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The Interplay Between the Gut Microbiome and Enteric Nervous System after Spinal Cord Injury

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B.S., B.A., North Carolina State University, 2017

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Abstract

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One of the most debilitating outcomes of spinal cord injury (SCI) is dysregulation of the gastrointestinal tract, known as neurogenic bowel dysfunction (NBD). NBD most often presents as severe constipation and can occur regardless of lesion location or severity. It is thought that a number of factors contribute to the impairment of gut function after injury, however, the exact etiology is currently unknown. Recent investigation has revealed that SCI not only influences the gut itself, but also the gut's microbial inhabitants, with the most significant dysbiosis associated with the most severe cases of NBD. In this dissertation I review the current literature regarding the physiology of the gut in the presence and absence of SCI and highlight how injury-associated changes in the nervous system, immune system, and gut microbiome influence one another and contribute to NBD. To determine if SCI induces any characteristic changes to the microbiome, we conducted a systematic review of existing human and animal post-injury microbiome datasets. Our analysis revealed a consistent loss of microbes that produce short-chain fatty acids (SCFAs), a byproduct of microbial fermentation of dietary fiber. Using a severe thoracic model of SCI, we found that providing injured mice with inulin, a dietary fiber, immediately after SCI significantly reduced the severity of gut dysbiosis and dysmotility, and improved locomotor outcomes. Further investigation revealed that inulin-mediated improvements to gut and locomotor outcomes are dependent on the anti-inflammatory cytokine IL-10, which is increased in response to butyrate, a microbially-produced SCFA. We next sought to determine if the SCI-associated microbiome was inherently detrimental to host health, even outside of the context of SCI. Using gnotobiotic models, we found that injury-associated microbes do influence intestinal and metabolic function, even in injury-naïve mice. Our findings reveal a microbiome-based pathway that can be targeted to improve health after SCI. NBD is most often associated with SCI but is also a component of numerous diseases and disorders of the nervous system and often comorbid with changes in the gut microbiome, suggesting that the findings presented herein could have wide reaching implications for treating aspects of various nervous system disorders.

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Figures and Tables.

Chapter 1: Introduction.

1.1 Brief overview of the branches of the nervous system.

The mammalian nervous system is composed of the central nervous system (CNS) and peripheral nervous system (Fig. 1.1). The CNS includes the brain, which is responsible for perceiving sensory information and dictating motor responses, among other functions, and the spinal cord, which primarily serves as a highway for trafficking information between the brain and the rest of the body (1). The peripheral nervous system is organized into the somatic nervous system and the autonomic nervous system (2). The somatic nervous system, also known as the voluntary nervous system, is composed of a network of cells that bring sensory information to the CNS via afferent nerves and carry motor information from the CNS to the body via efferent nerves (2).

The other branch of the peripheral nervous system, the autonomic nervous system, is further comprised of the sympathetic nervous system, the parasympathetic nervous system, and the enteric nervous system (3). The sympathetic and parasympathetic nervous systems work together in homeostasis to regulate automatic (or autonomous) functions, such as blood pressure, respiration, and heart rate (4). The sympathetic nervous system is responsible for the fight-or-flight response, while the parasympathetic nervous system is responsible for the more relaxed rest-and-digest response. The final branch of the autonomic nervous system is the enteric nervous system (ENS), which controls gastrointestinal (GI) function. The ENS largely operates autonomously, but its functions are mediated by extrinsic innervation from other branches of the peripheral nervous system (5). Nearly all aspects of the peripheral nervous system require the CNS to consolidate information and dictate the appropriate responses to maintain homeostatic balance, and nearly all of this information – sensory, motor, autonomic – passes through the spinal cord.

1.2 Anatomy of the spinal cord.

The adult human spinal cord is approximately 45 cm long and consists of cervical, thoracic, lumbar, sacral, and coccygeal sections (Fig. 1.2) (6). The cervical spinal cord contains 8 segments (C1-C8), which innervate distinct portions of the head, neck, and arms carrying sensory and motor information between the cord and the body. The thoracic spinal cord (T1-T12) innervates the upper torso, the lumbar (L1-L5), sacral (S1-S5), and coccygeal (Co1) cord innervates the lower torso and legs (Fig 1.2) (6). Despite the complex nature of the spinal cord, cross sections of these 31 segments have a relatively similar architecture. Each segment of the cord is flanked by a pair of spinal nerves, one on each side, which are connected to the cord by a pair of spinal nerve roots; sensory information is relayed through the dorsal root, and motor information through the ventral root (Fig. 1.3) (7). The cord itself is composed of gray and white matter. Gray matter is composed of neuronal cell bodies and unmyelinated axons, while white matter is composed of myelinated neuronal axons (6). The gray matter within each section of the cord is related to the spinal nerves entering or exiting the cord in that segment. The ventral horn contains somatic motor nuclei, the lateral horn contains autonomic efferent nuclei, and the dorsal horn contains visceral and somatic sensory nuclei (1). Information is trafficked between the gray matter nuclei and nuclei in supra-spinal centers via distinct tracts of myelinated axons, which run the length of the cord (6). These axons make up the white matter sections of the cord. The percentage of white matter is higher in the more proximal segments of the cord, because it contains axons carrying information to each of the more distal segments of the cord.

1.3 The gastrointestinal tract and the enteric nervous system.

The human gastrointestinal (GI) tract is composed of a consortium of organs responsible for digestion and nutrient absorption (8). The GI tract is composed of the main tube-shaped organs including the mouth, esophagus, stomach, small intestine, large intestine, and anus (Fig. 1.4). Additional digestive organs directly support GI function, including the liver, pancreas, and gallbladder, which produce and secrete hormones and digestive enzymes (8). The various organs of the GI tract have different roles in the digestive process. The mouth chews food, the stomach breaks down proteins, and the intestine propels material down the tract, absorbing nutrients along the way. The intestine itself is a relatively thin tube composed of layers of muscle and neurons surrounding the intestinal lumen (Fig. 1.5). The thickest layer of the intestine is the mucosa, which is in direct contact with the external environment containing microbes and other luminal contents (8). In adulthood, epithelial cells make up over 60% of the total cells in the gut, with the bulk of the remaining cells belonging to the immune system (9). The intestinal mucosa contains a variety of specialized epithelial cells, organized into finger-like projections known as villi (10). These projections drastically increase the surface area of the gut lining, to aid in digestion and absorption of nutrients. The large surface area and thin permeable tissue of the gut also leaves the gut susceptible to infection, as such the gut hosts a variety of tissue-resident immune cells as well as major lymphatic systems.

The bulk of the epithelial cells on intestinal villi are enterocytes, whose primary function is absorption of nutrients (8). Among the enterocytes are more specialized epithelial cells including Paneth cells, goblet cells, M cells, Tuft cells, enteroendocrine cells (EECs), and proliferating stem cells. Paneth cells contain granules of antimicrobial peptides that can be secreted to modulate the

gut microbiome and defend against pathogens (11). Goblet cells produce a protective mucus coating that lines the intestinal lumen, and also serve as antigen importing cells, closely linked with the immune system (12). M cells, or microfold cells, are another form of antigen importing cells in the gut and are closely associated with gut associated lymphoid tissue (GALT) (13). Tuft cells serve both chemosensory and anti-parasitic functions. Many Tuft cells have neuron-like projections known as neuropods (14), and roughly half of Tuft cells in the gut are closely associated with sensory nerve fibers, suggesting Tuft cells are involved in rapid communication of the gut environment to the nervous system (15). Tuft cells also initiate immune responses to non-bacterial members of the microbiome including protozoan and helminth parasites (16, 17). EECs make up approximately 1% of the gut epithelium, and primarily function as endocrine cells, producing and secreting hormones and neurotransmitters, with varying hormone composition based on their location within the GI tract (18). EECs serve a secondary role as sensory transducers in direct communication with sensory neurons of the ENS, vagus, and dorsal root ganglia (19). EECs play a significant role in modulation of GI motility via these endocrine and neuronal routes. Intestinal stem cells are found in the intestinal crypts, at the base of the villi, and are multipotent, capable of differentiating into any of the other epithelial cell types as needed (20).

Although epithelial cells have diverse specializations, outside of nutrient absorption, they often function as neuron-associated sensory transducers (Tuft cells and EECs) or as microbiome-modulating immune-associated cells (Paneth, Tuft, goblet, and M cells). These interactions highlight the importance of the epithelial layer at the interface between the microbiome and the immune and nervous systems and suggest a number of routes by which the microbiome might influence host physiology. Despite the various roles of the different components of the GI tract,

one key mediator responds to sensory information and dictates coordinated motor function throughout the tract, the enteric nervous system (ENS). Spanning from the esophagus to the anus, the ENS directs the propulsion of food down the GI tract by contracting and relaxing muscles in the intestinal walls (5). The ENS is composed of 20 neuronal subtypes including motor neurons, sensory neurons, and interneurons, among others (5). The neurons of the ENS are primarily found in the myenteric and submucosal plexuses, situated between muscle layers within the intestinal wall (Fig. 1.5). The myenteric plexus coordinates smooth muscle contraction in the gut lining, while the submucosal plexus is responsible for secretion and absorption (21). This motor activity, whose function is propelling material down the GI tract, is known as peristalsis.

Peristalsis involves multiple types of neurons, including sensory neurons, motor neurons, and interneurons. In brief, the sensory neurons of the ENS, intrinsic primary afferent neurons (IPANs), detect chemical and mechanical stimuli caused by a bolus of food in the gut (Fig. 1.5). In response to the sensory input, the IPANs signal to interneurons on either side of the bolus, ascending interneurons proximal to the bolus, and descending interneurons distal to the bolus (5). Evidence from the guinea pig colon suggests that IPANs primarily communicate with the excitatory interneurons and excitatory motor neurons, and that the inhibitory interneurons receive signals via cross talk with the excitatory interneurons (22) or are directly mechanosensitive (23), rather than receiving direct input from the IPANs themselves. Synaptic convergence between excitatory and inhibitory interneurons ensures that greater excitatory activation proximal to the bolus corresponds with greater inhibitory activation distal to the bolus (24). Ascending interneurons then signal to excitatory motor neurons, and the descending interneurons signal to inhibitory motor neurons. The smooth muscle of the GI tract is innervated by excitatory and inhibitory neurons, in contrast to

skeletal muscle that is only innervated by excitatory motor neurons (25). Excitatory motor neurons lead to contraction of circular and longitudinal muscles in the intestine proximal to the bolus, and inhibitory motor neurons lead to relaxation of circular and longitudinal muscle in the intestine distal to the bolus (26). The contraction of longitudinal muscle shortens the segment of intestine just proximal to the bolus increasing the pressure generated by the circular muscle contractions and, along with the relaxation of the muscles just distal to the bolus, propel the luminal contents slowly down the GI tract.

Excitatory motor neurons primarily signal using the neurotransmitter acetylcholine, while the inhibitory motor neurons primarily signal with the neurotransmitter nitric oxide (5). Peristalsis not only propels matter down the GI tract, but it also churns intestinal contents, aiding in digestion. A lack of myenteric neurons in mouse models results in mega colon and death due to the inability to generate coordinated muscle contractions (27). However, mice lacking half of their ENS neurons can still have coordinated GI motor movements and live a normal lifespan, suggesting that there are circumstances in which there can be extensive loss of the ENS before severe issues arise (28). A recent study found that enteric neurogenesis continues into adulthood in mice, with 85% of myenteric neurons being less than 2-weeks old, balanced by a loss of ~5% of myenteric neurons each day (29). Although controversial, this finding suggests that manipulation of the ENS environment could lead to restoration of ENS neuronal populations and improve GI motility disorders.

The ENS is a component of the autonomic nervous system (ANS), meaning animals do not have any voluntary control over GI function. GI function is truly autonomous, as the GI tract is the only organ system with its own nervous system, complete with intrinsic sensory and motor neurons, and can generate coordinated muscle contractions in the gut, even when the gut is removed from the body (30, 31). However, there are numerous intrinsic and extrinsic factors that influence GI function. Within the GI tract, interstitial cells of Cajal (ICCs) serve as pacemaker cells that coordinate and generate slow wave electrical activity, even when neuronal signaling is pharmacologically blocked (32). Mice without ICCs are unable to generate slow wave electrical activity but can intriguingly still generate propagating GI contractions (33, 34). These neuron-like cells also play roles in transduction of ENS motor neuron signals and are able to sense stretching of GI muscles (35).

The upper portions of the GI tract are also influenced by the migrating motor complex (MMC) which is a recurring GI motility pattern that takes place during periods of fasting (36). The MMC, in humans, occurs in a cyclic pattern, roughly every 2 hours, and is characterized by spontaneous contractions through the stomach and small intestine (36). The MMC, like much of the GI tract appears to be partially mediated by extrinsic innervation from the ANS. The majority of mass movement in the large intestine is regulated by the colonic migrating motor complex (CMMC), also known as high amplitude propagating contractions (HAPCs) (37). Although the exact cellular mechanisms are unknown, these mass movements appear to function very similarly to small intestine MMCs and occur in *ex vivo* preparations of human colon, suggesting that extrinsic innervation is not necessary for generating these colonic motor patterns (30).

The organs of the GI tract are innervated by the sympathetic and parasympathetic nervous systems, also components of the ANS (5). Sympathetic preganglionic neurons innervating the GI tract originate from the thoracic and lumbar regions of the spinal cord, while parasympathetic preganglionic neurons innervating the GI tract originate in the brainstem and sacral spinal cord (Fig. 1.6) (38, 39). Sensory information such as pain and distension are relayed to the central nervous system via vagal, thoracolumbar, and lumbosacral pathways, which help modulate homeostatic activity of the GI tract (39). Together these branches of the peripheral nervous system form long central circuits, which are a type of reflex circuitry that involves spinal sensory afferents and sympathetic motor efferents. An additional subset of neurons, intestinofugal neurons, are intrinsic ENS neurons with sensory characteristics, that form a shorter peripheral version of this reflex circuitry whereby intestinofugal neurons directly communicate with postganglionic sympathetic neurons (40). Along with specialized epithelial cells and the various associated branches of the nervous system, one other cell type plays a major role in GI function, bacteria.

