Changes in the rodent gut microbiome following chronic restraint stress and low-intensity rTMS

Bhedita J. Seewoo [a,b,c], Eng Guan Chua [d], Yasmin Arena-Foster [a,b], Lauren A. Hennessy [a,b], Anastazja M. Gorecki [a,b], Ryan Anderton [e], Jennifer Rodger [a,b,*]

[a] School of Biological Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA, 6009, Australia
[b] Perron Institute for Neurological and Translational Science, 8 Verdun St, Nedlands, WA, 6009, Australia
[c] Centre for Microscopy, Characterisation & Analysis, Research Infrastructure Centres, The University of Western Australia, 6 Verdun St, Nedlands, WA, 6009, Australia
[d] The Marshall Centre for Infectious Diseases Research and Training, The University of Western Australia, 35 Stirling Highway, Crawley, WA, 6009, Australia
[e] Institute for Health Research and School of Health Sciences, University of Notre Dame Australia, 33 Phillimore Street, Fremantle, WA, Australia

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ABSTRACT

Gut microbiome composition is associated with mood-relating behaviours, including those reflecting depression-like phenotypes. Repetitive transcranial magnetic stimulation (rTMS), a non-invasive neuromodulation technique, is an effective treatment for depression, but its effects on the gut microbiome remain largely unknown. This study assessed microbial changes from rat faecal samples longitudinally following chronic restraint stress (CRS) and 10 Hz low-intensity rTMS treatment. CRS increased abundance within the Proteobacteria (Deltaproteobacteria, Desulfovibrionales) and Firmicutes (*Anaerostipes, Frinsingococcus*), with decreases in Firmicutes family (*Acidaminococcaceae*) and genera (*Roseburia, Phascolarctobacterium* and *Fusicatenibacter*) persisting for up to 4 weeks post CRS. The decrease in Firmicutes was not observed in the handling control and LI-rTMS groups, suggesting that handling alone may have sustained changes in gut microbiome associated with CRS. Nonetheless, LI-rTMS was specifically associated with an increase in *Roseburia* genus that developed 2 weeks after treatment, and the abundance of both *Roseburia* and *Fusicatenibacter* genera was significantly correlated with rTMS behavioural and MRI outcomes. In addition, LI-rTMS treated rats had a reduction in apoptosis pathways and several indicators of reduced inflammatory processes. These findings provide evidence that the brain can influence the gut microbiome in a "top-down" manner, presumably via stimulation of descending pathways, and/or indirectly via behavioural modification.

1. Introduction

Major depressive disorder (MDD) is a debilitating neuropsychiatric syndrome with significant morbidity and mortality due to the risk of suicide. Whilst the exact cause of depression is still largely unknown, current research suggests that mental illnesses such as MDD are caused by a complex array of genetic, neurological, hormonal, immunological, environmental, and psychological factors. These factors may be integrated via the gut-brain axis, the bidirectional communication network between the central and enteric nervous systems consisting of neural and immune pathways as well as the hypothalamic-pituitary-adrenal axis (Capuco et al., 2020; Cruz-Pereira et al., 2020). There is

increasing evidence that gut-brain signalling is altered in MDD, with with an altered gut microbiome reported as well as a link between MDD and gut symptoms (e.g. IBS) (Kochar et al., 2018; Barandouzi et al., 2020). However the causal relationships between the "bottom" (gut, microbiome), and the "top" (mood and brain function) of the gut-brain axis remain unclear.

Currently, most studies have investigated the bottom-up effects of the gut microbiome on brain chemistry and relevant behaviour in depression. The gut, including the composition of the microbiome, influences mood, cognition and other neurological functions (Clapp et al., 2017; Makris et al., 2021). For example, a faecal microbiota transplant from stressed animals or patients with depression to healthy animals has

been shown to increase anxiety and depression-like behaviours in recipients, whereas the transplant of healthy microbiota to patients with depression conversely decreases depression symptoms (Chinna Meyyappan et al., 2020). Similarly, probiotic treatment has been shown to reduce anxiety- and depression-related behaviours (Bravo et al., 2011) and restore brain changes related to depression and emotion processing (Bercik et al., 2010; Tillisch et al., 2013). However, the evidence for a top-down influence of the brain on the microbiome is less clear. While the brain regulates gastrointestinal motility, secretory activity and immune responses (Mayer, 2011), and psychological stress can change microbiome composition (Galley et al., 2014), a direct influence of brain signalling on the microbiome has not been definitively demonstrated. Additionally, many antidepressant medications targeting brain function and mood are delivered orally and have antimicrobial properties (Valles-Colomer et al., 2019; Ait Chait et al., 2020), making it difficult to differentiate between potential top-down effects of neurological change on the gut, and the direct effect of oral medication on microbiome composition (Lukić et al., 2019; McGovern et al., 2019).

The use of brain-specific treatment methods can help to dissect the top-down effects of depression treatment on the gut microbiome. Non-invasive brain stimulation has exhibited potential positive effects on the gut microbiome in patients with weight disorders through subsequent improvements in weight (Artifon et al., 2020; Ferrulli et al., 2021). Here we use repetitive transcranial magnetic stimulation (rTMS), a non-invasive FDA-approved treatment for depression (O'Reardon et al., 2007) that uses electromagnetic induction to alter the excitability of the brain (George et al., 2000). Given the evidence for perturbed gut-brain signalling in depression, we hypothesised that this brain-specific treatment for depression would allow us to isolate any potential top-down effects of the brain on the gut microbiome. Because of the variability of human subjects not only in terms of the gut microbiome but also in their response to rTMS, here we use a well characterised chronic restraint intervention in rats to: 1) validate the effects of a chronic restraint stress model of depression on gut microbial composition and diversity in male Sprague-Dawley rats and 2) identify the effects of low-intensity rTMS treatment on the gut microbiome in this depression model.

2. Methods

All experimental procedures were approved by the University of Western Australia (UWA) Animal Ethics Committee (RA/3/100/1640) and conducted in accordance with the *National Health and Medical Research Council Australian code* for the care and use of animals for scientific purposes. All investigators were trained in animal handling by the UWA Programme in Animal Welfare, Ethics, and Science (PAWES) and had valid Permission to Use Animals (PUA) licenses.

2.1. Animals

The microbiome samples analysed here were collected from several cohorts of rats over a 2-year period. In addition to CRS and rTMS described in detail below, animals underwent behavioural testing and MRI imaging procedures to measure brain anatomy, resting-state functional MRI (rs-fMRI) and neurotransmitter levels (Magnetic Resonance Spectroscopy) (Fig. 1). The outcomes of MRI (including rs-fMRI, hippocampal volume and neurotransmitter levels) and behavioural tests are used in the present study for correlation analysis, and have been submitted for publication in full elsewhere. Summary of behavioural data are available on the Figshare repository (https://figshare.com/), with https://doi.org/10.6084/m9.figshare.16866790. Full raw behavioural and MRI datasets are available on request to the corresponding author.

