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Investigation of Microbiota Alterations and Intestinal Inflammation Post-Spinal Cord Injury in Rat Model

Gregory O'Connor,[1,*] Elisabeth Jeffrey,[1,*] Derik Madorma,[1] Alexander Marcillo,[2] Maria T. Abreu,[3] Sapna K. Deo,[1] W. Dalton Dietrich,[2] and Sylvia Daunert[1]

Abstract

Although there has been a significant amount of research focused on the pathophysiology of spinal cord injury (SCI), there is limited information on the consequences of SCI on remote organs. SCI can produce significant effects on a variety of organ systems, including the gastrointestinal tract. Patients with SCI often suffer from severe, debilitating bowel dysfunction in addition to their physical disabilities, which is of major concern for these individuals because of the adverse impact on their quality of life. Herein, we report on our investigation into the effects of SCI and subsequent antibiotic treatment on the intestinal tissue and microbiota. For that, we used a thoracic SCI rat model and investigated changes to the microbiota, proinflammatory cytokine levels, and bacterial communication molecule levels post-injury and gentamicin treatment for 7 days. We discovered significant changes, the most interesting being the differences in the gut microbiota beta diversity of 8-week SCI animals compared to control animals at the family, genus, and species level. Specifically, 35 operational taxonomic units were enriched in the SCI animal group and three were identified at species level; *Lactobacillus intestinalis*, *Clostridium disporicum*, and *Bifidobacterium choerinum*. In contrast, *Clostridium saccharogumia* was identified as depleted in the SCI animal group. Proinflammatory cytokines interleukin (IL)-12, macrophage inflammatory protein-2 (MIP-2), and tumor necrosis factor alpha were found to be significantly elevated in intestinal tissue homogenate 4 weeks post-SCI compared to 8-weeks post-injury. Further, levels of IL-1β, IL-12, and MIP-2 significantly correlated with changes in beta diversity 8-weeks post-SCI. Our data provide a greater understanding of the early effects of SCI on the microbiota and gastrointestinal tract, highlighting the need for further investigation to elucidate the mechanism underlying these effects.

Keywords: *Bifidobacterium choerinum*; *Clostridium disporicum*; *Clostridium saccharogumia*; intestinal microbiome; *Lactobacillus intestinalis*; quorum sensing molecules; spinal cord injury

Introduction

In the United States alone, there are approximately 250,000 people living with the devastating consequences of spinal cord injury (SCI). Each year, approximately 11,000–17,000 new SCIs occur, resulting in a spectrum of neurological impairments and disabilities that compromise the quality of life (QoL) of the individuals affected.[1] Although a significant amount of research has focused on the pathophysiology of SCI with the goal of neural protection and repair, there is limited information on the consequences of SCI on remote organs and systems.[2–5] Experimental and clinical data have shown that SCI can have significant negative effects on a variety of organ systems, such as the gastrointestinal (GI) tract, lungs, kidney, spleen, and liver,[5,6] affecting their function and compromising the QoL of individuals with SCI. Although the mechanisms underlying these remote changes are not fully understood, recent studies unveiled that SCI can produce systemic immune challenges and inflammatory events that critically influence the body's progressive response and metabolic status.[7–10]

GI complications are typically responsible for 11% of hospitalization in the SCI population[11,12] and are rated as serious problems compromising QoL.[13] GI motility disorders include delayed gastric emptying, abdominal pain, and diminished transit through the GI tract.[14–18] Importantly, 30% of people living with the detrimental consequences of SCI consider bowel disorders to be a greater concern than bladder or sexual dysfunction.[19] Despite this important consequence of SCI, limited studies have focused on understanding the acute and chronic effects of SCI on GI function and the underlying mechanisms responsible for these changes. In this regard, Kabatas and colleagues[3] described the spectrum of neural and anatomical abnormalities shown in the GI system after SCI in an experimental animal model, and suggested that such GI

[1]Department of Biochemistry and Molecular Biology, University of Miami, Miller School of Medicine, Miami, Florida.
[2]Miami Project to Cure Paralysis, Department of Neurological Surgery, University of Miami, Miller School of Medicine, Miami, Florida.
[3]Division of Gastroenterology, University of Miami, Miller School of Medicine, Miami, Florida.
*These authors contributed equally to this work.

disorders require therapeutic development to target specific underlying mechanisms.

The gut microbiome is a determinant of human health and, as such, an area of current intense research, especially given that it relates to chronic inflammatory diseases of the gut.[20] Despite this link between the intestinal microbiome and health, there are very limited data characterizing the gut microbiota in adult SCI patients with bowel dysfunction. In one recent clinical study, Gungor and colleagues[21] reported alterations in gut microbial patterns in stool samples of adult chronic SCI patients. Although this work is important in the field, additional research is required to clarify critical aspects of the relationship between the microbiota and SCI, such as investigations into potential intestinal inflammatory events post-SCI and identification of specific bacterial strains of interest that have been altered. Additionally, a study by Kigerl and colleagues[22] aimed at determining how the gut microbiome affects functional recovery of a mouse model of spinal cord injury up to 5 weeks post-injury. This study showed that administration of probiotics with lactic acid producing bacteria led to better recovery of function with neuroprotection, highlighting the importance of microbiome research post-SCI.