1.4 Overview of the gut microbiome in health and disease.

Humans are host to trillions of microbes inhabiting all environmentally exposed surfaces of our bodies, with the vast majority of them found within our GI tract. These microbes hail from various taxonomic kingdoms and include bacteria, archaea, fungi, protists, and viruses. The best studied and perhaps most influential organisms of the gut microbiome are bacteria. Humans are first colonized by bacteria immediately after birth, rapidly forming a complex community of microbes. Once the GI tract is colonized and the various niches are filled, the microbiome remains remarkably stable. However, microbiome composition can be altered by diet, pharmaceuticals, aging, disease, and injury (41). Changes in microbiome composition can be beneficial, detrimental,

or have no notable effect at all. Humans and microbes have co-evolved over millennia, leading to numerous adaptations whereby humans and microbes interact (42). Indigenous microbes modulate numerous aspects of host physiology, including immunity (43), metabolism (44), and neurotransmission (45).

Gut microbes influence the maturation, function, and activation of immune cells in the CNS and in the periphery (43), due in part to the presence of endotoxins that can readily enter the body and even cross the blood-brain-barrier (46). Microbes also modulate host metabolism, largely by modifying hormone and neurotransmitter release from EECs in the gut epithelium, which directly influences GI motility as well as metabolic processes such as fat storage, insulin sensitivity, and appetite (44, 47). The gut microbiome also influences endocrine systems that are not directly tied to EECs, such as the hypothalamic-pituitary-adrenal axis, which is responsible for production of cortisol, a stress hormone that influences numerous aspects of host physiology including inflammation and gut motility (48). The gut microbiome is essential for production of secondary bile acids which are involved in host metabolism and immune function (49). Gut microbes produce, or influence host production of, numerous neurotransmitters including glutamate, GABA, acetylcholine, dopamine, serotonin, and norepinephrine (45). These signaling molecules augment host neuronal signaling in both the ENS and the CNS by way of the vagus nerve and dorsal root ganglia (19, 50), with downstream influence on locomotion and GI motility (51). Importantly, the gut microbiome has also been implicated in the development and progression of neurological disorders, suggesting that microbial signals can confer both positive and negative physiological outcomes (52-56). Inflammation is influenced by microbial populations and has been shown to

contribute to intestinal dysmotility and permeability, both of which are increased after SCI (57-62).

1.5 The gut microbiome, fiber, & short-chain fatty acids.

Beyond the microbes themselves, microbial metabolites can have extensive influence on host physiology. Metabolites are produced as byproducts of fermentation of host dietary components, cross-feeding between microbial species, conversion of host products, and metabolites produced de novo (63). These metabolites include secondary bile acids, gases, alcohol, and polysaccharides, among others (63). Of particular interest are short-chain fatty-acids (SCFAs), which are produced by bacterial fermentation of dietary fiber, and are known to modulate numerous aspects of the host intestinal environment, including inflammation, metabolism, and GI motility (50, 64, 65). Fiber not only influences SCFA levels, but it also changes the composition of the gut microbiome and improves microbiome resiliency to perturbation and infection (66).

Inulin fiber has been shown to prevent bacterial penetration of the inner colonic mucus layer (67), prevent colonic atrophy associated with metabolic syndrome (68), and suppress *Clostridium difficile* infection (69) in mice. Inulin has also been shown to improve gut dysbiosis and neuronal recovery in mouse models of traumatic brain injury (70, 71). Different sources of fiber have differential SCFA profiles, but in general increased fiber leads to increases in the SCFAs acetate, propionate, and butyrate. These key SCFAs influence the host immune system by inhibiting histone deacetylases (HDACs) and promote generation of regulatory T cells via activation of the G-protein coupled receptors GPR43 and GPR41 (72-74). SCFAs also increase mucin production

by goblet cells and help maintain intestinal barrier integrity (75). In addition to fibers influence on the microbiome and microbial metabolites, fiber draws in water and increases stool bulk, which can trigger enteric reflex circuitry and increase mucin production, aiding in colonic motility (76).

1.6 A brief introduction to spinal cord injury.

Nearly 1 in 50 people in the United States is living with a form of paralysis, with traumatic SCI ranking second only to stroke (77). Traumatic SCI is a costly and debilitating condition, currently afflicting 9 million people globally, including 300,000 Americans, with thousands of new cases occurring each year (78, 79). Roughly 80% of the 18,000 annual cases of traumatic SCI, in the US, occur in men, and are most often the result of vehicle crashes, falls, or violence (80, 81). Most injuries occur at cervical or thoracic levels, with 40% of injuries resulting in paraplegia and 60% in tetraplegia (80, 82). In addition to the physical burden, the financial burden associated with SCI is immense. In the US, lifetime healthcare costs average $1.4 to $5.8 million dollars per SCI, with higher-level injuries and younger age at injury contributing to higher cost (80). Aside from healthcare expenses, the average annual financial loss is $89,000 in lost wages and productivity, with only 18% of people with SCI employed one-year post-injury (80). The burden expands beyond that experienced by the people with injury, with 2/3 of caregivers receiving no pay for care of people with injury. Providing cost-effective and non-invasive strategies to address the time-consuming tasks associated with everyday life after SCI are critical.

1.7 Physiological changes associated with spinal cord injury.

Spinal cord injury affects the entire body, with particularly detrimental effects on motor, sensory, and autonomic functions at and below the site of injury (83). Higher-level SCIs lead to more extensive dysfunction throughout the body, as less of the body is able to successfully communicate with the CNS. Some of the most outwardly apparent symptoms of SCI are the loss of motor functions in the limbs, with higher-level injuries (cervical) resulting in tetraplegia and lower-level injuries (L5 or higher) resulting in paraplegia (83). The extent of impairment is dependent on the extent or completeness of injury, with a complete injury being neurologically defined as no sensory or motor function below the level of injury, resulting in the highest level of impairment. Roughly 33% of injuries are considered complete (80).

In addition to the loss of motor and sensory function in the limbs, SCI results in significant dysfunction of the autonomic nervous system, which controls respiration, cardiac muscles, and other visceral organs (84). Autonomic dysfunction, similar to limb dysfunction, is more extensive for higher injury levels. For instance, injuries at C3 or higher result in the need for mechanical ventilation, T6 or higher can lead to life-threatening sudden changes in blood pressure (autonomic dysreflexia), while SCI at any level (sacral or higher) can result in bowel, bladder, and sexual dysfunction (83). Bowel dysfunction after SCI is considered one of the most burdensome aspects of post-SCI life but is understudied relative to other aspects of injury (85).

1.8 Gastrointestinal motility dysfunction after spinal cord injury.

Following spinal cord injury, nearly all aspects of physiology change. Some of the most detrimental changes occur in the GI tract, culminating in a condition known as neurogenic bowel. Severe constipation affects approximately 40-60% of persons with SCI (86, 87). As with all sequelae associated with SCI, the location and severity of the injury dictate the specific outcomes. One common theme, however, is slowed gastrointestinal motility, where people with injury experience 3-4 times slower gastric emptying, colonic transit, and whole gut transit times than uninjured people, with no significant differences between tetraplegic and paraplegic participants (88). People with SCI have significantly reduced baseline and postprandial colonic contractility, with no postprandial response in some parts of the colon, indicating a suboptimal but still functional ENS (89). Slowed GI motility tends to worsen with time and is more pronounced in patients with more chronic injury (90). These changes in GI function are one of the most impactful detriments to quality of life in the injured population, and restoration of normal bowel function often ranks higher in importance than recovery of leg function (85).

Changes in GI transit time also occur in animal models of SCI. A study in pigs uncovered increases in transit time and decreases in contractility pressure and frequency (91), which reflect changes seen in humans with SCI (88). Rodent models have also documented changes to GI motility and transit time. Both cervical and thoracic transections in rats lead to rapid alterations in GI transit and gastric emptying, significantly impaired as early as 30 minutes after injury (92). As with location of injury, severity of injury impacts duration and extent of GI outcomes. Rats with thoracic transection and rats with a less severe thoracic contusion both had slowed GI motility for at least 3-weeks post injury, but by 6-weeks rats with the less severe contusion injuries began to

functionally recover (93). However, despite the functional recovery, rats with contusion still lacked spontaneous gastric contractions, indicating that the GI tract might adapt to injury, but does not necessarily recover baseline function (93). It is important to note that rodents tend to recover more below-lesion function after SCI than humans do, meaning it is not necessarily expected that these improvements to GI function in chronic stages of contusive SCI would occur in humans with chronic injury.

1.9 The role of extrinsic neurons in neurogenic bowel.

The exact etiology behind GI motility dysfunction after SCI is still unknown, however many studies point to loss of descending supraspinal input as one causal mechanism. Studies in humans with SCI have found a correlation between GI transit time and low frequency heart rate variability, indicating involvement of the sympathetic nervous system, which is a known mediator of peristaltic activity (94). In agreement with this, one study found that sympathectomy of the inferior mesenteric plexus in rats with T8 SCI lead to reduced GI transit time and stronger colonic contractions, indicating that SCI increases sympathetic activity to the gut (95). Another study found that vagotomy of rats with SCI inhibited slowed GI transit, indicating parasympathetic involvement (96). However, that same study noted a similar inhibition of post-SCI GI dysmotility in rats with a celiac ganglionectomy, which could indicate sympathetic involvement as well, reinforcing that GI motility is regulated on multiple levels.

Another study found that rats with a 4-week-old T3 transection had slowed whole gut transit time and a decreased colonic contractile response to acetylcholine in both proximal and distal colon

(97). This suggests that colonic motility deficits occur in part due to inadequate sensitivity to parasympathetic (cholinergic) stimulation. Importantly, this insensitivity was most pronounced in the distal colon, where parasympathetic innervation comes from the pelvic nerves rather than the vagus (Fig. 1.6). However, this study failed to consider that acetylcholine is also the main neurotransmitter involved in excitatory signaling in the ENS and is released by numerous intrinsic neurons as well as those of the parasympathetic nervous system. Intriguingly a similar study in rats, with a T10 transection, found that 1-week post-SCI, acetylcholine led to significantly greater contractions in proximal colon, but a qualitatively diminished response in the distal colon, despite significant decreases in the pan-neuronal marker, PGP9.5, in both proximal and distal colon (98). Taken together these two studies indicate a temporal and/or injury level dependent shift in muscarinic sensitivity to parasympathetic inputs, characterized by cholinergic hypersensitivity at 1-wk post-SCI but hyposensitivity at 4-wks after injury. Collectively this indicates that loss of appropriate coordination between the branches of the autonomic nervous system plays a role in injury-induced dysmotility.

In addition to the changes in autonomic neuronal mechanisms, voluntary control over spinal defecation centers is lost after SCI (99). Loss of somatic control inhibits the relaxation of anal sphincters, preventing defecation (38). In many cases of SCI there is also loss of sympathetic tone, which keeps the anal sphincters contracted. Taken together this loss of tone and loss of somatic control of the anal sphincters leads to increased constipation along with increased incidence of fecal incontinence (99). The inability of people with SCI at or above T7 to increase abdominal pressure by contracting abdominal muscles is also thought to play a role in difficulty with bowel evacuation (38).

1.10 Intrinsic changes involved in neurogenic bowel.

In addition to loss of coordination of extrinsic autonomic innervation to the gut, the neuromuscular compartment of the gut itself changes significantly after SCI. Humans with SCI have reduced nerve fiber density and fewer neurons within the myenteric plexus of the ENS, with more significant neuronal loss in people with more severe GI dysfunction, as well as fibrosis in the longitudinal muscle layer (100). Rodent models of SCI have uncovered complementary findings. A study using a T3 transection model found that injury led to a significant loss of colonic myenteric neurons, reduced spontaneous colonic contractions, increased colonic inflammation, reductions in size of mucosal crypts, and increased colonic muscle thickness and collagen deposition (101). Further investigation by this group, using the same T3 transection model, confirmed loss of nitrergic and cholinergic neurons in the colon, with corresponding loss of inhibitory and excitatory junction potentials (102). Rats with a less severe midthoracic contusion injury presented with an impaired immune response throughout the whole colon, and a reduction of nitrergic neurons and reduced colonic contractility in the proximal but not distal colon (103). Similar to colonic findings, mucosal layer atrophy, loss of nitrergic neurons, and diminished inhibitory junction potentials were found in the stomach and duodenum of rats after a lower thoracic contusion injury (104). Collectively these findings, at both acute and chronic stages of injury, indicate that the GI tract undergoes significant changes after SCI, regardless of injury severity or location, largely related to inhibitory nitrergic neurons of the ENS.

Another key intrinsic mediator of gut motility are interstitial cells of Cajal (ICCs), which coordinate peristaltic function at the interface between the neurons and muscles of the GI tract. ICCs tend to be decreased in most GI motility disorders (105). However, the current data on ICCs in SCI is less clear. One study in humans noted a decrease in myenteric ICCs post-injury (100), while a recent study in rats observed a temporal increase in myenteric ICCs post-injury (106).

1.11 Current therapeutics and unmet need.

Various therapies exist to aid in bowel evacuation following SCI, including manual removal of stool, digital stimulation of the anal sphincter, and nerve-stimulating suppositories and enemas (107, 108). These techniques provide some efficacy but are time-consuming and diminish quality of life. Additional interventions include spinal implantation of electrical nerve stimulators and bowel resection surgeries, invasive procedures which pose inherent risk (107, 108). There is a critical need for an effective non-invasive treatment that minimizes risk. Targeted treatment of the gut microbiome has the potential to meet this need.

1.12 Fiber, SCFAs, and SCI.

Dietary fiber has been shown to rescue dysbiosis and improve associated pathology in numerous diseases and disorders (109-111), however, there has been limited investigation into fiber use after SCI (112, 113). Many bacterial taxa preferentially depleted following SCI are SCFA producers, suggesting that fiber may aid in the recovery of post-SCI intestinal dysfunction via upregulating production of SCFAs (60, 62, 114, 115). A recent study found that the SCFAs acetate, propionate, and butyrate were significantly lower in persons with SCI as compared to healthy controls (116).

Intriguingly these changes were more significant at more chronic timepoints and varied based on injury level and severity of injury, trends that are also seen with dysbiosis and GI motility dysfunction (114-119). Increased levels of SCFAs were positively correlated with improved locomotor outcomes and decreased intestinal permeability, after fecal microbiome transplant (FMT) in mice with SCI (120). Direct administration of SCFAs to mice with SCI has been shown to improve locomotor and inflammatory outcomes (116, 121, 122). There is a growing body of evidence that SCI leads to significant changes in the composition of the gut microbiome, characterized in part by a preferential loss of SCFA-producing bacteria. These changes in microbiome composition may serve as indicators of specific types of SCI-associated gut dysfunction or provide evidence as to what type of therapeutic is best suited for individual cases of injury. These concepts and the relevant literature are expanded upon in Chapter 2.

