A prospective study in severely injured patients reveals an altered gut microbiome is associated with transfusion volume

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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 and at 3, 7, 10, and 13 days (±2 days) following injury. Microbial DNA was isolated for 16s rRNA sequencing, and α and β diversities 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 β-diversity profile similar to that of healthy volunteers over time, it remained different from healthy controls. Alternatively, α diversity initially increased postinjury, 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 α diversity ($p < 0.001$) and a β diversity clustering closer to healthy controls.

CONCLUSION: 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 postinjury. (*J Trauma Acute Care Surg.* 2019;86: 573–582. Copyright © 2019 American Association for the Surgery of Trauma.)

LEVEL OF EVIDENCE: Prognostic and Epidemiological, level III.

KEY WORDS: Trauma; injury; gut microbiome; dysbiosis; transfusion.

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 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 multiple injuries, burn injury, traumatic brain injury (TBI) and spinal cord injury 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.

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These findings were presented in part at the Military Health System Research Symposium August 19–23, 2016. Data from this paper, in varying forms, have been presented and will be presented at: The 2018 Military Health System Research Symposium, Kissimmee, FL. The 77th Annual Meeting of the American Association for the Surgery of Trauma and the Clinical Congress of Acute Care Surgery and the 4th World Trauma Congress, San Diego, CA.

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TABLE 1. Characteristics of Injured Patients and Healthy Controls

	Control	Total
No. subjects	13	72
Age	43	44
No. Females	6 (46%)	23 (31.9%)
No. Blunt		57 (79%)
No. Penetrating		15 (21%)
Mean ISS		21
Mean Shock Index (HR/SBP)		0.96
Mean number RBCs (units) in 72 h		6
Mean total blood products (units) in 72 h		6
No. patients receiving ≥ 4 units RBCs in the ED		20 (28%)
No. patients receiving a MTP		7 (10%)
Transport time (min)		28.5

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 18 years or older, Injury Severity Score (ISS) >15, ground transport to UH from the scene, and admission to the UH Surgical Intensive Care Unit. Exclusion criteria included: prisoners, younger than 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