Along with investigating the intestinal microbiome, studies have begun to look at the role of bacterial quorum sensing (QS). Bacterial QS is the mechanism by which bacteria communicate through small molecules collectively known as quorum sensing molecules (QSMs). QS regulates various bacterial processes, including growth, biofilm formation, and virulence factor production; thus, QS has been implicated in various disease and infection states.[23–27]

Given the above encouraging studies and the need for a better understanding of the mechanism that leads to GI problems post-SCI, we decided to investigate the effect of changes in the intestinal microbiome and GI tract in SCI by using an established SCI rat model (moderately severe thoracic [T9] injury),[28] allowing for assessment of microbiota and inflammation post-injury. Specifically, we determined the changes in the intestinal microbiota post-SCI and antibiotic treatment, with species-level identification of certain altered species. By focusing on species-level identification, we can characterize the effects the altered levels of the identified species are potentially having on the health of the host. Further, we investigated the prospective underlying mechanisms by determination of the levels of proinflammatory cytokines and bacterial communication, specifically as it relates to quorum sensing and the levels of QSMs. The studies described herein aim at contributing to the microbiome, SCI, and GI fields by providing a better understanding of the effects post-SCI on the microbiota and GI tract.

Methods

Animals and spinal cord injury rat model

Adult female Fischer rats were housed according to National Institutes of Health guidelines and the Guide for the Care and Use of Animals. Only female rats were selected for this study to decrease potential microbiota variations related to differential recovery, because there are significant differences in locomotor performance and volumes of preserved gray and white matter within the injured cord segment between male and female rats.[28] All animal procedures were approved by the University of Miami Miller School of Medicine Institutional Animal Care and Use Committee. Animals were handled by a core facility, and all samples collected from animals were blinded before being transferred to laboratory personnel for processing and analysis. Before surgery, animals were weighed and anesthetized with a mixture of 2% isoflurane and 40% oxygen and prepared for surgery as previously described.[28] An adequate level of anesthesia was determined by monitoring the corneal and hindlimb withdrawal reflexes. Backs were next shaved and aseptically prepared with chlorhexidine and lacrilube ophthalmic ointment applied to eyes to prevent drying. Rats were placed on a warming pad to maintained body temperature at 37 ± 0.5°C as assessed by a rectal probe.

Rats were next subjected to a moderately severe contusion injury using the MASCIS (Multicenter Animal Spinal Cord Injury Study) weight-drop device (NYU Impactor).[29] A laminectomy at thoracic vertebra T9 was produced exposing the dura mater. Stabilization clamps were placed to support the column and the exposed spinal cord (T10) injured by dropping a 10.0-g rod from a height of 25.0 mm. After injury, muscles were sutured and the skin closed with metal clips. Rats were allowed to recover in a warmed cage (approximately 30°C) with water and food easily accessible for 24 h. Standard rodent diet was given with filtered, sterilized water (water bottles were fitted with long curved sipper tubes) and animals were kept on Alpha Dri® bedding (changed three times a week) in pairs. Post-recovery, rats were kept at approximately 24°C with 12-h on/off light cycles. All rats, both SCI and sham-operated controls, received antibiotic treatment (gentamicin, 5 mg/kg) for the first 7 days after surgery to prevent additional infections. The analgesic, Buprenex (0.03 mg/kg), was also given daily for 2 days. Bladders were manually expressed twice-daily until bladder function returned. Animals had access to flood and water *ad libitum*. Sham-operated animals that underwent all surgical procedures to expose the spinal cord, but were not injured, served as controls. Pairs were assigned after SCI and control surgery recovery, allowing for co-housing of injured and uninjured animals.

Tissue sampling

This study consisted of four groups of animals: 1) controls, that is, sham-operated but not injured rats, which were sacrificed 4 weeks post-sham surgery ($n = 8$); 2) SCI-injured rats, which were sacrificed 4 weeks post-SCI ($n = 8$); 3) controls, that is, sham-operated but not injured rats, which were sacrificed 8 weeks post-sham surgery ($n = 8$); and 4) SCI-injured rats sacrificed 8 weeks post-SCI ($n = 8$). Eight weeks post-injury would be used for the subsequent microbiome sequencing to model a long-term SCI injury,[30] whereas 4 weeks post-injury served as a time point for comparison of cytokine and QSM levels over time. The entire intestine, stomach excluded, was collected at sacrifice. Intestinal content was manually extracted from the whole intestine, by cutting open of the intestines and collecting the internal content, and mixed to homogenize prior to microbiome sequencing. In preparation for QSM and cytokine analysis, tissue from the small intestine was homogenized by douce homogenizer, in an equal amount (weight/volume) of distilled, deionized water, and intestinal content was diluted 1:100 in deionized water.