1.13 Figures.

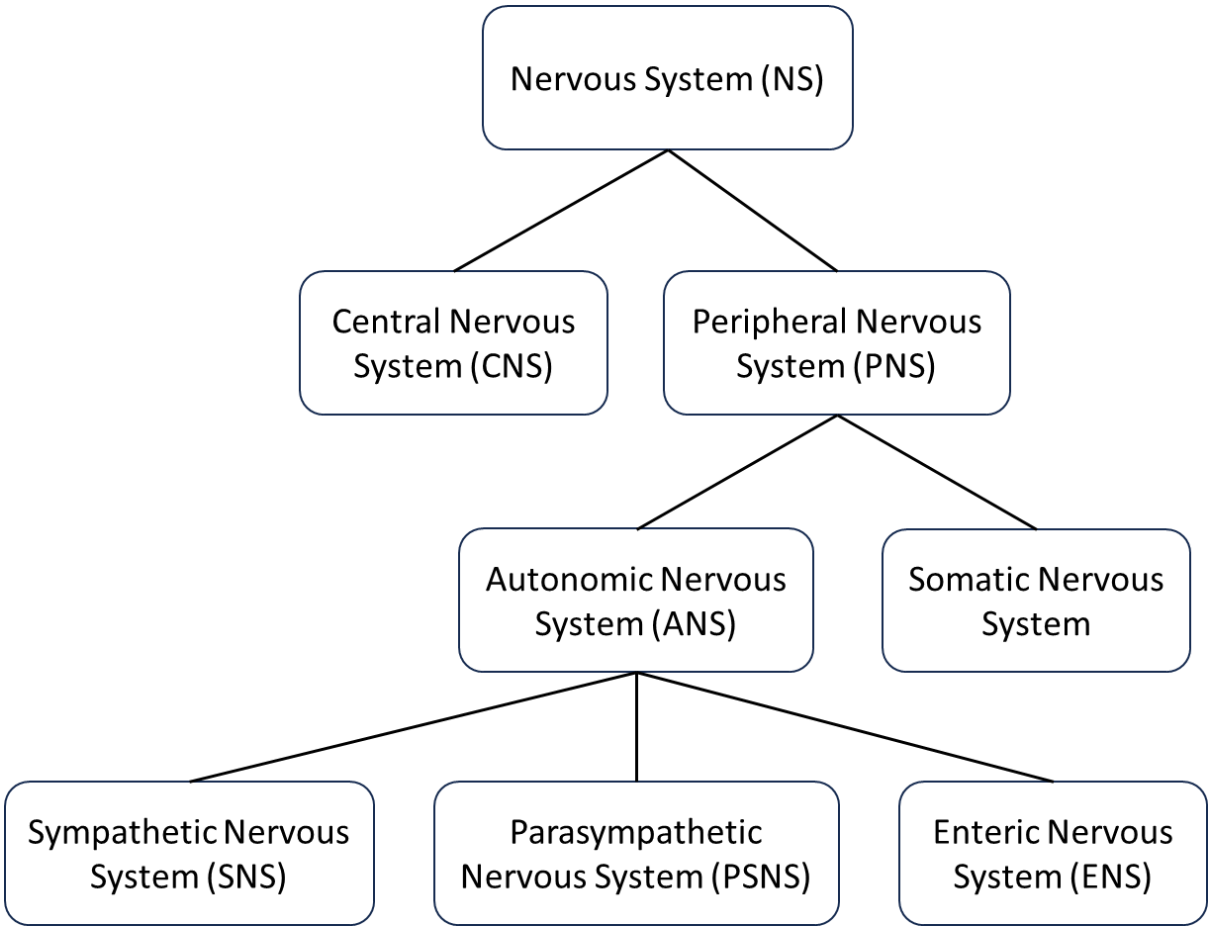

Figure 1.1. Branches of the nervous system.

Overview of the major branches of the nervous system and common initialisms in parentheses used throughout this dissertation to refer to the different branches. The CNS includes the brain and spinal cord, while the components of the PNS are found throughout the body. The ENS is found exclusively within the organs of the gastrointestinal tract. The somatic NS is also commonly referred to as the voluntary NS.

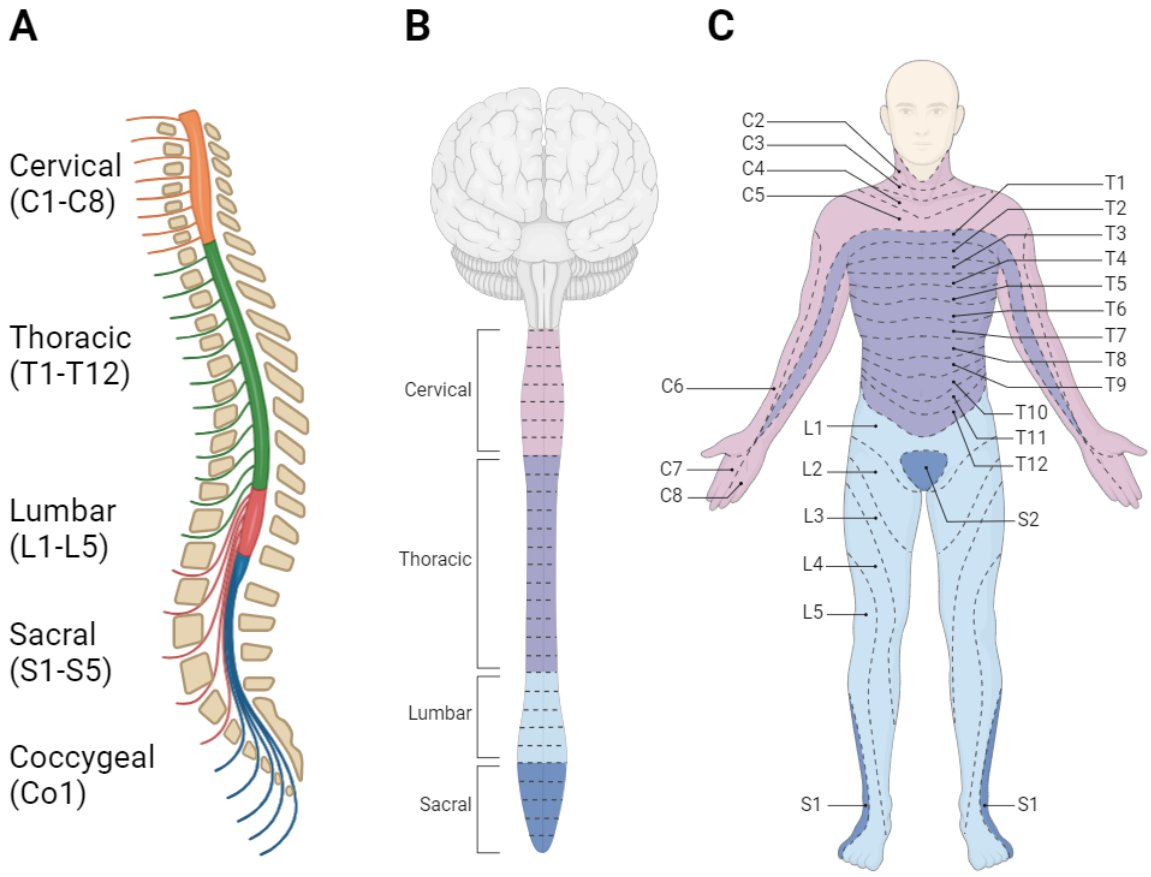

Figure 1.2. Spinal column anatomy and innervation patterns.

A) Schematic showing location of spinal cord segments and spinal nerves within the human vertebral column, where cervical=orange, thoracic=green, lumbar=red, sacral=blue, and coccygeal=black. **B, C**) Schematic detailing spinal cord segments (**B**) and the corresponding locations of skin (dermatome) innervated by each segment (**C**), where cervical=pink, thoracic=purple, lumbar=light blue, and sacral=dark blue. Although the dermatome is specifically a map of skin innervation patterns, it is generally representative of non-visceral sensory and motor innervation to the various parts of the body below the skin and is often used to determine the functional location and completeness of a SCI. For innervation patterns of visceral organs see Fig. 1.6. Created in BioRender.

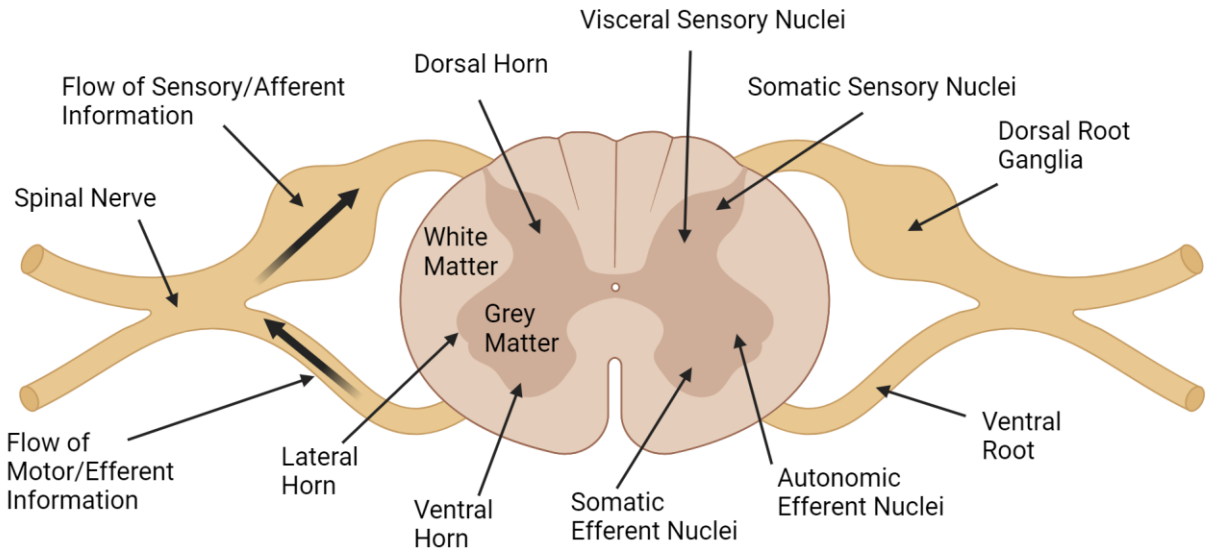

Figure 1.3. Spinal cord anatomy.

Cross section of a spinal cord segment detailing various anatomical structures as well as the direction of afferent and efferent neuronal signaling in nerve roots. Created in BioRender.

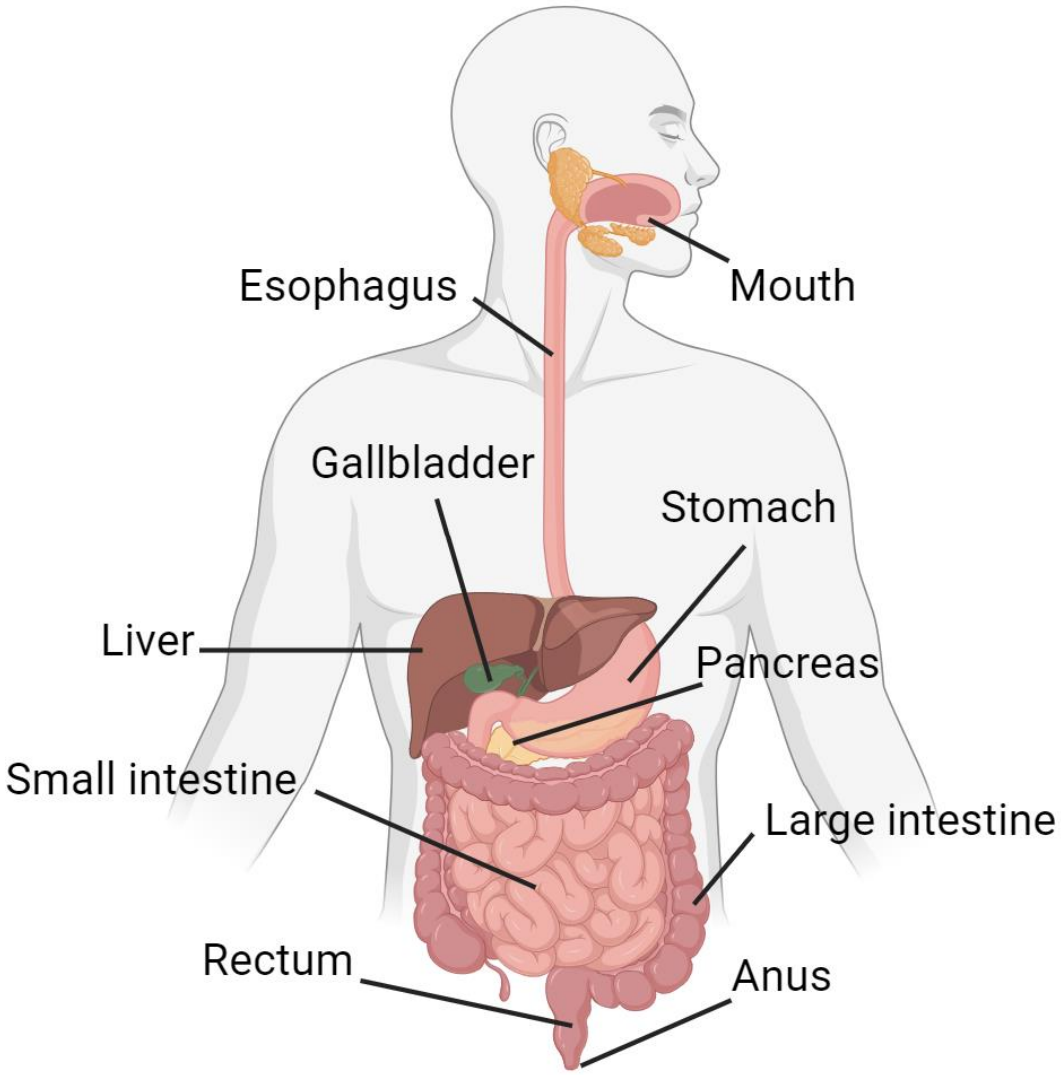

Figure 1.4. Components of the gastrointestinal tract.