Young adult male Sprague-Dawley rats (5–6 weeks old, 150–200g) from the Animal Resources Centre (Perth, Western Australia) were maintained in a temperature-controlled animal care facility on a 12-h light-dark cycle with food and water *ad libitum* (except during restraint). Animals were allowed to acclimatise to the facility for one week following their arrival. Cages contained two acidified water bottles (acidified as per safety regulations) and 500g of food pellets. To ensure the influence of diet on the gut microbiome was minimal, the diet for all animals was kept consistent and the weights of the animals were recorded weekly. Animals were randomly assigned to one of the following groups: 1) active group consisting of animals (n = 12) which underwent CRS and received accelerated 10 Hz LI-rTMS (10 min three times daily, 1 h apart, five days/week for two weeks) to the left hemisphere; 2) sham group consisting of (n = 12) animals which underwent CRS and sham LI-rTMS; and 3) control group consisting of animals (n = 7) which underwent CRS but did not receive any stimulation or extra handling during the treatment period (Fig. 1). Animals were housed in pairs, such that those within the same group were co-housed to prevent transmission of faecal dysbiosis (Sun et al., 2013). At the end of the study, animals were euthanised by intraperitoneal overdose of pentobarbitone (Lethabarb) anaesthesia (160 mg/kg i.p.).

The CRS procedure was carried out for 2.5 h/day for 13 consecutive days as described previously (Seewoo et al., 2020). Animals receiving LI-rTMS were habituated to handling and the coil as described
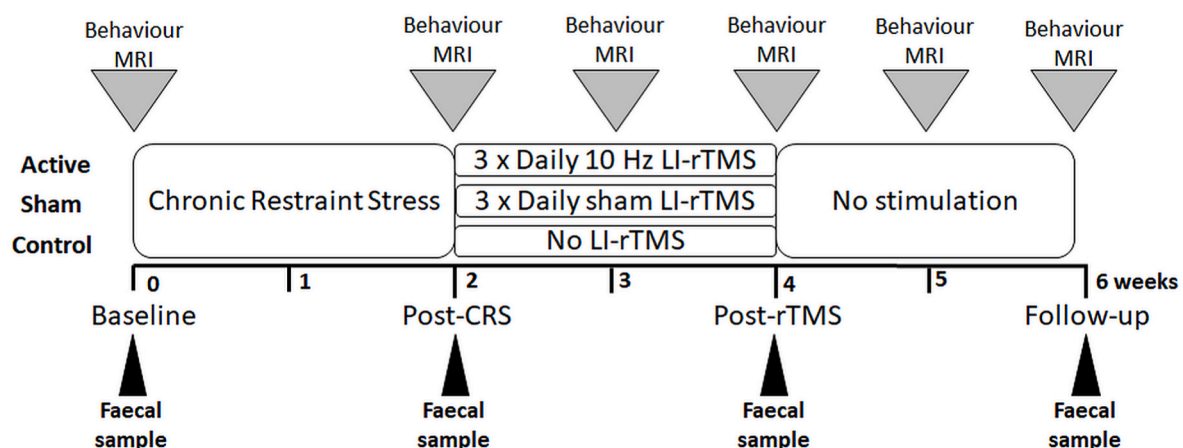

Fig. 1. Experimental timeline. The research design consisted of an initial one-week period of habituation upon arrival of the animal. Rats then underwent chronic restraint stress for 2.5 h daily for 13 consecutive days. Animals were randomly allocated to 3 groups: active animals received 10 min of 10 Hz LI-rTMS three times daily (1 h apart, five days a week for two weeks), sham animals received a sham version of the stimulation protocol and depression control animals received no stimulation or extra handling. Faecal samples were collected at baseline (before the start of CRS), after the end of the 13 days of CRS (Post-CRS), after the end of the treatment period (Post-rTMS) and two weeks after the end of the treatment period (Follow-up).

previously (Rodger et al., 2012; Makowiecki et al., 2014). LI-rTMS was delivered at a frequency of 10 Hz using a custom-built round coil at an intensity of approximately 13 mT at the surface of the cortex, which is below the rodent's motor threshold (Seewoo et al., 2018). Stimulation was delivered following an accelerated protocol: 3 times daily at 1-h intervals for 2 weeks (Seewoo et al., 2019, 2021). During the sham procedure, the coil was disconnected. Faecal pellets were collected from rats in all groups prior to CRS (baseline), after the last CRS procedure (post-CRS), at the end of the treatment period (post-rTMS) and two weeks after end of the treatment period (follow-up) (Fig. 1).

2.2. Sample preparation and sequencing

Between 2 and 6 faecal pellets per animal were collected four timepoints, each separated by two weeks: baseline, post CRS, post LI-rTMS and follow up (Fig. 1). Pellets were collected during behavioural testing sessions when they were spontaneously and abundantly expelled in a clean and controlled environment with no additional interventions. Pellets were frozen within 10 min of production at each collection timepoint and stored separately at −80 °C until the completion of the animal cohort. Using aseptic techniques to avoid cross contamination, rat faecal pellets were subsequently weighed into microcentrifuge tubes, and a 10 g mid-section of each pellet was sent to Australian Genome Research Facility (AGRF, Australia) for DNA extraction and sequencing of the *16S rRNA* gene V3–V4 region on an Illumina MiSeq platform using the 2 × 300 bp V3 chemistry. The V3V4 primers used by AGRF were a modified 341F (5′-CCTAYGGGRBGCASCAG-3′) and a modified 806R (5′-GGACTACNNGGGTAT CTAAT-3′) (Yu et al., 2005). The 16S raw sequencing data is publicly available at Figshare repository (https://figshare.com/), with https://doi.org/10.6084/m9.figshare.16866790.

2.3. 16S data processing

Raw sequencing reads were subjected to quality and adapter trimming using the bbduk.sh command available in the BBTools suite (version 38.87) (https://jgi.doe.gov/data-and-tools/bbtools/), with the following input parameters: qtrim = r trimq = 20 ktrim = r k = 23 mink = 11 minlen = 200 hdist = 1 tpe tbo. Next, the MeFiT pipeline (version 1.0) (Parikh et al., 2016) was run with default parameters to merge trimmed overlapping paired-end reads. The summary statistics of the trimmed and merged sequence reads are available in Table S1. For filtering and clustering of merged reads into operational taxonomic units (OTUs), the MICCA software (version 1.7.2) was used (Albanese et al., 2015). In detail, merged sequences of less than 420 bp were filtered using the *micca filter* command. Then, the *micca otu* command was run to perform *de novo* greedy clustering of the sequences into OTUs using a sequence identity threshold of 97%, along with the removal of chimeric sequences and singleton OTUs. For each representative OTU sequence, taxonomic information was assigned using the Bayesian LCA-based taxonomic classification method (version 2.3-alpha) with a minimum e-value of 1e-10 and 100 bootstrap replications, against the NCBI 16S ribosomal RNA database (Gao et al., 2017). A minimum confidence score of 80 was used to accept a taxonomic assignment at each level. Multiple sequence alignment of the OTU representative sequences was performed using PASTA (Mirarab et al., 2014), where the output alignment file was used for phylogenetic tree construction using FastTree (Price et al., 2010) under the GTR model with CAT approximation. The OTU table, as well as the taxonomic information, are available in Table S2.

