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## A PROSPECTIVE STUDY IN SEVERELY INJURED PATIENTS REVEALS AN ALTERED GUT MICROBIOME IS ASSOCIATED WITH TRANSFUSION VOLUME

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### Abstract

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#### Conflicts of Interest:

No competing financial interests exist.

**Background:** Traumatic injury can lead to a compromised intestinal epithelial barrier and inflammation. While alterations in the gut microbiome of critically injured patients may influence clinical outcomes, the impact of trauma on gut microbial composition is unknown. Our objective was to determine if the gut microbiome is altered in severely injured patients and begin to characterize changes in the gut microbiome due to time and therapeutic intervention.

**Methods:** We conducted a prospective, observational study in adult patients (n=72) sustaining severe injury admitted to a Level I Trauma Center. Healthy volunteers (n=13) were also examined. Fecal specimens were collected on admission to the Emergency Department (ED) and at 3, 7, 10, and 13 ( $\pm 2$ ) days following injury. Microbial DNA was isolated for 16s rRNA sequencing, and  $\alpha$ - and  $\beta$ -diversity were estimated, according to taxonomic classification against the Greengenes database.

**Results:** The gut microbiome of trauma patients was altered on admission (i.e., within 30 minutes following injury) compared to healthy volunteers. Patients with an unchanged gut microbiome on admission were transfused more RBCs than those with an altered gut microbiome ( $p < 0.001$ ). Although the gut microbiome started to return to a  $\beta$ -diversity profile similar to that of healthy volunteers over time, it remained different from healthy controls. Alternatively,  $\alpha$ -diversity initially increased post-injury, but subsequently decreased during the hospitalization. Injured patients on admission had a decreased abundance of traditionally beneficial microbial phyla (e.g., *Firmicutes*) with a concomitant decrease in opportunistic phyla (e.g., *Proteobacteria*) compared to healthy controls ( $p < 0.05$ ). Large amounts of blood products and RBCs were both associated with higher  $\alpha$ -diversity ( $p < 0.001$ ) and a  $\beta$ -diversity clustering closer to healthy controls.

**Conclusions:** The human gut microbiome changes early after trauma and may be aided by early massive transfusion. Ultimately, the gut microbiome of trauma patients may provide valuable diagnostic and therapeutic insight for the improvement of outcomes post-injury.

**Level of Evidence:** Level III

**Study Type:** Prognostic and Epidemiological

## Keywords

trauma; injury; gut microbiome; dysbiosis; transfusion

## Introduction:

The human microbiome contains 100-fold more genes than the human genome and encompasses a vast network of symbiotic microbes, which outnumber mammalian cells (1, 2). The human gut microbiome plays a vital role in host development and homeostasis, including cellular metabolism, nutrient digestion and absorption, development and maintenance of the immune system, and control of the inflammatory response (3, 4). The distribution of intestinal microbes changes with age and is also influenced by diet and disease (5, 6). The majority of gut microbes belong to the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* and are known contributors to the intestinal epithelial barrier (7–9). These microbes provide tonic stimulation to the innate immune system, via toll-like receptor (TLR) signaling, resulting in increased intestinal

motility, reinforcement of epithelial integrity, and increased production of metabolites (10, 11). This process ultimately leads to a preserved distribution of mutualistic and commensal organisms that protect against transiently invading pathogens.

Alterations in intestinal microbiome diversity have been identified in various disease states, including obesity, cardiovascular disease, asthma, inflammatory bowel disease, *Clostridium difficile* (*c.diff*) colitis and colorectal carcinoma cancer (12–15). Recent data also characterize the consequences of an altered distribution of gut microbes, termed dysbiosis. Rapid dysbiosis is seen in critical illness and worsens during a prolonged hospitalization (16). Intestinal dysbiosis has also been attributed to septic complications in critically ill patients, likely due to the critical role that symbiont organisms play in colonization resistance against acquired pathogens (17, 18). Furthermore, in critically ill patients with a prolonged hospitalization, the gut microbial composition dramatically changes causing pathogen communities of extremely low diversity to emerge, triggering further virulence (19).

Although reductions in intestinal microbial diversity have been linked to increased mortality in critically ill patients, less defined is the impact of trauma on the intestinal microbial community of severely injured humans and the consequences of dysbiosis in this population. Trauma patients are susceptible to multiple organ dysfunction syndrome, hospital acquired infection, and the systemic inflammatory response syndrome occurring days to weeks after injury (20, 21). Several early clinical studies in small patient populations have demonstrated phylogenetic changes among gut microbial populations following traumatic and burn injury, yet these studies have lacked the power and the inclusion of clinical intervention analysis to be conclusive (22–25). Preclinical data from various injury models including polytrauma, burn injury, traumatic brain injury (TBI) and spinal cord injury (SCI) also support the concept that traumatic injury alters the gut microbiome which impacts outcome (24, 26–31). Larger clinical studies are needed to address this gap and better understand the microbial changes occurring in the gut following injury especially in the context of early resuscitation.

