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Repeated mild traumatic brain injury affects microbial diversity in rat jejunum

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Traumatic brain injuries (TBI) manifest into post-traumatic stress disorders such as anxiety comorbid with gut ailments. The perturbations in gut microbial communities are often linked to intestinal and neuropsychological disorders. We have previously reported anxiety and abnormalities in gut function in mild TBI (MTBI)-exposed rats. The current study demonstrates the changes in gut microbiome of MTBI-exposed animals and discusses its implications in intestinal health and behaviours. The rats were subjected to repeated MTBI (rMTBI) and microbial composition in jejunum was examined after 6 h, 48 h and 30 days of rMTBI. Significant reduction in bacterial diversity was observed in the rMTBI-exposed animals at all the time points. Principal coordinate analysis based on weighted UniFrac distances indicated substantial differences in gut microbial diversity and abundances in rMTBI-exposed animals as compared to that in healthy controls. The abundance of Proteobacteria increased dramatically with reciprocal decrease in Firmicutes after rMTBI. At the genus level, *Helicobacter, Lactobacillus, Campylobacter,* and *Streptococcus* were found to be differentially abundant in the jejunum of rMTBI-exposed rats as compared to sham controls indicating profound dysbiosis from the healthy state. Furthermore, substantial depletion in butyrate-producing bacterial communities was observed in rMTBI-exposed animals. These results suggest that the traumatic stress alters the gut microbiome with possible implications in gut health and neuropsychopathology.

Keywords. Gut microbiota; gut–brain axis; mild traumatic brain injury; Proteobacteria; jejunum; 16S rRNA amplicon

1. Introduction

The advent of recent high-throughput sequencing technologies led to gain deeper insights into the understanding of various host–microbe interactions. On similar lines, studies on gut microbiota have attracted considerable attention in recent years, with a special emphasis on its impact on multiple aspects of host physiology. Gut microbiome plays an integral role in host metabolism, homeostasis maintenance, nutrition, and immune function (Tremaroli and Bäckhed 2012; Round and Mazmanian 2009). These microbes synthesize vitamins, amino acids, and metabolites such as short-chain fatty acids (SCFA; Bull and Plummer 2014). Therefore, characterization of the gut microbial communities holds paramount importance in understanding the host–microbe interplay in health and disease. Recently, a growing body of evidence linked perturbations in the gut microbiome to various metabolic and comorbid neuropsychiatric disorders such as autism, depression, schizophrenia, obesity, type 2 diabetes mellitus, rheumatoid arthritis, etc. (Grochowska *et al.* 2018; Shreiner *et al.* 2015).

The bacteria associated with the human host are reported to be of the same order as the count of human cells (Sender *et al.* 2016). The consortium of bacteria colonising the gut play a crucial role in regulating the gut–brain axis; hence it is also referred as gut–microbiome–brain axis (Cenit *et al.* 2017). The comorbidity of stress-induced neuropsychiatric disorders and gastroenteric ailment implicate the importance of bidirectional communication between the gut–brain axis in pathophysiology of stress (Carabotti *et al.* 2015). Disruption of the gut–microbiome–brain axis is recently reported in diseases such as irritable bowel syndrome (IBS), autism spectrum disorders, Parkinson's disease and disorders of mood, anxiety and chronic pain (Mayer *et al.* 2015). Specifically, alterations in gut microbiome have been associated to dysregulation of microglial activity, blood–brain

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barrier disruption along with impairment of neuropsychiatric activities leading to anxiety, abnormal behaviour and cognition (Logsdon *et al.* 2018; Cenit *et al.* 2017).

Tannock and Savage in 1974, have suggested that neuropsychological stress induced dysregulation of the hypothalamic–pituitary axis can influence gut microbiota (Tannock and Savage 1974). Traumatic brain injuries (TBI) including concussions result in psychological and physiological disturbances in the clinical population (Karr *et al.* 2014). The TBI is commonly found comorbid with abnormalities in gut functions such as gut motility, permeability, and inflammation (Mayer 2000; Drossman 2011). Gastrointestinal dysfunction is one of the most common, but neglected consequences of TBI (Kharrazian 2015; Zhu *et al.* 2018). Abnormalities in gut motility and mucosal alterations are frequently observed in TBI patients that can lead to ulceration and inflammation (Kao *et al.* 1998; Hang *et al.* 2003). According to a recent survey, 85% of TBI are characterized as MTBI graded on Glasgow Coma scale (Li *et al.* 2016). Usually the symptoms of MTBI resolve within 3 weeks of trauma; however, a few patients experience post-concussion syndrome (McInnes *et al.* 2017).

Weight drop on closed head induces MTBI in rodents and cause anxiety, depression and cognitive deficits similar to post concussion syndrome (Meyer *et al.* 2012; Mychasiuk *et al.* 2014; Zohar *et al.* 2003; Sagarkar *et al.* 2017a). Our previous studies showed that the repeated MTBI (rMTBI) induces anxiety-like behaviour in rats and decreases gut motility, specifically in the jejunal part of the gut (Sagarkar *et al.* 2017a, b). However, the perturbation in the microbial population colonizing the jejunum post-MTBI remains mostly unknown. The current study is aimed to investigate the changes in the microbial communities in jejunal mucosa post-MTBI, which may further help in understanding the microbial contribution to the imbalance in the gut–brain axis after physical traumatic stress. In this study, the rats were subjected to rMTBI at immediate and protracted time points, and changes in their gut microbiota composition were tracked. The changes in microbial communities at different levels of their classification and diversities are discussed in view of gut–brain pathophysiology post-rMTBI as previously reported (Sagarkar *et al.* 2017a, b, 2019).

2. Materials and methods

2.1 *Animal experiments*

Adult (75–90 days old) male Wistar rats weighing 200–225 g were housed under controlled light (12 h of light, followed by 12 h of darkness) and temperature (25 ± 2 °C) conditions. All protocols employed in the present study were carried out in accordance with National Institutes of Health (NIH), USA Guidelines under the strict compliance with Institutional Animal Ethics Committee (IAEC), Savitribai Phule Pune University, Pune, India. Food and water were provided ad libitum during the course of the study.

