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# Bacterial dysbiosis and decrease in SCFA correlate with intestinal inflammation following alcohol intoxication and burn injury

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## ABSTRACT

**Background** Patients intoxicated at the time of burn experience increased rates of sepsis and death compared with that observed in similarly sized burns alone. We sought to characterise changes in the intestinal microbiome and short-chain fatty acids (SCFAs) following alcohol intoxication and burn injury and to determine whether these changes are associated with intestinal inflammation.

**Methods** 10–12-week-old C57BL/6 male and female mice were subjected to ethanol intoxication and a 12.5% total body surface area scald burn injury. The following day, mice were euthanised and faecal contents from the caecum and small intestine (SI) were harvested for 16S sequencing for microbial analysis and caecum contents underwent high-performance liquid chromatography mass spectroscopy to assess SCFAs.

**Results** The intestinal microbiome of ethanol burn (EB) mice exhibited decreased alpha diversity and distinct beta diversity compared with sham vehicle (SV). EB faeces were marked by increased Proteobacteria and many pathobionts. EB caecum faeces exhibited a significant decrease in butyrate and a downward trend in acetate and total SCFAs. SCFA changes correlated with microbial changes particularly in the SI. Treatment of murine duodenal cell clone-K (MODE-K) cells with faecal slurries led to upregulation of interleukin-6 (IL-6) from EB faeces compared with SV faeces which correlated with levels of Enterobacteriaceae. However, supplementation of butyrate reduced faecal slurry-induced MODE-K cells IL-6 release.

**Conclusion** Together, these findings suggest that alcohol and burn injury induce bacterial dysbiosis and a decrease in SCFAs, which together can promote intestinal inflammation and barrier disruption, predisposing to postinjury pathology.

### WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Gut barrier dysfunction is often implicated in the pathology associated with burn as well as alcohol exposure.

### WHAT THIS STUDY ADDS

⇒ The findings show that alcohol and burn injury result in bacterial dysbiosis and decreased short-chain fatty acids. Additionally, this study identifies increased intestinal epithelial interleukin-6 release in response to faecal slurries from mice subjected to ethanol and burn, and that butyrate treatment may limit this induction of inflammation.

### HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study provides supporting justification for further studies to assess the consequences of microbial changes following ethanol and burn injury in hopes of improving outcomes for those suffering from burn injury and subsequent sequelae.

## INTRODUCTION

Burn injury is a common cause of trauma, resulting in high morbidity and mortality.[1–3] In the USA, over half of patients hospitalised for burns have positive blood alcohol content on admission.[4 5] Hospitalisations due to concurrent burn injury and alcohol intoxication are associated with poorer outcomes, including increased fluid requirements, more costly hospitalisations and longer stays, ultimately resulting in higher mortality.[5 6] Post burn, there is a breakdown of the gastrointestinal (GI) barrier, leading to inflammation and translocation of luminal contents into systemic circulation, which is further exacerbated by alcohol exposure.[7 8] In addition to physical damage to the GI barrier, ethanol and burn both delay GI motility, as assessed by fluorescein isothiocyanate (FITC)-dextran transit, with a more dramatic reduction when combined.[9–11] Combined ethanol and burn injury results in predominant overgrowth of Enterobacteriaceae, which contains many pathobionts.[7 12–14] In the setting of damaged intestinal epithelium, these bacteria are known to lead to pro-inflammatory signalling

and intestinal inflammation, which may further exacerbate the damage observed post burn.[15]

The human GI tract houses 10–100 trillion microbes.[16] Commensal bacteria keep pathogenic bacteria at bay by maintaining host epithelial barrier integrity, competing for epithelial binding sites, modulating host immune responses and through metabolites (both by generating beneficial metabolites for host cells and sequestering nutrients from pathobionts).[17 18] Alterations in GI microbiome composition, broadly termed dysbiosis, have been correlated with numerous disease states, including alcohol use, burn injury, inflammatory bowel disease (IBD), colon cancer and sepsis.[8 12 19 20]

One group of beneficial bacterial metabolites are short-chain fatty acids (SCFAs), namely acetate, propionate and butyrate.[21] Butyrate constitutes a major fuel source for differentiated intestinal epithelial cells (IECs) and is readily absorbed in both the large intestine and small intestine (SI), where it maintains intestinal barrier integrity by upregulating tight junction proteins and promoting terminal differentiation.[22] In addition to maintaining the intestinal barrier, butyrate and to a lesser extent propionate act in an anti-inflammatory manner both locally in the GI tract and systemically.[23] Butyrate supplementation has been promising in reducing inflammation and extending periods of remission in IBD in both murine models and patients.[24 25] Additional recent evidence suggests that SCFA restoration may offer a protective role in the context of alcohol consumption.[26]

In this study, we sought to understand how changes in the gut microbiome and the SCFAs they produce may contribute to intestinal inflammation following combined alcohol intoxication and burn injury to further our understanding of the contribution of the intestinal microbiome to pathophysiology observed.

## MATERIALS AND METHODS

### Animals

A total of 10–12-week-old C57BL/6 male and female mice were obtained from Charles River Laboratories and maintained in animal housing facilities at Loyola University Chicago Health Sciences Division, Maywood, Illinois, USA. All animal experiments were conducted in accordance with the guidelines set forth in the Animal Welfare Act and were approved by the Institutional Animal Care and Use Committee at Loyola University Health Sciences Division.

### Murine model of acute alcohol intoxication and burn injury

As previously described, a well-established model of acute alcohol intoxication and scald burn injury was used.[10 11 14 27–29] Briefly, mice were randomly assigned to two experimental groups: Sham injury and vehicle (water) or burn injury and ethanol. On the day of injury, mice were gavaged with 400 µL of 25% ethanol in water (2.9 g/kg) or 400 µL of water. Mice were given 1 mg/kg buprenorphine subcutaneously. Four hours following the gavage, mice were anaesthetised with a ketamine hydrochloride/xylazine cocktail (~80 mg/kg and ~1.2 mg/kg, respectively) given by intraperitoneal injection. The dorsal surface of the mice was shaved before placing the mice in a prefabricated template exposing ~12.5% of total body surface area (TBSA) calculated using Meeh's formula.[30] Burn group animals were immersed in ~85°C water bath for ~7 s to induce a full-thickness scald burn injury. Sham animals were placed in a 37°C water bath for 7 s. Following burn or sham injury, animals were dried gently and given 1.0 mL normal saline resuscitation by intraperitoneal injection. Animals were returned to their cages and allowed food and water ad libitum and monitored.