To further characterize the impact of traumatic injury on the human gut microbiome, we conducted a prospective, observational cohort study of severely injured patients. The aim of this study was to characterize differences in gut microbial communities in trauma patients, identify changes in gut bacterial composition across time in these patients, and begin to elucidate the potential impact of therapeutic intervention. We hypothesized that the gut microbiome is altered in severely injured patients, which is also differentially affected depending on the resuscitation strategy.

## Methods:

Approval was obtained from the University of Texas Health San Antonio Institutional Review Board to conduct this study. Adult patients (age ≥ 18 years; n=72) sustaining a severe injury from blunt or penetrating trauma admitted to University Hospital (UH), a Level 1 Adult and Pediatric Trauma Center in San Antonio, Texas were enrolled prospectively from 2015 to 2016. Enrollment criteria included age ≥ 18 years, Injury Severity Score (ISS) >15, ground transport to UH from the scene, and admission to the UH Surgical Intensive

Care Unit (SICU). Exclusion criteria included: prisoners, age < 18 years, pregnancy, and patients transferred from outside hospitals. Patients were initially enrolled under a waiver of consent on admission to the UH Emergency Department (ED). Consent to participate and continue the study was obtained from the patient or a legal authorized representative as soon as possible following admission. Healthy volunteers (n=13) were also enrolled for comparison.

### Sample collection

Fecal specimens were collected on admission to the UH Emergency Department (Day 0) by rectal swab (COPAN, California, USA) on routine trauma evaluation. Stool was then collected on days 3, 7, 10 and 13 ( $\pm 2$  days) following injury at the time of defecation using a sterile collection method. All fecal samples were stored at  $-80^{\circ}\text{C}$  within 20 minutes of sampled collection for DNA isolation at a later time. Extensive demographic, injury, clinical and outcome data were prospectively collected on all patients. We stratified the number of total blood products (RBCs, fresh frozen plasma, platelets and cryoprecipitate) that patients received into the following groups: none, low (1-5 units), medium (6-10 units), high (11-19 units), and extreme ( $\geq 20$  units). Similarly, we stratified the number of RBCs transfused into none, low (1-3 units), medium (4-6 units), and high ( $\geq 7$  units).

### Gut microbiome analysis

Microbial DNA was isolated from all fecal samples using the QIAGEN QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany). DNA was quantified using the Thermo Scientific NanoDrop 1000 Spectrophotometer. Extracted genomic DNA was then used to amplify the V1-V2 variable region of the 16S rRNA genes with custom-designed primers (F27/R355) using PCR. The Forward Bosshard sequence was AGAGTTTGTGATCMTGGCTCAG (27F) and the Reverse Bosshard sequence was GCTGCCTCCCGTAGGAGT (355R) with the amplicon size of V1-V2 about 340bp (355-27). Subsequently, raw data were processed through the software package Quantitative Insights Into Microbial Ecology (QIIME). Samples were sequenced in duplicate runs to increase available data; since no differences in  $\alpha$  or  $\beta$  diversity were seen, these runs were combined for analysis (data not shown). Libraries for all samples were prepared and sequenced by Paired-end sequencing ( $2 \times 300$  bp) using the Illumina MiSeq platform. A mean of 164,813 pair-end raw reads (median of 165,738 pair-end raw reads) per sample were generated with read length of 301bps. Raw sequences were quality trimmed by removing reads shorter than 200 bases, resulting in a median quality score of 36 for forward reads, and 30 for reverse reads. The operational taxonomic units (OTU) were clustered based on at 97% similarity. Taxonomic classifications were made using the QIIME-formatted Greengenes (gg\_13\_8) 16S rRNA gene database according to standard phylogenetic methods. The OTU table was further filtered by removing OTUs found in only one sample. Rarefaction was performed to a depth of 28000 base pairs, which allowed inclusion of all samples.

Alpha ( $\alpha$ ) diversity, or the intra-population diversity (microbial diversity within individual patients at each time point), was estimated by calculating the number of observed OTUs (richness), evenness of OTU abundance, and diversity using the Faith\_PD and Shannon

Diversity Indices. The Kruskal-Wallis test was used to identify differences. Beta ( $\beta$ ) diversity, or the inter-population diversity (the microbial diversity between patients at each time point), was estimated by constructing principal coordinate analysis plots for the following  $\beta$ -diversity measures: weighted and unweighted UniFrac distances, Bray-Curtis, and Jaccard Indices using QIIME. Statistical analysis of these measures were performed with a permutational analysis of variance (PERMANOVA) for overall significance, with post-hoc pairwise PERMANOVAs run to assess differences across groups. Two way ANOVA with Tukey's multiple comparisons was used to perform statistical analyses on remaining data. Alpha < 0.05 was considered significant for all analyses. QIIME, STAMP and GraphPad Prism were used for the visualization and the statistics of the comparative metagenomics data sets.