2.2 *rMTBI experiments*

The closed-head weight drop paradigm was used to simulate MTBI in rats as described previously (Sagarkar *et al.* 2017a, b). Briefly, the trauma simulator has a hollow guide tube (100 cm) with an inner diameter of 15 mm and a cylindrical metal weight of approximately 200 g that results in a contact area of 5–7 mm^2 on the skull surface. The rats were mildly sedated using diethyl ether and placed on a platform made of aluminium foil. The weight was dropped on the intact skull, from the height of 30 cm by releasing the key. The drop was made at the intersection of lines connecting ears and eyes from opposite sides of the skull to ensure uniform impact on the head. The animals from trauma-induced group were subjected to 5 impacts with a recovery time of 48 h between each injury. The sham control (n=2; C1MTBI and C2MTBI) animals were not subjected to head trauma but underwent the rest of the procedures, including anaesthesia. The animals were allowed to recover from anaesthesia (2–3 h) and righting reflex, beam walking tests were performed. Animals from each of the three MTBI groups (n=2 per group) were decapitated at different time points; i.e. 6 h (6h1MTBI, 6h2MTBI), 48 h (48h1MTBI, 48h2MTBI) and 30 days (30d1MTBI, 30d2MTBI) post-trauma under deep anaesthesia induced by intraperitoneal (IP) injection of thiopentone sodium (60 mg/kg, Neon Laboratories Ltd, Mumbai). Before sacrificing, animals were deprived of food for 6 h to empty portions of the gastrointestinal tract. Rat jejunum samples were carefully dissected from the rest of the small intestine and stored at −80 °C until further use. The jejunums were thawed at room temperature; the jejunal mucosa was collected by scrapping the internal lining after longitudinally cutting the jejunum in aseptic conditions. The animal experimental paradigm is summarized in figure 1.

2.3 *Sample processing and DNA extraction*

DNA was isolated from jejunum scrapings of animals using Fast DNA spin kit for faeces (6570-200, MP bio, USA) as per manufacturer's instructions. Further, the qualitative and quantitative analysis was performed using Nanodrop 2000C spectrophotometer (Thermo Scientific, USA) (supplementary table 1).

2.4 *Amplicon sequencing and bioinformatics analysis*

The amplicon libraries were prepared using the Nextera XT index Kit as per 16S amplicon library preparation protocol by Illumina Inc. Amplification of the V3–V4 region of the

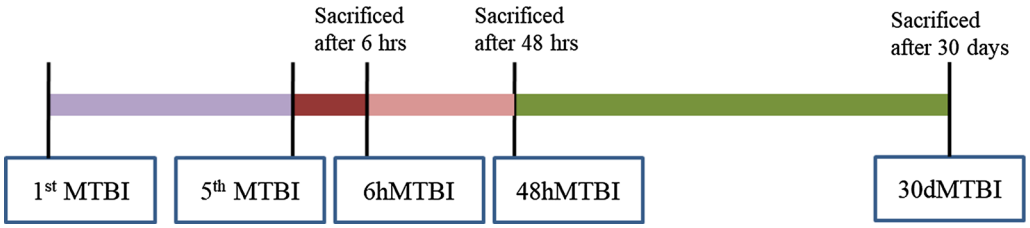


Figure 1. Experimental paradigm explaining the time course of study. The figure summarizes the sacrifice time points of each group post repetitive MTBI series.

16S rRNA gene was carried out and PCR products were resolved on 1.2% agarose gel. The amplicons with the Illumina adaptors were amplified using i5 and i7 primers as per standard Illumina protocol. The amplicon library was purified by 1X AMPure XP beads and quantified by Qubit fluorometer. The amplified libraries were analysed on 4200 Agilent Tape Station system (Agilent Technologies) as per the manufacturer's instructions. The samples were sequenced using Illumina MiSeq with 2*300 paired-end chemistry that generates at least 0.1 million reads per sample. The primers used for V3–V4 hypervariable region amplification are as follows: Forward primer 5′ GCCTACGGGNGGCWGCAG 3′ and reverse primer 3′ ACTACHVGGGTATCTAATCC 5′. After sequencing, the quality of raw paired-end reads was checked by FASTQC tool (Andrews 2010) and pre-processing of sequences (primers and barcodes trimming) was performed using Cutadapt (Martin 2011). Trimmed sequences were assembled by PEAR with default parameters (Zhang *et al.* 2014). Microbiome_helper package was used for pre-processing of the data such as trimming, chimera removal and conversion of Fastq to Fasta files (Comeau *et al.* 2017). The pre-processed Fasta sequences were merged into a single Fasta file using QIIME v1.8 (Quantitative Insights Into Microbial Ecology) (*http://qiime.org/*) (Caporaso *et al.* 2010). Sequence reads were assigned to operational taxonomic units (OTUs) by using a reference-based OTU picking approach with Greengenes database (DeSantis *et al.* 2006). The OTU picking was performed using UCLUST method with a similarity threshold of 97% (Kopylova *et al.* 2016). The RDP naïve Bayesian classifier (version rdp_classifier_2.10.1) (Wang *et al.* 2007) was used for taxonomic assignment. Using QIIME pipeline, alpha and beta diversity analysis was performed. Alpha diversity indices, i.e., ACE, Chao1, Simpson, Shannon index and Goods coverage were estimated using QIIME pipeline. Data normalization was carried out at 99,138 reads per sample, at the lowest sequence count. Beta diversity was also calculated within the QIIME pipeline using weighted and unweighted UniFrac distances (Lozupone *et al.* 2011). From these estimates, jackknifed Principal Co-ordinates (PC) were computed to compress dimensionality into three-dimensional principal coordinate analysis (PCoA) plots. Further statistical analysis was performed using in-house R scripts and R package. The raw reads have been deposited in the sequence read archive (SRA) and are available under the BioProject ID PRJNA51195.