Components of the gastrointestinal tract (mouth, esophagus, stomach, small intestine, large intestine, rectum, and anus) and support organs (liver, gallbladder, and pancreas), and their relative location within the body. Created in BioRender.

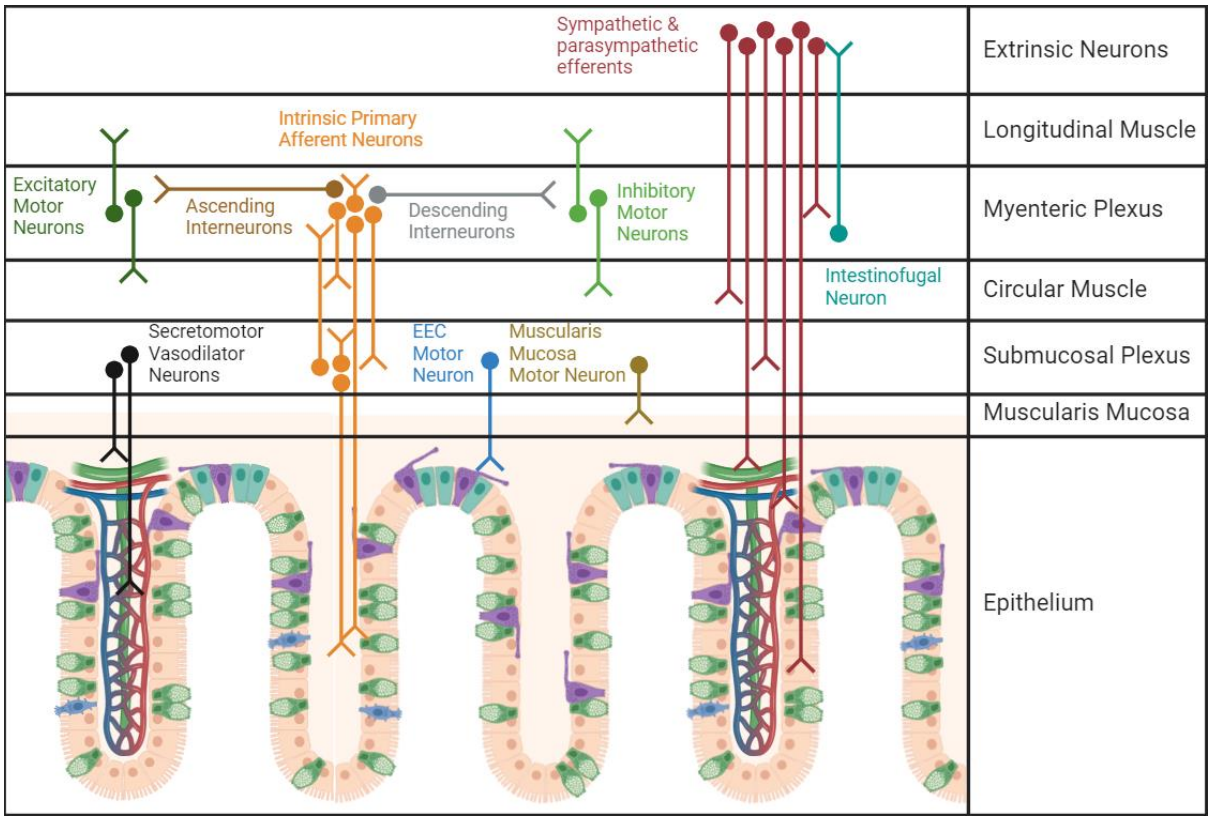

Figure 1.5. Enteric neuroanatomy and layers of the intestine.

Cross sectional view of the layers of the intestine and the locational of neuronal populations within each layer. The majority of enteric neuronal cell bodies are situated in ganglionic clusters within the myenteric plexus and submucosal plexus, situated between distinct layers of muscle. Created in BioRender.

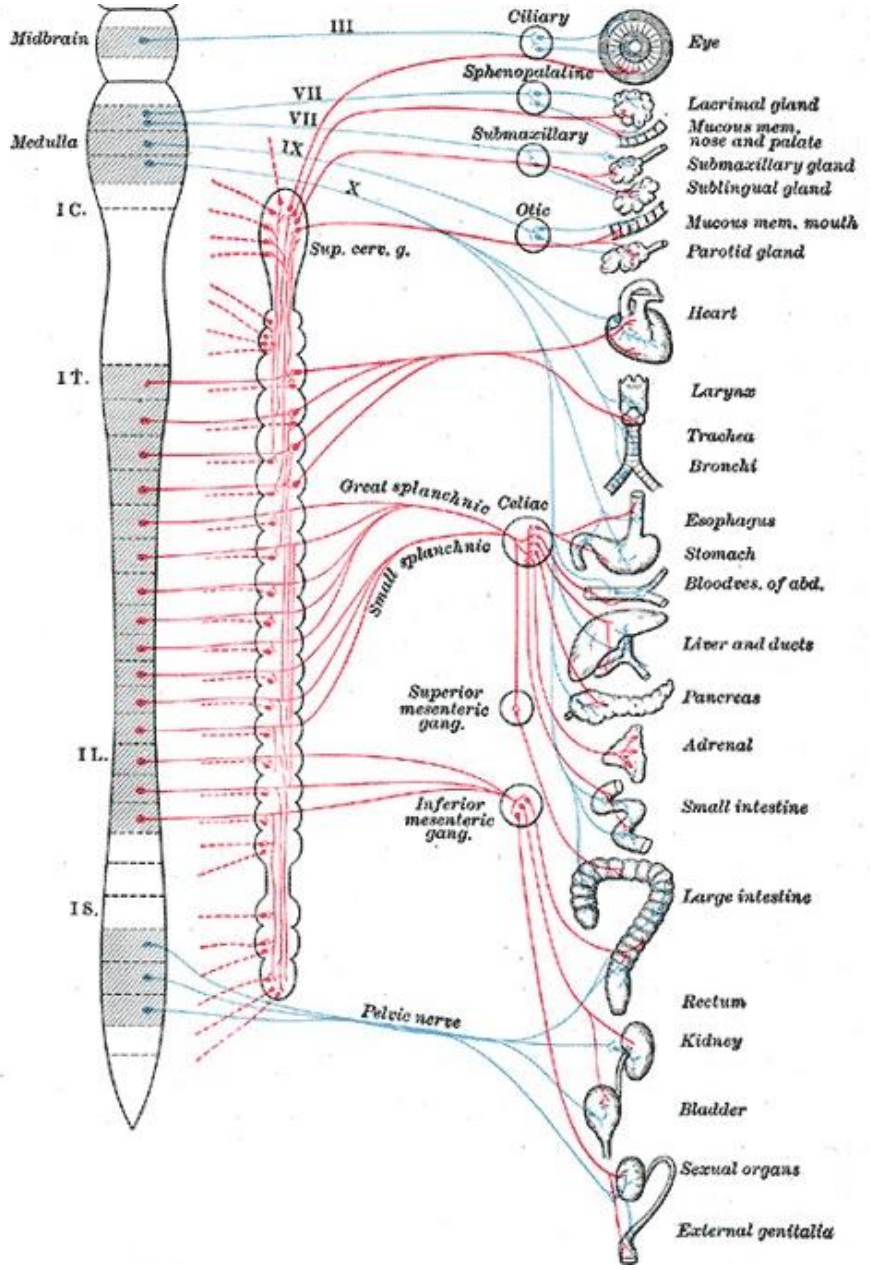

Figure 1.6. Autonomic innervation of visceral organs.

Parasympathetic (blue) and sympathetic (red) innervation of the organs, including those of the GI tract. Sympathetic innervation of the GI tract originates in the thoracic and lumbar spinal cord, parasympathetic innervation of the proximal colon and upper portions of the GI tract originate in the midbrain, and parasympathetic innervation of the distal colon originate in the sacral spinal cord. Figure from Anatomy of the Human Body (123).

Chapter 2: Traumatic spinal cord injury and the contributions of the post-injury microbiome.

This chapter is a reformatted version of the following publication:

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2.1 Abstract.

Spinal cord injuries are an enormous burden on injured individuals and their caregivers. The pathophysiological effects of injury are not limited to the spine and limb function but affect numerous body systems. Growing observations in human studies and experimental models suggest that the gut microbiome is altered following spinal cord injury. Given the importance of signals derived from the gut microbiome for host physiology, it is possible that injury-triggered dysbiosis subsequently affects aspects of recovery. Here, we review emerging literature on the role of the microbiome following spinal cord injury. Specifically, we highlight findings from both human and experimental studies that correlate taxonomic changes to aspects of injury recovery. Examination of both observational and emerging interventional studies supports the notion that future therapeutic avenues for spinal cord injury pathologies may lie at the interface of the host and indigenous microbes.

2.2 Introduction.

Spinal cord injuries (SCI) are traumatic occurrences with an annual incidence of roughly 15,000 new cases in the United States, and almost half a million world-wide (124). Due largely to injuries resultant from ground transportation incidents or falls (124), SCI leads to life-long impairment across numerous body systems. It further imposes an enormous mental, physical, and financial burden on the injured individual and their caregivers. In the US, estimates of financial costs that individuals and their caregivers will incur over the post-injury lifetime is $1.2–$5 million per person, excluding lost wages, in conjunction with significant decreases in overall quality of life (124).

Injuries themselves are highly variable in their etiology (*e.g.* contusion, transection, *etc.*), severity (*e.g.* depth of contusion, duration of compression/distraction, completeness of severance), and level of injury (*e.g.* cervical, thoracic, lumbar). These etiological factors lead to a wide range of both primary injury phenotypes and secondary sequelae. Certainly, para- and tetraplegia are visible, well-known, and debilitating indications of SCI, however dysfunction in the gastrointestinal (GI), genitourinary, and cardiac systems are also apparent both acutely and chronically following SCI (85, 125, 126). Further, long term effects on mood disorders and somatosensation (*i.e.* nociception) arise (127-129).

Given the broad and systemic effects of SCI across physiological systems, emerging studies in the last 5-6 years have begun to examine potential contributions of the indigenous microbiome to SCI recovery. As the microbiome has established capacity to modulate inflammatory and metabolic responses (43), two critical players in setting the tone of SCI recovery, it is likely that microbiome-dependent activities impart effects on recovery. In fact, a number of recent experimental studies establish concrete associations with microbiome-mediated contributions to recovery after SCI in rodent models.

Additionally, a wide array of intrinsic GI dysfunctions (*e.g.* neuropathy) are observed acutely following injury (130). We, and others, predict that these acute alterations to GI physiology drive changes to the structure of the microbiome. Across both rodent models and human incidences of SCI, shifts in particular microbial taxa of the gut microbiome have been observed (61, 115, 131-140). This dysbiosis (a detrimental disruption to the microbiome community) has the capacity to feed into the physiological pathways that impact recovery following injury. From these emerging

observational and experimental studies, a more complete picture of SCI recovery is being developed. The drastic and acute, whole-body, physiological dysfunctions that arise following SCI triggers disturbances in the intestinal environment, influencing both the composition and functionality of the microbiome. As microbiome structure and activities change, altered detrimental signals derived from this dysbiotic community differentially modulate inflammatory activation and metabolic input across all body systems, leading to effects on recovery both within and outside the spinal cord.

2.3 Remodeling of the gut microbiome after SCI.

Growing evidence indicates that the gut microbiome undergoes significant alteration following SCI and other traumatic injuries. Effects of medical treatment (*i.e.* antibiotics, steroids, analgesics), as well as secondary injuries, confound assigning primacy of SCI itself to those microbiome shifts observed in humans. However, experimental models which can limit these confounding variables also note substantial injury-dependent effects on microbiome structure.

To date, eight independent cohorts of persons with SCI have had microbiome assessments performed, with six of these studies publishing detailed genus-level gut microbiome analysis. While true metanalysis of taxonomic alterations is difficult at this juncture, owing to the low number of individuals (relative to the larger variability of the human microbiome), as well as technical considerations (region of 16S sequenced, amplicon database used, etc.), injury-dependent taxonomic alterations can be qualitatively combined (Table 2.1). Collapsing these different variables across studies (anatomical level of injury, time post-injury) to assess those taxa altered by injury reveals some consistent differences. Common taxa changes associated with SCI

are increases in the genera *Akkermansia*, *Alistipes*, *Bifidobacterium*, *Lactobacillus*, and *Phascolarctobacterium* and decreases in the genera *Coprococcus, Dialister, Faecalibacterium, Lachnospiraceae, Megamonas, Prevotella, Roseburia,* and *Subdoligranulum.* Interestingly, many of those taxa that are depleted are those which ferment complex polysaccharides to beneficial short-chain fatty acids (SCFAs) and appear vulnerable to perturbation in other inflammatory neurological conditions such as Parkinson's disease (141), as well as non-traumatic SCI, including spina bifida (142).

Animal models of SCI allow for greater control over injury and recovery conditions, enabling investigators to elucidate not only temporal changes in microbiome composition, but those that are specific to a predetermined injury level and severity. However, the reported taxa altered post-injury have not been consistent across studies, likely due to differences in methodologies, timing, and facility-level variations in murine gut microbiomes prior to perturbation/injury. Generally, the most consistent alterations to the post-SCI microbiome are characterized by an increase in the genus *Bacteroides* (5/9 studies identified with genus-level data) and a decrease in the genus *Lactobacillus* (observed in 6/9 studies) (Table 2.2).

Within studies, control for initial microbiome composition (comparing pre- and post-injury status) has indicated progressive changes to the microbiome that appear acutely after injury and progressively become more pronounced. The earliest significant alterations that have been reported occur 3 days post-injury, that become more engrained at later stages, between 7-40 days post-injury. (60, 143-145). However, some studies have reported a recovery of the microbiome, with the most significant changes occurring in the acute phase post-injury, and a subsequent

restoration of specific taxa to their pre-injury abundances (143, 146-148). Differences in injury severity and level may account for discrepancies between those that observe progressive dysbiosis and those studies which observe a microbiome recovery in conjunction with injury recovery. In one recent study, early (3 days post-injury) increases were observed in the orders Bifidobacteriales, Lactobacillales, Erysipelotrichales, and Verrucomicrobiales, with significant decreases in the genus *Bifidobacterium* (143). Conversely, a loss of the genus *Akkermansia* appeared later at ~13 days post-injury. While the alterations of these organisms reached statistical significance at the acute timepoints, they were qualitatively more reduced throughout recovery following injury, in a more severely injured group of animals, and showed longitudinal changes in magnitude out to 41 days post-injury (143). Collectively, these data indicate not only temporal alterations, but also a "dose-response" to injury severity with more severe injuries leading to greater impacts on the composition of the gut microbiome.