Microbiome sequencing

Intestinal content samples from rats at 8 weeks post-SCI or sham surgery were sequenced by 16S V4 ribosomal RNA (rRNA) gene sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA). Operational taxonomic units (OTUs) were considered significant if the false discovery rate (FDR)-corrected p value was less than or equal to 0.05 and the absolute value of the $\log_2$ fold change was greater than or equal to 1. Microbiome data processing and multi-variate statistical analyses were performed utilizing PhyCA-Stats™ analysis software. Probe intensities were background subtracted and scaled to Control Mix. Hybridization scores were calculated as $\log_2$ (mean probe fluorescence intensity) $\times$ 1000. OTUs were defined by high 16S rRNA gene sequence similarity, with the majority demonstrating >99% intra-OTU concordance. Before classification analysis, data reduction was done by multiple filtering steps, and taxa-sample intersections were calculated using abundance (AT) and binary matrices (BT). Pair-wise BT and AT dissimilarity scores were computed using Unifrac and weighted Unifrac (Wunifrac) distance metric. Wunifrac

metric considers OTU abundance in addition to phylogenetic distance between OTUs. Hierarchical clustering by average-neighbor and principal coordinate analysis (PCoA) were used to graphically summarize intersample relationships according to AT and BT dissimilarity scores. Unsupervised classification using the nearest shrunken centroid method was used to identify OTUs with most significant differences in abundance between groups. A randomization/Monte Carlo permutation–based test (Adonis test) was utilized for significance testing of differences between discrete and continuous variables.

Proinflammatory cytokines

Concentrations of proinflammatory cytokines interleukin (IL)-1β, IL-12, macrophage inflammatory protein 2 (MIP-2), and tumor necrosis factor alpha (TNF-α) were determined in small intestine homogenates from all four animal groups (described in *Blood and tissue sampling* section) by using commercial enzyme-linked immunosorbent assay kits from Life Technologies (Carlsbad, CA) as per the manufacturer's instructions.

Quorum sensing molecules

Biosensors based on *Escherichia coli*–containing plasmids that include the genes of a transcriptional regulator of the short-chain AHL (pSB406 plasmid) and long-chain AHL (pSB1075) QS communication pathways along with a bioluminescent reporter gene were used in these studies as described previously.[26] Additionally, a biosensor for AI-2 was based upon genetically modified *Vibrio harveyi* (MM32) as described previously.[24] Briefly, overnight cultures of cells were refreshed in LB broth (pSB406 and pSB1075) or AB media (MM32) and grown to OD600 0.45 (pSB406 and pSB1075) or 0.05 (MM32). Dose-response curves to determine response to analyte were generated from the cognitive analyte for each sensor (C6-AHL for pSB406, C12-AHL for pSB1075, and AI-2 for MM32). Analyte (10 μL) or intestinal content (1% weight per volume in distilled, deionized water) was added to 90 μL of sensor and incubated for 2 h before determination of bioluminescent intensity.

Statistical analysis

Microbiota data were analyzed as described in the above Microbiome Sequencing section. Cytokine and QSM analysis data were expressed as the mean ± the standard deviation of mean and analyzed in GraphPad Prism 7.04. Statistical significance was assessed with Student's *t* test, unless otherwise stated.

Results

Microbiome sequencing

Intestinal content samples from 8-week post-SCI and control animal groups were sequenced by 16S V4 rRNA gene sequencing on the Illumina MiSeq platform. A total of 5,048,814 reads were obtained from 23 (20 experimental, three control) samples with a mean of 219,513 reads per sample. Alpha diversity and Shannon analysis showed similar richness in read diversity between groups (Fig. 1). Rarefaction curve analysis indicated that samples were sequenced to adequate depth to capture microbiome composition. We discovered differences in the microbiota beta diversity of 8-week SCI animals compared to control animals at the family, genus, and species level. The most abundant families were *Lactobacillaceae*, *Turibacteraceae*, and *Bifidobacteriaceae* (Fig. 2; Table 1). OTUs were significantly different in samples from SCI animals within families of *Bifidobacteriaceae* and *Clostridiaceae* and significantly higher in abundance compared to animals in the control group (Table 1). Specifically, from 785 classifiable OTUs, 59 OTUs were significantly different in samples from the SCI animal group (Fig. 3). From these 59 significant OTUs, 35 OTUs were enriched in the SCI animal group and three were identified at species level; *Lactobacillus intestinalis*, *Clostridium disporicum*, and *Bifidobacterium choerinum* (Table 2). Additionally, *Clostridium saccharogumia* was identified as depleted in the SCI