### 16S bacterial DNA sequencing

One day after injury, mice were euthanised and distal SI and caecum lumen contents were collected. Faecal bacterial DNA was isolated using the PowerFecal Pro DNA Kit (Qiagen). DNA concentration and quality were assessed via a NanoDrop 2000 Spectrophotometer (Thermo Scientific). 16S rRNA PCR and sequencing were performed at the Loyola Genomics Facility. Briefly, the V4 region of the 16S rRNA gene was amplified with 27f and 1492r primers.[31] Sequencing was performed on an Illumina MiSeq using a V3 600 bp flowcell, yielding 300 bp paired end reads. Raw FASTQ files were analysed using QIIME2 and publicly available plugins. In short, sequences were demultiplexed using the demux plugin and then denoised for quality control using DADA2. Alpha and beta diversity metrics were calculated using the core-metrics-phylogenetic pipeline of the q2-diversity plugin. Features were annotated for taxonomic classification using the classify-sklearn plugin with a naïve Bayes classifier trained on reference sequences from the SILVAv132 database. Subsequent bacterial taxa and their read counts were used for further analysis. Demultiplexed paired-end sequences were deposited in the NCBI Sequence Read Archive (BioProject accession ID: PRJNA1207030).[32]

### Sequencing data analysis

Using QIIME2, within-sample variance (alpha diversity) was assessed by calculating Shannon's diversity index for each sample, which considers both the number of taxa present (richness) and the distribution of their abundances (evenness). To evaluate the contributions of richness and evenness separately, Chao1 and Pielou's were calculated, respectively. Differences between male versus female and ethanol burn (EB) versus sham vehicle (SV) were determined using the non-parametric Kruskal-Wallis significance test. Variance between samples (beta diversity) was measured by weighted UniFrac with principal coordinate analysis (PCoA) and assessed using PERMANOVA statistical analysis. Bacterial taxa were analysed by raw read counts (phyla and families) and relative abundance (phyla) via GraphPad Prism. Two samples were removed due to low total read counts (<100), then outliers were assessed and removed. Data are presented

as mean raw reads±SEM or mean relative abundance and analysed via Kruskal-Wallis with Dunn's multiple test or Mann-Whitney U test, where applicable.

### SCFA quantification

A small portion of each caecal content sample was snap frozen and sent to the University of Illinois mass spectrometry core for SCFA quantification via high-performance liquid chromatography mass spectrometry (HPLC-MS). Concentrations of each SCFA (acetate, propionate and butyrate) and combined total SCFAs are assessed as nmol SCFA per mg faeces.

### SCFA-bacterial correlation

Multiple regression analysis was performed using RStudio between bacterial family read counts averaging >500 in both the SI and caecum with caecum SCFA concentrations of individual samples for acetate, propionate, butyrate and combined SCFAs taking into consideration injury and sex. Adjusted R-squared values for each family and SCFA are represented in a heatmap with accompanying p-values overlayed.

### Small IEC culture and faecal slurry treatment

Murine duodenal cell clone-K (MODE-K) cells (generously provided by Dr. Jin Mo Park, HMS, Mass General, Charlestown, Massachusetts, USA) were cultured at 37°C with 5% $CO_2$ in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% Antibiotic-Antimycotic Solution (Hyclone) and 5% fetal bovine serum (Life-Technologies). SI faecal samples isolated 1 day following alcohol and burn injury were homogenised in 1 mL PBS per 100 mg faeces and centrifuged at 10 000 rpm for 10 min to generate an unfiltered 'faecal slurry'. MODE-K cells were seeded into 6-well plates for 48 hours. In a subset of experiments, cells were treated with filter-sterilised Sodium Butyrate (Sigma Aldrich) in PBS at a concentration of 2 mM or vehicle (PBS) 1 hour before faecal slurry. Cells were treated with faecal slurries diluted at 1:500 or PBS alone into culture media for 24 hours before media supernatant and cells were collected.

### Faecal bacterial qPCR

Genomic DNA was isolated from small intestinal faecal samples isolated 1 day following alcohol and burn injury using QIAamp PowerFecal Pro DNA Kit (Qiagen) according to manufacturer's instructions. DNA concentration was measured using the NanoDrop 2000 Spectrophotometer (ThermoScientific) and equal amounts were loaded for quantitative polymerase chain reaction (qPCR) reaction using 1X iTaq Universal SYBR Green Supermix (BioRad) with 200 nM of forward and reverse primers. Primers—total bacteria: F: (ACTCCTACGGGAGGCAGCAGT), R: (ATTACCGCGGCTGCTGGC); and Enterobacteriaceae: F: (GTGCCAGCMGCCGCGGTAA), R: (GCCTCAAGGGCACAACCTCCAAG). Level of Enterobacteriaceae is expressed as a ratio of Enterobacteriaceae/total bacteria.

### RNA isolation and RT-qPCR

RNA was isolated from collected MODE-K cells using the RNeasy Mini Kit (Qiagen). The concentration and quality of isolated RNA were determined by NanoDrop 2000 spectrophotometer (ThermoScientific), and cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (Life-Technologies). Expression of IL-6 mRNA was analysed by reverse transcription qPCR (RT-qPCR) using TaqMan Fast Advanced Master Mix (Life Technologies) and predesigned TaqMan primer probes (Life Technologies). Data were calculated via the $\Delta\Delta$Ct method with target gene Ct values normalised to beta actin housekeeping control.

### ELISA

Conditioned media supernatant interleukin-6 (IL-6) was assessed using a DuoSet ELISA kit (BD Biosciences). The data were quantified as pgIL-6/mL conditioned media. Data were normalised to the average concentration of conditioned media from vehicle treated (no Tx).

### Statistics

The data, wherever applicable, were presented as means±SEM and were analysed using one-way analysis of variance (ANOVA) with Tukey's post-hoc test, Student's t-test, Mann-Whitney U test or $\chi^2$ test (GraphPad Prism, V.10). Multiple regression was performed on RStudio (V. 2024.04.2+764, Posit Software, Boston, Massachusetts, USA). A p value of <0.05 was considered statistically significant. Unless noted otherwise, significance is represented as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

## RESULTS

### Ethanol and burn injury significantly alter faecal microbiome diversity and composition

Pairwise comparison of the microbiome from EB versus SV samples revealed an association with significantly reduced richness in the SI (Chao1 p<0.05) (figure 1A), but not the caecum following combined injury (figure 1B), while significantly reduced evenness was seen in both the SI (Pielou, p<0.05) and caecum (Pielou, p<0.01). Shannon's diversity demonstrated reduced bacterial diversity in the SI (Shannon, p<0.05) but not caecum (figure 1A, B). No significant differences were found from pairwise analysis of male versus female samples from either the SI or caecum (online supplemental figure 1A, B). Weighted UniFrac PCoA was performed to further assess differences in the microbial composition of EB samples compared with SV. EB samples exhibited separate clustering from SV, assessed by Pairwise PERMANOVA analysis (p<0.01), indicating distinct microbial compositions following ethanol and burn injury in both the SI and caecum (figure 1C, D).