Overall, the published data to date show that traumatic SCI results in a progressive shift in the gut microbiome in experimental models and suggest injury-dependent alterations in human incidences of SCI as well. However, the precise mechanisms that alter the intestinal environment and mediate impacts on the microbial community are not yet known. Robust physiological dysfunctions, such as hypoxia of intestinal tissues and increased inflammatory signaling are known to occur acutely post-injury, and likely lead to diminished intestinal motility and increased permeability (125, 130). These acute pathologies likely give rise to an altered intestinal environment that promotes selection of certain organisms, which through competition, depletes others. These effects may become more pronounced with time post-injury (60, 125, 149), suggesting the possibility that these altered and dysbiotic microbiomes become engrained in a feed-forward loop of dysregulation. Given the

substantial roles for certain members of the microbiome on inflammatory and metabolic responses (43), it is likely then that this cycle of dysfunction ultimately contributes to impaired recovery of numerous organ systems.

2.4 Interactions between the gut microbiome and SCI-induced neurogenic bowel.

The progressive shift in the gut microbial community following SCI is likely the result of multiple factors. Certainly, loss of skeletal muscle control will lead to varied effects on dietary choices and food intake (150, 151), inducing taxonomic alterations due to nutritional changes (152, 153). Further, depending on injury level, SCI may limit abdominal and rectal control of fecal evacuation, impairing overall intestinal motility, and impacting the intestinal environment (99, 108, 154, 155). These, and other, post-SCI effects on the gut including enteric neuropathy and hypoperfusion (130), lead to neurogenic bowel dysfunction (NBD), a post-injury condition characterized by constipation and/or fecal incontinence (156), that also likely impacts the microbiome (149, 157, 158).

Across numerous surveys, bowel dysfunction has proven to be one of the most detrimental aspects of SCI, in terms of quality of life (85). Despite this, its exploration and therapeutic avenues remain understudied compared to other, more visible, components of SCI recovery. While the microbiome is likely influenced by neurogenic bowel, growing evidence indicates that microbiome signals promote intestinal motility through neurogenesis and hormonal signaling (154, 155, 159-163). However, evaluation of the efficacy of microbiome-based interventions to ameliorate SCI-associated NBD in human injuries has not yet occurred.

To our knowledge, no studies to date have established cause-effect relationships between microbiome composition and SCI-triggered NBD. Some, however, have correlated certain microbial taxa with aspects of NBD and/or have modulated outcomes with microbiome manipulations (114, 120, 164-168). Comparisons between severity of injury, bowel dysfunction, and gut microbiome dysbiosis have revealed that these attributes are positively correlated (166). One observation of a human cohort compared microbiome compositions between individuals with upper motor neuron (UMN) bowel dysfunction and those with lower motor neuron (LMN) bowel dysfunction following SCI (114). UMN bowel dysfunction is characterized by an inability to defecate due to increased sphincter and colonic tonus, but a preservation of reflex circuitry between the ENS and the spinal cord (157, 158). In contrast, LMN bowel dysfunction is characterized by a loss of reflex circuitry, inhibiting peristalsis, and a lack of tonus within the anal sphincter and colon (157, 158). These deficits are associated with either constipation or constipation with incontinence, respectively. Significant alterations were observed in both UMN and LMN groups compared to uninjured individuals, for instance a decrease in SCFA-producing microbes. Both groups of people with injury displayed significantly reduced levels of the genus *Pseudobutyrivibrio*, that produces lactate, butyrate, and formate, as well as reduced levels of the genus *Megamonas*, that produces acetate and propionate (114). Interestingly, microbiome composition has been observed to differ based on injury level in this study, a trend seen by others as well (115, 140, 166-168). Those with UMN (constipation alone) had significantly decreased *Marvinbryantia* and *Dialister* species, while those with LMN (constipation with fecal incontinence) had significantly decreased *Roseburia* (114). These findings suggest that SCI remodels the gut microbiome in an injury-level dependent manner, reflective of the physiological status of the gut.

It is interesting to note that there is a relatively small body of pre-clinical observations of NBD in experimental models of SCI (130), and even more limited associations with the gut microbiome at this time. Rodent models of SCI recapitulate enteric neuropathy and diminished total intestinal transit that appears in human injuries (88, 101, 102, 120, 169), and microbiome contributions to intestinal motility post-injury were recently described (120). Following a fecal microbiome transplant (FMT) from a healthy, uninjured mouse, mice with a midthoracic contusion SCI showed improved parameters of intestinal motility (120). However, in this study, FMT did not alter intestinal motility in uninjured controls, indicating the microbiome contribution to this deficit may be specific to animals with SCI. Additional studies to specifically investigate cause-effect relationships between the microbiome and SCI-induced NBD are needed.

2.5 SCI-triggered local and systemic immune responses.

While trauma to the spinal cord results in significant inflammatory responses at the site of injury, an ever-growing body of literature indicates that SCI also results in drastic inflammatory effects across numerous body systems (170-172). This includes a range of peripheral inflammatory responses that are quite physically distant from the local injury originating within the cord (173). In human incidences, examination of acute peripheral inflammation is largely confounded by the fact that SCI does not often occur alone and instead arises in conjunction with trauma at other body sites (174). Experimental rodent models of injury have allowed for the isolation of peripheral effects after injury, solely due to the injury within the spinal cord (175). Even when isolated to a single injury event within the cord itself, SCI results in dramatic changes to the inflammatory status at peripheral body sites (170-173, 176).

The timing and magnitude of both systemic and CNS-resident inflammation in humans and experimental models' post-injury is dynamic and is dependent on the level, completeness, and severity of injury. In general, an acute increase in pro-inflammatory cytokine signals and infiltration of circulating monocytes is observed locally within the cord, as well as systemically. Following SCI, immune cells are recruited to the site of injury and infiltrate the cord, in an injury severity-dependent fashion (143). This was exemplified in mice through experimental comparison between a spinal cord contusion and a more severe contusion with spinal compression. Substantial differences in pro-inflammatory cytokine expression were observed in the spinal cord based on injury severity, with a more robust response observed in the more severely injured group (143). Acutely following injury, both IL-1β and TNF expression were elevated in the compression/contusion group, compared to contusion alone. Both injury groups displayed increased lymphoid and monocyte/macrophage infiltration into spinal cord than sham controls. However, spinal cords of the more severe contusion/compression injury group had significantly more monocytes/macrophages (particularly $Ly6C^+$ infiltrating cells), as well as a substantial increase in total and phagocytic ($F4/80^+$) microglia than those with less severe injury and sham controls. During chronic points of recovery, >40 days post-injury, in the dorsal root ganglia of animals with more severe contusion/compression injuries, but not those with less severe injury, $Ly6C^+$ macrophages remained present.

In higher-level injury, this acute local induction is followed by a dramatic decrease in peripheral immune function, SCI-induced immune depression syndrome (SCI-IDS) (172, 177-180). This loss of immune inflammatory capacity and increase in regulatory immune cells is thought to be beneficial to prevent autoimmunity against CNS-derived antigens released following traumatic

injury (172). However, the significant immune impairment from SCI-IDS leads to increased infection susceptibility, ultimately hampering recovery. Conversely, longer-term increased inflammation can be detrimental to multi-organ systems including the nervous system (pain hypersensitivity, mood impairments) and gastrointestinal tract (neurogenic bowel) (172, 181-183). Therefore, understanding the molecular contributions to these distal inflammatory events outside the spinal cord is critical for furthering an understanding of secondary effects of injury and limiting their impact on recovery outcomes.

2.6 Contributions of the microbiome to SCI-associated inflammation in humans.

The gut microbiome has a critical role in the development and dynamics of the immune system, not only in the intestinal tract, but the periphery, and CNS (55, 184-186). This modulation of inflammatory responses is critical for mediating recovery from infections, predisposing to neuroinflammatory insults, and prevention of autoimmunity. It is therefore not surprising to envision that these microbiome-dependent activities may also influence the inflammatory signals that arise following SCI and other traumatic CNS injuries. Indeed, inflammatory outcomes after injury have been associated with microbiome composition in both humans and in experimental models.

To the best of our knowledge (and at the time of this writing), only one published human dataset sought to directly correlate microbiome composition to inflammatory outcomes, after SCI. This recent observational study compared groups of individuals with SCI who were either pre- or type 2- diabetic (P/DM) to those with SCI but normal glucose tolerance (NGT). Interestingly, none of the participants had previously been diagnosed with diabetes prior to this study, suggesting that

specific pathophysiology arising after SCI may trigger metabolic inflammation and glucose intolerance. It was observed that those with SCI and post-injury P/DM status had an increase in circulating inflammatory cytokines, including IL6 and TNF compared to individuals with SCI but NGT (187). This is notable as these cytokines are known to be particularly elevated in individuals with type 2 diabetes (188-190).

Since the composition of the gut microbiome has the capacity to differentially modulate lipid and glucose metabolism (191), it is possible that SCI-induced microbiome disruptions contribute directly to these metabolic and inflammatory outcomes and that the microbiome alterations arising following SCI are themselves responsible for triggering metabolic inflammation and glucose intolerance (192). Examination of the microbiome by 16S rRNA profiling revealed substantial differences in both alpha and beta diversity of the fecal microbiome between the P/DM and NGT groups. Notably, individuals with SCI and P/DM also had a lower abundance of the order Clostridiales and a higher abundance of the genus *Akkermansia* (187). These observations are somewhat contradictory to prior studies linking microbiome alterations to metabolic inflammation (193). Others have instead observed increased Clostridiales abundance following SCI in experimental models (Table 2.2) (60, 191). Additionally, *Akkermansia* is highly correlated with beneficial outcomes in many metabolic disorders, including obesity and diabetes (194-196). This organism has been experimentally shown to be beneficial against metabolic syndrome in both animal models and human trials of these disorders (197, 198), and generally presents in lower abundance in patients with metabolic disorders (199). Given the role of *Akkermansia* in metabolizing intestinal mucosa and modulating intestinal integrity (200), and its response to both dietary fiber and antibiotics (200, 201), understanding how this and other organisms function in

the distinct context of SCI (with altered host intestinal physiology and microbiome structures) is necessary. Indeed, many other microbially-impacted metabolic parameters are altered following SCI. Traumatic SCI is associated with decreased levels of circulating high-density lipoprotein (HDL) (167), and within those with chronic SCI, glucose, HDL, and creatinine have been correlated with microbiome composition (166-168), including positive association with the phylum Actinobacteria. Further, both studies report an increase in the genus *Bifidobacterium* and decreases in *Prevotella*, *Dialister*, and *Faecalibacterium* genera (166-168), suggesting potential metabolism-mediating roles of these taxa. Thus, use of experimental systems will be critical towards understanding how these organisms contribute to metabolic-inflammation and delineating their roles in recovery from SCI.

2.7 Microbiome contributions to SCI-associated inflammation in experimental models.

While associations between SCI-triggered inflammation and microbiome composition in humans are more limited, numerous experimental animal studies have begun linking these intrinsic factors. By assessing the inflammatory changes, arising both systemically and locally within the spinal cord, in conjunction with changes to microbiome composition that arise after injury, one may begin identifying key players in the microbial community that impart beneficial or detrimental effects on recovery. These may not only reveal new, underlying biology of systemic interactions following traumatic CNS injury, but more coercively, establish potential probiotic organisms with therapeutic benefit towards recovery.

A recent study examining inflammatory outcomes in the rat model of thoracic SCI identified a strong correlation between three specific cytokines (IL-1β, IL-12, and MIP-2) and specific gut

microbial taxa (62). The strongest correlations were found between IL-1β and 23 distinct operational taxonomic units (OTUs). Eight OTUs that were able to be characterized to the species level were each negatively correlated with circulating IL-1β levels, suggesting that these particular organisms may modulate levels of this inflammatory cytokine. One of these specific taxa was *Faecalibacterium prausnitzii*, which was negatively correlated with IL-1β. This organism is well established as an SCFA-producing member of the microbiome, converting dietary fiber preferentially to butyrate (202). This is notable because one common occurrence in the SCI-associated microbiome is the general loss of SCFA-producing bacteria (see Tables 2.1 and 2.2). *Faecalibacterium* is of particular note as it was found to be decreased in 5/6 studies of humans with SCI, that reported genus-level data (Table 2.1). Since butyrate potently dampens inflammatory activation (203, 204), the loss of *F. prausnitzii* and other SCFA-producing organisms, including other members of the *Faecalibacterium* genus, suggests that the SCI-associated microbiome has decreased capacity to limit detrimental inflammatory responses. In fact, three distinct studies have evaluated the efficacy of SCFA-based therapeutics for recovery from SCI, and all three studies found improvements to SCI-associated inflammation in animal models in animals provided increased SCFAs (122, 205, 206).

While peripheral inflammation arises following injury, emerging data indicate the microbiome has capacity to modulate immune responses within the CNS itself, including the spinal cord. For instance, recent experimental studies have identified components of signaling pathways activated by microbes, as modulators of spinal cord inflammation following SCI. One receptor, Toll-like receptor 4 (TLR4) has garnered recent attention. This pattern recognition receptor detects and responds to bacterial lipopolysaccharide (LPS) derived from Gram-negative bacterial cell

envelopes, leading to significant inflammatory responses. Following SCI in mice, a robust activation of the TLR4 signaling cascade was observed to occur in both intestinal tissue and the spinal cord (207). This includes, the TLR4 adaptor protein MyD88, the p65 and IκBα signaling molecules, leading to increased NFκB activation, the transcription factor for numerous inflammatory cytokines and chemokines. While only an association, this implicates a role for microbial triggers of post-SCI inflammation via a TLR4-dependent pathway.