Prior to analysing both alpha and beta diversities using the phyloseq (McMurdie and Holmes, 2013) and amplicon (https://rdrr.io/github/microbiota/amplicon) R packages, rarefaction of the OTU table was performed at 8462 sequences per sample. For alpha diversity analysis, four indices were evaluated, including the number of observed OTUs, abundance-based coverage estimator (ACE), Shannon entropy and inverse Simpson. Comparison of alpha diversity between groups and

different timepoints was performed using ANOVA with post-hoc Tukey test. For beta diversity analysis, principal coordinates analysis (PCoA) was performed at the OTU level using both weighted and unweighted UniFrac distance metrics, followed by the analysis of similarity test (ANOSIM) to test for differences in microbial communities between groups and different timepoints. Volatility analysis was performed for each group of rat subjects by comparing the Aitchison distances for OTUs between successive samples collected from the same animal over time (Bastiaanssen et al., 2021). Prediction of the microbial communities for KEGG functional pathways was performed using PICRUSt2 (version 2.3.0-b) (Douglas et al., 2020). The generated KEGG pathway abundances are available in Table S3.

2.4. Statistical analysis

To identify significant bacterial taxa and KEGG pathways between groups and different timepoints, the analysis of composition of microbiomes (ANCOM) procedure (Lin and Peddada, 2020) was performed on unfiltered absolute abundance data, adjusted for cage and study batch effects. The *p*-values were adjusted using the Benjamini-Hochberg procedure at the significance level of 0.05. Significant bacterial taxa and KEGG pathways were declared by using ANCOM's W-statistic with a threshold of 0.7. All ANCOM test results are available in Table S4. For each differentially abundant taxa and KEGG pathways identified by ANCOM, further statistical testing was performed on the centered log-ratio (CLR)-transformed raw taxa and KEGG abundance values as described below.

2.4.1. Effect of CRS

Bacterial diversity and composition were similar between groups at baseline. Due to the small sample size, data from the baseline and post-CRS timepoints were pooled from the three groups receiving restraint to determine the effects of CRS on the gut microbiome. The difference in bacterial composition between baseline and post-CRS timepoints was examined by the Wilcoxon signed rank test (n = 29/timepoint).

2.4.2. Effect of LI-rTMS

To determine the effects of LI-rTMS on the gut microbiome, the Quade test was used to determine if there was a significant effect of timepoint (post-CRS, post-rTMS and follow-up) in each group, followed by *Post hoc* Wilcoxon signed rank test to determine paired differences between timepoints. The Benjamini-Hochberg false discovery rate (FDR) method was used to correct for multiple comparisons at the significance level of 0.05.

2.4.3. Correlation analysis

Magnetic resonance imaging (MRI) and behavioural data were acquired at the four timepoints using previously published methods (Seewoo et al., 2020). Spearman correlations were performed between the microbiome data and the following MRI and behavioural measures from unpublished results: GABA and glutamate concentration (as a ratio to total creatine concentration) obtained from proton magnetic resonance spectroscopy (1H-MRS) within the sensorimotor cortex; hippocampal volume obtained from T2-weighted anatomical MRI data; cingulate cortex functional connectivity obtained from seed-based analysis of resting-state functional MRI (rs-fMRI) data; functional connectivity within the interoceptive network, salience network and default mode network obtained from independent component analysis of rs-fMRI data; number of exits from the open arms of the elevated plus maze (EPM); time spent immobile during the forced swim test (FST); time spent swimming during FST; and latency, which is the time taken to exhibit the first immobility behaviour during FST.

3. Results

3.1. Effect of CRS on the gut microbiome

CRS did not induce any changes in alpha diversity or beta diversity (Supplementary Figs. S1–S10). However, class-level analyses revealed a significant increase in Deltaproteobacteria post-CRS (V = 102, p = 0.011) which was accompanied by an increase in the corresponding Desulfovibrionales order (V = 98, p = 0.009; Fig. 2). Genus-level analysis showed marked increases in both *Anaerostipes* and *Frisingicoccus* of the *Clostridia* class (V = 68, p < 0.001; V = 86, p = 0.004 respectively; Fig. 2). There were no significant changes in any other taxa or KEGG pathways.

3.2. Effect of LI-rTMS on the gut microbiome

LI-rTMS did not induce any changes in alpha diversity or beta diversity (Supplementary Figs. S1–S10). Class-level analyses revealed a significant decrease in the Negativicutes class over time in the depression control group ($F_{(2,12)} = 12.20$, p = 0.001; $p_{FDR} = 0.031$ for all; Fig. 3). This decrease was accompanied by a significant decrease in the corresponding Acidaminococcales order ($F_{(2,12)} = 12.20$, p = 0.001; $p_{FDR} = 0.047$ for all). Within the Negativicutes class, *Acidaminococcaceae* family also significantly decreased over time in the depression control group ($F_{(2,12)} = 20.37$, p < 0.001; $p_{FDR} = 0.031$ for Post-CRS vs Post-rTMS, $p_{FDR} = 0.023$ for Post-CRS vs Follow-up and Post-rTMS vs Follow-up), as did the corresponding *Phascolarctobacterium* genus ($F_{(2,12)} = 14.14$, p < 0.001; $p_{FDR} = 0.023$ for Post-CRS vs

Post-rTMS and Follow-up, $p_{FDR} = 0.031$ for Post-rTMS vs Follow-up). Additionally, there was a significant effect of timepoint for the *Roseburia* genus from the Clostridia class in the active ($F_{(2,22)} = 4.569$, p = 0.022) and depression control groups ($F_{(2,12)} = 5.243$, p = 0.023). There was a trend for an increase in *Roseburia* genus between the post-rTMS and follow-up timepoints ($p_{FDR} = 0.081$) in the active group while *Roseburia* genus decreased significantly at the follow-up timepoint compared to both post-CRS and post-rTMS timepoints in the depression control group ($p_{FDR} = 0.047$ for both). Furthermore, within the Clostridia class in the control group, there was a significant decrease in *Fusicatenibacter* genus from the post-CRS timepoint to the post-rTMS and follow-up timepoints ($F_{(2,12)} = 15.23$, p < 0.001; $p_{FDR} = 0.047$ for both) and a trend for an increase in *Intestinimonas* genus from the post-CRS timepoint to the post-rTMS and follow-up timepoints ($F_{(2,12)} = 7.868$, p = 0.007; $p_{FDR} = 0.07$ for both). There were several significant changes in KEGG functional pathways (Table 1, Fig. 4).