Taxonomic analysis of the six most abundant bacterial phyla in the SI and caecum, respectively, revealed no significant changes in total read counts from male or female samples following ethanol and burn injury

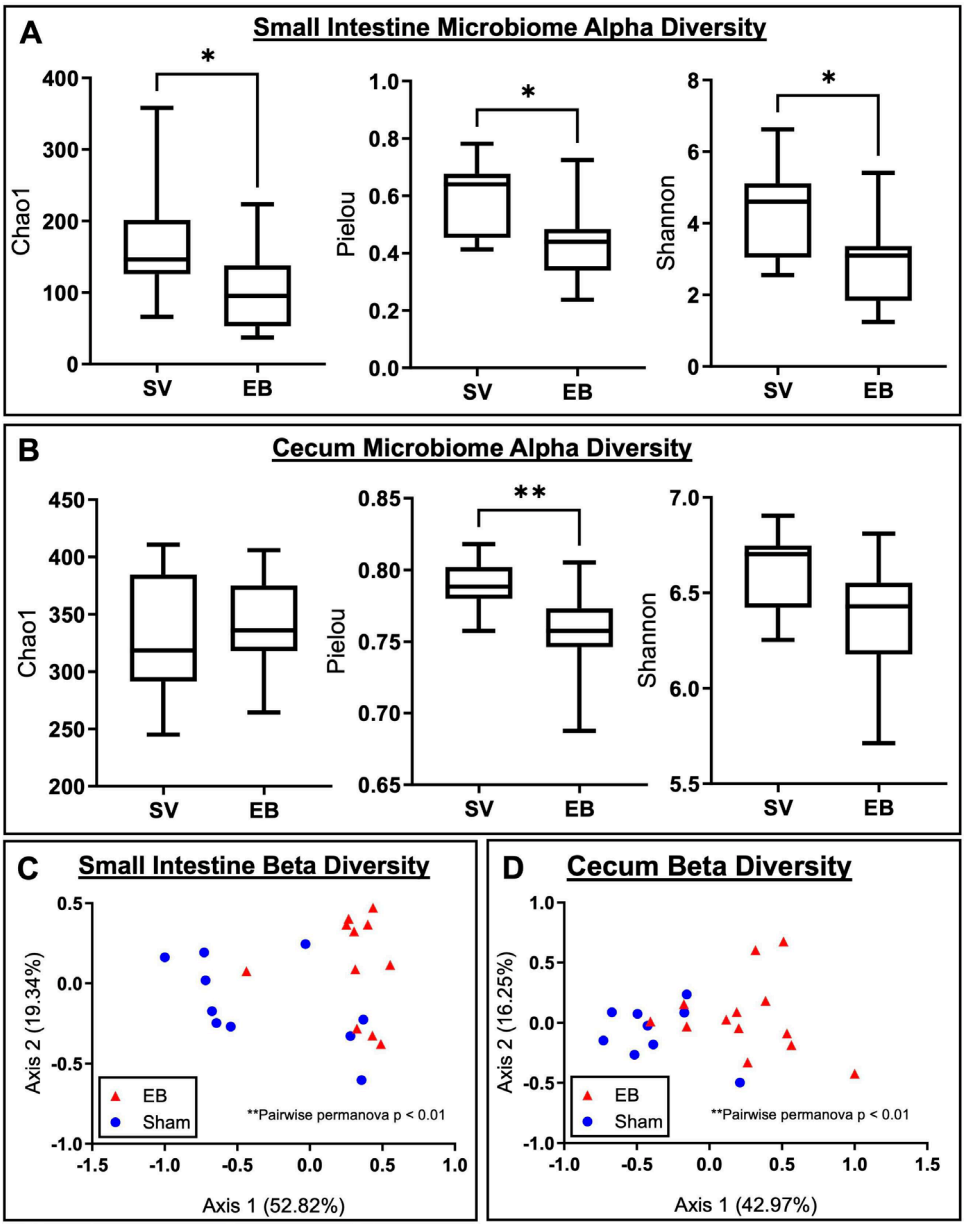


**Figure 1** Alpha and beta diversity metrics comparing SV and EB samples. Small intestinal and caecal contents were collected 1 day after ethanol and burn injury, and faecal bacterial DNA was isolated for 16S sequencing. (A) Alpha diversity metrics for the small intestine and (B) caecum were individually assessed for SV versus EB sample comparison. Significance was analysed via Kruskal-Wallis pairwise analysis, n=10–13 animals per group, *p<0.05, **p<0.01. (C, D) Weighted UniFrac principal coordinate analysis of (C) small intestinal or (D) caecal samples. Statistical analysis via Pairwise PERMANOVA confirms significant differences in the clustering of SV versus EB samples in both the small intestine and caecum, **p<0.01. EB, ethanol burn; SV, sham vehicle.

(online supplemental figure 1C, D). Following ethanol and burn injury, in the SI, there was a significant reduction in the relative abundances of Actinobacteria, Bacteroidetes and Verrucomicrobiota, while Proteobacteria was significantly elevated (figure 2A, B). In the caecum, the relative abundance of Firmicutes was significantly reduced in EB caecum samples compared with SV, while Bacteroidetes and Proteobacteria were significantly increased after injury (figure 2C, D). Male and female mice showed consistent trends in the relative abundances of bacterial phyla following ethanol and burn injury, with Proteobacteria remaining the most significantly altered phylum in both the SI and caecum (figure 2B, D).

We have previously shown increased intestinal inflammation and overgrowth of pathobionts following combined alcohol and burn injury.[10] Herein, we sought to characterise intestinal dysbiosis following alcohol and burn injury through 16S sequencing. We used taxonomic analysis to assess changes in bacterial families and genera. Enterobacteriaceae is one such family from the Proteobacteria phyla and was the most abundantly increased in both the SI (figure 3A) and the caecum (figure 3B)