3. Results

High-throughput sequencing resulted in generation of approximately 3 million raw reads from eight samples with average 368,869 ± 50,449 reads per sample. After assembly of paired-end reads, 2.3 million reads were retained from all the samples. Finally, 1,362,785 high-quality sequences were used for taxonomic assignment resulting in 2005 operational taxonomic units (OTUs) in all samples (supplementary table 2). Good's coverage for all the control and trauma samples was found to be 0.998 ± 0.001 (mean ± SD) indicating that the majority of the bacterial diversity was captured in all the samples. Alpha diversity measures such as Chao1, observed and Shannon indicated significant diversity reduction in rMTBI samples as compared to sham controls (table 1; figure 2). A linear decrement in diversity was observed with increase in days post-rMTBI implying that rMTBI decreases bacterial diversity in a time-dependent manner. A similar observation was made using rarefaction curve which represents number of OTUs as a function of number of sequences in the samples (supplementary figure 1). Species richness was also found to be higher in control samples as compared to rMTBI samples with lowest species richness post 30 days (figure 3).

Beta diversity analysis using PCoA ordination plots based on weighted and unweighted UniFrac metrics considering all

Table 1. The following table depicts alpha diversity measures in rats from different groups (C1MTBI, sham control; C2MTBI, sham control; 6 h 1MTBI, MTBI-6h; 6 h 2MTBI, MTBI-6h; 48 h 1MTBI, MTBI-48h; 48 h 2MTBI, MTBI-48h; 30 days 1MTBI, MTBI-30 days; 30 days 2MTBI, MTBI-30 days)

Sample ID	Observed species	Chao1	Shannon	Simpson
C1MTBI	836	1189.33	5.00	0.92
C2MTBI	608	929.31	5.65	0.95
6h1MTBI	496	691.66	4.44	0.91
6h2MTBI	302	436.12	3.91	0.88
48h1MTBI	516	745.52	4.50	0.90
48h2MTBI	437	665.80	4.10	0.84
30d1MTBI	307	549.00	3.64	0.82
30d2MTBI	358	662.94	4.26	0.89

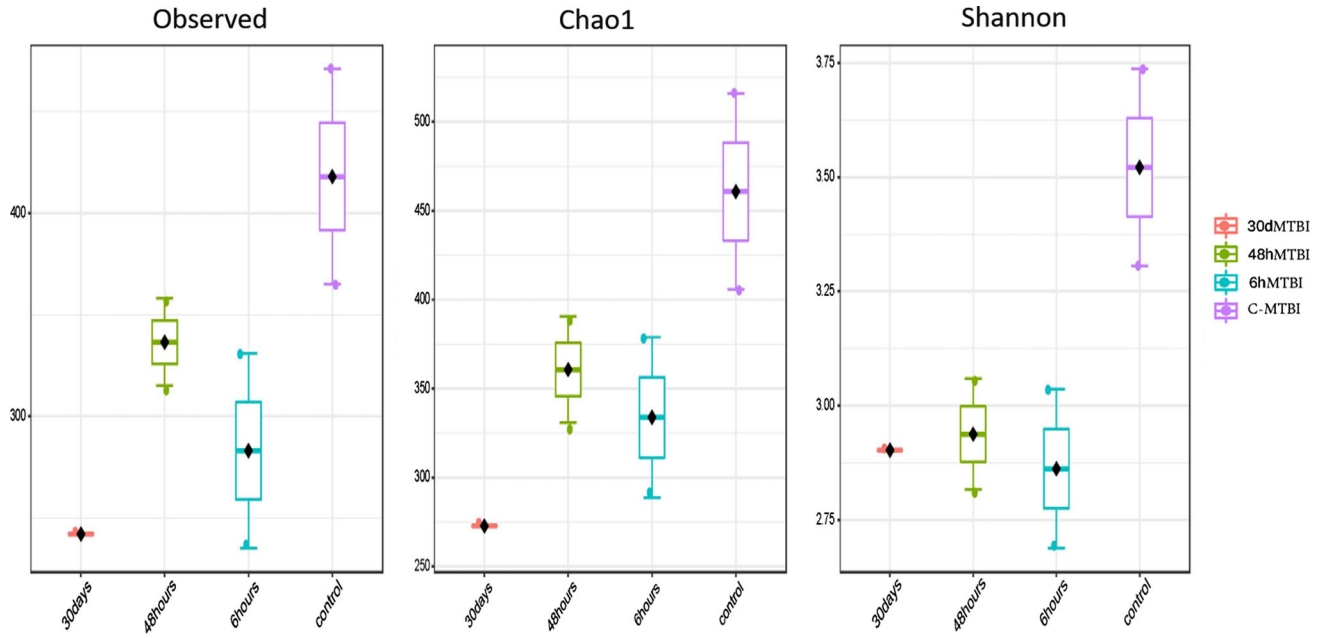


Figure 2. Box plot of alpha diversity measures viz. Observed species, Chao1 and Shannon index in trauma and control samples. 30 days, 48 h, 6 h represents time points after MTBI. Observed species, Chao1 and Shannon index showed substantial differences in microbial diversity between trauma and control samples.