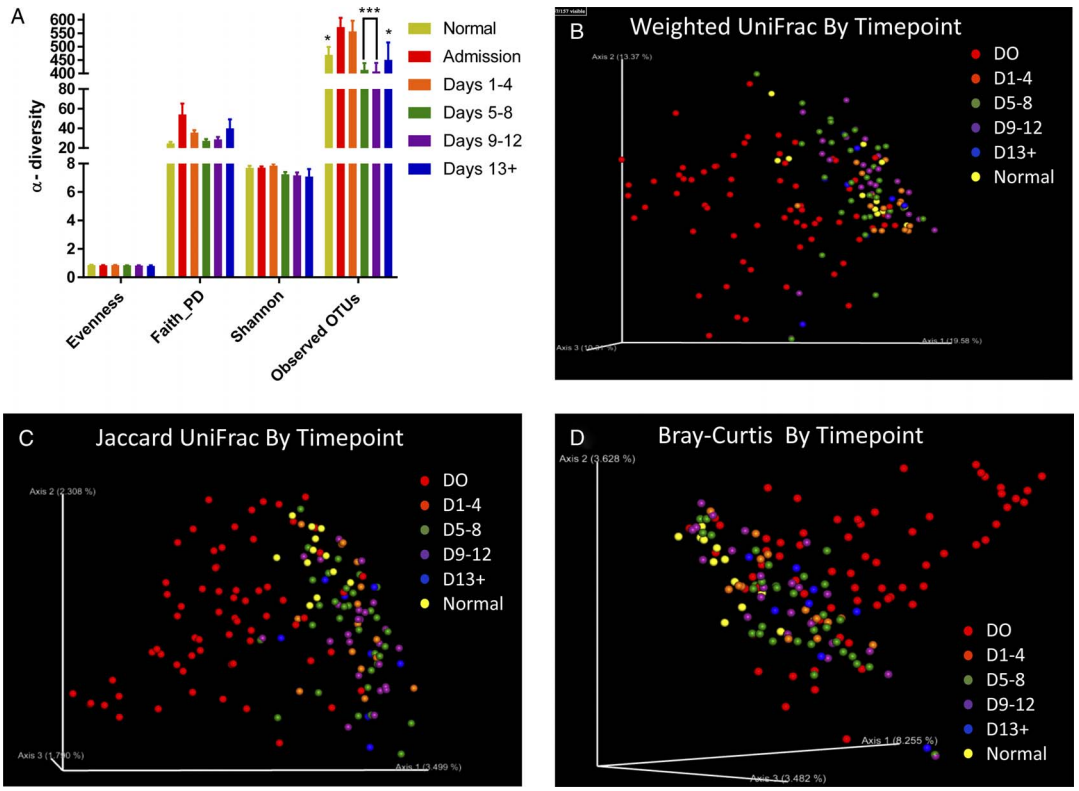

Figure 1. Alpha (α) and Beta (β) diversity for all injured patients over time and healthy controls. *A*, α diversity, as measured by observed OTUs, was significantly increased on admission (day 0) compared with healthy controls and subsequently decreased at day 5 ($p < 0.05$). β diversity is represented by PCA 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%). β diversity was significantly different between injured patients and healthy controls at all timepoints and compared with 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 to 4 (orange dots), there are still significant differences on days 5 to 8 (green dots), days 9 to 12 (purple dots) and days 13+ (blue dots).

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
No. subjects	53 (74%)	19 (26%)	
Age	45	43	0.89
No. females	17 (32%)	6 (32%)	1.00
No. blunt	42 (79%)	15 (79%)	1.00
No. 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 h	2	7	0.0014
Total blood products (units) in 12 h	3	14	0.0014
No. patients receiving ≥ 4 units RBCs in the ED	12 (22.6%)	7 (36.8%)	0.23
No. patients receiving a MTP	1 (1.8%)	6 (31.5%)	0.0002
Transport time, min	27	27	1.00

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 ED (day 0) by rectal swab (COPAN, Murrieta, CA) on routine trauma evaluation. Stool was then collected on days 3, 7, 10, and 13 days (±2 days) following injury at the time of defecation using a sterile collection method. All fecal samples were stored at −80°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 (≥20 units). Similarly, we stratified the number of RBCs transfused into none, low (1–3 units), medium (4–6 units), and high (≥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 AGAGTTTGATCMTGGCTCAG (27F) and the Reverse Bosshard sequence was GCTGCCTCCCGTAGGAGT (355R) with the amplicon size of V1-V2 about 340 bp (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 α or β 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 × 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 301 bps. 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 28,000 base pairs, which allowed inclusion of all samples.

Alpha (α) diversity, or the intrapopulation 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 (β) diversity, or the interpopulation diversity (the microbial diversity between patients at each time point), was estimated by constructing principal coordinate analysis plots for the following β-diversity measures: weighted and unweighted UniFrac distances, Bray-Curtis, and Jaccard Indices using QIIME. Statistical analysis of these measures was 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 less than 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

TABLE 3. *p* Values for Each of the Measured β-diversity Indices for Total Blood Products, Total RBCs, Shock Index, ISS, and Blunt vs Penetrating Trauma