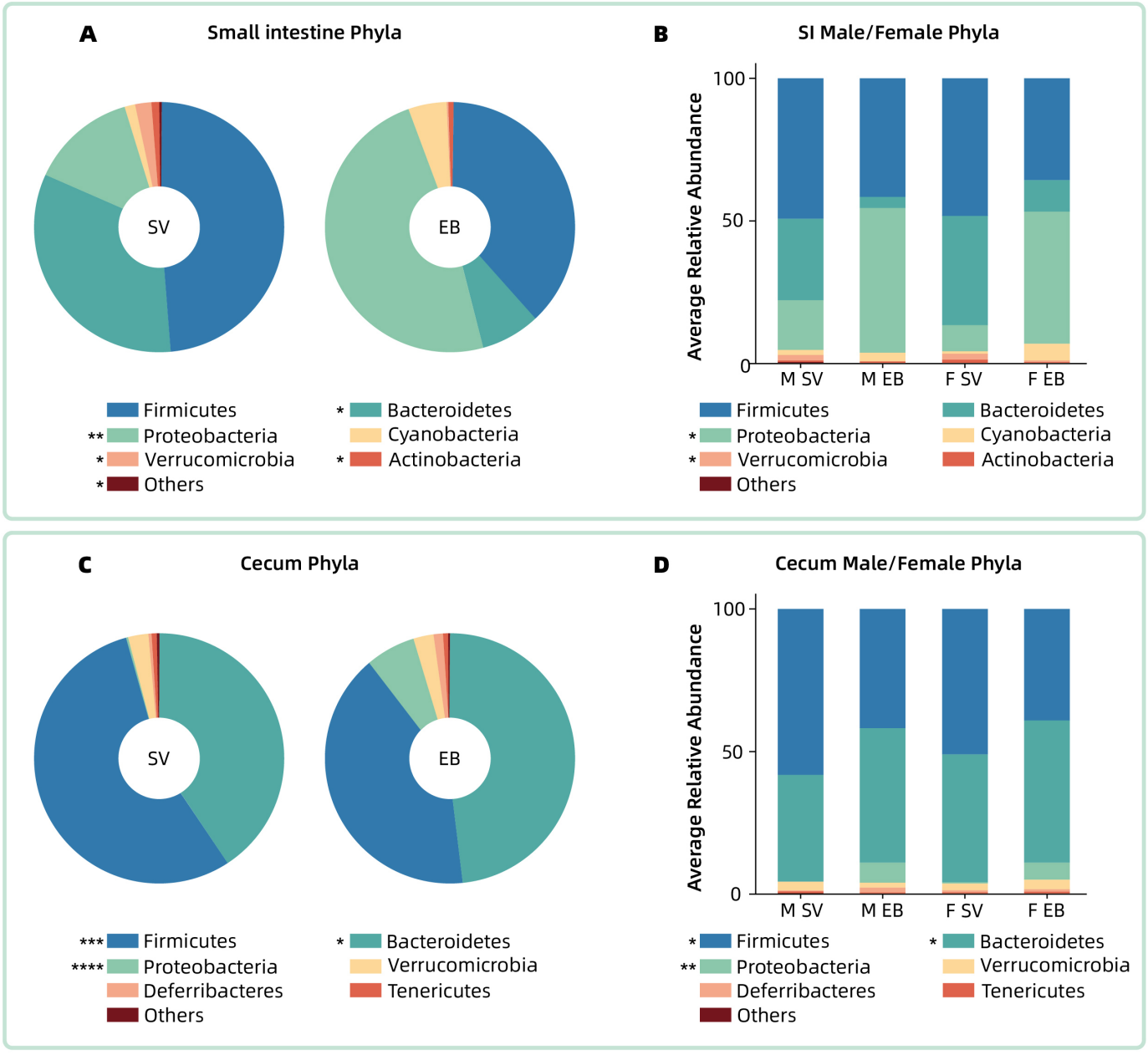


**Figure 2** Ethanol and burn injury alter relative abundance of bacterial phyla in the small intestine and caecum. Small intestinal and caecum luminal faecal contents were collected 1 day after ethanol and burn injury, and faecal bacterial DNA was isolated for 16S sequencing. Schematics represent the abundance (read count) of individual bacterial phyla relative to total bacterial read counts and averaged across the groups depicted. (A) Parts of a whole plot of SV and EB small intestinal faecal phyla, respectively. (B) Bar graphs comparing small intestinal SV and EB with male and female samples separated. (C) Parts of a whole plot of SV and EB caecum faecal samples phyla respectively. (D) Bar graphs comparing caecum SV and EB with male and female samples separated. (A, C) Parts of a whole chart combine male and female samples together to compare SV to EB overall. Significance was analysed by Mann-Whitney U test, n=10–13 animals per group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (B, D) Statistical analysis via non-parametric Kruskal-Wallis test (significance shown in colour legend) with on graph significance depicting Dunn's multiple comparisons test with associated male or female SV samples, n=5–7 animals per group, *p<0.05, **p<0.01. EB, ethanol burn; SV, sham vehicle.

following ethanol and burn injury. The Enterococcaceae family, also from the Proteobacteria phyla, was significantly increased following ethanol and burn injury in both the SI (figure 3A) and the caecum (figure 3B).

Following analysis of bacterial populations known to contain pathobionts, we investigated changes in probiotic bacteria. Phyla-level analysis revealed a decrease in Verrucomicrobiota in the SI but not caecum following ethanol and burn injury (figure 2). Verrucomicrobiota contains *Akkermansia*, an SCFA-producing bacterium known to confer protective properties to the intestinal epithelium.[33] Further investigation revealed decreases in multiple SCFA-producing bacteria following ethanol and burn injury, including the Lachnospiraceae family,