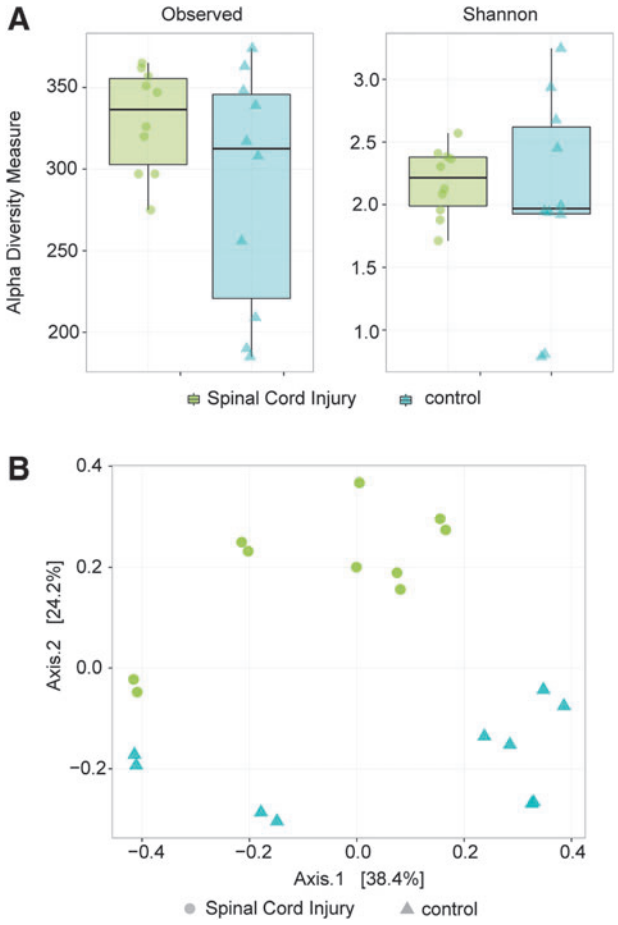

FIG. 1. Diversity of OTUs in 8-week intestinal content. (**A**) Alpha diversity of OTUs in intestinal content of 8-week sham control and SCI rats: observed diversity based on number of OTUs present in each sample and Shannon diversity index to account for richness and evenness of OTUs within a sample. (**B**) Dimensional reduction of Bray-Curtis distance between samples using the PCoA ordination method. OTUs, operational taxonomic units; PCoA, principal coordinates analysis; SCI, spinal cord injury.

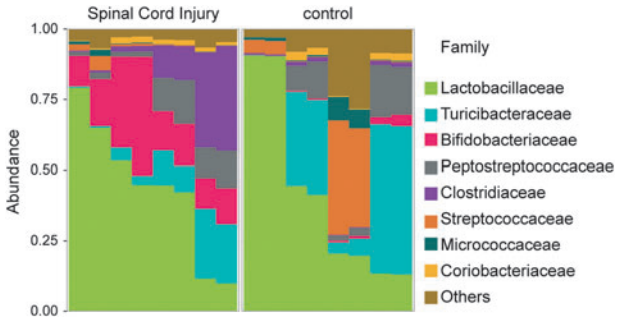

FIG. 2. Differences in microbiota. Most abundant taxa in intestinal content of sham control and SCI rats at the family level. SCI, spinal cord injury.

Table 1. Most Abundant Families

Family	*Chi-square*	p *value*	*SCI mean*	*Control mean*
Lactobacillaceae	0.0993	0.75	43.8	41.7
Turicibacteraceae	0.5404	0.46	9.54	22.8
Bifidobacteriaceae	**11.2941**	**<0.001**	**19.3**	**1.2**
Peptostreptococcaceae	0	1	7.26	7.74
Clostridiaceae	**5.8346**	**0.02**	**12.5**	**0.961**
Streptococcaceae	0.5404	0.46	1.2	10.7
Micrococcaceae	3.1875	0.07	0.535	2.31
Coriobacteriaceae	0.0441	0.83	1.41	1.43

The top eight most abundant bacterial families in samples 8 weeks post-SCI or sham surgery by Kruskal-Wallis' rank-sum test including percent relative abundance of each family.

Bolded rows indicate the values that are statistically significant (p-value less than 0.05).

SCI, spinal cord injury.

animal group. Interestingly, *Lactobacillus intestinalis* accounted for 15.5% of all sequences.

Proinflammatory cytokine levels

Small intestines from SCI and controls animals were harvested at the 4- and 8-week time points, homogenized, and assessed for proinflammatory cytokines IL-1β, IL-12, MIP-2, and TNF-α (Fig. 4). IL-1β was increased in the SCI animal group 4 weeks post-SCI, with levels returning to basal by the 8-week time point (Fig. 4A). IL-12 was statistically significantly elevated in SCI animals compared to control animals at the 4-week time point, as well as significantly higher in the SCI animal group at the 4-week compared to 8-week time points (Fig. 4B). Levels of MIP-2 and TNF-α were significantly higher for both SCI animals at the 4-week time point as compared to 8 weeks (Fig. 4C,D).

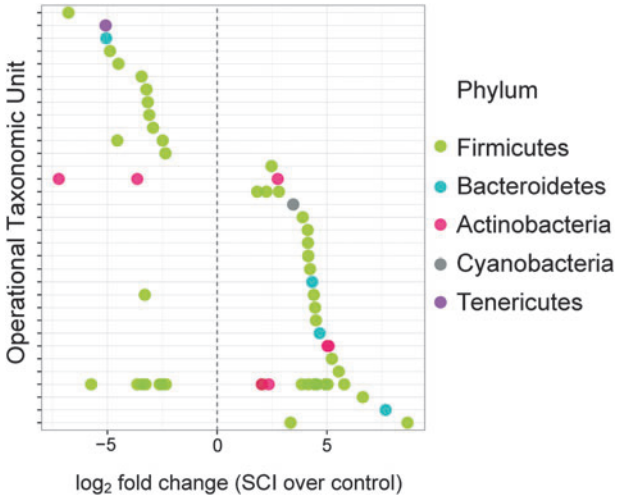

FIG. 3. Differentially abundant OTUs. Plot shows differentially abundant OTUs in intestinal content of SCI animals compared to sham control. Each point represents an OTU belonging to each genus. Features were considered significant if their FDR-corrected p value was less than or equal to 0.05, and the absolute value of the $\log_2$ fold change was greater than or equal to 1. OTUs, operational taxonomic units; FDR, false discovery rate; SCI, spinal cord injury.