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

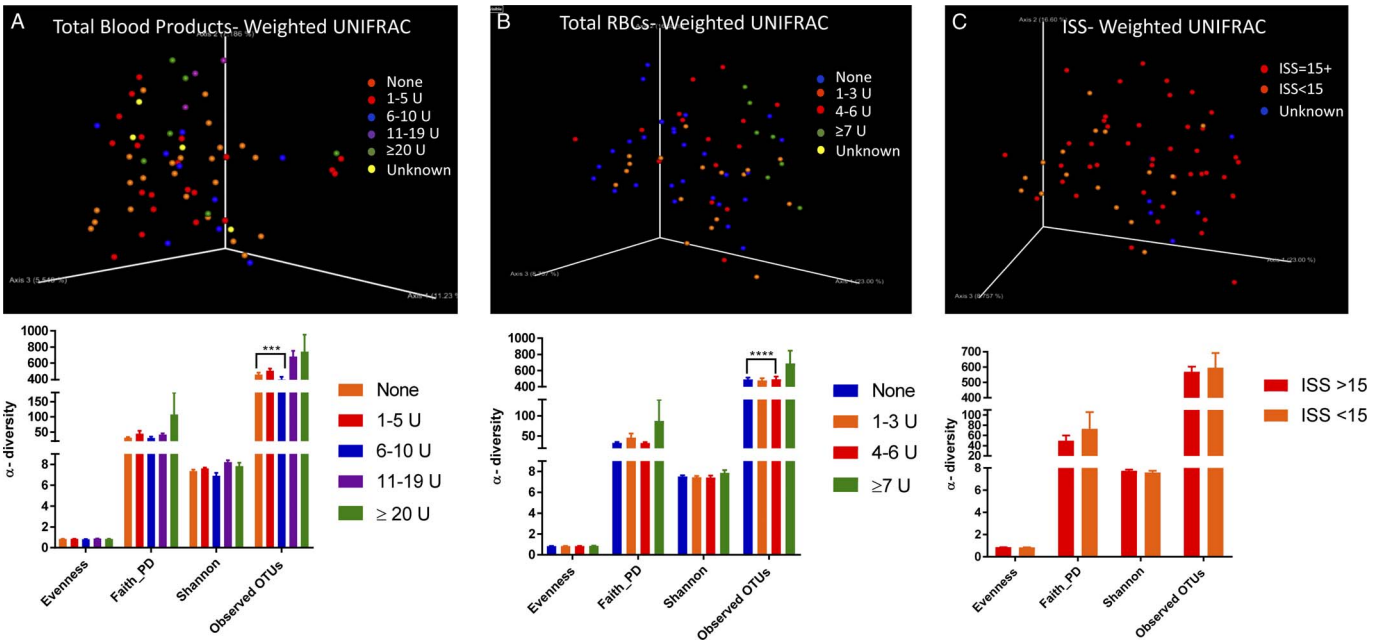

Figure 2. Alpha (α) and Beta (β) diversity for all injured patients for total blood products, RBCs, and ISS. Top row illustrates β diversity represented by the Weighted UniFrac PCA plots, while the bottom row shows α diversity (i.e., observed OTUs) for total blood products (*A*), RBCs (*B*), and ISS 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, β 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%). α diversity also differed according to blood products and RBCs infused, but not by ISS score.

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

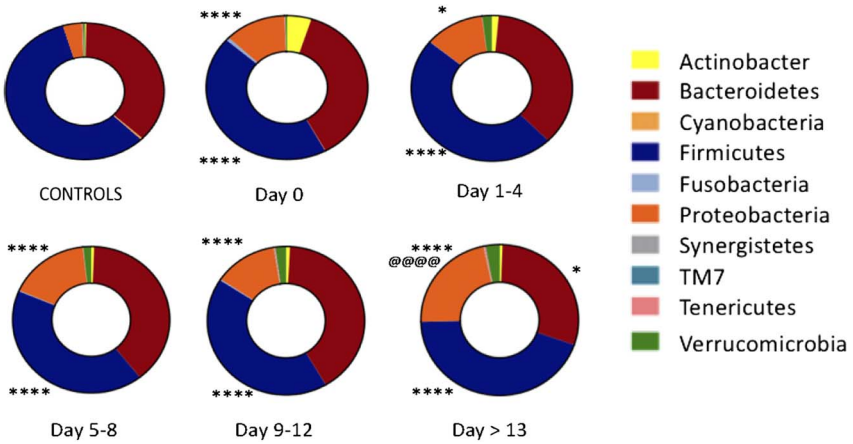

Figure 3. Gut microbial composition following injury over time and healthy controls characterized by phyla. The dominant phyla in both healthy controls and the postinjury groups at all timepoints were *Firmicutes* and *Bacteroidetes*. Relative abundance of the phylum *Firmicutes* was significantly decreased at days 0, days 1 to 4, days 5 to 8, days 9 to 12, and >13 days (*$p < 0.05$, ****$p < 0.0001$) compared with 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 ($^{@@@@}p < 0.0001$).

protocol (MTP), defined as 10 or more units of RBCs transfused in 24 hours, while 28% received 4 units or greater of RBCs in the ED on admission.

α and β Diversity Analyses

The α diversity, intrapopulation diversity as measured by observed OTUs, demonstrated an initial increase compared to healthy controls but decreased significantly by days 5 to 8 and remained lower compared with 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 α diversity did not reach significance (Fig. 1A). Conversely, differences in β diversity (interpopulation 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, β diversity remained significantly different in the injured patients over all time points compared with both healthy controls and day 0 for patients by all β diversity measures (Bray Curtis, (un)weighted UniFrac, Jaccard; Fig. 1B-D). The 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 β 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, α diversity and β diversity were estimated for all samples according to RBCs, total blood products transfused, ISS, shock index and blunt versus penetrating trauma. The PERMANOVA values for all β 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 β 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 to 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 to 3 units of RBCs ($p = 0.005$), and 4 to 6 units of RBCs ($p = 0.047$). Similarly, α 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 β 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, α diversity was similar between severely injured and less severely injured patients (Fig. 2C). While there was no difference in β diversity by shock index or by blunt versus penetrating trauma (Table 3), patients sustaining a blunt trauma had a significantly lower α diversity than those with a penetrating injury ($p = 0.0025$, Supplemental Digital Content 1, tiff file of graphs of α diversity by ISS and blunt versus penetrating trauma, http://links.lww.com/TA/B272).