## Results:

### Patient population

Characteristics of enrolled patients are shown in Table 1. A total of 72 patients and 13 healthy controls were enrolled with a similar age. The majority of the patients were male with a mean ISS of 21 and suffered from primarily blunt trauma. The mean shock index was 0.95. The mean total blood products transfused was 6 units within the first 72 hours for all of the patients enrolled. The mean total RBCs transfused for all of the enrolled patients was also 6 units within 72 hours. Of note, the majority of the blood and products was transfused in the first 12 hours of admission with only 8 units of RBCs and 2 units of platelets in total given after 12 hours for all of the enrolled patients. Ten percent of the injured patients received a massive transfusion protocol (MTP), defined as 10 or more units of RBCs transfused in 24 hours, while 28% received 4 units of RBCs in the ED on admission.

### $\alpha$ and $\beta$ diversity analyses

The  $\alpha$ -diversity, intra-population diversity as measured by observed OTUs, demonstrated an initial increase compared to healthy controls but decreased significantly by days 5-8 and remained lower compared to admission for the duration of the stay (Fig 1A). The Faith\_PD and the Shannon Indices demonstrated a trend toward lower number of species by day 5, but this measure of  $\alpha$ -diversity did not reach significance (Fig 1A). Conversely, differences in  $\beta$ -diversity (inter-population diversity) and microbial profile, as depicted in the principle components analysis (PCA) plots, were observed on day 0 for patients (depicted in red in Fig 1B-D) versus controls (yellow in Fig 1B-D). While the microbial profile in the injured patients shifted to more closely resemble the microbiome of the healthy controls over time,  $\beta$ -diversity remained significantly different in the injured patients over all time points compared to both healthy controls and day 0 for patients by all  $\beta$  diversity measures (Bray Curtis, (un)weighted UniFrac, Jaccard; Fig 1B-D). PERMANOVA analysis confirmed a significant effect longitudinally and compared to healthy controls ( $p < 0.05$ ). These PCA results suggest that injury disrupts the gut microbiome as early as 30 minutes from the time of injury since samples were taken on arrival to the ED and mean transport time was less than 30 minutes.

The gut microbiome in 26% of the injured patients more closely resembled the microbial profile of the healthy controls as demonstrated by the  $\beta$ -diversity (Fig 1). When these patients were separated (Table 2), the patients whose gut microbiome was similar to controls received significantly more units of RBCs and total blood products in 12 hours versus those whose microbiome differed (7 units RBCs and 14 units of total blood products versus 2 units RBCs and 3 units total blood products respectively,  $p < 0.05$ ; Table 2). Conversely, there were no differences between these groups in the proportion of blunt injuries, ISS, or Shock Index.

To confirm this observation,  $\alpha$ - and  $\beta$ -diversity were estimated for all samples according to RBCs, total blood products transfused, ISS, shock index and blunt vs penetrating trauma. PERMANOVA values for all  $\beta$ -diversity indices on these metrics are shown in Table 3. The weighted UniFrac Index was significantly different according to amount of both RBCs and total blood products transfused (Table 3, Fig 2A-B). When post-hoc pairwise testing was performed for weighted UniFrac  $\beta$  diversity, patients that received over 20 units of blood products were significantly different than patients that received no products ( $p = 0.002$ ), 1-5 units ( $p = 0.02$ ), and 6-10 units ( $p = 0.017$ ), but not different than patients who received 11-19 units ( $p = 0.171$ ). More specifically, similar results were observed for the amount of RBCs in that patients who received greater than 6 units of RBCs had a microbial profile that was significantly different than patients who received no RBCs ( $p = 0.001$ ), 1-3 units of RBCs ( $p = 0.005$ ), and 4-6 units of RBCs ( $p = .047$ ). Similarly,  $\alpha$ -diversity, (i.e., observed OTUs) was significantly greater in patients receiving more than 6 units RBCs and at least 10 units of total blood products (Fig 2A-B).

These results suggest early massive transfusion is associated with preservation of species diversity. Table 3 reveals that  $\beta$ -diversity also differed according to ISS by Weighted UniFrac (Fig 2C) and Bray-Curtis analyses (Plot not shown) suggesting that injury severity also affects the diversity of the gut microbiome. However,  $\alpha$ -diversity was similar between severely injured and less severely injured patients (Fig 2C). While there was no difference in  $\beta$ -diversity by shock index or by blunt versus penetrating trauma (Table 3), patients sustaining a blunt trauma had a significantly lower  $\alpha$ -diversity than those with a penetrating injury ( $P = 0.0025$ , Supplemental Digital Content 1, **.tiff file of graphs of  $\alpha$ -diversity by ISS and blunt versus penetrating trauma**).