Table 2. Species-Level Identification

Strain name	*Interpretation*	*Adjusted* p *value*
Lactobacillus intestinalis	Enriched in case	9.87×10^{-7}
Clostridium disporicum	Enriched in case	2.69×10^{-7}
Bifidobacterium choerinum	Enriched in case	1.39×10^{-11}
Clostridium saccharogumia	Enriched in control	2.35×10^{-4}

OTU, species-level identification, and adjusted p value for the four species found differentially abundant in SCI animals compared to the sham control.

OTU, operational taxonomic unit; SCI, spinal cord injury.

In the interest of elucidating connections between these changes in proinflammatory markers and the reported changes in gut microbiome composition, we conducted permutational multivariate analysis of variance analysis to determine covariate significance. We found that three of the proinflammatory cytokine variables contributed significantly to the beta diversity of the samples. That is, cytokines IL-1β, IL-12, and MIP-2 were found to be significantly correlated with microbiome changes in SCI animals. IL-1β was significantly correlated with 23 OTUs by a $\log_2$ fold change per relative unit. Of these, eight were identified at species level and all negatively correlated with IL-1β, including *Streptococcus acidominimus*, *Clostridium sp.40*, *Ruminococcus bromii*, *Faecalibacterium prausnitzii*, *Gemmiger formicillis*, *Ruminococcus obeum*, *Dorea longicatena*, and *Corynebacterium mastitidis* (Table 3). For IL-12, from the 12 OTUs found to be significant, five of these had a positive $\log_2$ fold change per relative unit of IL-12. Three of the 12 OTUs were identifiable at species level as *Lactobacillus intestinalis*, *Bifidobacterium choerinum* (negative $\log_2$ fold change), and *Clostridium saccharogumia* (positive $\log_2$ fold change). MIP-2 significantly correlated with microbiome diversity differences, but there were no significant OTUs detected; 78 OTUs had an unadjusted p value less than 0.05, but failed to meet the absolute value $\log_2$ fold change threshold of 1.

Microbiome quorum sensing molecules levels

To investigate the effects of SCI on the bacterial signaling molecules of the microbiota, intestinal content from animals that had underwent SCI or sham surgery was collected at 4 and 8 weeks post-procedure. Specifically, we investigated the levels of short- and long-chain *N*-acyl homoserine lactones (AHLs), used by Gram-negative bacterial QSM communications, as well as autoinducer-2 (AI-2), used by both Gram-positive and -negative bacterial QSM communications, in the intestinal content (Fig. 5). The levels of short-chain and long-chain AHLs remained similar between animals in SCI and control groups at both the 4- and 8-week time points. In contrast, levels of AI-2 were similar at the 4-week time point, but were significantly elevated 8 weeks post-SCI as compared to the control group. Additionally, levels of AI-2 in SCI animals at 8 weeks were significantly higher compared to those of the same rats 4 weeks post-SCI.

Discussion

In this study, we investigated whether SCI can induce early intestinal inflammation and alteration in the microbiota post-SCI and gentamicin treatment. To address these questions, we conducted a series of experiments using an SCI rat model of