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 postinjury groups at all timepoints 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 to 4 ($p = 0.0015$), days 5 to 8 ($p < 0.0001$), days 9 to 12 ($p < 0.0001$), and greater than 13 days ($p < 0.0001$) compared with 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 more than 13 days compared with 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 with 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 an MTP compared with 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 with 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 with 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 β 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 β diversity is congruent with these same patients receiving more blood; however, there was no difference in α 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.

AUTHORSHIP

S.E.N. participated in the study design and idea, literature search, data collection and patient enrollment, data generation, data analysis, data interpretation, article drafting and critical revision, project oversight;.D.M.B. participated in the data and statistical analysis and bioinformatics, data interpretation, critical revision, generation of figures. T.R.J. participated in the literature search, data collection, and article drafting. Y.Z. participated in the data analysis and bioinformatics. Z.L. participated in the metagenomic sequencing and data analysis. S.S. participated in the data collection, sample preparation, laboratory analysis, and data generation. M.D.R. participated in the data collection and patient enrollment, study coordinator for patient enrollment and study completion. R.B.J. participated in the data collection and patient enrollment, study coordinator for patient enrollment and study completion. D.R.M. participated in the data collection and patient enrollment, sample preparation, laboratory analysis. C.Z. participated in the data collection and patient enrollment, literature review, and critical revision. E.S. participated in the data interpretation and critical revision. R.M.S. participated in the critical revision, and mentorship. M.G.S. participated in the data interpretation, critical revision, mentorship. D.H.J. participated in the data interpretation, critical revision, mentorship. B.J.E. participated in the critical revision and mentorship.

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DISCLOSURE

The authors have no conflicts of interest to disclose.
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DISCUSSION

Mitchell J. Cohen, M.D. (Denver, Colorado): That's the Ernest E. Moore Shock Trauma Center, I might add. Dr. Agarwal and Dr. deMoya, it's my honor to be asked by the AAST to discuss this excellent work that was nicely presented by Dr. Nicholson.

Indeed, it's my greatest honor to be part of AAST and the important science our organization supports. I am equally honored to be invited to discuss this particular paper which I should say is very important.

I think this science is, indeed, the future and this work is very good. I am very much an admirer of both this work and the group from San Antonio.

In this work the outstanding and very prolific group from San Antonio is centered around the very important topic of the microbiome after injury, in particular their insight about not only the infectious biology but, more importantly, the mechanistic insights into both what drives the biome and also what the biome drives, which is separate from the commonly discussed infectious disease sequelae.

In this work the group follows on from work from many groups, including ours, which has shown in a few papers that both the lung and the gut biome are affected by injury.

In the lung work our group, when I was in San Francisco, showed that smoking exposure predisposes the biome to make more likely acute injury after trauma. And in the gut work we showed a narrowing and increased virulence very quickly after injury as a result of trauma.

In that work our group struggled with what the control group should be. Indeed, there is a huge body of work in the biome outside of trauma showing vast differences in the biome depending on social and health factors.

Your living situation, your socio-economic status, what you ate, who you live with and your acute and chronic health status all radically change what your biome looks like.

When we looked at uninjured, healthy volunteers and uninjured trauma patients we saw vast differences between these control groups. Right? Those of us just walking around the hospital who were willing to give a sample and the patients who came to our trauma center and weren't severely injured.

And because of that in our previous paper we used uninjured actual patients because we felt that they more closely represented our trauma cohort than did the healthy volunteers that were walking around the hospital.

And to that end, with that preface, I have some concerns about the methodology in this paper of picking the control group from healthy volunteers. The authors used these healthy controls which I worry don't reflect an appropriate control to the trauma group. So I have a few questions.

What do we know about these healthy controls? Are they similar in socio-economic or health status to the trauma group? Were they on antibiotics? Did they have any health problems?