## Organism classifications

The representative and most abundant phyla included *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Verrucomicrobia*, and *Tenericutes* in all samples, irrespective of the time after injury (Fig 3). The dominant phyla in both healthy controls and the post-injury groups at all time points were *Firmicutes* and *Bacteroidetes*. These two phyla comprised 95% of the bacteria in the healthy control group and total levels of these two phyla were decreased by 14% in the injured patients at day 0. Combined levels of *Firmicutes* and *Bacteroidetes* further decreased in patients hospitalized longer than 13 days with both phyla comprising 74% of the bacteria present. There were significant decreases in relative abundance at the phylum level seen in *Firmicutes* at days 0 ( $p < 0.0001$ ), days 1-4 ( $p = 0.0015$ ), days 5-8 ( $p < 0.0001$ ), days 9-12 ( $p < 0.0001$ ), and  $> 13$  days ( $p < 0.0001$ )



compared to healthy controls (Fig 3). Conversely, an increased relative abundance in the phylum of *Proteobacteria* was observed at day 0 following injury and at each time point compared to controls (day 0  $p=0.0003$ , days 1-4  $p=0.0166$ , days 5-8  $p<0.0001$ , days 9-12  $p=0.0008$ , and days >13  $p<0.0001$ ; Fig 3). Furthermore, there was a significant increase in *Proteobacteria* in patients hospitalized for >13 days compared to patients on admission ( $p=0.001$ ; Fig 3). There was also an overall significant loss in *Bacteroidetes* in patients with a prolonged hospitalization greater than 13 days compared to patients on admission ( $p=0.03$ , Fig 3).

## Discussion:

The role of the gut microbiome in health and disease has been increasingly recognized with recent attention given to its role in critical illness and injury. However, there is insufficient clinical evidence to further characterize this association. To our knowledge, we have compiled the largest prospective study using non-culture based sequencing techniques of injured patients to date. We demonstrated that severe injury alters the gut microbiome within 30 minutes of injury with continued dysbiosis during a prolonged hospitalization. Despite these distinct differences, there was a subpopulation of patients whose microbiome more closely resembled that of an uninjured individual. Patients in this subpopulation received more RBCs and total blood products within the first 12 hours with a greater percentage of patients receiving a MTP compared to patients whose microbiome differed from uninjured individuals.

These data have similarities to recently published clinical pilot studies, however, our methods and results have some notable differences that provide guidance for developing future studies. Howard et al. found changes in phylogenetic composition and relative abundance within 72 hours of injury in a population of 12 severely injured trauma patients (22). However, they found no difference in composition on admission compared to their controls. Our study had the power to detect a difference; however, it is important to note that we also found a small subpopulation of patients in whom the gut microbiome was not different compared to our healthy controls. Moreover, Howard et al. utilized patients that sustained a trauma but were not found to have injuries as their controls as opposed to our control population of healthy, uninjured volunteers. In this light, it can be speculated that the stress of transport to the hospital has effects on microbial flora in the gut. Future studies might incorporate both this control group and normal healthy individuals to explore this possibility. The findings from our study concerning changes with hospital longevity are also consistent with previous studies performed in critically ill patients with prolonged hospitalization demonstrating dysbiosis and the emergence of low diversity communities (16–19).

We also found that massive transfusion was associated with significant changes in  $\beta$  diversity and demonstrated increased species diversity with more units transfused. This suggests that early massive transfusion may preserve the gut microbiome by increasing gut perfusion. This discovery holds the possibility to inform resuscitative strategies by initiating prehospital transfusion at an early point in time and potentially adjusting resuscitation according to patients' gut microbial profile. Alternatively, these differences may represent a

physiological response from a less injured patient with an intact immune response. However, there was a significantly increased species diversity in patients receiving more blood with a microbial profile more closely resembling that of a healthy control. The finding that increased ISS has similar changes in  $\beta$ -diversity is congruent with these same patients receiving more blood; however, there was no difference in  $\alpha$ -diversity according to ISS.

Traditionally, the phylum *Firmicutes* contains more “health-promoting” bacteria whereas the phylum *Proteobacteria* contains more pathogenic bacteria. Our data reveal rapid dysbiosis seen on admission following trauma. Injured patients already exhibited elevated levels of pathogenic bacteria (*Proteobacteria*) at day 0 and loss of beneficial bacteria (*Firmicutes*) compared to healthy controls. This trend continued during the hospitalization also indicating sustained dysbiosis following injury and hospitalization. As Krezalek et al. have suggested, this supports the development of a pathobiome following injury, critical illness and prolonged hospitalization that could significantly alter patient outcomes and contribute to morbidity and mortality. While their study found that this difference was more pronounced in patients that die later in their hospital stay, whether this is true in the trauma population requires investigation (32).