3.3. Correlation between the gut microbiome and brain and behavioural measures

Spearman's rank correlation test using data from all groups and timepoints revealed significant correlations of microbiome composition and KEGG pathways with several MRI and behavioural measures (Fig. 5). However, none of these correlations survived multiple comparison correction in the active and depression control groups. In the sham group, GABA was positively correlated to Negativicutes class (r = 0.47, p = 0.005, $p_{FDR} = 0.037$), Acidaminococcales order (r = 0.48, p = 0.005, $p_{FDR} = 0.037$), *Acidaminococcaceae* family (r = 0.45, p = 0.007,
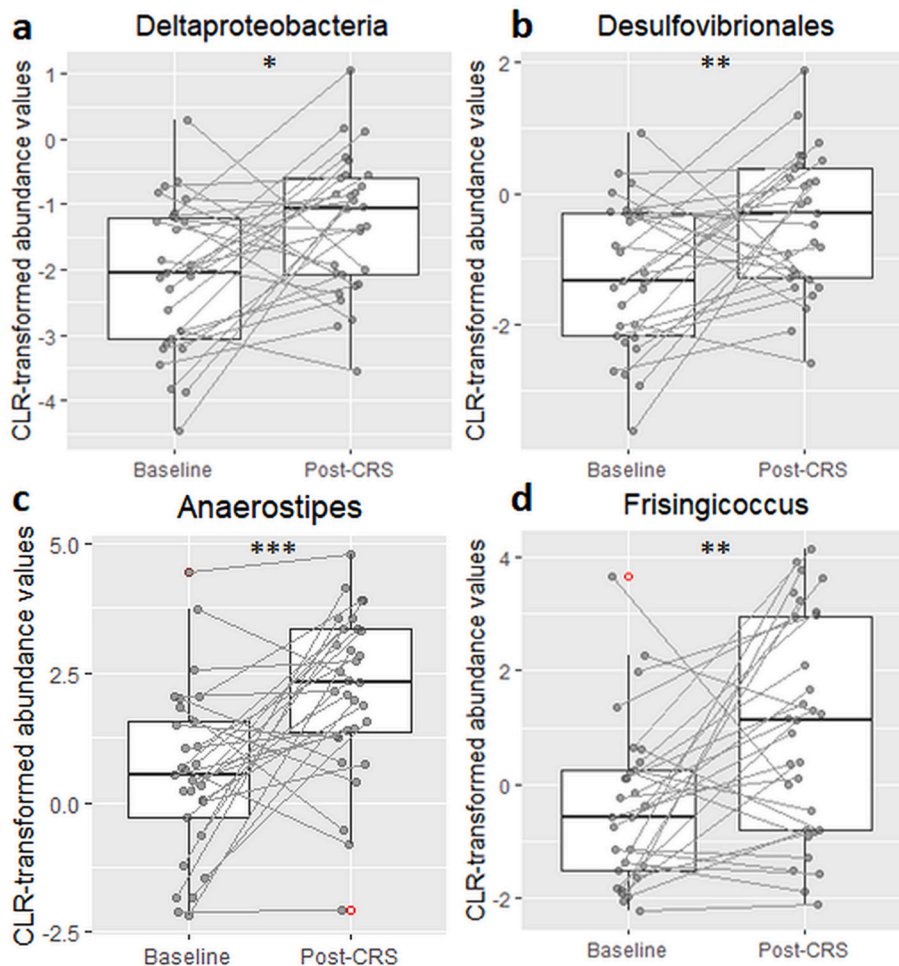

Fig. 2. Effect of chronic restraint stress on abundance of Deltaproteobacteria class (a), and its corresponding Desulfovibrionales order (b), and *Anaerostipes* (c), and *Frisingicocus* (d) genera of the Clostridia class . The figure shows boxplots of centered log-ratio transformed taxa abundances for taxa identified as significant in the ANCOM's W-statistic (threshold of 0.7). Between-timepoint comparisons were made by Wilcoxon signed rank test. *p < 0.05; **p < 0.01; ***p < 0.001.
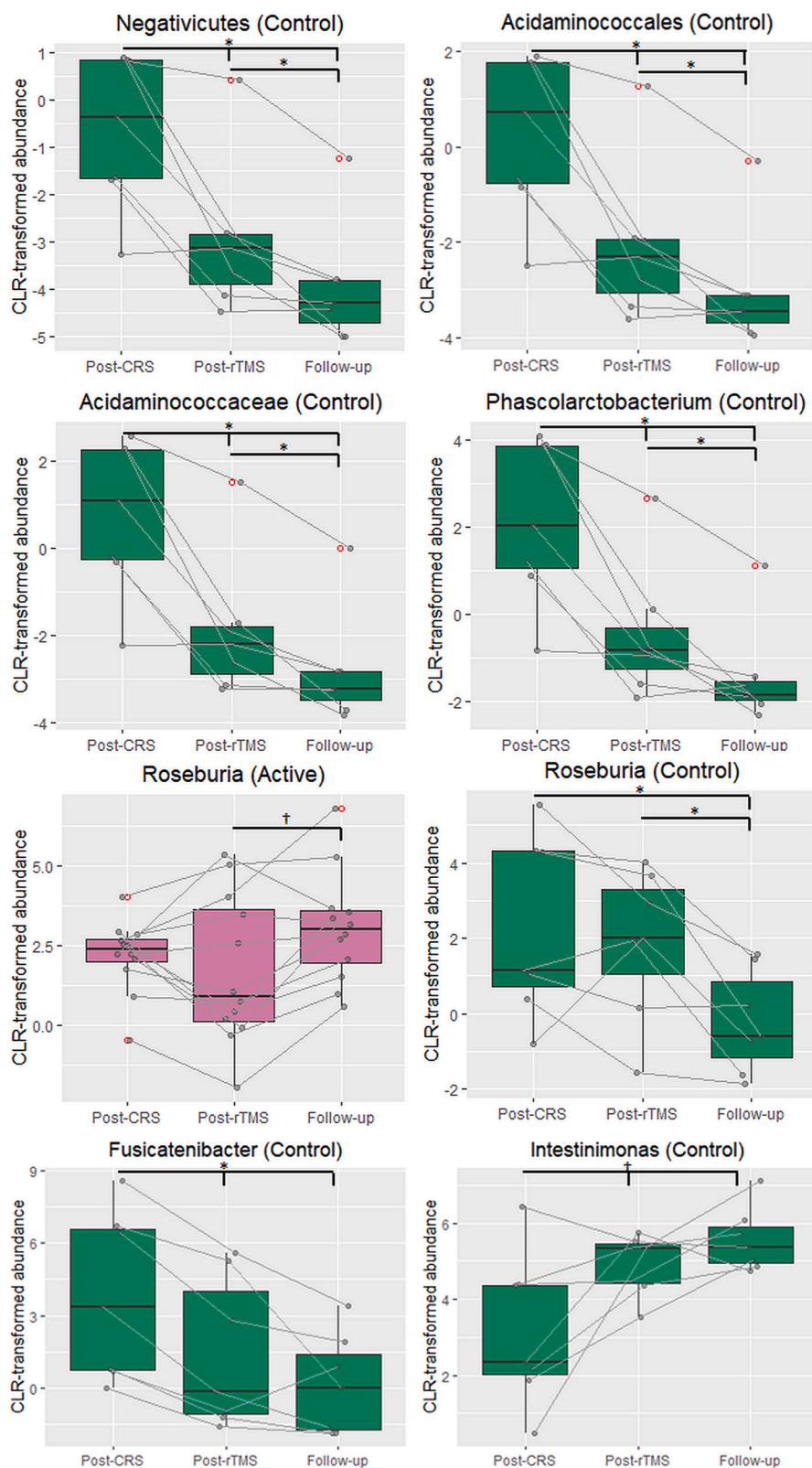
Fig. 3. Changes in abundance of taxa between timepoints within the active, sham and depression control groups. The figure shows boxplots of centered log-ratio transformed taxa abundances for taxa identified as significant in the ANCOM's W-statistic (threshold of 0.7). Quade test was used to determine if there was a significant effect of timepoint (post-CRS, post-rTMS and follow-up) in each group. Between-timepoint comparisons were made by *Post hoc* Wilcoxon signed rank test with Benjamini-Hochberg FDR correction. †p < 0.1; *p < 0.05.