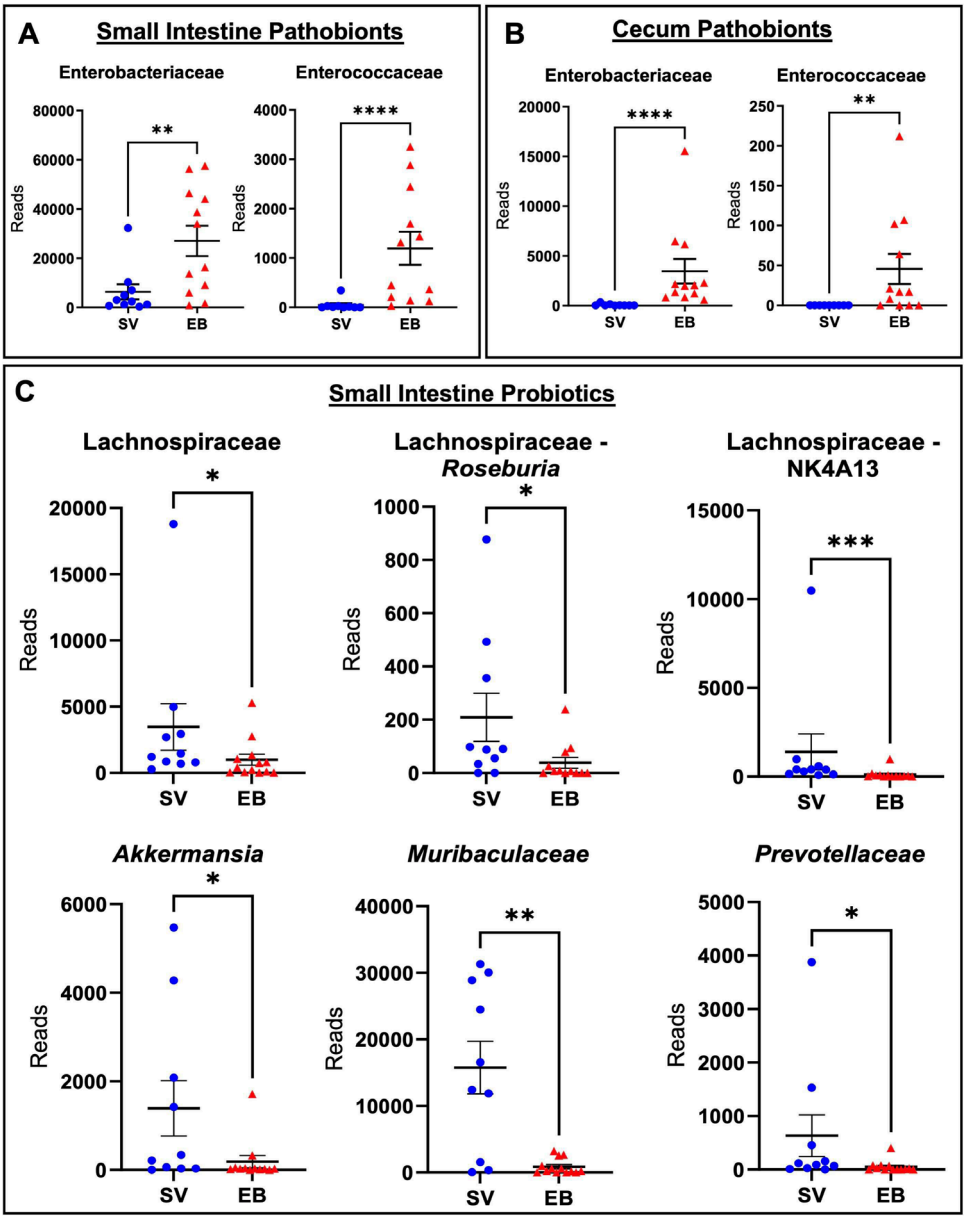


**Figure 3** Ethanol and burn injury increase pathobionts and decrease SCFA-producing bacteria. Small intestinal and caecal contents were collected 1 day after ethanol and burn injury, and faecal bacterial DNA was isolated for 16S sequencing. Graphs representing (A) small intestinal or (B) caecal 16S sequencing read counts of pathobionts, Enterobacteriaceae and Enterococcaceae comparing SV with EB. (C) Small intestinal read counts of SCFA-producing bacteria: Lachnospiraceae, *Roseburia*, Lachnospiraceae NK4A13, *Akkermansia*, *Muribaculaceae* and *Prevotellaceae*. Significance was analysed by Mann-Whitney U test, n=10–13 animals per group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. EB, ethanol burn; SCFA, short-chain fatty acid; SV, sham vehicle.

in particular *Roseburia* and Lachnospiriaceae NK4A13, in addition to *Akkermansia*, *Muribaculaceae* and *Prevotellaceae* (figure 3C). There were no significant differences between SV and EB in the caecum for any of these probiotic bacteria (online supplemental figure 2).

### Faecal content from ethanol and burn injury promotes epithelial cell release of IL-6

Due to elevated levels of pathobionts and decreases in potentially beneficial bacteria in the SI, we hypothesised that the SI faecal content from EB mice would be more pro-inflammatory than that of SV mice. Treatment of the murine small IEC cell line, MODE-K, with bacterial products, including lipopolysaccharide, has been shown to stimulate pro-inflammatory cytokine production via toll-like receptor signalling.[34] The pro-inflammatory cytokine IL-6 has been demonstrated to promote intestinal inflammation, tissue damage and gut barrier disruption following alcohol and burn injury.[7 8] To assess the inflammatory capacity of the faecal microbiome following ethanol and burn injury, MODE-K cells were treated with faecal slurries isolated from SI faeces of EB or SV mice. MODE-K cell gene expression of IL-6 was significantly