Extensive evidence supports the gut as an immune organ, especially given its intimate relationship with the gut microbiota (9, 33–35). Trauma-induced injury to the gastrointestinal system can produce profound effects on the gut microbiome and the immunoinflammatory response with resultant consequences on clinical outcome. Increased inflammation at the intestinal level and decreased antimicrobial peptides appear to influence the pathophysiologic processes following injury (24). Damage to the intestinal wall leads to mucosal barrier inflammation, resulting in higher gut nitrate levels and an abnormal mucosal oxygen gradient (36–38). These environmental and metabolic changes lead to proliferation of pathogenic microbes in the *Proteobacteria* phylum (including *Pseudomonas aeruginosa* and *Escherichia coli*), in addition to pathogenic species from the normally health-promoting *Firmicutes* phylum (including *Staphylococcus aureus* and *Enterococcus* spp.) (38–40). This new unstable microbiome ecosystem that emerges more closely resembles that of an infectious state with low diversity microbial communities (38). Targeting this pathobiome with alternatives to antibiotics (e.g., probiotic adjuncts or virulence directed medications) also has the potential to improve outcomes.

Physiologic stressors such as hypoperfusion and vasoconstriction also impair gut motility and alter the intestinal flora. The resulting ischemia reperfusion injury has been shown to induce changes in ileal and colonic microbiota (41–43). Thus, our finding that resuscitation seemingly protects gut microbiota is intriguing. However, the brain-gut axis and central nervous system dysfunction may also impact the gut microbiome through bidirectional vagal pathways between the CNS and the gut, neuroendocrine signaling, immunologic signaling, and the effects of microbe-derived metabolites such as butyrate on the blood brain barrier (27, 44–46). In other types of trauma, intestinal permeability allows for translocation of certain types of bacteria, which may be related to ZO-1, occluding, or mucin levels (24, 47, 48) Additional insults during the hospitalization such as subsequent episodes of hypoxia, prolonged exposure to medications (e.g., antibiotics, opiates, vasopressors, steroids, proton pump inhibitors), multiple procedures and trips to the operating room, and periods of



inadequate or artificial nutrition can all further disturb the gut microbiota (32). These aberrations may then influence clinical outcomes such as mortality from late onset sepsis and inflammatory disorders, hospital and ICU length of stay, infection rates, and inflammatory disorders.

There are several limitations to our study. While associations can be inferred, the findings do not prove causality. Future preclinical studies could elucidate some of the mechanisms involved that may be causing the changes observed in the gut microbiome following injury. In our study, controls were healthy volunteers with some included from hospital personnel. Future clinical studies could expand the control population to include patients sustaining trauma but found to be uninjured. Also, subsequent samples after admission swabs were taken from stool at the time of defecation; this led to inexact time points, which we subsequently pooled. While rectal swabs may provide consistency in the future, it has been shown that rectal swabs have the same integrity of isolated DNA (49, 50). Furthermore, the use of antibiotics was not accounted for in the current study which would almost assuredly influence the gut flora during the hospitalization. The microbiome of antibiotic exposed subjects (trauma patients) is likely different than healthy volunteers not exposed to antibiotics. In addition, massively transfused subjects are known to be depleted of infused drugs, especially antibiotics, which could affect the transfusion-related differences. Of note, no antibiotics were given in the prehospital setting prior to admission to the ED and rectal swabs: therefore, antibiotics likely had little effect admission samples. Future microbial diversity analyses could attempt to account for antibiotic usage.