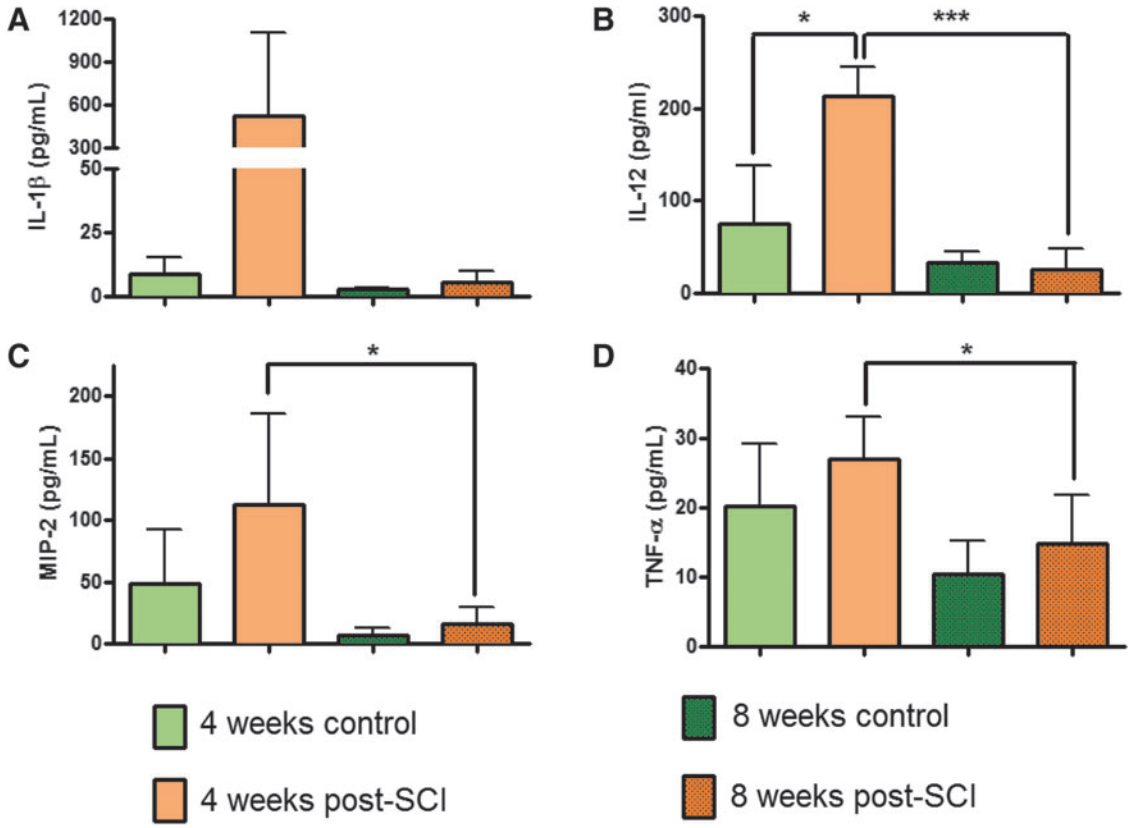

FIG. 4. Cytokines in intestinal homogenate. Changes in proinflammatory cytokines (**A**) IL-1β, (**B**) IL-12, (**C**) MIP-2, and (**D**) TNF-α measured in intestinal homogenate of 4- and 8-week post-sham or -SCI animals (two-tailed *t* test between each of the four groups, $*p < 0.05$; $***p < 0.0001$). IL, interleukin; MIP-2, macrophage inflammatory protein-2; TNF-α, tumor necrosis factor alpha.

moderately severe thoracic [T9] injury. Specifically, our study focused on evaluating alterations in the gut microbiome by microbiome sequencing, with a focus on identifying specific bacterial species with altered levels, after inducing SCI or sham surgery control in animals; *L. intestinalis*, *C. disporicum*, and *B. choerinum* were significantly enriched in samples coming from animals with SCI, whereas *C. saccharogumia* was significantly depleted (Table 2).

Table 3. Species Correlated with IL-1β

Strain name	*Log_2 fold change*	*Adjusted* p *value*
Streptococcus acidominimus	–4.41	0.0217
Clostridium sp. 40	–4.02	0.0279
Ruminococcus bromii	–4.72	0.0212
Faecalibacterium prausnitzii	–4.82	0.024
Gemmiger formicilis	–3.99	0.0279
Ruminococcus obeum	–4.98	0.0148
Dorea longicatena	–6.55	0.00387
Corynebacterium mastitidis	–7.23	0.00143

OTU, species information, fold change, and adjusted *p* value shown for taxa significantly correlated with changes in IL-1β.

Bolded row indicates a bacterial strain of interest that is further discussed in the Discussion section.

OTU, operational taxonomic unit; IL-1β, interleukin-1 beta.

Our analysis revealed a high prevalence of the *Lactobacillaceae* family post-SCI. This family is mainly responsible for fermenting carbohydrates into lactic acid, is commonly found in the GI microbiome, and has been shown to promote recovery post-SCI.[22] Specifically, work by Kigerl and colleagues,[22] which focused on immune changes and motility post-injury, revealed that probiotic administration of lactic acid producing bacteria aided in functional recovery in mice post-SCI. Additionally, it has been shown that low levels of *L. intestinalis* may lead to obesity in rats; thus, the substantial increase in abundance of *L. intestinalis* that we discovered (Table 2) is likely beneficial to the host animal. In addition, we found significantly higher levels of the bacterial QSM AI-2 in the intestinal content from animals 8 weeks post SCI compared to controls (Fig. 5A). Production of AI-2 has been identified as a contributing factor to persistence of *Lactobacillaceae* in the gut.[31] Therefore, the increased abundance of *L. intestinalis* is likely being supported by the increase in AI-2 QS that we observed. This may also help to explain the unusually high prevalence of *L. intestinalis* species (15.5% of all sequences) in this study.

Little is known about *Clostridium disporicum*, which was found to be significantly more abundant in the SCI group compared to the control (Table 2), given that it is not usually dominant in the healthy human gut. Interestingly, and in contrast, *C. disporicum* has been found in the microbiota of patients with other GI dysfunctions such as Crohn's disease[32]; however, its role in this disease is not known. Examination of other species from the *Clostridiaceae* family does not help to elucidate whether this species confers positive or negative effects given that some members of this family are harmless