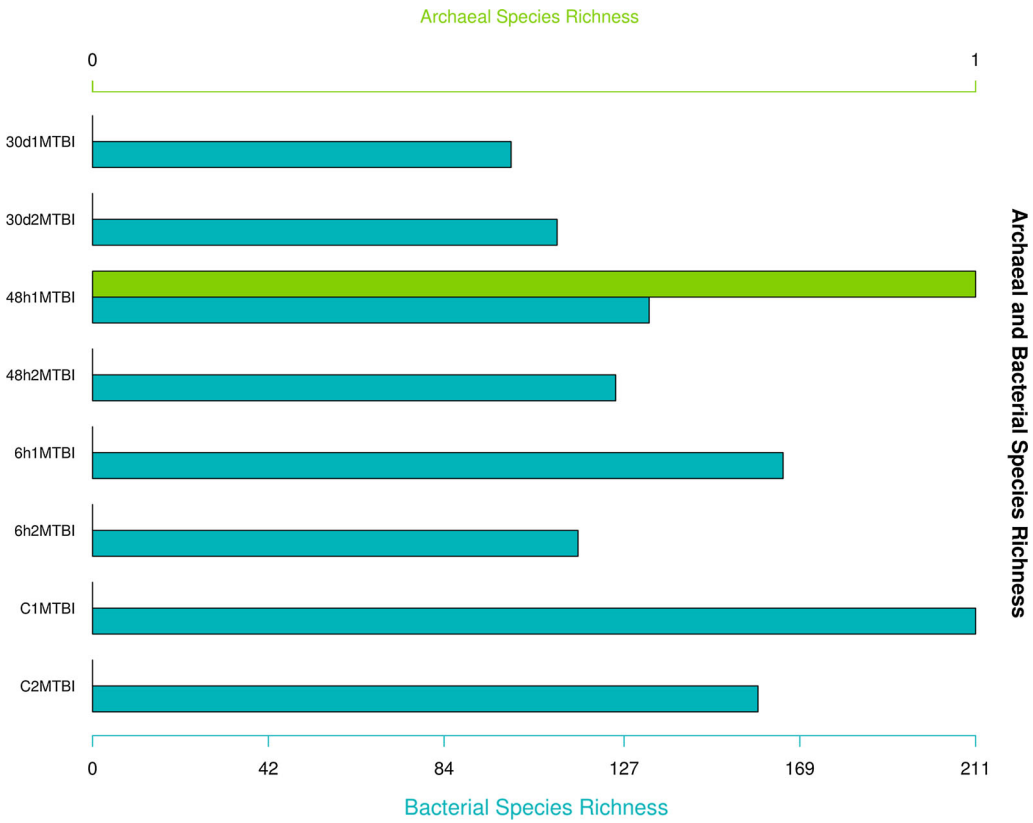


Figure 3. Bar chart representing total bacterial and archaeal species richness across all samples.

the OTUs demonstrated that samples from control and rMTBI groups segregated into distinct clusters based on their abundances and presence/absence of OTUs (figure 4). Control samples formed a separate cluster showing less interindividual variation between the samples. However, rMTBI samples showed higher interindividual variations.

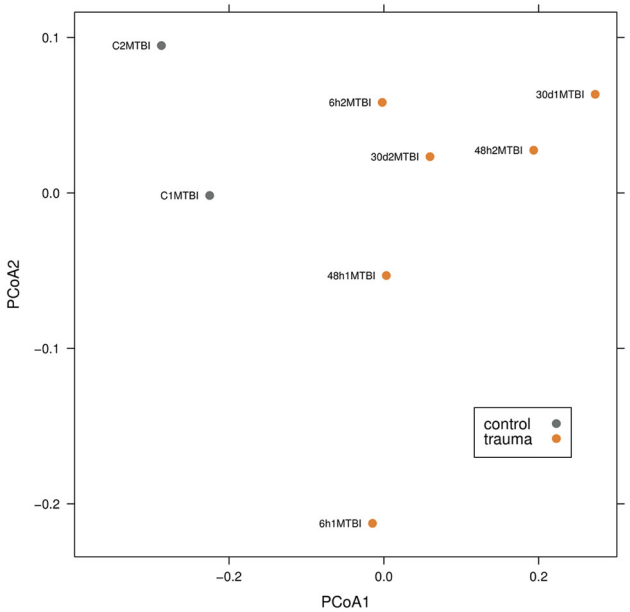


Figure 4. Principal coordinate analysis (PCoA) based on weighted UniFrac metrics among trauma and control samples. Proportion of variance explained by each axis is denoted in the corresponding axis labels. Each circle (designated by the color) represents control and trauma group and labels represent sample IDs.

Analysis suggests immediate changes in the gut microbiota after rMTBI (6 h) and the perturbation with reduction in bacterial diversity was persistent until 30 days.

A total of 15 bacterial phyla were detected in all the samples. Bacterial phyla Proteobacteria (57.2%), Firmicutes (33.4%), Tenericutes (4.43%) and Fusobacteria (2.3%) were most abundant in rMTBI samples and constituted for 97% of the total microbiome in rMTBI group. Similarly, Firmicutes (64.1%), Proteobacteria (27.8%), Bacteroidetes (3.1%), TM7 (2.1%) and Actinobacteria (1.2%) were most abundant in sham controls and constituted for 98.4% of the total microbiome (supplementary table 3). The relative abundances of top 10 phyla and top 30 genera across all samples were calculated and the results are represented in figure 5a and b respectively. Figure 5a indicates waxing and waning of abundance of Proteobacteria and Firmicutes in rMTBI samples respectively, as compared to controls. A total of 11 genera were detected in MTBI samples and 10 genera were detected in control samples with relative abundance above 1% (supplementary table 4). Moreover, the most abundant genus in rMTBI exposed animals was *Helicobacter* (29.2%) followed by *Lactobacillus* (21.7%) and *Campylobacte*r (17.7%), while in controls the most abundant genus was *Lactobacillus* (40.3%) followed by *Helicobacter* (11.5%) and *Streptococcus* (9.8%) (figure 5b). We observed a gradual increase in relative abundance of genus *Helicobacter* (29.2% ± 4.9) in trauma samples from 6 h to 30 days, but decrease in abundance of the genus *Lactobacillus* was independent of time post-trauma (21.7% ± 12.3). Overall, a substantial reduction in the relative abundance of *Lactobacillus* followed by *Streptococcus* and an increase in the abundance of the *Helicobacter* and *Campylobacter* was observed in the rMTBI group.

A hierarchical clustering using ward method was employed to analyse the clustering between the rMTBI groups with microbial abundance at phylum (figure 6a) and family level (figure 6b). The hierarchical clustering showed clear separation between rMTBI and control samples, but no clear separation was observed within distinct rMTBI groups, i.e. 6 h-MTBI, 48 h-MTBI, and 30 days-MTBI. Firmicutes, Actinobacteria, and TM7 were found to be abundant in control rats, whereas Proteobacteria was abundant in rMTBI-exposed rats (figure 6a). At family level, *Campylobacteraceae* and *Helicobacteraceae* were most abundant in rMTBI-exposed rats, especially post 30 days of rMTBI. On the contrary, these bacterial families were least abundant in the jejunum of control rats. Similarly, families such as *Actinomycetaceae, Erysipelotrichaceae,* and *Lachnospiraceae* were abundant in control samples as compared to rMTBI samples (figure 6b). Discriminant Analysis was conducted to find the biomarkers associated with trauma (figure 7). The linear discriminant analysis predicted *Campylobacteraceae, Helicobacteraceae, Mycoplasmataceae,* and *Aerococcaceae* as the biomarkers of trauma.