$p_{FDR} = 0.047$) and *Phascolarctobacterium* genus ($r = 0.46$, $p = 0.006$, $p_{FDR} = 0.045$). Glutamate was positively correlated to Negativicutes class ($r = 0.58$, $p < 0.001$, $p_{FDR} = 0.005$), Acidaminococcales order ($r = 0.58$, $p < 0.001$, $p_{FDR} = 0.004$), *Acidaminococcaceae* family ($r = 0.59$, $p < 0.001$, $p_{FDR} = 0.004$) and *Phascolarctobacterium* genus ($r = 0.60$, $p < 0.001$, $p_{FDR} = 0.003$), but negatively correlated to the *Intestinimonas*

genus ($r = -0.46$, $p = 0.005$, $p_{FDR} = 0.037$) and ko03450 ($r = -0.48$, $p = 0.003$, $p_{FDR} = 0.033$) and ko00941 ($r = -0.49$, $p = 0.003$, $p_{FDR} = 0.029$) pathways. For the forced swim test, time spent immobile was positively correlated to the *Phascolarctobacterium* ($r = 0.44$, $p = 0.007$, $p_{FDR} = 0.048$) and *Fusicatenibacter* genera ($r = 0.51$, $p = 0.002$, $p_{FDR} = 0.020$), but negatively correlated to the *Intestinimonas* genus ($r = -0.47$, $p =$

Table 1

Summary of changes in abundance for KEGG functional pathways following chronic restraint stress (CRS) and between timepoints within the active, sham and depression control groups. Active animals received 10 min of 10 Hz LI-rTMS three times daily (1 h apart, five days a week for two weeks), sham animals received a sham version of the stimulation protocol with the coil turned off and control animals received no stimulation or extra handling.

KEGG	Functional pathway	Group	Quade test	Timepoint comparison	Mean ± SD
ko04210	Apoptosis	Active	$F_{(2,22)} = 8.272$, p = 0.002	Post-CRS vs Post-rTMS p_{FDR} = 0.031 Post-rTMS vs Follow-up p_{FDR} = 0.028	Post-CRS = -2.71 ± 0.53 Post-rTMS = -3.32 ± 0.83 Follow-up = -2.46 ± 0.37
		Sham	$F_{(2,22)} = 7.286$, p = 0.003	Post-CRS vs Follow-up p_{FDR} = 0.078 Post-rTMS vs Follow-up p_{FDR} = 0.037	Post-CRS = -3.25 ± 0.96 Post-rTMS = -3.60 ± 1.29 Follow-up = -2.67 ± 0.82
ko00930	Caprolactam degradation	Sham	$F_{(2,22)} = 5.016$, p = 0.016	Post-CRS vs Post-rTMS p_{FDR} = 0.007 Post-CRS vs Follow-up p_{FDR} = 0.007	Post-CRS = -5.00 ± 0.67 Post-rTMS = -3.87 ± 0.53 Follow-up = -3.88 ± 0.84
ko00312	beta-Lactam resistance	Sham	$F_{(2,22)} = 6.790$, p = 0.005	Post-CRS vs Follow-up p_{FDR} = 0.031 Post-rTMS vs Follow-up p_{FDR} = 0.010	Post-CRS = 1.85 ± 0.42 Post-rTMS = 1.66 ± 0.56 Follow-up = 1.17 ± 0.74
ko03450	Non-homologous end-joining	Control	$F_{(2,12)} = 7.455$, p = 0.008	Post-CRS vs Post-rTMS p_{FDR} = 0.070 Post-CRS vs Follow-up p_{FDR} = 0.070	Post-CRS = -2.94 ± 1.35 Post-rTMS = -1.75 ± 0.69 Follow-up = -1.28 ± 0.45
ko00941	Flavonoid biosynthesis	Control	$F_{(2,12)} = 5.243$, p = 0.023	Post-CRS vs Post-rTMS p_{FDR} = 0.070 Post-CRS vs Follow-up p_{FDR} = 0.070	Post-CRS = -4.37 ± 1.77 Post-rTMS = -2.74 ± 0.91 Follow-up = -2.15 ± 0.67
ko05131	Shigellosis	Control	$F_{(2,12)} = 17.71$, p < 0.001	Post-CRS vs Post-rTMS p_{FDR} = 0.023 Post-CRS vs Follow-up p_{FDR} = 0.023 Post-rTMS vs Follow-up p_{FDR} = 0.031	Post-CRS = -7.58 ± 0.41 Post-rTMS = -6.57 ± 0.66 Follow-up = -7.25 ± 0.42

0.004, p_{FDR} = 0.037) and ko03450 (r = –0.56, p < 0.001, p_{FDR} = 0.007) and ko00941 (r = –0.54, p < 0.001, p_{FDR} = 0.010) pathways. Latency to first immobility behaviour was positively correlated to the ko03450 pathway (r = 0.56, p < 0.001, p_{FDR} = 0.007), but negatively correlated to the Negativicutes class (r = –0.62, p < 0.001, p_{FDR} = 0.002), Acidaminococcales order (r = –0.63, p < 0.001, p_{FDR} = 0.002), *Acidaminococcaceae* family (r = –0.65, p < 0.001, p_{FDR} = 0.001) and both *Phascolarctobacterium* and *Fusicatenibacter* genera (r = –0.66, p < 0.001, p_{FDR} = 0.001 for both).

4. Discussion

There is increasing evidence that the gut-brain axis plays a key role in the aetiology and treatment of depression. Our study confirmed previously reported changes in the gut resident bacteria composition following CRS in rats and adds that handling the rats after CRS caused a significant effect on the abundance of Firmicutes phylum. Our main finding was that LI-rTMS increased the abundance of the anti-inflammatory *Roseburia* genus, which was correlated with behavioural improvement in the forced swim test. Taken together with the decrease in apoptosis pathways following LI-rTMS, as well as the correlation of caprolactam pathway with functional outcomes only in rTMS treated animals, our results suggest that LI-rTMS may have anti-inflammatory and protective effects on the gut microbiome that are associated with its therapeutic effects.

4.1. Diversity and volatility

Diversity of the gut microbiome is often used as an index for health, with an increase in overall diversity and species richness being a good indicator of health and a reduction or lack of diversity often signalling disease (Shreiner et al., 2015). However, there is no consensus in the literature regarding whether or how the gut microbiota richness and diversity changes in human depression and preclinical models (Barandouzi et al., 2020; Simpson et al., 2021). In the present study, neither CRS nor LI-rTMS induced changes in alpha or beta diversity parameters, or in volatility; in the literature, there is evidence for no change (Naseribafrouei et al., 2014; Zheng et al., 2016), a decrease (Kelly et al., 2016; Huang et al., 2018) and an increase in diversity (Jiang et al.,

2015). This disparity is likely due to the high individual variability in gut microbiome composition together with underpowered studies and the presence of confounders such as effect of antidepressants or other oral prescription medications. The ability to control for potential effects of diet, lifestyle and environment on the gut microbiome is an important advantage of performing animal studies, and can potentially increase the sensitivity of detecting microbiome changes that can then be validated in humans (Bear et al., 2020).