FMT allows the ability to directly assess the contribution of particular microbiomes to outcomes of interest, such as TLR4-mediated inflammation. Using this approach, microbiomes derived from either mice with SCI or sham injury were introduced into conventionally-colonized mice with SCI (207). Injured mice treated with SCI-derived microbiomes displayed increased inflammatory cytokines relative to injured mice without an FMT. Further, mice treated with sham-derived FMT showed reduced inflammation, strongly suggesting that the microbiome that arises post-SCI is sufficient to exacerbate injury-induced inflammation and that the two differential microbiome communities (sham-derived or SCI-derived) have discrete impacts on inflammatory signaling (207, 208). While unable to elucidate specific bacterial populations that directly associate with changes in the inflammatory status (likely due to an insufficient sample size) TLR4-signaling was further implicated by the observation that those molecular components downstream of the TLR4 activation cascade were concurrently upregulated. Thus, suggesting (although not directly testing) that the SCI-associated microbiomes have enhanced capacity to stimulate TLR4 and exacerbate inflammatory signaling.

Additional implication of microbial-stimulated TLR4 activation following SCI has come from the study of cAMP-specific phosphodiesterase 4B (PDE4B) activation on SCI recovery. PDE4B is a downstream and parallel component of TLR4 activation and promotes cytokine activation (144, 209, 210). Comparison of SCI outcomes between wild-type and PDE4B null mice revealed a substantial decrease in inflammatory markers including GFAP, CD11b, Iba1, and Cox2 in injured mice lacking PDE4B, implicating this signaling pathway in post-SCI inflammation. Intriguingly, PDE4B null mice also showed improved locomotor recovery following SCI and did not present with other SCI-triggered pathophysiologies, such as gut microbiome alterations, bacterial overgrowth, or endotoxemia, which the wild-type animals robustly displayed. In fact, wild-type mice displayed a substantial increase in TLR4-stimulating *Proteobacteria* following SCI, perhaps suggesting that the bloom of these microbes potentiates post-injury inflammation. These data further support a broader model, whereby acute inflammatory signaling triggers microbiome alterations, which then lead to a chronic feed-forward cycle and exacerbation of systemic inflammation and neurological impairment, leading to additional dysbiosis and endotoxemia. Rolipram, a nonspecific PDE4 inhibitor, is widely available and has been used to treat a variety of human conditions (211), and has shown efficacy for recovery in preclinical models of SCI (212-214).

2.8 SCI microbiome association with gut permeability after injury.

One largely embraced theory surrounding how gut bacteria may lead to detrimental systemic and neuro- inflammatory responses, relevant to SCI, is through disruption of intestinal epithelial tight junctions and subsequent increased translocation of pro-inflammatory bacteria and/or their metabolites. Not just observed in traumatic neurological injury, various physiological disturbances

can give rise to impaired barrier function and increased bacterial translocation from the gut, leading to an exacerbated inflammatory response throughout the body after injury. These ultimately result in systemic effects that can have drastic impacts on recovery from injury. SCI has long been established to trigger increased gut permeability and bacterial translocation, leading to quantifiable levels of bacterial endotoxin in the blood within 24hrs, and within various tissues and organ systems within 48hrs of SCI (215). Recent studies have further validated and characterized these observations with combinations of both functional assessments (such as direct measurement of bacterial burden in peripheral tissues following injury, and absorption of typically non-absorbable molecules- *e.g.* FITC-dextrin) and molecular characterizations (*e.g.* expression and production of tight junction proteins in the intestinal epithelium). Overall, across experimental models, SCI results in an acute disruption of the intestinal epithelial barrier with diminished barrier function, increased permeability, enhanced bacterial translocation, and increased circulating endotoxin, all within 7 days post-injury (60, 120, 144, 164).

To combat this, prior recommendations entailed immediate administration of broad-spectrum antibiotics after injury. However, as our understanding of the complex interactions between host and microbiome after traumatic injury have evolved, it is becoming apparent that a more targeted approach is essential. A number of recent studies have indicated that broad-spectrum antibiotics prior to, or immediately post-injury, can adversely affect recovery in experimental models (60, 164). Therefore, while broad antibiotic treatment may prevent translocation, as well as limit secondary infections that are a significant risk post-injury, these benefits may be outweighed by their effects on the native microbiome. Rather than using broad-spectrum antibiotics to rescue the negative effects of bacterial translocation, a number of recent studies have evaluated microbiome-

based approaches to restore inflammatory responses by shifting the composition of the microbiome to a less disrupted state.

2.9 Microbiome manipulations with therapeutic potential for SCI recovery.

Recovery of locomotor function is perhaps the most visible and most targeted component of SCI recuperation. To a greater extent than human injuries, rodents with SCI are able to recover limb functions following a period of paralysis. With a set of highly standardized behavioral assays (*e.g.* Basso, Beattie, Bresnahan [BBB] and Basso Mouse Scale [BMS]) to measure injury-induced locomotor dysfunctions, experimental rodent models of injury provide a dynamic system to measure how limb function can be restored or impaired following SCI (216, 217).

Through its impacts on inflammation (perhaps via modulation of gut permeability), the gut microbiome represents a potential therapeutic target for SCI recovery. The gut microbiome is readily accessible and potentially modifiable through a number of different methodologies. Such methods range from whole microbiome FMT that reconstitute a complex microbial community, to probiotic therapy with specified organisms, or prebiotic treatments that select for certain taxa or induce a specific function. Further advances are also being made in the field of "postbiotics," in which microbial-derived metabolites are introduced directly to mediate host and/or microbiome physiologies. A wide variety of emerging studies have begun to explore these potential avenues for benefit following SCI.

2.9.1 Fecal microbiome transplants.

Fecal microbiome transplants are notable as they have the capacity to elucidate specific microbiome-mediated effects on various host physiologies in experimental settings, to determine potential key contributors to desired outcomes. A recent study investigated the efficacy of a healthy donor-derived FMT following a T10 contusive SCI in mice (120). Injured mice, when provided a daily, oral FMT, displayed significantly decreased levels of NFκB in the spinal cord and colon four weeks after injury, compared to injured animals provided a vehicle gavage. Corresponding to this inflammatory marker, SCI animals receiving FMT also displayed improved locomotor scores (120). In conjunction with previously described studies, this suggests that microbiome transplantation with a healthy microbiome may have beneficial effects on inflammatory responses post SCI. Conversely in a milder rat injury model, FMT from an uninjured donor did not result in significant improvement to locomotor recovery in the animal with SCI (148). However, FMT did reduce anxiety-like behaviors in recipient animals (148), suggesting microbiome-dependent effects beyond inflammatory and locomotor recovery and emphasizing a need for careful selection of FMT donors (147).

FMT has also been observed to improve motor evoked potentials (MEP) in the gastrocnemius muscle after SCI (120). FMT was able to restore the amplitude of the MEP and a trend toward shorter latency (faster response) when compared to untreated mice with SCI, suggesting a capacity of microbiome-derived signals to modulate post-injury neuronal physiology beneficially and at a highly refined level. In fact, FMT treated animals with SCI also displayed greater grip strength and higher numbers of motoneurons, synapses, and neuronal axons in spinal cord sections compared to those untreated animals with SCI (120). This was further validated with observations

that FMT from uninjured animals improved outcomes in white matter sparing, neurotrophic factors, and other critical physiological parameters in the cord, following SCI in mice (208). Thus, not only does the gut microbiome contribute to central aspects of recovery from SCI, but modulation of the community as a whole with FMT (to restore to a pre-injured taxonomic structure), has capacity to promote therapeutic outcomes following SCI.

2.9.2 Probiotic therapeutics.

While FMT has garnered a great deal of attention due to its efficacy and ability to broadly impact the entirety of the gut community, efforts to identify specific microbial contributors may reveal keystone taxa that are sufficient to drive beneficial immune responses after SCI. One such study, by Kigerl et al., noted a significant increase in inflammatory immune cell activation within the gut-associated lymphoid tissues (GALT) after a T9 contusion SCI (60). A shift in immune cell activation was observed, with increased B-cells, macrophages, $CD3^+$ T-cells, and dendritic cells within GALT following injury. This coincided with an increased expression of cytokines, both pro- and anti-inflammatory, including IL-10, TGFβ, TNFα, and IL-1β after injury. Administration of the probiotic cocktail VSL#3 (now Visbiome, consisting of *Streptococcus thermophilus, Bifidobacterium breve, B. longum, B. infantis, Lactobacillus acidophilus, L. plantarum, L. paracasei, and L. deslbrueckii* subsp. *Bulgaricus*) significantly diminished this immune activation in the GALT following SCI, suggesting a therapeutic benefit from certain organisms not necessarily requiring a full FMT.

These beneficial effects of select bacterial taxa were not confined to the intestinal inflammatory environment. Those animals receiving VSL#3 also displayed substantially improved inflammation

in the spinal cord, with fewer infiltrating macrophages, and increased dendritic cells and $FOXP3^+$ regulatory T-cells (60). Notably, antibiotic treatment to alter the gut microbiome prior to SCI, resulted in increased markers of inflammation in the cord compared to SCI alone, including macrophages and $CD3^+$ T-cells. These inflammatory parameters correlated directly with survival of white matter in the injured cord tissues, with VSL#3 treated animals having substantially more white matter spared following injury. Locomotor recovery and the percentage of spared white matter in mice receiving VSL#3 probiotics were also significantly improved. While these data strongly implicate the immune response in the cord and periphery with recovery, they also further support the notion of a gut microbiome contribution to SCI recovery. In fact, VSL#3 has recently shown efficacy in promoting functional recovery following traumatic peripheral nerve injury, in contrast to impaired recovery following antibiotic pre-treatment (218). These observations together indicate that specific microbial taxa can be beneficial not solely in the local intestinal environment, but within the spinal cord itself at the site of injury and even the peripheral nervous system. Thus, association with specific microbes may have a tremendous impact on long-term neurological recovery following SCI.

2.9.3 Selective antibiotic treatment.

Administration of a broad-spectrum cocktail of antibiotics *prior to* injury has been shown to greatly diminish recovery after T9 contusion SCI in mice- including impaired hindlimb function, increased inflammation, and greater lesion volume at the site of injury (60, 164). However, the use of selective antibiotics post-injury may provide a mechanism to prevent pathobionts from blooming and allow for beneficial microbes to become enriched. Certain antibiotics have shown promise for reducing inflammation and improving outcomes in a wide range of neuroinflammatory

diseases, although whether their activities are dependent on an intact microbiome (as opposed to "off-target effects" on host factors) is not fully established.

One such antibiotic is minocycline, which has been shown to confer anti-inflammatory and neuroprotective effects in rodents (219-222), as well as modest motor improvement in humans (223), after SCI. The therapeutic value of minocycline was tested in a rat injury model of unilateral cervical (C5) SCI (145). At this cervical lesion level, but not at lower thoracic injury sites such as those described in prior sections, a significant immune depression occurs, with decreases in abundance of a number of cytokines and chemokines (172, 177-180). Irrespective of minocycline treatment, these injured animals all displayed significantly depressed cytokine responses compared to their baseline at 5 days post-injury (145). However, minocycline treatment post-injury rescued immune suppression by 28 days following injury, restoring cytokine profiles to pre-injury levels. Rats with a C5 SCI, but no minocycline treatment remained in a prolonged immune suppressed state. Not surprisingly, minocycline treatment resulted in acute, although transient, changes to the microbiome that were more robust than those alterations induced by injury alone. These microbiome alterations were characterized predominantly by a significant decrease in the Firmicutes/Bacteroidetes ratio (145). These acute changes to the microbiome preceded the effect of minocycline on cytokines and chemokines, implicating the microbiome in minocycline-dependent immune regulation following SCI. However, examination of minocycline's effects in germ-free animals or reconstitution of minocycline-shifted microbiomes would help to provide evidence towards causality, rather than correlation of microbiome alterations during treatment. It is important to note that this more mild, unilateral injury model did not observe an effect on locomotor recovery following minocycline treatment. Minocycline has additional impacts on

neuroprotection and immune regulation in cell culture systems (224, 225), suggesting that its beneficial effects *in vivo* may be more complex than modifying the microbiome alone. Although technically challenging, studies of SCI and minocycline treatment in germ free animals may be necessary to assess the neuroprotective role of minocycline independent of the microbiome.

2.9.4 Beneficial microbiome-related metabolites.

While FMT, probiotic, and antibiotic experiments provide evidence that the presence or absence of select microbes (or the disruption of the community) can modulate SCI outcomes, small molecules derived from these gut communities (so-called "postbiotics") may allow more precise tuning of physiological responses during SCI recovery. One such class of beneficial metabolite is the short chain fatty acids (SCFAs). Following therapeutic FMT in a mouse T10 contusion model of SCI, roughly half of the microbial taxa that were depleted post-injury returned to normal levels (120). Notably, these included *Blautia*, *Anaerostipes*, and *Butyricimonas* all of which are capable of producing the beneficial SCFA, butyrate (226-228). Given its broad roles in energy homeostasis, anti-inflammatory signaling, and epigenetic modification (through histone deacetylase modulation), butyrate and other SCFAs represent a physiologically beneficial microbiome metabolite (229-232). Post-SCI, many SCFAs are reduced in the gut, including propionate, butyrate, isobutyrate, and caproic acid- with butyrate the most robustly affected of the SCFAs (depleted >50%) (120). Despite the drastic effects of SCI on SCFA levels, FMT from an uninjured donor led to a >40% increase in butyrate levels (120). Interestingly, SCFA levels were only altered following FMT into mice with SCI and not when introduced into uninjured controls. This may indicate a homeostatic effect of SCFAs in a healthy microbiome, with beneficial impacts only observed in the presence of an existing deficit (*i.e.* SCI). SCFAs have also been shown to promote

barrier integrity in both the intestine and at the blood-brain barrier (233, 234). Following FMT, tight junction protein expression in the gut and pericyte coverage in the spinal cord were restored. Gut permeability and blood-spinal cord-barrier integrity were concurrently improved, suggesting a potential mechanism by which FMTs ameliorate inflammatory outcomes in SCI (120, 208). These early data help to support a notion that butyrate and other SCFAs may be providing benefit to recovery post-injury, and provide a foundation for uncovering specific, modifiable microbial factors as therapeutics.

Other SCFA-derived pharmaceuticals have also shown efficacy in reducing inflammation and improving locomotor recovery (122, 205, 206). For instance, D-β-hydroxybutyrate (DBHB) treatment resulted in improved locomotor function, reduced microglial and glial activation, suppressed NLRP3 inflammasome activation, and decreased protein levels of IL-1β and IL-18 in the spinal cord of injured mice (206). Treatment with other SCFAs, including sodium butyrate (122) or sodium propionate (205), has also been observed to greatly improve locomotor outcomes in rodent models of SCI in a dose-dependent fashion. These SCFAs additionally reduced inflammation in the spinal cord, with both diminished proinflammatory cytokine levels and cellular markers of immune activation (122, 205).