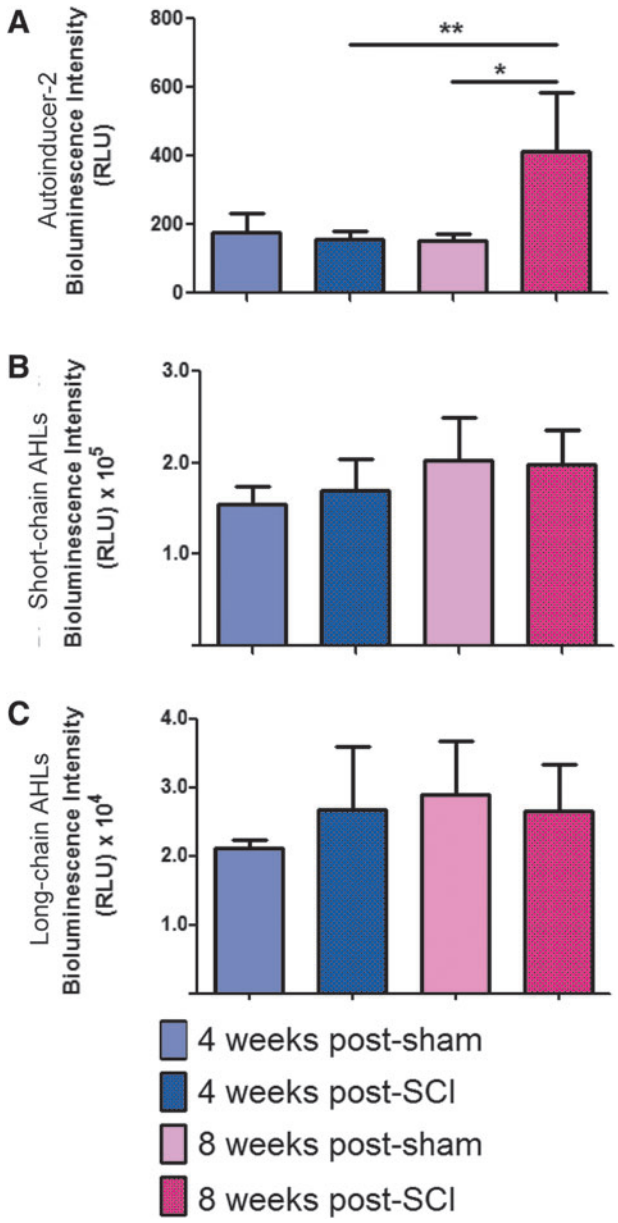

FIG. 5. Quorum sensing molecules in intestinal content. Levels of (**A**) autoinducer-2, (**B**) short-chain (C6) *N*-acyl homoserine lactone, and (**C**) long-chain (C12) *N*-acyl homoserine lactone in the intestinal content of 4- and 8-week sham control and 4- and 8-week SCI rats by bioluminescence (two-tailed *t* test between each of the four groups, $*p < 0.05$; $**p < 0.001$). SCI, spinal cord injury.

whereas others are notorious for being harmful; the premier example of a ''bad'' species is *Clostridium difficile*, a member of this family that is commonly found in GI infections in hospitalized individuals.[33] In contrast, others such as *Clostridium ramosum* are known to contribute to the production of short-chain fatty acids in the colon and hence promote GI and immune health.[33,34] Recent work by Favier and colleagues has shown that *C. disporicum* is one of the first, most dominant bacterial species that colonizes the intestines of healthy infants.[35] Although its function is not known, its presence at the initiation of microbiome colonization in healthy infants led us to hypothesize that it is possible that the increased level of *C. disporicum* in the intestines has a positive effect on the intestinal health of animals post-SCI.

Another bacterial species found to be significantly more abundant in the SCI group compared to control was *Bifidobacterium choerinum* (Table 2). It is well established that the family of *Bifidobacteriaceae*, commensal bacteria present in the gut of healthy individuals, exerts positive health effects on the host, an example being *B. choerinum*, which has demonstrated probiotic effects.[36] In terms of the role of *B. choerinum* in disease states, a study by Spichalova and colleagues points out to a beneficial role of this bacterial species. In their study, the researchers infected piglets with *B. choerinum* and then subsequently infected the same piglets with *Salmonella enterica*.[37] They found that whereas the probiotic effects of *B. choerinum* were limited post-*Salmonella* infection, the abundance of *S. enterica* was lower in the dual infected animals, indicating that *B. choerinum* was protective against the pathogenic *S. enterica*. In our work, we found that *B. choerinum* is significantly enriched post-SCI, which has led us to hypothesize that this species may have a positive effect on the host post-SCI through its known probiotic and antipathogenic effects.

Contrary to the previous species discussed, *Clostridium saccharogumia* was found to be highly depleted post-SCI (Table 2). Literature reports have shown that *C. saccharogumia* converts plant lignans, chemical compounds found in edible plant materials such as flaxseed, into the bioactive molecule enterolactone *in vivo*.[38] This is of importance given that enterolactone has been postulated to have a series of benefits on human health ranging from anticancer properties to improving cardiovascular health.[39] Thus, the depletion of *C. saccharogumia* is likely to prove to have a deleterious effect in the host. Interestingly, the probably negative effects of the loss of *C. saccharogumia* are in contrast to the three previously discussed upregulated bacterial species (*L. intestinalis*, *C. disporicum*, and *B. choerinum*), which likely have a beneficial influence on the host. It is important to note that the three bacterial species discussed above, *L. intestinalis*, *C. disporicum*, and *C. saccharogumia*, have not been previously implicated in having a role-related SCI, and specifically any role in the bowel dysfunction suffered by individuals with SCI.