However, the timing of our study may have contributed to the lack of changes in diversity and volatility after CRS: in our studies the animals received CRS for only 2 weeks, which may not be sufficient for differences to appear. Other studies in mice performed CRS daily for 5 weeks and found more extensive changes compared to those reported here (Yang et al., 2021). rTMS treatment was also short because we applied an accelerated treatment (3x daily for 2 weeks) instead of the conventional single daily treatment for 4–6 weeks. The trend towards a reduction in volatility over time in our data suggests that more time may be required for the microbiome to change in response to environmental stressors and treatment, and our data may reflect overlapping changes due to CRS and treatment.

Despite the lack of change in overall diversity, our results identify changes in the sub-levels of two of the major phyla (Proteobacteria and Firmicutes) following CRS, similar to changes described in human patients with depression. We observed an increase in Deltaproteobacteria class of the Proteobacteria phylum, principally due to an increase in the Desulfovibrionales order. Proteobacteria have consistently been shown to be upregulated in a chronic unpredictable mild stress mouse model (Tian et al., 2019) and in patients with depression (Barandouzi et al., 2020; Cheng et al., 2020; Simpson et al., 2021), matching our findings.

4.2. CRS and LI-rTMS increase abundance of Firmicutes

The most significant changes in our study were in the Firmicutes which is one of the most abundant phyla in the microbiome of rats and humans and have been linked to a wide range of human health conditions including obesity and mental illness (Barandouzi et al., 2020). Several studies have shown that patients with depression have altered abundance of Firmicutes, but results remain inconsistent: both increases and decreases in the overall abundance of Firmicutes have been reported
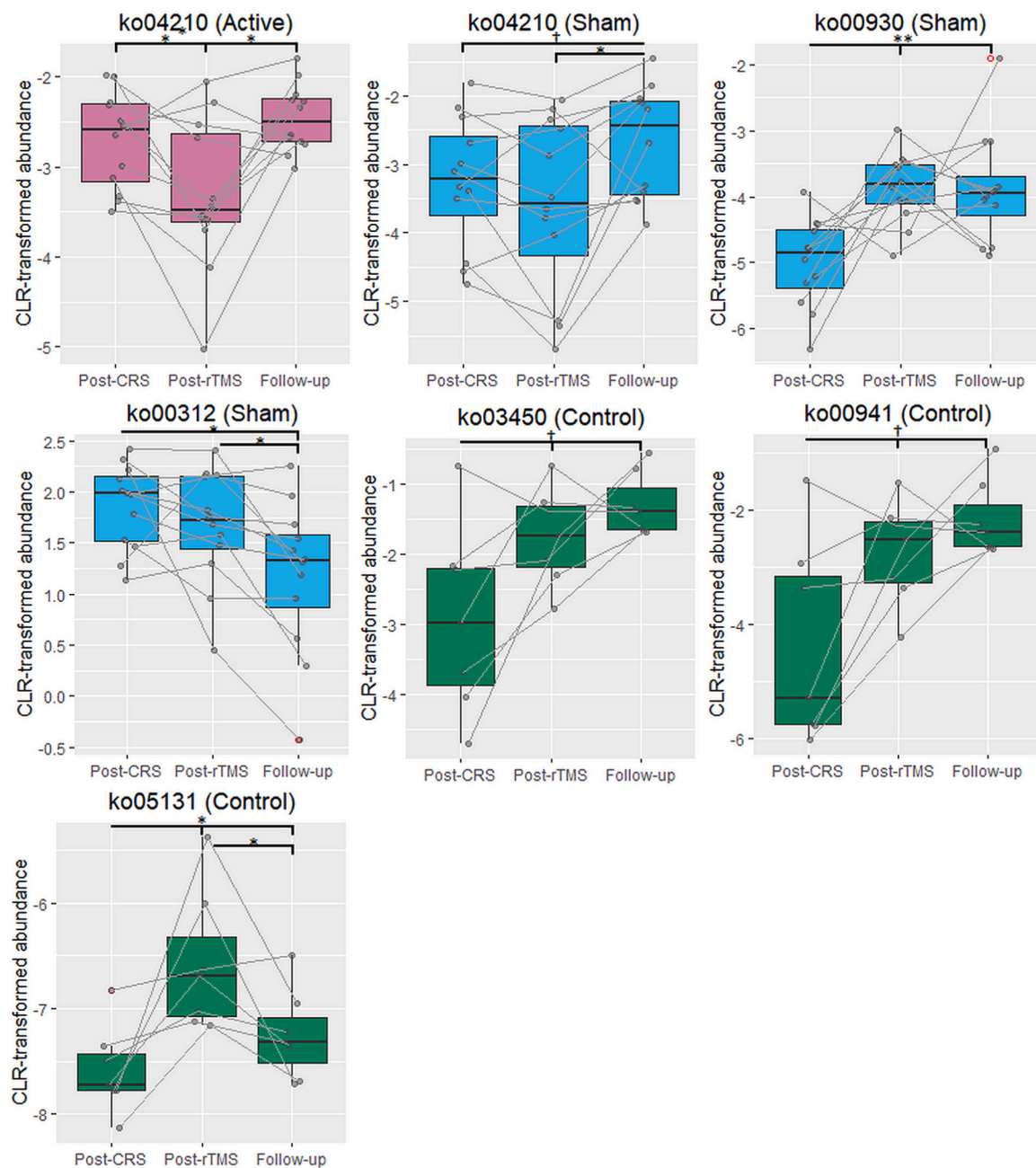
Fig. 4. Changes in KEGG pathways between timepoints within the active, sham and depression control groups. The figure shows boxplots of centered log-ratio transformed KEGG pathway abundances for pathways identified as significant in the ANCOM's W-statistic (threshold of 0.7). Quade test was used to determine if there was a significant effect of timepoint (post-CRS, post-rTMS and follow-up) in each group. Between-timepoint comparisons were made by *Post hoc* Wilcoxon signed rank test with Benjamini-Hochberg FDR correction. †p < 0.1; *p < 0.05; **p < 0.01.

in patients with depression (Barandouzi et al., 2020; Capuco et al., 2020). Other analysis approaches such as the Firmicutes/Bacteroidetes ratio have been related to several neurological diseases and inflammatory conditions (Zhang et al., 2021), including obesity (Crovesy et al., 2020), autism spectrum disorders (Coretti et al., 2018) and inflammatory bowel diseases (Stojanov et al., 2020), with a reduced ratio associated with improvement. However more recent studies have highlighted the difficulty in associate the Firmicutes/Bacteroidetes ratio with health status, not only because of the challenges of controlling for confounders (Magne et al., 2020), but also because of the variation of this ratio across the human lifespan (Mariat et al., 2009).