In conclusion, traumatic injury has an early and profound effect on the gut microbiome with continued dysbiosis (i.e. loss of health promoting microbes and increased pathogenic bacteria) throughout the hospital stay. Differences in diversity are also seen with massive blood transfusion compared to limited or no transfusion implying that early transfusion may confer a protective effect on the gut by improving perfusion and limiting reperfusion injury. Further understanding of the gut's response to traumatic injury holds the potential to inform resuscitative strategies and offer therapeutic strategies such as early transfusion, fecal transplant, administration of probiotics or prebiotics, other nutritional interventions, and the development of virulence directed medications to limit antibiotic usage.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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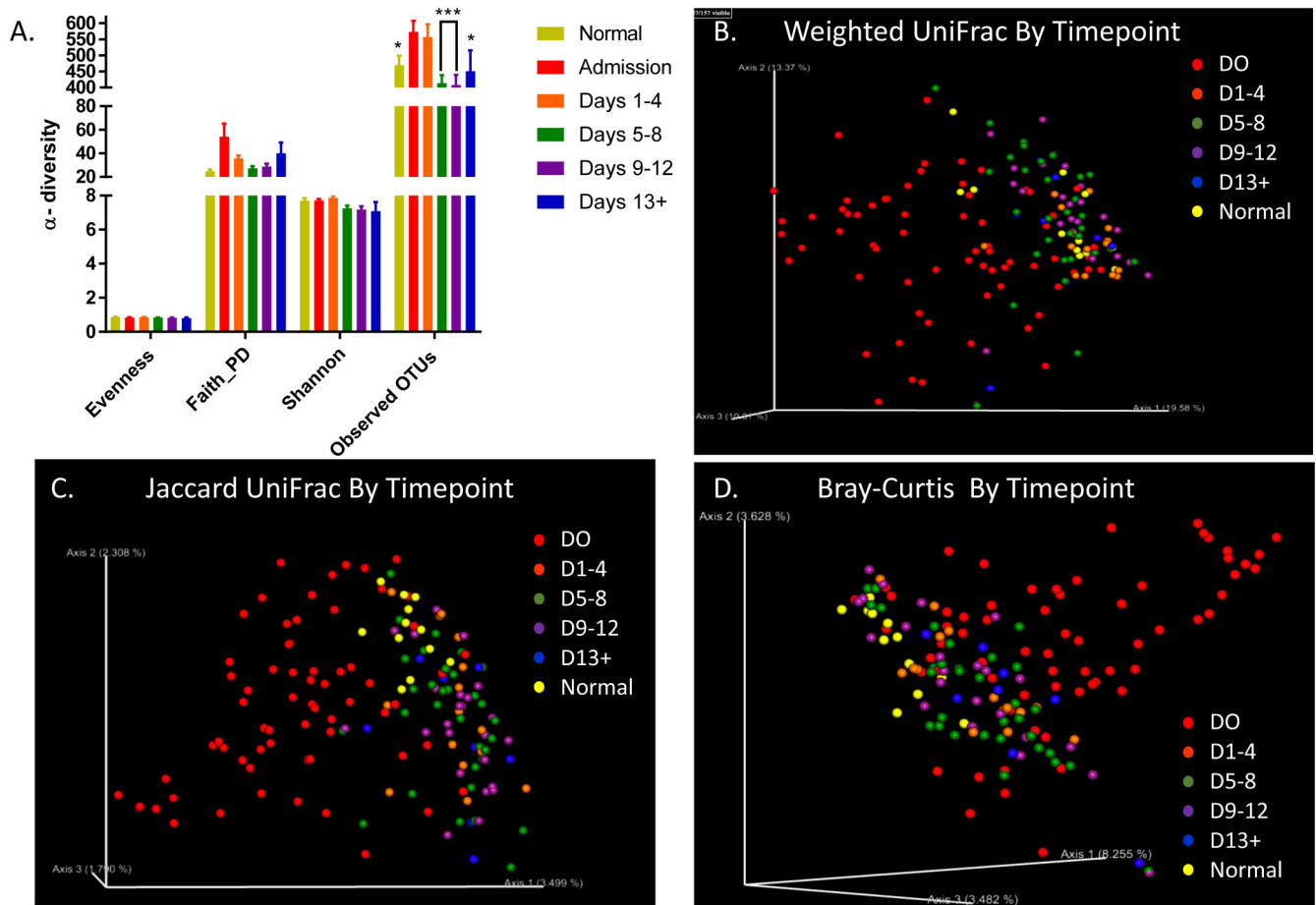
These findings were presented in part at the Military Health System Research Symposium August 19-23, 2016. Kissimmee, Florida and will be presented at the 77<sup>th</sup> Annual Meeting of the American Association for the Surgery of Trauma and the Clinical Congress of Acute Care Surgery and the 4<sup>th</sup> World Trauma Congress, San Diego, CA. The project described was supported by the National Center for Advancing Translational Sciences, National Institutes of Health, through Grant **KL2 TR001118**. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the Department of the Army and the Department of Defense. Support was also received by the University of Texas Health San Antonio Military Health Institute and the Bob Kelso Endowment awarded to the University of Texas Health San Antonio Department of Surgery. The authors would like to thank the following individuals for their support: Basil A. Pruitt, Jr., Dawn Garcia and Korri S. Weldon for 16S sequencing sample processing and data generation, Yidong Chen, PhD for bioinformatics support.