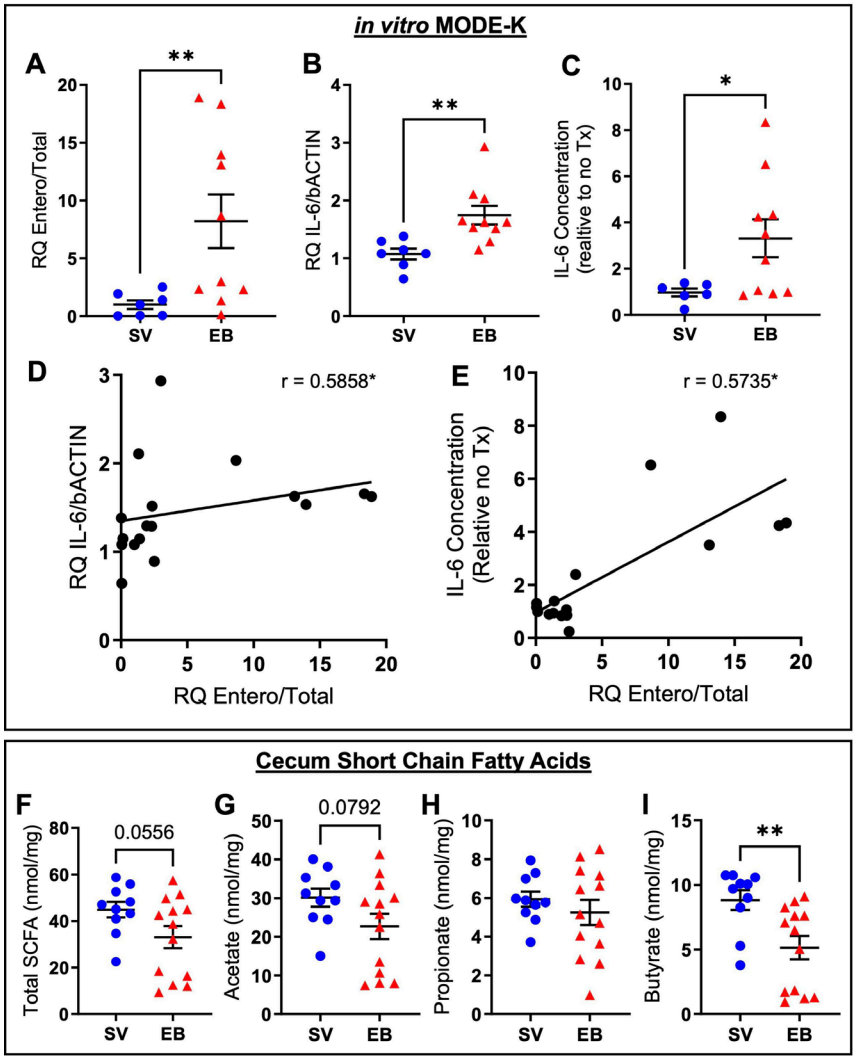


**Figure 4** (A–E) Elevated small intestinal Enterobacteriaceae levels after ethanol and burn correlate with pro-inflammatory potential. Small intestinal faecal contents were collected from SV (n=7) or EB (n=10) mice 1 day after combined injury. Faecal bacterial DNA was isolated from a portion of each sample while remaining faecal contents were homogenised in PBS to generate faecal slurry. (A) Bacteria were quantified via bacterial qPCR with the level of Enterobacteriaceae in each sample being expressed as a ratio of Enterobacteriaceae/total bacteria and depicted relative to an experiment's average SV sample. (B) MODE-K cells were treated with small intestinal faecal slurry diluted in culture media for 24 hours and then IL-6 and gene expression were quantified via RT-qPCR with beta actin used as housekeeping. (C) Conditioned media was collected for protein quantification of IL-6 via ELISA and normalised to conditioned media with no treatment. Graphs depict the average result from the faecal slurry of individual mice as a single point in addition to group means±SEM. Statistical analysis of SV versus EB groups via Welch's t-test depicted as *p<0.05, **p<0.01. (D, E) Correlation of small intestinal faecal samples (n=17) from individual mice comparing Enterobacteriaceae levels to (D) IL-6 gene expression or relative protein concentration following 24 hours of faecal slurry treatment assessed via Spearman correlation analysis. R values displayed with significance of correlation depicted as *p<0.05. (F–I) Ethanol and burn injury reduce faecal SCFA. SCFAs were quantified from caecal contents collected 1 day after ethanol and burn injury via high-performance liquid chromatography mass spectroscopy. Graphs depict concentrations of (A) total SCFAs, (B) acetate, (C) propionate and (D) butyrate in nmol/mg faeces with male and female samples combined to compare SV to EB overall. Individual mice represented by each dot in addition to group mean±SEM, n=10–13. Significance was analysed via Welch's t-test, n=10–13 animals per group, **p<0.01. EB, ethanol burn; IL-6, interleukin-6; MODE-K, murine duodenal cell clone-K; qPCR, quantitative polymerase chain reaction; RT-qPCR, reverse transcription qPCR; SCFA, short-chain fatty acid; SV, sham vehicle.

increased by treatment with EB faecal slurries compared with treatment with SV faecal slurries (figure 4B). EB faecal slurries also promoted more IL-6 protein expression compared with SV faecal slurries (figure 4C). Bacterial qPCR was used to confirm a significant increase in SI Enterobacteriaceae levels relative to total bacteria following ethanol and burn injury (figure 4A), consistent with the 16S sequencing results (figure 3A). To further assess the relationship between Enterobacteriaceae and small IEC inflammation, Spearman correlation analyses were performed between the relative quantity of Enterobacteriaceae in a sample and the level of IL-6 induced by

that sample at the mRNA or protein level. Both IL-6 gene expression (figure 4D) and protein (figure 4E) exhibited significant positive correlations with Enterobacteriaceae levels.

### Reduced caecal SCFA content correlates with reduced levels of SCFA-producing bacteria in the gut

In addition to direct interactions of bacteria with the gut barrier, metabolites produced by bacteria, particularly SCFAs, maintain the gut barrier and provide a major source of energy for the intestinal epithelial barrier.[35 36] To study how changes in bacterial composition after alcohol and burn injury may impact levels of these bacterial metabolites, we quantified SCFAs (acetate, butyrate and propionate) via HPLC-MS in caecal contents of both male and female mice subjected to SV or EB. Overall, there were non-significant decreasing trends in the concentration of total SCFAs and acetate in the caecum following ethanol and burn injury (figure 4F, G), which was similar in both male and female mice (online supplemental figure 3A, B). There were no significant changes observed in propionate concentration (figure 4H) (online supplemental figure 3C). The only significantly differential SCFA after ethanol and burn injury was butyrate, which exhibited significantly reduced concentrations (figure 4I) with non-significant trending decreases in both male and female mice (online supplemental figure 3D). Due to the possibility of type-II error caused by smaller sample sizes when being stratified into male and female mice, more robustly powered studies are warranted in order to confirm if the butyrate findings observed in pooled samples remain significantly different between SV and EB for both male and female mice.

To begin to elucidate how changes in bacterial composition after ethanol and burn injury may contribute to the changes in SCFA concentration observed, multiple variable correlation analysis considering injury and sex was performed between bacterial family read counts and SCFA concentrations of individual samples. Heatmaps depicting the corresponding adjusted R-squared values were then generated to visualise the significance and degree of correlation. To investigate correlations within the same sampling location, SCFA concentrations were first compared with caecal bacterial family read counts. Surprisingly, caecal bacterial levels showed few strong correlations with SCFA concentrations (figure 5A). Interestingly, small intestinal bacterial families demonstrated more significant correlations with caecal SCFA concentrations, particularly of butyrate, which was the most significantly altered SCFA (figure 5B). In both the SI and caecum, few bacterial families correlated positively with propionate concentrations (figure 5A, B). The majority of small intestinal bacterial families that correlated with downstream caecum SCFA levels exhibited positive correlations, for many as bacterial levels decreased in the SI, there was a concurrent decrease in SCFA levels in the caecum, especially of butyrate.

### Butyrate treatment can limit IEC release of IL-6 induced by faecal content from ethanol and burn

Butyrate is known to act in an anti-inflammatory manner in the intestines and is an imperative bacterial metabolite for maintenance of the host intestinal epithelial barrier.[25] Herein, we sought to investigate if butyrate can limit inflammation induced by the dysbiotic microbiome following ethanol and burn injury. To assess the impact of butyrate on the inflammatory capacity of the faecal microbiome, MODE-K cells were treated with butyrate and then faecal slurry isolated from small intestinal faeces of EB or SV mice. As expected, treatment with butyrate reduced IL-6 produced by IECs (figure 6A). Treatment with butyrate prevented the induction of IL-6 on treatment with both SV and more significantly EB faeces (figure 6B, C).