Despite the lack of change in overall diversity, we observed differences between groups in abundance of specific sub-taxa. Members of the

Lachnospiraceae family (Clostridia class: *Anaerostipes* and *Frisingicoccus genera)* significantly increased following CRS, supporting previous findings that link increased Firmicutes with pro-inflammatory processes also implicated in depression (Huang et al., 2018; Barandouzi et al., 2020). However, the changes in Firmicutes that occurred in the weeks following CRS are more difficult to interpret. The most consistent changes were detected in the control group, with downregulation of the *Lachnospiraceae* family, suggesting a spontaneous decrease in the effects of CRS over time once the restraint intervention was stopped. However, the decreases were not observed in the active and sham groups, suggesting that handling the rats during LI-rTMS procedures had a significant impact on their microbiome, potentially sustaining the effects of CRS. While we cannot rule out that bacteria were directly transmitted
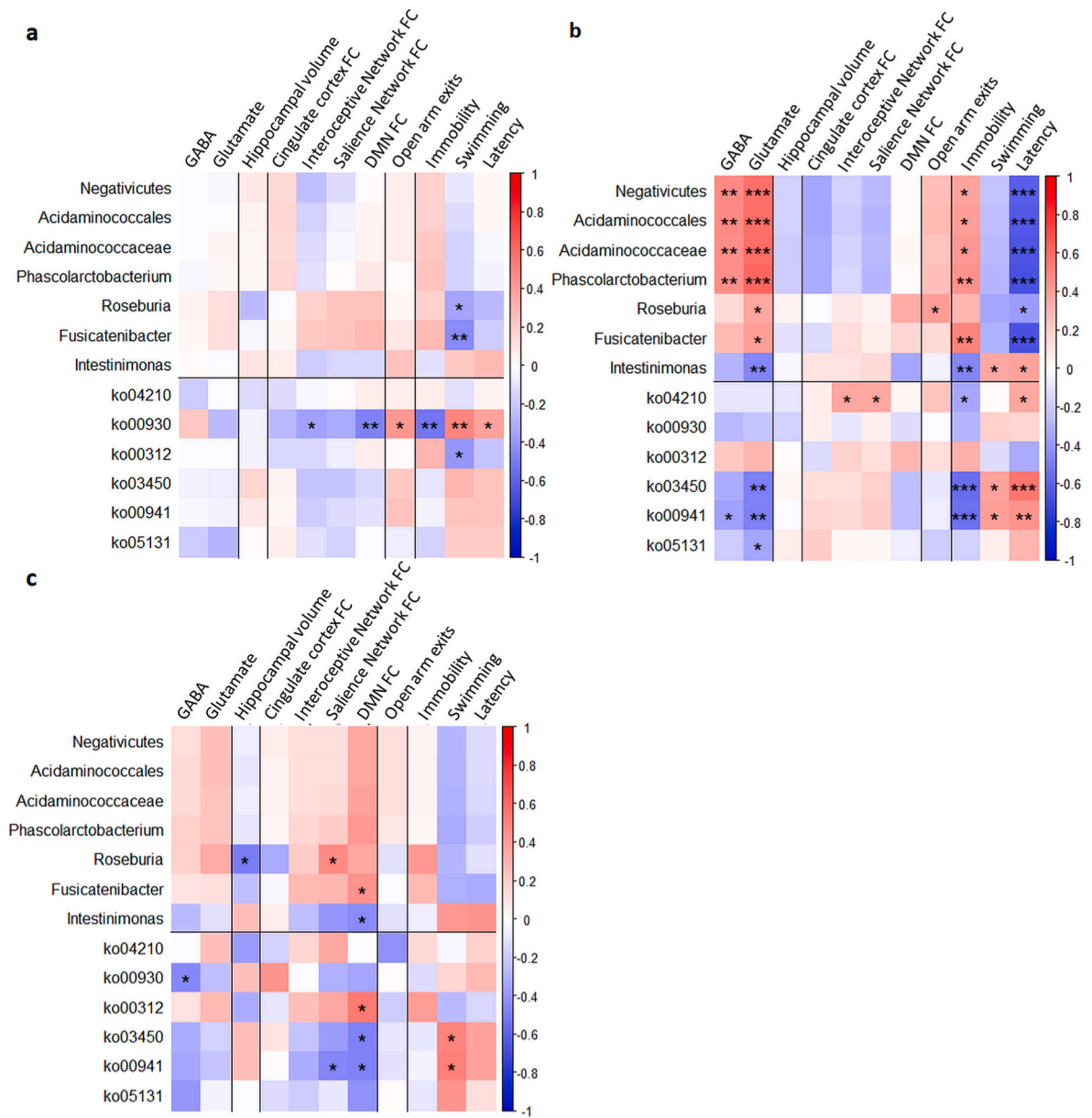
Fig. 5. Correlations between microbiome data and MRI and behavioural measures. Centered log-ratio transformed abundances of taxa and KEGG pathways for taxa and pathways identified as significant in the ANCOM's W-statistic (threshold of 0.7) were correlated to the following parameters by Spearman correlations: GABA/tCr and Glutamate/tCr ratios from 1H-MRS data; hippocampal volume; connectivity (average z-scores) of the cingulate cortex, interoceptive network, salience network and default mode network from the rs-fMRI data; number of exits from the open arms from elevated plus maze data; and time spent immobile (Immobility) and swimming, and latency time to exhibit first immobility behaviour from forced swim test data. *p < 0.05, **p < 0.01, ***p < 0.001 (no multiple comparison correction).

from the researchers to the rats, this is unlikely because of the strict physical containment and personal protection equipment protocols in place in the animal care facility (PC2). Rather, handling has been shown to have significant effects on rodent behaviour and wellbeing, most often reducing anxiety (Schmitt and Hiemke, 1998). However, some studies have shown that handling may have different outcomes depending on the animal's experiences and environment (Pritchard et al., 2013). It is therefore possible that the handling associated with delivering active and sham LI-rTMS sustained the stress induced by CRS.

While the data suggest that the handling component of rTMS is primarily responsible for maintaining high abundance of Firmicutes genera in the weeks following CRS, an increase in *Roseburia* genus only in the rTMS group identifies a specific effect of rTMS. In contrast to other Firmicutes genera, *Roseburia* is considered a beneficial bacterium that

has a positive impact on the immune system: the flagellar system of *Roseburia intestinalis* reduces intestinal inflammation by suppressing IL-17 in the host (Zhu et al., 2018), and its abundance has been reported to decrease under various disease conditions (Patterson et al., 2017; Tamanai-Shacoori et al., 2017). However, recently, a higher abundance of *Roseburia* has also previously been linked to changes in gut microbiome in mice and to cerebral hypometabolism (Sanguinetti et al., 2018). Interestingly, previous studies of the gut microbiome following brain stimulation (using different protocols in patients with weight disorders) have shown an increase in Clostridia, the class to which *Roseburia* belongs, and these were interpreted as being anti-inflammatory and therefore beneficial (Artifon et al., 2020; Ferrulli et al., 2021), although different taxonomic sub-levels were involved compared to our study. Our study highlights that it is difficult to

interpret changes in bacteria at the phyla level because taxa within the Firmicutes phylum have different functions.