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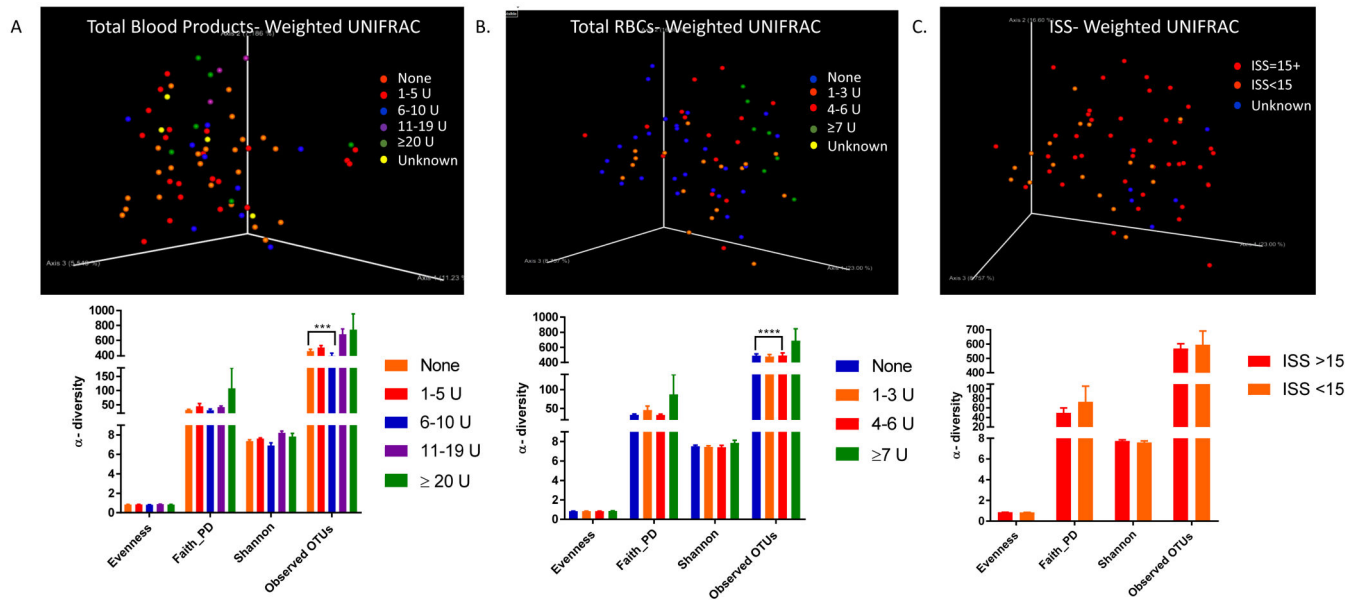
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**Figure 1: Alpha (α) and Beta (β) diversity for all injured patients over time and healthy controls.**

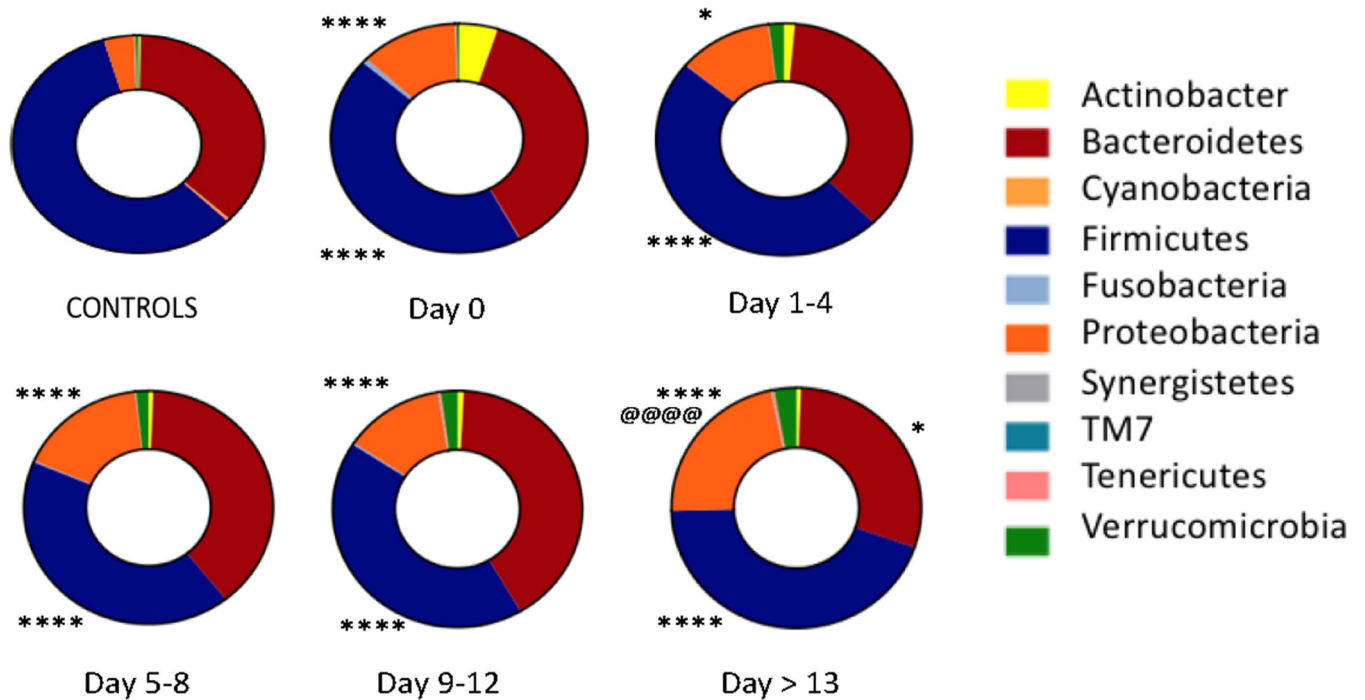
**A.** α-diversity, as measured by observed OTUs, was significantly increased on admission (day 0) compared to healthy controls and subsequently decreased at day 5 ( $p < 0.05$ ). β-diversity is represented by principle components analysis plots for all injured patients over time and healthy controls for the following indices: **B.** Weighted UniFrac (Variability accounted for PC1=19.6%, PC2=13.4%, PC3=10.3%); **C.** Jaccard UniFrac (Variability accounted for PC1=3.5%, PC2=2.3%, PC3=1.8%); **D.** Bray-Curtis (Variability accounted for PC1=8.3%, PC2=3.6%, PC3=3.5%). B-diversity was significantly different between injured patients and healthy controls at all time points and compared to day 0 ( $p < 0.05$ ). Specifically, admission samples (red dots) displayed a massive spatial shift compared to healthy controls (yellow dots) indicating substantial dissimilarity in gut flora. While there is a general shift for the gut microbiome of trauma patients to resemble healthy controls as early as days 1-4 (orange dots), there are still significant differences on days 5-8 (green dots), days 9-12 (purple dots) and days 13+ (blue dots).