Based on the previous literature, members of the families *Lachnospiraceae* and *Ruminococcaceae* (Vital *et al.* 2014), *Veillonellaceae* (Esquivel-Elizondo *et al.* 2017) and *Erysipelotrichaceae* (Pozuelo *et al.* 2015) are considered as potential butyrate producers. We conducted imputed analysis on our dataset which revealed the differential abundance of members of these families as shown in figure 8. Moreover, the relative abundance of butyrate-producing bacterial families was decreased in rMTBI samples. A sharp decline in relative abundance of *Lachnospiraceae, Ruminococcaceae*, and *Erysipelotrichaceae* was seen at 6 h post-rMTBI compared to control, and such a trend was persistent up to 30 days.

4. Discussion

Increasing evidences are showing that the intestinal microbiota contributes to the host physiology and maintains homeostasis. A plethora of studies relate the microbial communities to immunity mechanisms and inflammatory challenges in the intestine. Therefore, intestinal microbiota is linked to gut pathologies such as inflammatory bowel disease and cancer (Ayres *et al.* 2012; Dupont and Dupont 2011; Le Chatelier *et al.* 2013; Yoshimoto *et al.* 2013). Not only intestinal health but in recent years the perturbations in gut microbiota have been implicated in neurodevelopmental and neurobehavioral disorders such as depression, anxiety, and autism (Hsiao *et al.* 2013; Kang *et al.* 2013). The present study characterizes the alterations in microbial communities in jejunal mucosa of the rMTBI-induced rats using high-

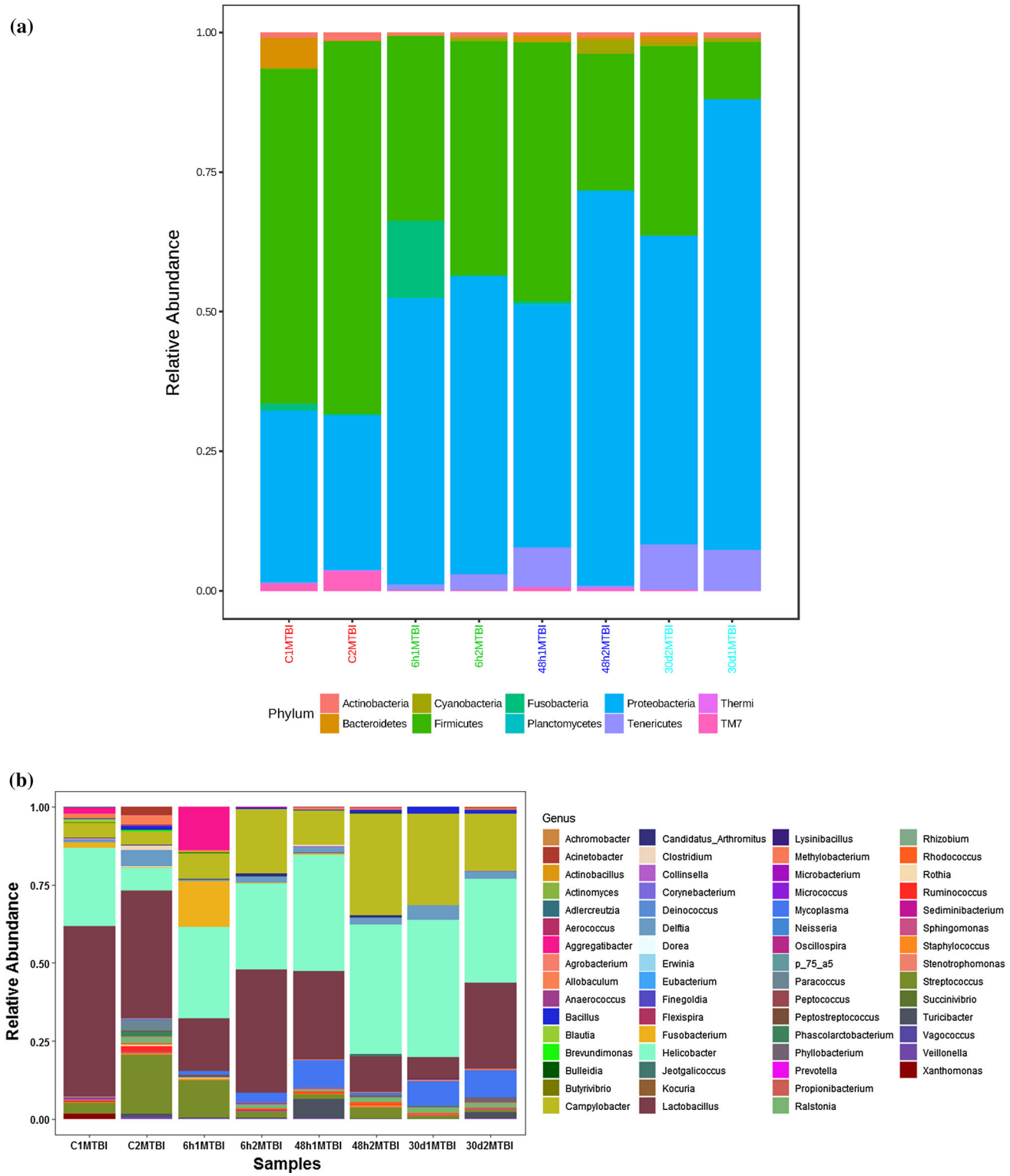


Figure 5. Bar plot showing relative abundance of bacterial taxa. (**a**) Bar plot for relative abundances of top 10 phyla between control and MTBI groups. (**b**) Bar plot for relative abundances of top 30 genera between control and MTBI groups.

