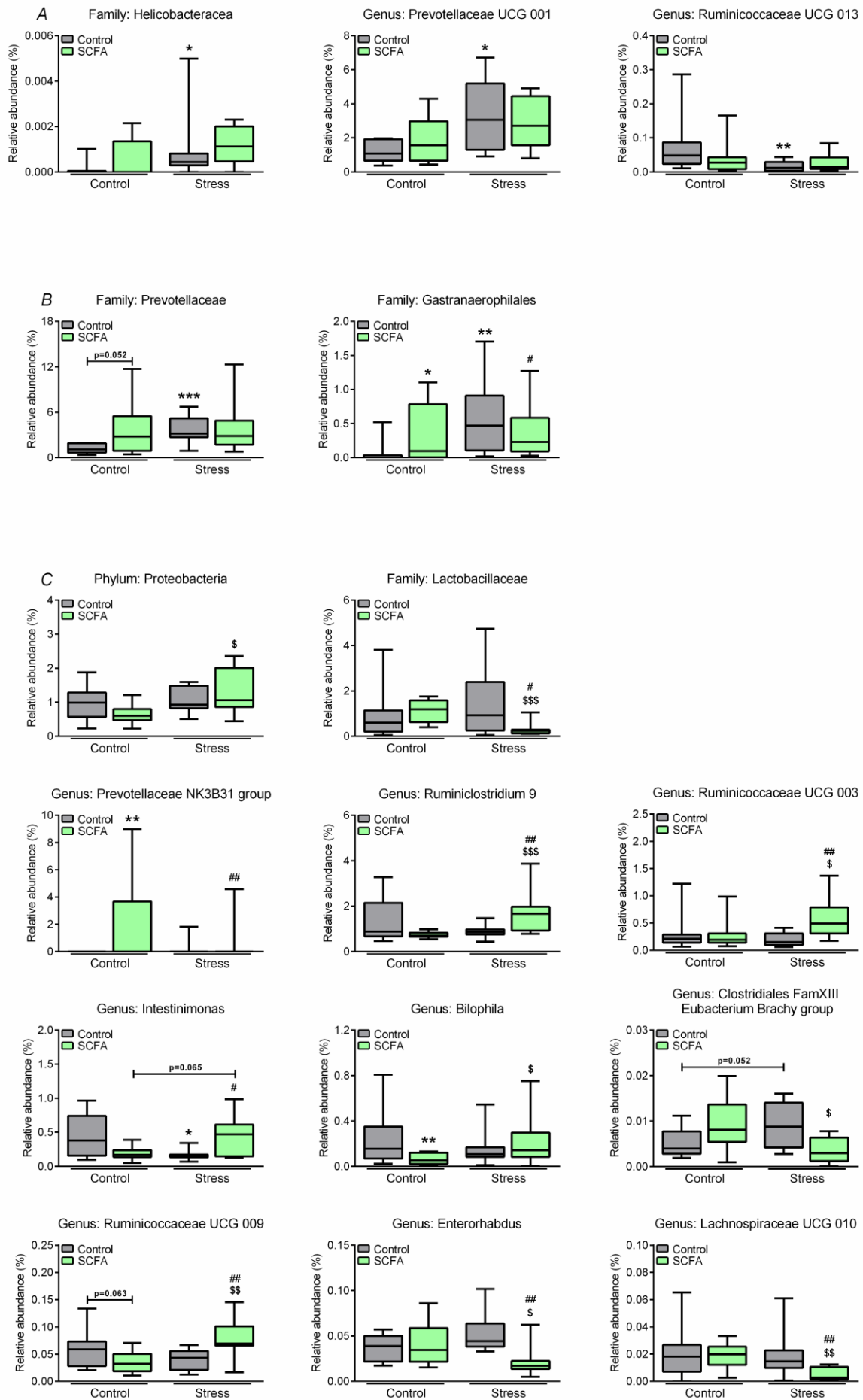
Figure 7. Stress induces intestinal permeability, which was rescued by SCFA treatment. In vivo intestinal permeability was assessed five weeks post stress (A). Colonic genes involved in intestinal permeability of which gene expression was investigated were claudin-1 (Cldn1), tight junction protein 1 (Tjp1), occludin (Ocln) and mucus 2 (Muc2) (B). Gene expression analysis of genes involved glucocorticoid signalling were corticotropin-releasing hormone receptor 1 and 2 (Crhr1 and Crhr2 respectively), mineralocorticoid receptor (Mr) and glucocorticoid receptor (Gr) (C). All data were non-parametrically distributed and analysed using the Kruskal-Wallis test, followed by the Mann-Whitney test. Significant differences are depicted as: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$; Control/Control compared to SCFA/Control or Control/Stress, [$]$p < 0.05$ and [$$]$p < 0.01$; SCFA/Control compared to SCFA/Stress, [#]$p < 0.05$ and [##]$p < 0.01$; Control/Stress compared to SCFA/Stress. All data are expressed as mean ± SEM (n = 8-10). Dots on each graph represent individual animals.
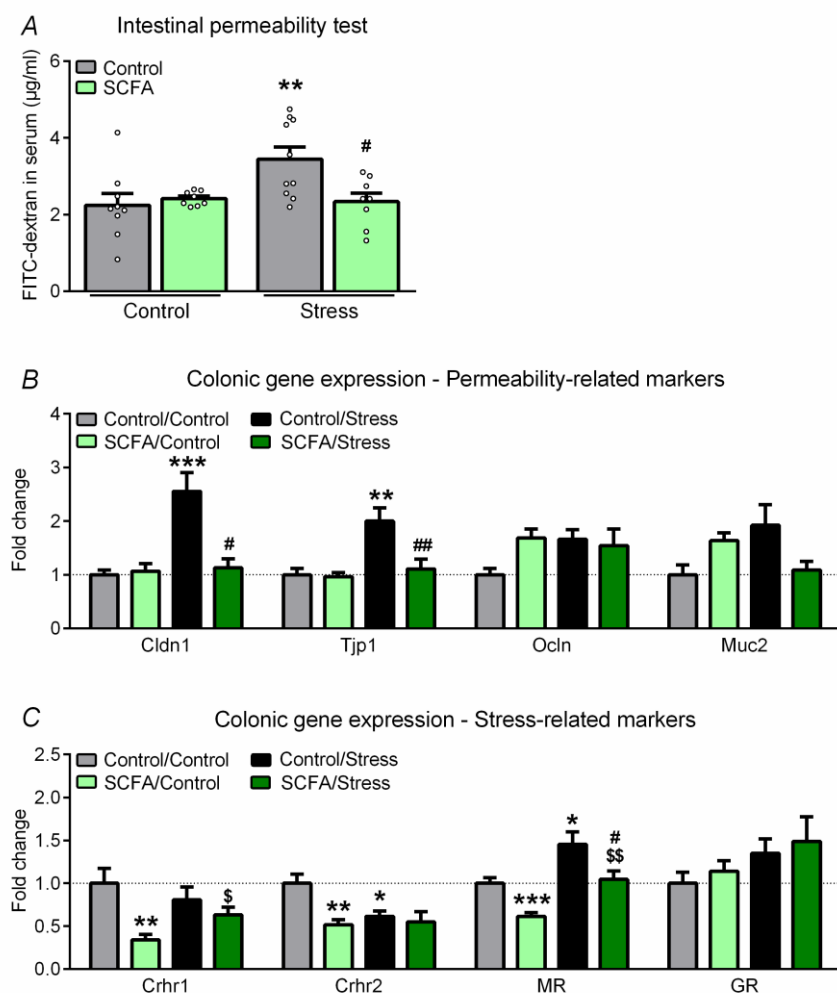