**Figure 2: Alpha ( $\alpha$ ) and Beta ( $\beta$ ) diversity for all injured patients for total blood products, RBCs, and injury severity score (ISS).**

Top row illustrates  $\beta$ -diversity represented by the Weighted UniFrac principle components analysis plots, while the bottom row shows  $\alpha$ -diversity (i.e., observed OTUs) for total blood products (A), RBCs (B), and ISS score (C). The weighted UniFrac Index was significantly different according to amount of total blood products infused (Variability accounted for PC1=11.2%, PC2=7.2%, PC3=5.5%), wherein patients receiving none (orange dots) or low (red dots) amounts of total blood products transfused had significantly different microbiome than those getting large (purple dots) or extreme (green dots) of blood products. The same can be said RBCs (Variability accounted for PC1=23.0%, PC2=16.6%, PC3=8.8%), wherein patients receiving no RBCs (blue dots), or a low amount (orange dots) were significantly different than those receiving large amounts of RBCs (green dots). Additionally,  $\beta$ -diversity was different for those with an ISS score above 15 (red dots) versus those with an ISS under 15 (orange dots) (Variability accounted for PC1=23.0%, PC2=16.6%, PC3=8.8%).  $\alpha$ -diversity also differed according to blood products and RBCs infused, but not by ISS score.





**Figure 3: Gut microbial composition following injury over time and healthy controls characterized by phyla.**

The dominant phyla in both healthy controls and the post-injury groups at all time points were *Firmicutes* and *Bacteroidetes*. Relative abundance of the phylum *Firmicutes* was significantly decreased at days 0, days 1-4, days 5-8, days 9-12, and >13 days (\*-p<0.05, \*\*\*\*-P<0.0001) compared to healthy controls. The relative abundance in the phylum of *Proteobacteria* was increased at day 0 following injury and at each time point compared to admission samples (@@@@-p<0.0001).

**Table 1.**

Characteristics of injured patients and healthy controls.

	Control	Total
# of subjects	13	72
Age	43	44
# of Females	6 (46%)	23 (31.9%)
# of Blunt		57 (79%)
# of Penetrating		15 (21%)
Mean ISS		21
Mean Shock Index (HR/SBP)		0.96
Mean number RBCs (units) in 72 hours		6
Mean Total blood products (units) in 72 hours		6
Number of patients receiving 4 units RBCs in the ED		20 (28%)
Number of patients receiving a MTP		7 (10%)
Transport Time (min)		28.5

**Table 2.**

Characteristics of injured patients in whom the gut microbiome was different from controls in injured patients with a gut microbial profile similar to controls.

	Microbiome Different than Controls	Microbiome Similar to Controls	p value
# of subjects	53 (74%)	19 (26%)	
Age	45	43	0.89
# of Females	17 (32%)	6 (32%)	1.00
# of Blunt	42 (79%)	15 (79%)	1.00
# of Penetrating	10 (19%)	5 (26%)	0.52
ISS	20	22	0.34
Shock Index (HR/SBP)	1.02	0.84	0.18
RBCs (units) in 12 hours	2	7	0.0014
Total blood products (units) in 12 hours	3	14	0.0014
Number of patients receiving 4 units RBCs in the ED	12 (22.6%)	7 (36.8%)	0.23
Number of patients receiving a MTP	1 (1.8%)	6 (31.5%)	0.0002
Transport Time (min)	27	27	1.00

**Table 3.**

p-values for each of the measured  $\beta$ -diversity indices for total blood products, total RBCs, shock index, ISS and blunt vs penetrating trauma.

$\beta$ - diversity Measure	Total Blood Products	Total RBCs	Shock Index	ISS	Blunt vs. Penetrating
Bray-Curtis	p=0.098	p=0.117	p=0.732	p=0.05	p=0.247
Jaccard	p=0.074	p=0.097	p=0.706	p=0.088	p=0.123
Unweighted UNIFRAC	p=0.065	p=0.12	p=0.732	p=0.273	p=0.368
Weighted UNIFRAC	p=0.017	p=0.01	p=0.905	p=0.015	p=0.219