## DISCUSSION

Large burn results in dysbiosis of the GI microbiome.[8] The murine model used here is a relatively small burn injury (~12.5% TBSA), which by itself does not severely impact the GI system and does not alter the microbiome.[7 14] When this small burn injury is combined with ethanol, or another predisposing factor such as advanced age, the injury results in severe intestinal pathology marked by tight junction disruption, reduced stemness, impaired intestinal transit/motility and increased leakage of FITC-dextran from the intestines into systemic circulation.[7 14 27 29 37–40] We demonstrate a perturbation of the intestinal microbiome and SCFAs they produce following combined alcohol intoxication and burn injury. The microbiome of both the SI and caecum were disturbed; however, the SI microbiome exhibited more disruption of both alpha and beta diversity metrics than the caecum (figure 1). One explanation for the dramatic disruption of the SI microbiome may be in part due to the delay in GI motility well described in the murine model of acute ethanol intoxication and burn injury used.[10 11] Slowed transit or dysmotility can result in a backup of faecal contents into the SI, resulting in small intestinal bacterial overgrowth and dysbiosis, which are well documented in alcohol use and burn both individually and together.[7 41] The SI exhibited a significant overgrowth of Proteobacteria following alcohol and burn (figure 2A, B), which was also observed, but to a lesser extent, in the caecum (figure 2C, D). Proteobacteria is a phylum that contains many pathobionts or potentially pathogenic bacteria, including Enterobacteriaceae, which is significantly increased following the combined injury (figure 3A, B). Pathobionts are ubiquitous microorganisms that, at baseline, are not inherently pathogenic to their host but can function as opportunistic pathogens when their populations are expanded.[42] Further studies are necessary to understand if the intestines are serving as a reservoir for pathogenic bacteria leading to increased rates of sepsis and bacteraemia observed in patients intoxicated at the time of burn injury.[7 8]

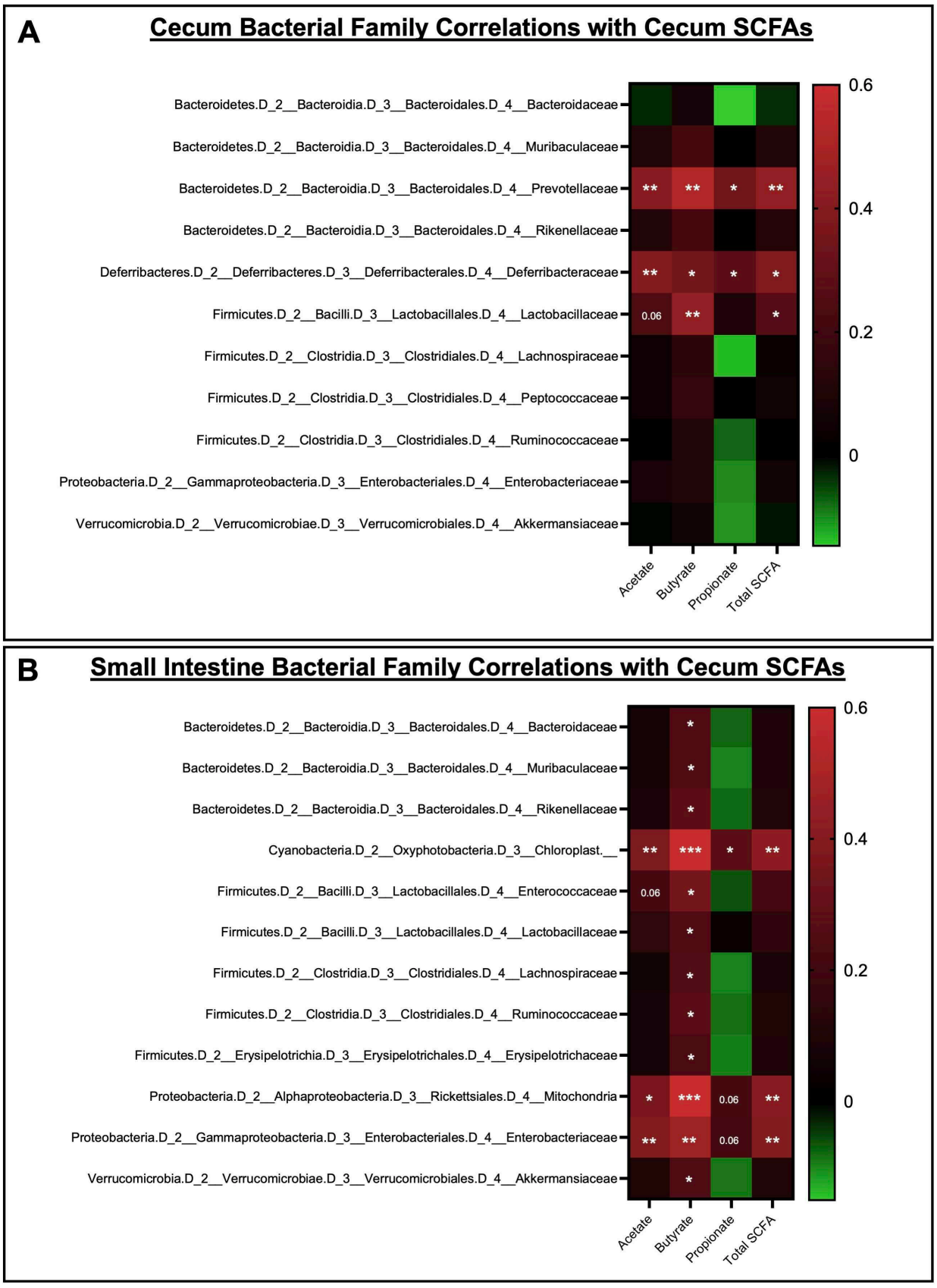


**Figure 5** Reduced SCFA concentration after ethanol and burn injury correlates with bacterial families. Heat map of multiple regression adjusted R-squared values comparing acetate, butyrate, propionate or total SCFA concentration with the read counts of abundant (>500 average reads across all samples) bacterial families identified in (A) caecal contents or (B) small intestinal contents isolated 1 day after ethanol and burn injury. Red is used to display positive correlations, while negative correlations are displayed in green. Significance of each correlation is shown as *p<0.05, **p<0.01, ***p<0.001. SCFA, short-chain fatty acid.