Figure 8. Neither stress nor SCFAs affected caecal microbiota diversity. Microbial alpha-diversity was determined using Chao1, Simpson, Shannon metrics, as well as the number of species detected (A). Beta-diversity of the overall composition was depicted using principle component analysis (B). Alpha-diversity metrics were non-parametrically distributed and analysed using the Kruskal-Wallis test. Data is depicted as median with IQR and min/max values as error bars (n = 9-10).
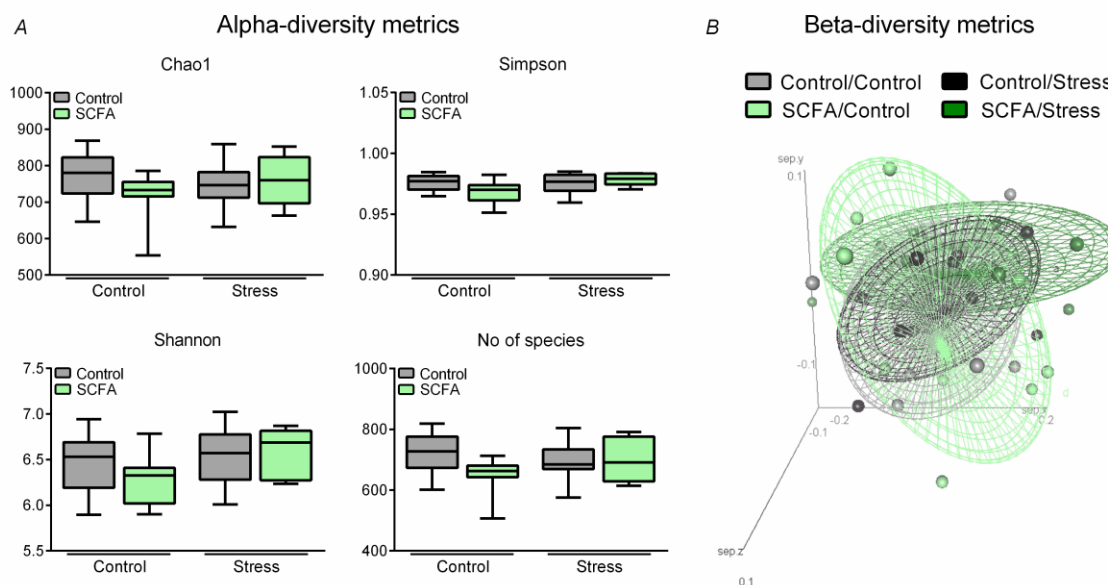
Figure 9. Only minor differences in caecum microbiota on family, genus and phylum levels were detected. The 16S sequencing revealed few significant differences in microbiota composition on phylum, family and genus level. Data is organised on stress-effect (A), concurrent SCFA- and stress-effect (B), and combined SCFA and stress effect (C). Data were non-parametrically distributed and analysed using the Kruskal-Wallis test, followed by the Mann-Whitney test. Significant differences are depicted as: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$; Control/Control compared to SCFA/Control or Control/Stress, $^\$p < 0.05$, $^{\$\$}p < 0.01$ and $^{\$\$\$}p < 0.001$; SCFA/Control compared to SCFA/Stress, $^\#p < 0.05$ and $^{\#\#}p < 0.01$; Control/Stress compared to SCFA/Stress. Data are depicted as median with IQR and min/max values as error bars (n = 9-10).

A

Family: Helicobacteracea

Genus: Prevotellaceae UCG 001

Genus: Ruminicoccaceae UCG 013

B

Family: Prevotellaceae

Family: Gastranaerophilales

C

Phylum: Proteobacteria

Family: Lactobacillaceae

Genus: Prevotellaceae NK3B31 group

Genus: Ruminiclostridium 9

Genus: Ruminicoccaceae UCG 003

Genus: Intestinimonas

Genus: Bilophila

Genus: Clostridiales FamXIII Eubacterium Brachy group

Genus: Ruminicoccaceae UCG 009

Genus: Enterorhabdus

Genus: Lachnospiraceae UCG 010

Marcel van de Wouw is a Dutch PhD student in his third year working at APC Microbiome Ireland at University College Cork under the supervision of Prof. John Cryan and Ted Dinan. His research involves the investigation of how short-chain fatty acids can affect microbiota-gut-brain axis signalling and how this relates to behaviour. He completed his undergraduate training focussed on how ghrelinergic signalling can influence food intake, which involved a 10-month internship at University College Cork under the supervision of Dr. Harriet Schellekens and Prof. John Cryan.