4.3. Functional and pathway implications of microbiome changes

Although we found no changes in KEGG pathways following CRS, the characteristics of the different bacterial sub-taxa can suggest mechanisms whereby microbial changes might contribute to depression after chronic stress. Notably, genera in the Desulfovibrionales order are sulphate reducing and have been implicated in gastrointestinal inflammation through hydrogen sulphide accumulation in the gut (Dordević et al., 2021). In addition, the expression of lipopolysaccharides on the outer cell membrane of this order are thought to stimulate a host immune response through pro-inflammatory receptor stimulation and cytokine release (Hakansson and Molin, 2011). An extensive body of literature supports the association between depression and a chronic low-grade inflammatory response (Berk et al., 2013) and therefore, a higher abundance of these pro-inflammatory bacteria may play a role in development and/or maintenance of depression (Barandouzi et al., 2020; Kunugi, 2021).

Only one KEGG pathway was significantly altered following active LI-rTMS: apoptosis was decreased following treatment but returned to post-CRS levels at the 2 week follow up. Interestingly, anti-apoptotic effects of LI-rTMS have been reported in the brain: LI-rTMS has been shown to decrease genes related to apoptosis and inflammation (Grehl et al., 2015; Clarke et al., 2021) and when delivered at higher intensities promotes survival signalling pathways after a stroke (Caglayan et al., 2019). rTMS also increases the pro-survival factors such as BDNF, not only in the brain but also peripherally in serum (Makowiecki et al., 2014; Niimi et al., 2016; Heath et al., 2018; Feng et al., 2019). Taken together, our data showing a decrease in the apoptotic pathway and an increase in the anti-inflammatory *Roseburia* genus, specifically following active LI-rTMS, suggest that this brain-specific treatment may reduce inflammation and dysbiosis in the gut. It will be important to determine in future studies whether the impact of TMS on the microbiome is due to vagal nerve activation, or if circulating factors such as cytokines and/or BDNF are involved.

4.4. Correlations of microbiome with behaviour and MRI

Using data collected from the same animals and published in previous studies (Seewoo et al., 2019, 2021), we correlated behavioural and MRI outcomes with the sub-taxa of interest identified in our current analysis. A key finding was that in the active LI-rTMS group, *Fusicatenibacter* and *Roseburia* were negatively correlated with swimming behaviour in the FST. These genera are both members of the *Lachnospiraceae* family which has been consistently negatively correlated with symptoms of depression in humans (Vacca et al., 2020). Correlation of KEGG pathways with behavioural and MRI outcomes identified only the caprolactam pathway in active LI-rTMS (Rampelli et al., 2020). The caprolactam pathway is involved in the degradation of carbon compounds found in man made products such as nylon, synthetic fibres and plasticisers. Increases in the caprolactam pathway are interesting because they have been associated with longevity in humans, and high levels of caprolactam metabolising bacteria may provide a protection against toxins in the environment. Environmental toxins such as plastics and synthetic organic compounds have an impact on mental health (Daniel et al., 2020; Minatoya and Kishi, 2021; Schirmer et al., 2021) and our results raise the possibility that LI-rTMS may facilitate the growth of micro-organisms that can breakdown these compounds, potentially provide a protective effect. It will be important in future studies to determine whether the bacterial changes identified in this study actively contribute to the changes in behaviour, or are merely a consequence of the different interventions.

4.5. Study limitations and future directions

The current study has several limitations. Firstly, we did not include a control group of rats that had no intervention (ie. Non-CRS and non-rTMS group). While this group would have been useful to track the spontaneous changes in gut microbiome over the 6 weeks of the study, our aims and statistical approach were to compare animals longitudinally over time to explore how the microbiome changes over time in individual animals in response to CRS and rTMS. This longitudinal approach is useful for understanding individual changes that are likely to occur in humans. Secondly, only male rats were utilised in this study, as the CRS model is most reliable in male Sprague Dawley rats. Given that there are strong sex differences in depression aetiology and treatment and in the gut-brain axis, future studies should expand the applicability of present results by investigating microbiome changes in female rats (Eid et al., 2019; Holingue et al., 2020). Thirdly, the rats in this study were young adults. Age has been shown to have a significant effect on the composition of the gut microbiome (Agans et al., 2011; Radjabzadeh et al., 2020). Six months or older rats that have fully completed cerebral development (Mengler et al., 2014) could be used in future studies to exclude potentially confounding effects of brain-maturation with age. Lastly, avoiding the effects of co-housing animals is difficult. While animals in the same treatment group were co-housed in the present study to avoid microbial transfer between study groups, the fact that co-housed animals tend to have more similar microbiome could mask or exaggerate the results.

We also acknowledge the widely recognised challenges and limitations of modelling and measuring depression-like behaviour in rats: depression is diagnosed in humans based on subjective measures that are difficult to evaluate in rats. In choosing our behavioural tests, we selected tests that are considered the gold standard in the literature (EPM and FST) and test for anxiety and learned helplessness/stress responses respectively (Seewoo et al., 2020). Although performing additional tests could potentially provide more information about the models and interventions, we believe that our design represents an acceptable trade-off that optimises the quality of the data without redundancy (Cryan and Mombereau, 2004; Shoji et al., 2016) or "over testing" that could increase stress levels and compromise outcomes.

4.6. Conclusion

In conclusion, this study is the first of its kind to investigate the effects of CRS and LI-rTMS treatment on the gut microbiome longitudinally in male Sprague Dawley rats. Specifically, we found that chronic stress induced gut microbial dysbiosis, which was associated with high levels of anxiety and depression as well as brain changes. Our results after CRS highlight that although handling had a major impact on microbiome composition, there was evidence supporting a top-down effect of rTMS on the gut microbiome, with the main outcome being an increase in abundance of the *Roseburia* genus. The change in the microbiome was observed only in the LI-rTMS group, and not in the sham or no-handling control groups, suggesting that it was a consequence of altering brain activity with LI-rTMS. However, it remains unclear whether the increase in *Roseburia* is a direct effect via stimulation of neural pathways that connect the brain to the gut, or whether it is an indirect effect, resulting from the behavioural (mood) improvements. Given that *Fusicatenibacter* abundance was also significantly correlated with behaviour, but was not significantly altered by rTMS at the group level, and that handling alone had a significant impact on the microbiome, it is likely that there is complex feedback between microbiome, behaviour (including stress levels) and brain function: future studies should investigate faecal transplants and vagal nerve transection interventions to further explore the interplay of the gut brain axis components with behaviour and mood.

CRediT authorship contribution statement

Bhedita J. Seewoo: Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Eng Guan Chua:** Data curation, Formal analysis, Methodology, Visualization, Writing – review & editing. **Yasmin Arena-Foster:** Data curation, Investigation, Writing – original draft, Writing – review & editing. **Lauren A. Hennessy:** Investigation, Methodology, Project administration, Writing – review & editing. **Anastazja M. Gorecki:** Data curation, Investigation, Methodology, Writing – review & editing. **Ryan Anderton:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. **Jennifer Rodger:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The author(s) declare no competing financial and/or non-financial interests in relation to the work described.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2022.100430.

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