Postbiotics, such as SCFAs, may themselves serve as modulators of therapeutic physiologies, however changing the functional output of the microbiome also represents an easily accessible pharmaceutical target- *i.e.* drugging the microbiome. This avenue has shown promise in models of cardiovascular and Parkinson's disease, with small molecules developed to act directly on bacterial metabolic activities within the microbiome which then affect host functions (235, 236). In the

context of SCI, one such example is melatonin, a signaling molecule with activities both on the host and on the microbiome (237). Melatonin is produced primarily by enterochromaffin cells of the GI tract and has capacity to influence the composition of the gut microbiome as well as the integrity of the intestinal barrier (238-240). A small number of studies in various models of traumatic injury have observed beneficial neuroprotective effects of melatonin administration (241-244). In a mouse model of midthoracic SCI, intraperitoneal administration of melatonin was found to reduce colonic inflammation following injury (164). SCI-triggered increases in colonic concentrations of the proinflammatory cytokines IL-17, IFNγ, and MCP-1 were all reversed by melatonin treatment, indicating its anti-inflammatory benefits. Correspondingly, systemic administration of melatonin also resulted in some restoration of SCI-induced gut microbiome alterations (164). Consistent with other studies (see Tables 2.1 & 2.2), following SCI, representatives of the Lactobacillales and Bifidobacterium orders were substantially decreased, while members of the order Clostridiales were increased. Intriguingly, levels of the cytokine MCP-1 positively correlated with abundance of Clostridiales, and negatively correlated with abundances of Lactobacillales, suggesting an association between these taxa and gut inflammatory responses. Members of the order Lactobacillales are known to produce lactocepin, a potent anti-inflammatory protease (245), which others have identified as downregulated after experimental SCI (246). Increased inflammatory state was also associated with a disrupted and more permeable intestinal barrier, suggesting that increased translocation of gut bacterial components may drive systemic inflammation. In fact, increased gut permeability was correlated with levels of Clostridiales but inversely correlated with levels of Lactobacillales implicating select taxa in this physiological dysfunction. Melatonin treatment restored some components of the gut microbiome, including increased levels of Lactobacillales and decreased levels of Clostridiales, but had no impact on

Bifidobacterium. Intestinal tight junction protein expression and intestinal barrier integrity were also restored following melatonin administration post-SCI, as was intestinal motility. Overall these data indicate that melatonin is capable of limiting SCI-induced dysbiosis as well as molecular markers of intestinal function, either by mediating host intestinal physiology or through direct action on the microbiome.

The beneficial effects of melatonin post-SCI also corresponded with substantially improved locomotor recovery in melatonin treated mice, following SCI. Notably, co-administration of melatonin and broad-spectrum antibiotics in mice with SCI partially negated the protective effects of melatonin treatment on locomotor recovery, suggesting, in part, the necessity of the microbiome for melatonin-based improvements in many aspects of SCI recovery. The association between melatonin-mediated benefit to SCI recovery and microbiome alterations may therefore be directly related. However, it is unclear if melatonin acts independently on inflammatory pathways or if this activity requires an intact microbiome.

While still in early pre-clinical, experimental studies, these emerging investigations provide the foundational proof-of-concept for microbiome-related modulation of SCI recovery. Through FMT, select probiotic organisms, or microbiome-derived metabolites, these may provide more accessible avenues for SCI therapy and uncover new biological pathways involved in traumatic neurological injury. Further understanding of diet-microbiome interactions may also provide new direction for methods to improve microbiome resilience in the face of injury-induced perturbation and prevent the feed-forward cycle of inflammatory dysfunction that occurs following SCI.

2.10 Looking broadly into the future at microbiome effects on SCI.

SCI results in life-long dysregulation of numerous body systems, much more than the visible impacts on limb function. As discussed in prior sections, gastrointestinal dysfunction is a major quality of life issue for those individuals recovering from SCI (85, 247). In addition, rates of anxiety, depression, and other affective disorders are considerably higher in persons with SCI than in the able-bodied population (127, 128, 248). While the mechanisms to explain this phenomenon are likely highly multifactorial and complex, a plethora of data indicate contributions of indigenous microbes to mood disorders in both humans and experimental models (249-253). Emerging data in experimental models of SCI, evaluating the onset of anxiety-like behaviors have suggested that microbiome manipulations post-injury (for instance FMT or minocycline antibiotics) can rescue various rodent behaviors that mirror anxiety (145, 147, 148). Given broad, whole-body impacts of microbiome manipulation, it is hopeful that future microbiome-targeted therapeutics will alleviate some of the multi-system physiological burdens experienced by those with SCI and their caregivers. Since the microbiome is readily accessible for modulation, translation to clinical studies are highly tempting with the growing body of pre-clinical data. In fact, an ongoing, randomized clinical trial has early reported findings demonstrating a restoration of the SCI-associated gut microbiome with a low carbohydrate / high protein (LC/HP) diet, including an increase in SCFA-producing bacterial species, suggesting the viability and potential efficacy of such therapeutic avenues (254, 255).

Deeper understanding of the large-scale microbial community alterations that occur following injury will be necessary to generate further hypotheses regarding specific organisms that may contribute to dysfunction following SCI. Metagenomic analysis of the gut microbial community

has revealed further insights into SCI-triggered dysregulation of not only the well-studied bacterial taxa, but also viral organisms present in the gut (246), and the ability to begin to assess eukaryotic microbes as well. The use of metagenomics, in combination with metabolomics studies are needed to begin to draw direct lines between the metabolic and immunologic output of the microbiome and the microbiome composition itself. Current studies inferring genetic content and metabolic output from 16S sequencing lack the specificity and depth that metagenomics provides.

Most importantly however, experimental validation in gnotobiotic models will be key to addressing both the contribution of certain gut microbes to SCI-relevant physiologies and to establish direct functional connections between specific organisms and their beneficial outcomes. By combining gnotobiotic techniques with experimental models of SCI, and informed by growing human microbiome datasets, the microbiome is poised to be a valuable therapeutic target for recovery of traumatic SCI.

2.11 Acknowledgments.

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2.12 Tables.

Table 2.1. Microbiome alterations in incidence of human spinal cord injury.

Phylum	2021 Yu	2016 Gungor	2018/19 Zhang	2021 Bazzocchi	2020 Lin	2020 Li
(A)synergistetes/Synergistota	↑	N/A	=	N/A	N/A	N/A
Actinobacteria	↑	N/A	↑	N/A	N/A	N/A
Bacteroidetes	↓	N/A	=	↓	N/A	N/A
Cyanobacteria	↓	N/A	=	N/A	N/A	N/A
Firmicutes	N/A	N/A	↓	=	↓	N/A
Fusobacteria	N/A	N/A	↑	N/A	N/A	N/A
Proteobacteria / Pseudomonadota	↓	N/A	↑	N/A	N/A	N/A
Saccharibacteria	N/A	N/A	=	N/A	N/A	N/A
Tenericutes	N/A	N/A	=	N/A	N/A	N/A
Verrucomicrobia	N/A	N/A	↑	N/A	N/A	N/A
Genus	**2021 Yu**	**2016 Gungor**	**2018/19 Zhang**	**2021 Bazzocchi**	**2020 Lin**	**2020 Li**
Akkermansia	N/A	↑	↑	↑	N/A	N/A
Alistipes	N/A	=	↑	N/A	↑	↑
Anaerostipes	N/A	=	N/A	N/A	N/A	N/A
Anaerotruncus	N/A	=	N/A	N/A	↑	↑
Bacteroides	N/A	=	↑	↓	N/A	N/A
Barnesiella	N/A	N/A	N/A	N/A	↑	N/A
Bifidobacterium	↑	↑	↑	N/A	N/A	N/A
Bilophila	N/A	N/A	N/A	N/A	↑	N/A
Blautia	N/A	=	↑	N/A	N/A	N/A
Butyricicoccus	↓	N/A	N/A	N/A	N/A	N/A
Campylobacter	N/A	N/A	N/A	N/A	N/A	↓
Christensenella	↑	N/A	N/A	N/A	↑	N/A
Cloacibacillus	↑	N/A	N/A	N/A	N/A	N/A
Clostridium	↑	=	N/A	↓	↓	N/A
Collinsella	N/A	↑	N/A	N/A	N/A	N/A
Coprobacillus	↑	N/A	N/A	N/A	↑	N/A
Coprococcus	↓	↓	N/A	↓	N/A	N/A
Coriobacteriaceae	↑	N/A	N/A	↑	N/A	N/A
Desulfovibrio	↓	N/A	N/A	N/A	N/A	N/A
Dialister	↓	↓	↓	↓	↓	N/A
Dorea	↑	=	N/A	N/A	N/A	N/A
Eggerthella	↑	N/A	N/A	N/A	↑	↑
Eisenbergiella	N/A	N/A	N/A	N/A	N/A	↑
Enterococcus	N/A	↑	N/A	↑	N/A	N/A
Erysipelatoclostridium	N/A	N/A	N/A	N/A	N/A	↑
Escherichia	N/A	↑	↑	N/A	N/A	N/A
Eubacterium	↑	=	↓	N/A	N/A	↑
Faecalibacterium	↓	↓	↓	↓	↓	N/A
Flavonifractor	N/A	=	N/A	N/A	↑	N/A
Gemmiger	↓	N/A	N/A	N/A	N/A	N/A
Gordonibacter	N/A	N/A	N/A	N/A	↑	N/A
Haemophilus	N/A	N/A	N/A	N/A	↓	N/A
Holdemania	N/A	N/A	N/A	N/A	↑	N/A
Intestinimonas	N/A	N/A	N/A	N/A	↑	N/A
Klebsiella	N/A	N/A	↑	↑	N/A	N/A
Lachnoclostridium	N/A	N/A	↑	N/A	N/A	↑
Lachnospira	N/A	N/A	N/A	N/A	↓	N/A
Lachnospiraceae (various genera)	↓	↓	N/A	↓	↓	N/A
Lactobacillus	N/A	↑	↑	↑	N/A	N/A
Leuconostoc	N/A	N/A	N/A	N/A	↓	N/A
Marvinbryantia	N/A	↓	N/A	N/A	N/A	N/A
Megamonas	N/A	↓	↓	N/A	↓	N/A
Megasphaera	N/A	N/A	↑	N/A	↓	N/A
Methanobrevibacter	N/A	N/A	N/A	↑	N/A	N/A
Odoribacter	N/A	N/A	N/A	N/A	N/A	↑
Oscillospira	N/A	=	N/A	↓	N/A	N/A
Oscillibacter	N/A	N/A	N/A	N/A	N/A	↑
Parabacteroides	N/A	N/A	N/A	N/A	↑	N/A
Pasteurellaceae	↓	N/A	N/A	N/A	N/A	N/A
Phascolarctobacterium	N/A	N/A	↑	N/A	↑	↑
Prevotella	↓	↓	↓	↓	N/A	N/A
Pseudobutyrivibrio	N/A	↓	N/A	N/A	↓	N/A
Pseudoramibacter	↑	N/A	N/A	N/A	N/A	N/A
Rhodococcus	N/A	N/A	N/A	N/A	↓	N/A
Roseburia	↓	↓	=	N/A	↓	N/A
Ruminococcus	N/A	=	=	↓	↓	N/A
Sarcina	N/A	↑	N/A	N/A	N/A	N/A
Streptococcus	N/A	=	N/A	↑	N/A	N/A
Subdoligranulum	N/A	↓	↓	N/A	↓	N/A
Sutterella	↓	N/A	N/A	N/A	N/A	↑
Thalassospira	N/A	=	N/A	N/A	N/A	N/A
Veillonella	N/A	N/A	N/A	N/A	↓	N/A

Key

Higher in Controls / Lower in SCI (↓)
Lower in Controls / Higher in SCI (↑)
Data Not Available / Not Reported **(N/A)**
Data Reported, No Different Between Groups **(=)**

Phyla Higher in SCI (↑), in 2 or more studies.

Actinobacteria

Genera Higher in SCI (↑), in 2 or more studies.

Akkermansia
Alistipes
Anaerotruncus
Bifidobacterium
Christensenella
Coprobacillus
Eggerthella
Enterococcus
Escherichia
Klebsiella
Lachnoclostridium
Lactobacillus
Phascolarctobacterium

Phyla Lower in SCI (↓), in 2 or more studies.

Bacteroidetes
Firmicutes

Genera Lower in SCI (↓), in 2 or more studies.

Coprococcus
Dialister
Faecalibacterium
Lachnospiraceae (genus: N/A)
Megamonas
Prevotella
Pseudobutyrivibrio
Roseburia
Ruminococcus
Subdoligranulum

Table 2.2. Alterations to the gut microbiome in experimental spinal cord injury.