And, Number 2, why not use uninjured healthy volunteers? Clearly we all have an over-triage rate, patients that get brought to our trauma center as high-level trauma activations who were totally uninjured. Why not use those? To make that the center of comparison would probably be better.

Number 3. Tell us about the time points. I know that you got these at Time Zero and then at multiple different times but I see from the manuscript that they were convenient samples when patients had bowel movements. That makes the timing very different. Why not use a digital rectal exam to do the rectal swab and then you get really comparable time points?

Number 4. There is a claim that the biome is changed within 30 minutes after injury. This, by my read, is a comparison of those trauma patients to those healthy controls so it very well may be that the trauma patients are just different than the healthy controls. I absolutely believe that the biome changes quickly but may not that quickly.

And so I am wondering do different injury severity types change the alpha diversity? What about the comparison to less-injured or uninjured trauma patients, as I keep harping on? Does that difference persist in your data set?

And, lastly, I have some question about the finding of blood transfusion being protective of beta diversity. While it's an important finding and it clearly fits my bias, is it controlled for all of the other factors, including injury severity, shock, and individual patient characteristics? This needs to be done in a multivariate way rather than a single variate way.

Overall, I have to say that I congratulate the authors on this very important and well-done study. To be clear that they have my scientific admiration and I think this is really important work. I really do think it is the future and stuff we are going to be talking about for many years to come.

My questions are not meant to suggest otherwise, but only to foster scientific discussion and to get to the bottom of this crucial biology and topic.

I thank the AAST for the privilege of discussing this paper.

Susan Evans, M.D. (Charlotte, North Carolina): You used ISS as your way to determine whether or not you were going to include patients in the study. How did you do ISS?

Most of us I think don't have access to ISS while in the trauma bay. Can you tell us, do you do that or do you have registrars who are immediately ready for that information? Thank you.

Susannah E. Nicholson, M.D. (San Antonio, Texas): I'd like to thank Dr. Cohen and Dr. Evans for their questions and for the important discussion.

So to answer the question about the controls, certainly that is a very important observation. But we, you know, in this early pilot study we did, we picked healthy volunteers in this study.

And I think certainly worth including uninjured trauma patients is a great suggestion for future studies and would like to incorporate that into future work. That being said, our controls that were used were not on any antibiotics, overall were fairly healthy.

Most of the patients, in reviewing that data – although I would have to go back in and do more of a statistical analysis for my patient data – in terms of health problems I do have some of that but most of the patients were actually fairly healthy, as well. We tried to limit patients that were on antibiotics.

Also, in terms of the fecal samples, again, another great point. You know in designing this study I think if I had to do it over again I would go ahead and try to use the rectal swab on the subsequent days so to take that out of the equation.

That being said, so a rectal swab, there is literature to support the rectal swab as the same as a stool sample but definitely the timing part of the stool sample would be helpful to have that more standardized.

In terms of the 30-minute, that question, again, we certainly looked at ISS and there was no difference in alpha diversity in ISS or however there was a difference in the severely-injured patients compared to the less-severely-injured patients.

There was no difference in beta diversity in blunt versus penetrating trauma and no difference in shock index in beta diversity. However, there was a difference in alpha diversity in patients that had penetrating trauma did have a higher, actually had a higher diversity.

Again, more work is needed to better delineate that time period. Also, you know, this study obviously does not show, does not discuss causality but some more preclinical work is needed to also address this.

In regards to – in regards also to the ISS or in looking at some of the other factors in some of our analysis, so with the beta diversity the analysis for that is _____ in nature and is not discreet.

So we did – that being said, we did do multiple _____novas for the ISS for shock index for blunt and penetrating trauma in addition to the numbers of blood products and our RVCs first received.

To address your point, we definitely could incorporate in looking at alpha diversity since it incorporates specific numeric data points we could certainly incorporate some of the other factors into an additional analysis.

For the beta diversity, also going back to with the transfusion, there was a significant difference in the beta diversity

And this was in the absence of those healthy controls in the patients that on Day Zero that received more blood versus those that received less blood. In the alpha diversity they had a more diverse microbiome which has traditionally been seen as a more of a beneficial thing.

So to sum all of that up, it is definitely great to, a great area where we want to do more research.

Oh, and, also, to address Dr. Evans' question, how we determined ISS, our group has developed a table, basically, that helps us define you know based on CT findings. And we estimate to the best of our knowledge the ISS and then can go back and confirm based on the trauma registry.