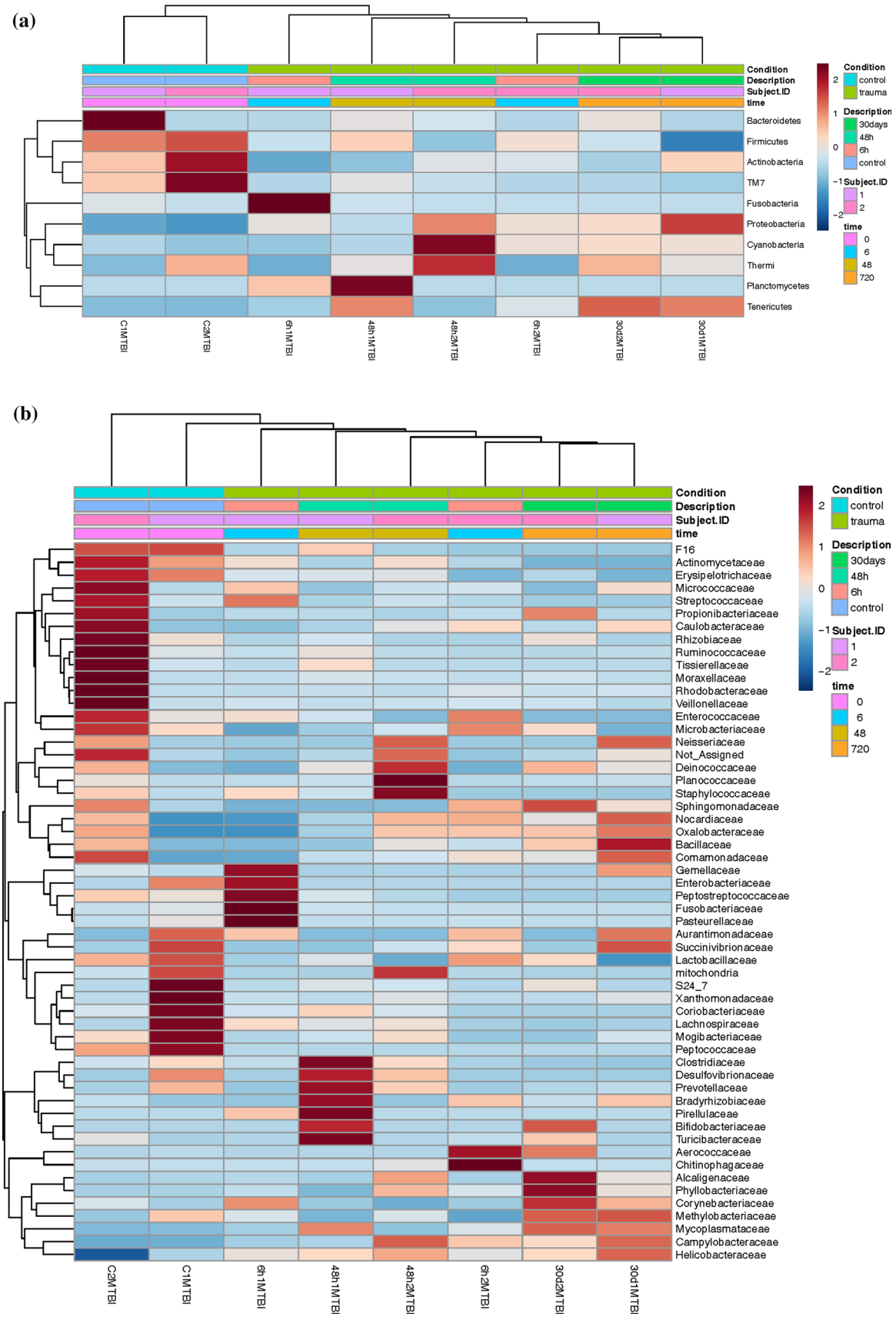
throughput targeted amplicon sequencing of the 16S rRNA gene. Although this study is based on a small cohort size, which is one of the shortcomings of our findings, the results for the first time link rMTBI to perturbations in microbial communities in jejunum as early as 6 h post-trauma, and it persisted through 48 h and continued until 30 days. Whereas rMTBI induced the increase in phylum Proteobacteria, owing to rise in the abundance of *Helicobacter* and

Campylobacter; levels of Firmicutes declined persistently from 6 h to 30 days post-trauma as compared to healthy controls.

Recently, Nicholson and colleagues detailed the effect of moderate TBI on the faecal microbiota composition and reduction in bacterial alpha diversity post-TBI, similar to what we have observed in the jejunal microbiota. Controlled cortical impact model, wherein the animals underwent craniotomy, was employed in their study (Nicholson *et al.* 2018); whereas we have applied a closed-head injury paradigm. In yet another study, dramatic changes are reported in the caecal microbiota in response to TBI in mice (Houlden *et al.* 2016). These changes were observed post 72 h of stroke in mice; similar to that found in trauma patients (Howard *et al.* 2017). We have noticed the changes in microbiome in jejunum as early as 6 h which persisted until 48 h and 30 days time points. In mice, the norepinephrine release in the gut as a consequence to TBI was suggested as causal to the perturbation in caecal microbiome (Houlden *et al.* 2016). In this connection, the findings by Singh and colleagues are noteworthy in which brain injuries due to a stroke caused gut microbial dysbiosis and reduced gastrointestinal motility in mice (Singh *et al.* 2016). Using the same rMTBI model as in this study, we have previously reported the slowing in gut motility at the level of jejunum in rats at identical time points of 48 h and 30 days (Sagarkar *et al.* 2017b). These observations suggest the concurrence between microbial dysbiosis and gut motility. However, bacterial communities belonging to Bacteroidetes phylum was overcrowded in faeces after the stroke (Singh *et al.* 2016). On the contrary, we have observed the increased abundance of Proteobacteria in the jejunum of rMTBI-exposed rats. The discrepancies in observations in these two studies could be related to (1) species differences (mice versus rats), (2) injury paradigm (stroke versus rMTBI), and (3) microbial sample (faeces versus jejunual mucosa). A recent study elucidated the link between TBI and gut bacterial dysbiosis in mice (Treangen *et al.* 2018). Although they have observed an elevation of *Eubacterium and Marvinbryantia* post brain injury, *Helicobacter* and *Campylobacter* dominated in our trauma groups. Again, these variations could be ascribed to different experimental protocols and models. Proteobacteria are known to be associated with inflammation and dysbiosis (Bäckhed *et al.* 2012). With the increase in Proteobacteria in the gut of rMTBI-exposed rats, intriguing would be to examine the inflammatory changes therein, in addition to reductions in gut motility as reported earlier (Sagarkar *et al.* 2017b).