In addition to the alterations to the microbiota composition, levels of proinflammatory cytokines IL-1β, IL-12, MIP-2, and TNF-α in the small intestines of the animals at 4 weeks post-SCI (Fig. 4) indicated mild inflammation. This aligns with previous research showing intestinal damage and decrease in the thickness of ileum mucosa in rats given a severe SCI injury[3] and an increase of mRNA for Toll-like receptors 1, 2, 4, 5, and 7 post-SCI in mice.[40] Further, we determined that the cytokines, IL-1β, IL-12, and MIP-2, were found to be significantly correlated with microbiome changes in the animals with SCI. In that regard, we observed that eight OTUs significantly correlate with IL-1β (Table 3). Specifically, these OTUs, which were found to decrease with increasing IL-1β, are taxa involved in butyrate production in the GI tract. Butyrate has strong anti-inflammatory effects on macrophages and can suppress ongoing inflammation in the central nervous system[21]; therefore, it is probable that decreases in butyrate-producing bacteria contribute to inflammation in the gut. Of these eight OTUs, of special interest is *Faecalibacterium prausnitzii*, a butyrate producing commensal bacterium. Oral administration of this species has shown to protect against induced GI disease states in mice, suggesting that its role in gut health is significant enough to prevent GI damage.[41] Thus, at the 4-week post-SCI time point, when we noted an increased level of IL-1β, we can also correlate it with a decrease in

butyrate producing bacteria, including *F. prausnitzii*. This decrease may then have contributed to the statistically significant increased levels of IL-12 in the SCI animal group when compared to controls at the same 4-week time point (Fig. 4).

This study highlights the importance of the time frame of intestinal inflammation in the context of microbiota changes. Research has shown that host-mediated inflammatory responses alter the colonic microbiome community and vice versa[42,43]; thus, the more time points post-SCI that are investigated, the greater information that can be extracted. While only assessing the 4- and 8-week post-SCI, we determined temporary increases in intestinal inflammation post-SCI that likely alter the microbiome and can lead to a complex interplay between the host and its microbiome. This complexity can be compounded by additional factors, including sex differences and antibiotic administration post-injury. By focusing only on female rats, while eliminating potential microbiota alterations attributed to differential healing between female and male rats,[28] we have introduced a bias toward gonadal sex hormones, which have been implicated in aiding recovery post-SCI in humans and mice.[44,45] Additionally, microbiota variability within each group (Fig. 2) may have been enhanced because of in-cage coprophagia across groups and potential synergistic effects of SCI-induced dysbiosis with gentamicin.

In this study all animals, both SCI and control, were treated with gentamicin for 7 days after injury to prevent infections, most commonly urinary tract infections (UTIs), which occur in impactor-based SCI-contusion rodent models.[28] As part of the translational goal of treatments for patients, this antibiotic treatment was included because people with SCI have a greatly increased risk for UTIs attributed to common development of neurogenic bladder. Whiteneck and colleagues report an annual 20% incidence of UTI in SCI, or almost 2 cases per person per year, in catheter-free patients with 10–15% of SCI mortality attributed to urinary sepsis.[46] Broad-spectrum antibiotic treatment is the most common method of treatment for SCI patients with symptomatic UTI. In terms of diversity changes in gut taxa induced by antibiotic treatment, work by Tulstrup and colleagues[47] investigated the effects of 9-day antibiotic treatment, using various antibiotics, on the intestinal microbiota of rats. Although this study did not specifically investigate gentamicin treatment, it showed that microbiota alterations of bacterial relative abundance were specific to each antibiotic. Thus, there is the potential for a synergistic affect where the dysbiosis induced from SCI combined with insult from antibiotics may alter the natural course of gut microbiota repopulation beyond SCI alone. This potential interaction will likely be of increasing interest as the field transitions toward potential therapeutics because of the use of antibiotic treatment in individuals with SCI.

In recent work, Gungor and colleagues sequenced the microbiome of patients with SCI to investigate the differences between two injury groups.[21] This clinical study was important in highlighting that SCI induces changes in the gut microbiome in chronic SCI subjects, but did not specifically investigate other parameters aside from microbiome composition. Interestingly, the findings of this study correlate with those observed in our preclinical work in the rat model, including the presence of the bacterial species *F. prausnitzii* and *B. choerinum* in subjects with SCI. Although further investigation is needed to fully translate our work to human patients, we believe that our microbiome and inflammation observations and analysis contribute to understanding the effects of SCI on the gut microbiome and highlight fidelity in the correlation between the microbiome in SCI murine models and human patients.[48–51] Although limited by the rodent model used, time points post-SCI injury, sex bias, and antibiotic treatment, our data provide a better understanding of the effects of SCI on the microbiota and GI tract and have identified specific bacterial species of interest. We believe that this knowledge will be critical in the design and development of potential bacterial-based therapeutic interventions post-SCI aimed at improving bowel dysfunction and enhancing QoL of patients suffering from SCI.

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Author Disclosure Statement

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Address correspondence to:
Sylvia Daunert, PharmD, PhD, MS
Department of Biochemistry and Molecular Biology
University of Miami
Miller School of Medicine
1011 NW 15th Street
Gautier Building 239D
Miami, FL 33136

E-mail: sdaunert@med.miami.edu