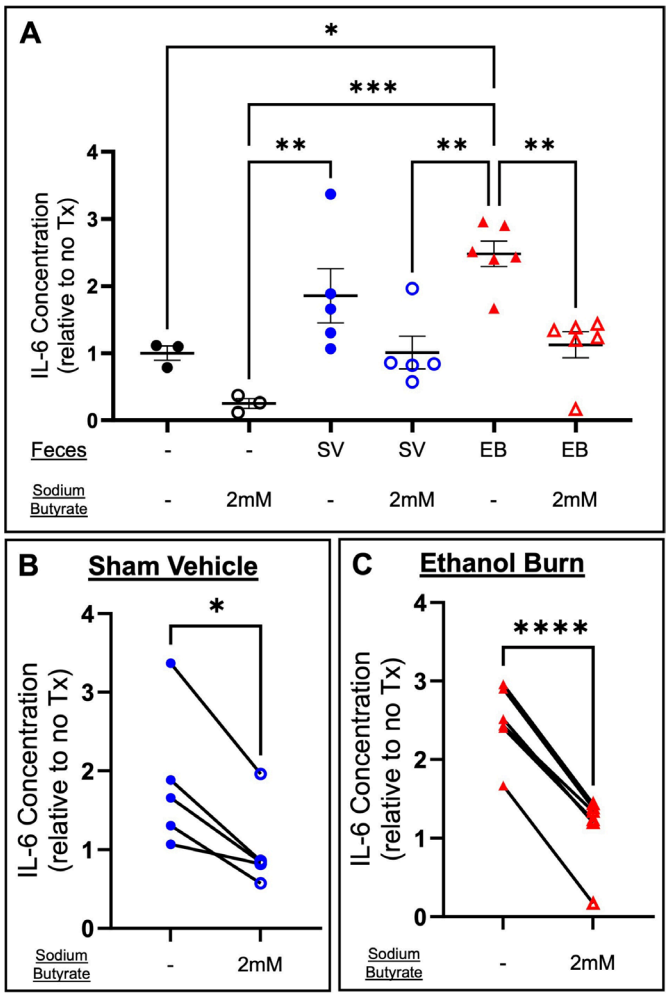


**Figure 6** Butyrate treatment limits inflammatory induction by intestinal faeces. Small intestinal faecal contents were collected from SV (n=5) or (n=6) mice 1 day after combined injury. Faecal contents were homogenised in PBS to generate faecal slurry. MODE-K cells were treated with 2 mM sodium butyrate in PBS or PBS alone; 1 hour later, small intestinal faecal slurry was added for 24 hours and then conditioned media was collected for protein quantification of IL-6 via ELISA and normalised to conditioned media with no treatment. Graphs depict the average result from the faecal slurry of individual mice as a single point in addition to group means±SEM. Significance was analysed by one-way ANOVA with Tukey's multiple comparisons *p<0.05, **p<0.01, ***p<0.001. (B, C) Paired t-test of MODE-K conditioned media with or without 2 mM sodium butyrate and addition of (B) SV faeces slurry n=5, or (C) EB faeces slurry n=6. Significance was analysed by paired t-test. *p<0.05, ****p<0.0001. ANOVA, analysis of variance; EB, ethanol burn; IL-6, interleukin-6; MODE-K, murine duodenal cell clone-K; SV, sham vehicle.

Functionally, the SI microbiome of mice subjected to ethanol and burn injury induced greater IL-6 production in MODE-K cells compared with that of SV mice (figure 4B, C). The levels of IL-6 induced by murine faecal slurries correlated with the level of Enterobacteriaceae identified in the faecal samples (figure 4D, E). This observation provides preliminary evidence that the microbial perturbations following combined ethanol and burn injury may have a functional consequence on intestines. However, more studies are necessary to substantiate these observations with additional microbial targets and metabolites and their subsequent impact on intestinal inflammation.

SCFAs are microbial metabolites, including acetate, propionate and butyrate, that play important roles in homeostasis of both the intestinal barrier and systemic inflammation.[43] In particular, butyrate serves as the primary fuel source for differentiated IECs and is imperative for maintaining tight junctions and, therefore, barrier integrity separating the host from luminal bacteria.[35 36] In this study, we identified decreases in SCFAs, with a particularly significant decrease in butyrate levels following ethanol and burn (figure 4I). Interestingly, the changes observed in caecal bacteria had very few positive correlations with the changes in SCFAs (figure 5A); however, the upstream changes in SI bacteria robustly positively correlated with changes seen in SCFAs, particularly of butyrate (figure 5B). More studies are needed to better understand the underlying mechanism of such a differential response in SCFAs and bacterial changes in caecum and SI.

We used IL-6 as a surrogate marker for intestinal inflammation. IL-6 has been shown to be elevated following ethanol and burn injury in multiple organs, including the intestines, lungs, liver and systemic circulation.[7 34 40 44] Importantly, it is necessary to interpret these data within the context that elevated IL-6, which correlates with adverse outcomes after injury, remains vital for healing and repair of damaged intestinal tissues and burn wound repair.[45–47] Further study is warranted to understand how to properly modulate levels of inflammatory mediators, including but not limited to IL-6, in reducing inflammatory-mediated tissue damage while still allowing for necessary tissue regeneration and repair. In multiple murine studies following combined ethanol and burn injury, intestinal IL-6 levels were blunted in total intestinal tissue as well as isolated IECs in response to various treatments that offered protective effects; therefore, we assessed IL-6 *in vitro*.[10 34 44] MODE-K treatment with butyrate limited the faecal microbiome's induction of IEC IL-6, particularly from faecal slurries of mice subjected to ethanol and burn injury. Further studies are warranted to investigate *in vivo* if SCFAs, specifically butyrate supplementation, may mitigate inflammation resulting from microbial dysbiosis and if SCFAs can reduce intestinal barrier disruption and inflammation, which are classically observed post alcohol and burn injury.

## Strengths and limitations

The purpose of this study was to assess the impact of combined alcohol and moderately sized burn injury on gut bacterial composition. Our previous studies evaluating 12.5% TBSA burn alone, or a single dose of alcohol alone did not significantly influence microbial communities as assessed by qPCR, compared with sham, but significant changes were observed in combined alcohol

and burn injury.[7 14] Previous animal and patient studies have shown perturbations of the microbiome following large burn injury or in a moderate burn combined with an additional insult such as advanced age or, in this case, alcohol.[7 8 14 48 49] Therefore, this study only evaluated differences in microbiome composition between SV mice and those subjected to combined alcohol and burn injury, but not either alone, which we recognise as a significant limitation.

While the *in vitro* data presented provide preliminary evidence that butyrate may limit MODE-K IL-6 production in response to faecal slurries, more studies are necessary to functionally parse out the components of faecal samples, including but not limited to bacteria, metabolites, proteins, microRNAs, and their respective impact on intestinal inflammation and if butyrate is protective to gut epithelium following alcohol and burn injury.

This study reveals perturbations in the microbiome and SCFAs they produce following alcohol and burn injury. While the microbiome in both the caecum and SI were affected by alcohol and burn injury, the SI microbiome was dramatically altered, and these changes correlated with decreases in SCFAs, especially butyrate. Future *in vivo* studies are warranted to assess if restoring butyrate levels and/or microbial composition can mitigate intestinal barrier disruption and subsequent development of pathology in those suffering from burn injury with or without alcohol exposure.

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