In the similar model of rMBTI in rats, we have observed the deficits in learning and memory (Sagarkar *et al.* 2019) and expression of anxiety-like behaviours as measured by light dark box (LDB) exploration test (Sagarkar *et al.* 2017a), comorbid with reduced gastrointestinal motility (Sagarkar *et al.* 2017b) at 48 h which persisted until 30 days. The TBI has been shown to induce alterations in inflammatory responses (Chen *et al.* 2008), contractility (Olsen *et al.* 2013), motility (Smith 2013) and permeability (Bansal *et al.* 2009) in the gut. Clinically, severe TBI has also been reported to be comorbid with food intolerance due to reduced gastrointestinal motility and absorption (Kao *et al.* 1998; Tan *et al.* 2011). A growing body of evidence also associates gut dysbiosis with neuropsychiatric ailments (Bansal *et al.* 2009; Dinan and Cryan 2017; Evrensel and Ceylan 2015). Several studies have associated stress and imbalance of hypothalamic-pituitary axis with short term as well as long term modulations in the gut microbial composition (Cryan and Dinan 2012). Exposure to early life stressor such as maternal separation caused persistent alterations in the composition of faecal microbiota of adult rats (O'Mahony *et al.* 2009). In addition, germ-free mice exhibited elevated anxiety like-behaviours as compared to gnotobiotic mice, which were alleviated by monocolonization with *Blautia coccoides* (Nishino *et al.* 2013). On the contrary, reduction in the anxiety-like behaviours of germ-free mice as accessed by LDB and elevated plus maze (EPM) tests were also reported in three independent studies (Clarke *et al.* 2013; Heijtz *et al.* 2011; Neufeld *et al.* 2010). In view of the above, the gut microbial changes in rMTBI-exposed rats are expected to be causal to the intestinal pathologies such as motility and inflammation, and the psychological manifestations of the rMTBI. Therefore, future studies are warranted to discern the specific microbial communities linked to rMTBI-induced imbalance of gut–brain axis and molecular mechanisms therein. For example, the expression of brain-derived neurotrophic factor (BDNF), key synaptic plasticity regulating neuropeptide and NMDA receptor subunit 2A (NR2A) was decreased in the cortex and hippocampus of germ-free mice (Sudo *et al.* 2004). Moreover, infection of gut pathogen (*T. muris*) also reduced BDNF mRNA expression in the hippocampus precipitating into anxiety-like behaviours which were restored by *Bifidobacterium longum* administration (Bercik *et al.* 2010). On similar lines, we have previously reported the reduction in the BDNF levels in the amygdala of rMTBI-induced rats at 48 h and 30 days post-trauma. While on one hand the gut microbiome is linked with the BDNF expression and anxiety levels in mice (Sudo *et al.* 2004; Bercik *et al.* 2010); on the other hand, we have observed the trauma-induced changes in gut microbiome and BDNF expression in amygdala coincident with anxiety-like behaviours (Sagarkar *et al.* 2017a). Therefore, it may be speculated that the healthy microbial communities in jejunum might confer a neuroprotective role via regulation of neuropeptides such as BDNF; the imbalance of which may be implicated in trauma-induced neuropsychiatric behaviours.

Proteobacteria have been listed previously as a marker of gut microbial dysbiosis (Shin *et al.* 2015). According to the spatial distribution of the bacteria in the gut, the *Helicobacter* are predominantly housed in stomach (Andersson *et al.* 2008) with an extremely low abundance in the small intestine and colon (Jandhyala *et al.* 2015). However, rMTBI increased the abundance of the Proteobacteria in the jejunum


(a)
Condition
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Bacteroidetes
Firmicutes
Actinobacteria
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Proteobacteria
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Tenericutes
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Actinomycetaceae
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Micrococcaceae
Streptococcaceae
Propionibacteriaceae
Caulobacteraceae
Rhizobiaceae
Ruminococcaceae
Tissierellaceae
Moraxellaceae
Rhodobacteraceae
Veillonellaceae
Enterococcaceae
Microbacteriaceae
Neisseriaceae
Not_Assigned
Deinococcaceae
Planococcaceae
Staphylococcaceae
Sphingomonadaceae
Nocardiaceae
Oxalobacteraceae
Bacillaceae
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Gemellaceae
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Peptostreptococcaceae
Fusobacteriaceae
Pasteurellaceae
Aurantimonadaceae
Succinivibrionaceae
Lactobacillaceae
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S24_7
Xanthomonadaceae
Coriobacteriaceae
Lachnospiraceae
Mogibacteriaceae
Peptococcaceae
Clostridiaceae
Desulfovibrionaceae
Prevotellaceae
Bradyrhizobiaceae
Pirellulaceae
Bifidobacteriaceae
Turicibacteraceae
Aerococcaceae
Chitinophagaceae
Alcaligenaceae
Phyllobacteriaceae
Corynebacteriaceae
Methylobacteriaceae
Mycoplasmataceae
Campylobacteraceae
Helicobacteraceae
C2M1TBI
C1M1TBI
6h1M1TBI
48h1M1TBI
48h2M1TBI
6h2M1TBI
30d2M1TBI
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Condition
control
trauma
Description
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control
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1
2
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0
6
48
720

◀**Figure 6.** (**a**) Hierarchical clustering and heatmap visualization at phylum level using trauma and control group as experimental factors. Hierarchical clustering was based on ward linkage algorithm using Euclidean distance as a distance measure. Heatmap is used to show taxa abundance in a particular sample. (**b**) Hierarchical clustering and heatmap visualization at family level using trauma group at different time points and control group as experimental factors. Hierarchical clustering was based on ward linkage algorithm using Euclidean distance as a distance measure. Heatmap is used to show taxa abundance in a particular sample.

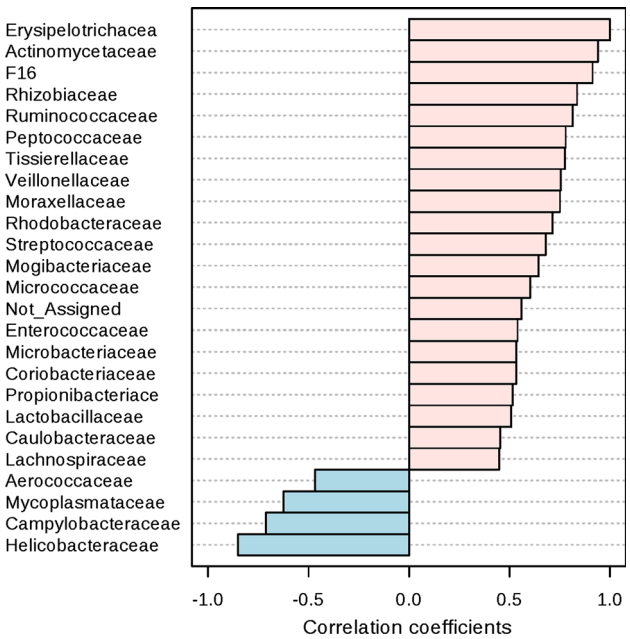

Figure 7. Uniqueness and divergence of jejunal microbial diversity in MTBI and control group was determined by Linear Discriminant Analysis (LDA) of effect size using LEFSe in MicrobiomeAnalyst. Blue colored bars denote taxa which were higher in MTBI group and pink colour denotes taxa which were higher in control groups.

suggesting bacterial translocation from adjoining areas of intestine. The notion is further supported by the increased abundance of *Helicobacter* and *Campylobacter* after trauma. While *Campylobacter* has been shown to be elevated in the fecal and intestinal samples of patients with Crohn's disease (Man *et al.* 2009), Helicobacter levels were found to be elevated in duodenal ulcers and gastric cancer (Sheh and Fox 2013). On the contrary, *Helicobacter* are proposed to have a protective role in intestinal bowel disease (Papamichael *et al.* 2014; Bartels *et al.* 2015). Therefore, the proposed model of rMTBI can be further utilized to draw the causal potential of these bacterial communities in traumatic stress-induced intestinal ailments including inflammation.

We have noticed the depletion of butyrate-producing communities in the gut of trauma-induced rats, which were abundant in control animals. The healthy gut is believed to harbour butyrate producers, mostly belonging to *Clostridium*

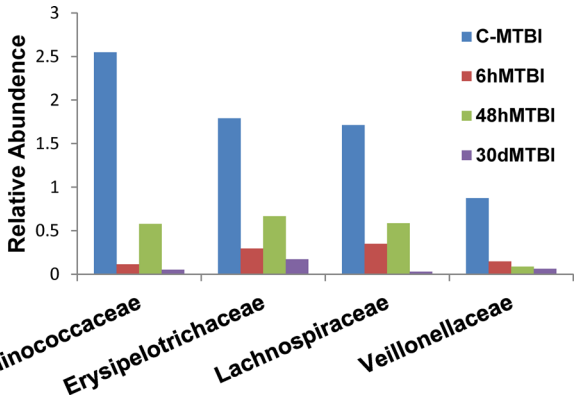

Figure 8. Bar chart showing relative abundance of potential butyrate producing families at each time point in MTBI groups and control group. Families *Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae,* and *Veillonellaceae* were selected based on available literature.

cluster IV and XIVa (Barcenilla *et al.* 2000; Rivière *et al.* 2016). Jejunum is known for its ability to absorb small molecules like butyrate and other SCFAs from the food (Schmitt *et al.* 1976). Butyrate is known for its effects as a histone deacetylase (HDAC) inhibitor (Bourassa *et al.* 2016). Previous studies have elucidated the action of microbiota-derived butyrate in HDAC inhibition in intestine (Furusawa *et al.* 2013; Waldecker *et al.* 2008). Inhibition of HDACs in brain by molecules such as butyrate, valproic acid, and trichostatin A has been linked to reduction in anxiety and fear (Whittle and Singewald 2014). Therefore, the reductions in butyrate-producing microbial communities might be suggested to be causal to increased anxiety levels and reduced gut motility in rMTBI-induced rats (Sagarkar *et al.* 2017a). Previous studies in rat and human support the potential of Bifidobacteria (a butyrate producer) and other probiotics in treating neurological ailments (Messaoudi *et al.* 2011). Recently, a study elaborated on the neuroprotective effects of *Clostridium butyricum* (a butyrate producer) in alleviating the impact of TBI, when trauma mice were treated intragastrically with the mentioned bacterium (Li *et al.* 2018). Hence, using the faecal microbiota transplantation (FMT) in rMTBI-induced rats, future studies could discern the contribution of butyrate-producing bacterial communities in maintaining the healthy homeostasis in gut-microbiome-brain axis.

In conclusion, the results of the present study potentially relate rMTBI-induced microbial changes in jejunum to the comorbid conditions of gut dysfunction and neuropsychiatric readouts. However, additional studies involving molecular and cognitive phenotyping are required to establish a causal link between trauma, behaviour and microbial perturbations. The depletion of butyrate-producing bacteria after the trauma may alter the epigenome in the specific areas of the brain relevant to emotional behaviours such as amygdala. These observations further offer a plausible explanation for the persistent anxiety-like behaviours

comorbid with reduced gut motility. The rMTBI model used in the present study, therefore, may prove useful in investigating the relevance of gut psychobiome in stress-induced neuropathologies.

4.1 *Study limitation*

The current study was carried out using only two samples per group which remains as a limitation of the study. Although the results show profound changes in the microbial composition in the jejunum of rMTBI-induced rats as compared to control rats, the degree of statistical significance cannot be provided in view of a limited number of samples. Although non-significant, a slight reduction in body weights of rMTBI-induced rats at the 30 days time point was observed. Therefore, the possibility may not be excluded that the reduced gut motility and consequent changes in feeding habits might underlie the profound differences in microbiome post-rMTBI. Future studies are warranted to scrutinize the relevance of gut microbiome changes in rMTBI-induced neuropsychiatric and intestinal ailments or vice versa to draw causal relationships between consequences.

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