Phylum	2018 O'Connor	2020 Schmidt	2021 Schmidt	2021 Jing	2019 Jing	2021 Cheng	2021 Du	2021 Rong	2021 Bannerman	2021 Doelman
Actinobacteria	N/A	N/A	N/A	N/A	N/A	N/A	↑	N/A	N/A	N/A
Bacteroidetes	N/A	N/A	=	↑	N/A	N/A	N/A	↑	N/A	↑ (acute)
Cyanobacteria	N/A	N/A	↑	N/A	N/A	N/A	N/A	N/A	N/A	↓
Epsilonbacteraeota	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	↓ (acute)
Firmicutes	N/A	N/A	=	↓	N/A	↓	↓	↓	N/A	↑ (acute)
Proteobacteria / Pseudomonadota	N/A	N/A	↓	N/A	N/A	↑	N/A	↑	N/A	↓
Tenericutes	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	↓ (acute)
Spirochaetes	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	↑

Genus	2018 O'Connor	2020 Schmidt	2021 Schmidt	2021 Jing	2019 Jing	2021 Cheng	2021 Du	2021 Rong	2021 Bannerman	2021 Doelman
Akkermansia	N/A	N/A	=	↑	N/A	N/A	N/A	N/A	↓	N/A
Alistipes	N/A	N/A	=	N/A	↑	N/A	N/A	N/A	N/A	N/A
Allobaculum	N/A	N/A	N/A	N/A	N/A	↑	N/A	N/A	N/A	N/A
Alloprevotella	N/A	N/A	N/A	N/A	N/A	↓	N/A	N/A	N/A	N/A
Anaerostipes	N/A	N/A	N/A	↓	N/A	N/A	N/A	N/A	N/A	N/A
Anaerotruncus	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Azospirillum sp. 47_25	N/A	N/A	↑	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Bacteroides	N/A	N/A	=	↑	↑	↑	↑	↑	N/A	N/A
Bifidobacterium	↑	↓	N/A	N/A	↑	N/A	N/A	N/A	↓	N/A
Bilophila	N/A	N/A	N/A	↑	N/A	N/A	N/A	N/A	N/A	N/A
Blautia	N/A	↓	N/A	↓	N/A	↑	N/A	↑	N/A	N/A
Butyricicoccus	N/A	↓	↑	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Butyricimonas	N/A	N/A	N/A	↓	N/A	N/A	N/A	N/A	N/A	N/A
Christensenellaceae	N/A	N/A	↓ (R-7 Group)	↓	N/A	N/A	N/A	N/A	N/A	N/A
Clostridium	N/A	↓	N/A	N/A	N/A	N/A	↓	N/A	N/A	N/A
Coprococcus	N/A	↓	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Dialister	N/A	↓	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Dorea	N/A	N/A	N/A	N/A	N/A	↑	N/A	N/A	N/A	N/A
Eggerthella	N/A	↓	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Enterobacter	N/A	N/A	N/A	N/A	N/A	N/A	N/A	↑	N/A	N/A
Enterococcus	N/A	↓	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Escherichia-Shigella	N/A	↓	N/A	N/A	N/A	N/A	N/A	↑	N/A	N/A
Eubacterium	N/A	↑	N/A	N/A	N/A	N/A	↑	N/A	N/A	N/A
Faecalibacterium	N/A	↓	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Flavonifractor	N/A	N/A	N/A	N/A	N/A	N/A	↑	N/A	N/A	N/A
Helicobacter	N/A	N/A	N/A	N/A	N/A	↓	N/A	N/A	N/A	N/A
Intestinimonas	N/A	N/A	↓	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Klebsiella	N/A	N/A	N/A	↓	N/A	N/A	N/A	↑	N/A	N/A
Lachnoclostridium	N/A	N/A	N/A	↑	N/A	↑	N/A	N/A	N/A	N/A
Lachnospiraceae/Lachnospira	N/A	↓	↓ (ND3007)	↓	↑	N/A	↑	N/A	N/A	N/A
Lactobacillus	N/A	N/A	↓	↓	↓	↓	↓	↓	N/A	N/A
Lactococcus	N/A	↑	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Megamonas	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Mogibacterium	N/A	↑	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Muribaculaceae (fam for CAG)	N/A	N/A	N/A	↓	N/A	N/A	N/A	N/A	N/A	N/A
Oscillibacter	N/A	N/A	↑	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Parabacteroides	N/A	↓	N/A	↑	N/A	N/A	N/A	N/A	N/A	N/A
Prevotella	N/A	N/A	↓	N/A	N/A	N/A	↓ (CAG-1031)	N/A	N/A	N/A
Romboutsia	N/A	N/A	↑	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Roseburia	N/A	↓	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Ruminiclostridium 6	N/A	N/A	↑	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Ruminococcaceae UCG-005	N/A	N/A	↓	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Ruminococcus 1	N/A	N/A	↑	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Slackia	N/A	↑	N/A	N/A	N/A	N/A	↑	N/A	N/A	N/A
Turicibacter	↓	N/A	N/A	N/A	N/A	N/A	↓	N/A	N/A	N/A
Weissella	N/A	↑	N/A	N/A	N/A	N/A	↑	N/A	N/A	N/A

Key

Higher in Controls / Lower in SCI (↓)
Lower in Controls / Higher in SCI (↑)
Data Not Available / Not Reported (N/A)
Data Reported, No Different Between Groups (=)

Phyla Higher in SCI (↑), in 2 or more studies.

Bacteroidetes

Genera Higher in SCI (↑), in 2 or more studies.

Bacteroides
Eubacterium
Lachnoclostridium
Slackia
Weissella

Phyla Lower in SCI (↓), in 2 or more studies.

Firmicutes

Genera Lower in SCI (↓), in 2 or more studies.

Christensenellaceae
Clostridium
Lactobacillus
Prevotella
Turicibacter

Chapter 3: Diet-microbiome interactions promote enteric nervous system resilience following spinal cord injury.

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3.1 Abstract.

Spinal cord injury (SCI) results in a plethora of physiological dysfunctions across all body systems, including intestinal dysmotility and deterioration of the enteric nervous system (ENS). Typically, the ENS has capacity to recover from perturbation, so it is unclear why intestinal pathophysiologies persist after traumatic spinal injury. With emerging evidence demonstrating SCI-induced alterations to the gut microbiome composition, we hypothesized that modulation of the gut microbiome could contribute to enteric nervous system recovery after injury. Here, we show that intervention with the dietary fiber inulin prevents ENS deterioration and limits SCI-induced intestinal dysmotility in mice. However, SCI-associated microbiomes and exposure to specific SCI-sensitive gut microbes are not sufficient to modulate injury-induced intestinal dysmotility. Intervention with microbially-derived short-chain fatty acid (SCFA) metabolites prevents ENS dysfunctions and phenocopies inulin treatment in injured mice, implicating these microbiome metabolites in protection of the ENS. Notably, inulin-mediated resilience is dependent on signaling by the cytokine IL-10, highlighting a critical diet-microbiome-immune axis that promotes ENS resilience following SCI. Overall, we demonstrate that diet and microbially-derived signals distinctly impact recovery of the ENS after traumatic spinal injury. This protective diet-microbiome-immune axis may represent a foundation to uncover etiological mechanisms and future therapeutics for SCI-induced neurogenic bowel.

3.2 Introduction.

Spinal cord injury (SCI) disrupts numerous aspects of physiology, most notably impacting autonomic and voluntary systems at or below the level of lesion (125, 126). The gastrointestinal

(GI) tract is no exception. Up to 60% of injured persons present with myriad intestinal symptoms including constipation, fecal incontinence, and slowed GI transit time, collectively termed neurogenic bowel dysfunction (NBD) (156, 158). NBD consistently ranks among the most burdensome aspects of SCI – often prioritized over lower limb function (85, 256); with complications resulting in significant hospitalizations and mortalities (257, 258). Rodent injuries recapitulate SCI-induced enteric dysfunctions (88, 89), including reductions in GI transit time, and enteric nervous system (ENS) atrophy and hypoactivity (97, 100-104). Despite the ability of the healthy ENS to recover following perturbation (259), these pathologies persist (90, 149). Thus, we hypothesize that the intestinal environment that arises after injury may inhibit ENS recovery.

One component of the intestinal environment is the indigenous gut microbiome. This complex microbial community plays pivotal roles in host physiology, including the development and modulation of the ENS (159-161). Emerging data highlight an association with the gut microbiome and locomotor recovery after SCI (60, 62), however, the potential pathogenicity of the injury-associated microbiome and precise contributions to recovery are unknown. After SCI, the gut microbiome composition is markedly shifted (260). While there is not yet a characteristic gut microbiome composition across SCI studies, some generalities are emerging. A reduction of short chain fatty acid (SCFA)-producing bacteria appears in both experimental and human injuries (260). SCFAs, produced by bacterial fermentation of dietary fiber, modulate the host intestinal environment, including promoting beneficial immune responses and GI motility (50). Despite the microbiome's ability to promote enteric neurogenesis in healthy individuals (159, 160), it is unknown whether the post-injury gut microbiome contributes to recovery of the GI tract following SCI. Dietary fiber interventions enhance the production of SCFAs and promote microbiome

resilience which impact ENS physiology (75, 109). We therefore sought to determine whether the microbiome-fermented dietary fiber inulin can limit enteric pathologies following SCI.

Here, we used a murine T9/10 contusion model of SCI, that recapitulates hallmark aspects of NBD (164). We find that inulin supplementation improves gut transit and prevents the loss of ENS neuronal processes. Employing gnotobiotic approaches, we observe that neither the injury-associated microbiome itself nor probiotic interventions with bacterial species lost after SCI were sufficient to modulate enteric pathologies. However, we identify the microbially-derived SCFA metabolite, butyrate, as one factor that limits SCI-induced enteric pathology, suggesting diet-microbe interactions facilitate ENS recovery. Since both inulin and SCFA interventions specifically increased the production of the multi-trophic cytokine IL-10, we investigated its involvement in enteric resilience post-injury. Indeed, injured mice lacking the IL-10 receptor did not respond to dietary inulin, highlighting this immune pathway in prevention of ENS deterioration. Overall, our data elucidate a critical microbiome-neuroimmune interaction elicited by diet that facilitates enteric neuronal and physiological resilience, prevents intestinal dysmotility, and improves enteric pathologies post-SCI.

3.3 Results.

3.3.1 Inulin limits NBD pathology following mid-thoracic SCI in mice.

As observed in human injuries, thoracic SCI in rodents triggers significant enteric dysfunction, including colonic dysmotility, ENS atrophy, and microbiome alterations (97, 100-102, 104). To investigate diet and microbiome dependent effects on SCI-induced NBD, we performed severe contusion injuries (~70 kilodyne, ~1.5mm displacement) at the T9/T10 mid-thoracic spinal cord

(vertebrae T7-T8) (261) in mice, in comparison to identical sham laminectomies. Injured animals were immediately provided with either water (vehicle) or water containing 1% soluble inulin (Fig. 3.1.A) – a fiber, dose, and route previously demonstrated to promote resilience of the microbiome (69). Regardless of dietary intervention, all injured mice quickly displayed significant hind-limb locomotor dysfunction, as measured by the Basso Mouse Scale (BMS) (217) and weight loss (Fig. 3.1.B, C). At 14-days post-injury (DPI), a timepoint of early recovery rather than acute injury, injured mice displayed slowed intestinal motility, which was significantly improved in those treated with dietary inulin (Fig. 3.1.D). Total intestinal transit is regulated by signals extrinsic to the GI tract (spinal and hormonal, for instance) as well as intrinsically by the ENS. To test the functionality of the ENS in the colon, as the site where inulin would largely be fermented, we recorded colonic contractions in an *ex vivo* preparation. We found that colons derived from injured mice treated with inulin displayed a higher frequency of contractions in the distal colon (Fig. 3.1.E-G). Thus, we hypothesized that inulin acts on intrinsic intestinal physiology to prevent SCI-induced dysmotility.

Subsequent molecular characterization of enteric neuronal markers in colonic tissues post-SCI validated prior reports of ENS dysfunction (97, 101-104). We observed a substantial decrease in PGP9.5 protein post-SCI, as well as in nNOS, but not in ChAT by western blot analysis (Fig. 3.1.H-J), highlighting the sensitivity of nitrergic enteric signaling in the post-injury environment. Dietary inulin intervention resulted in substantial increases of PGP9.5 and nNOS protein levels (Fig. 3.1.H, I), indicating it limits the loss of these markers. Correspondingly, immunofluorescence imaging of the colonic myenteric plexus revealed a substantial reduction of enteric neuronal processes post-SCI, that was significantly prevented by inulin intervention (Fig. 3.1.K, L). Critically, we note

minimal alterations to total neuronal number in the colonic myenteric ganglia (Fig. 3.1.M-O). Together with diminished protein abundances, this implicates a loss of neuronal networks, rather than an overt loss of neurons. Overall, immediate supplementation with inulin is able to diminish this deterioration of the ENS following SCI and maintain total intestinal transit.

3.3.2 Dietary inulin prevents SCI-triggered gut dysbiosis.

The gut microbiome readily responds to dietary inputs, and dietary fiber specifically promotes its resiliency (69). SCI in rodents has demonstrated progressive changes to overall gut microbiome composition beginning at ~14-DPI (60). We therefore investigated whether inulin intervention prevented SCI-induced microbiome compositional alterations using 16S rRNA profiling. At 14-DPI, we note limited impacts to alpha-diversity of the fecal microbiome (Fig. 3.2.A, B), but a substantial alteration to community structure by beta-diversity assessment (Fig. 3.2.C, D and Tables 3.1 & 3.2). A fully independent cohort was impacted similarly, although not identically, following SCI (Fig. 3.6 and Tables 3.3, 3.4, & 3.5). Across these independent studies, a small number of bacterial taxa, including *Bacteroides*, *Clostridium*, *Lactobacillus*, and *Turicibacter* were consistently sensitive to SCI, with increased or decreased relative abundances (Fig. 3.2.E and Table 3.3). Inulin intervention did not completely prevent an SCI-induced impact on the microbiome (Fig. 3.2.A-E and Fig. 3.6), and in fact largely created a community structure more unique to itself, rather than sham or vehicle-treated mice. We did observe a select set of SCI-sensitive taxa that were less affected by SCI, when in the presence of inulin. *Bacteroides sp.* and *Lactobacillus johnsonii* were increased by inulin post-SCI, while others such as *Clostridium celatum* and *Turicibacter sanguinis* remained similar to vehicle-treated, injured mice (Fig. 3.2.E-K). These data

demonstrate an effect of inulin on post-injury microbiome composition, corresponding to its ability to prevent SCI-triggered behavioral and molecular pathologies in the colon.

3.3.3 Injury and diet-associated gut microbiomes are not sufficient to induce or prevent NBD.

Enteric physiology and neurogenesis are modulated by metabolic and immune responses derived from signals of the gut microbiome (159-161). Given our finding that dietary fiber could prevent aspects of NBD, we sought to determine if the injury- or diet-associated microbiomes alone were sufficient to impact intestinal transit. We used an injury-naïve, gnotobiotic mouse model, reconstituting fecal microbiomes derived from mice with SCI-with or without inulin- or sham injured controls (Fig. 3.3.A). Surprisingly, there was no recapitulation of donor phenotypes in the recipients' intestinal transit (Fig. 3.3.B), indicating that the microbes themselves are not sufficient to drive intestinal transit in injury-naïve mice, nor is the benefit of inulin solely due to restructuring of the microbiome composition. Although the fecal microbiome was not sufficient to recapitulate the intestinal motility phenotypes of their donors in recipient mice, a number of colonic and circulating metabolic and inflammatory signals were directly affected by the microbiome (Fig. 3.7.A-G). This included elevated levels of colonic CXCL1 and IL-12p70 (Fig. 3.7.A-C), as well as reduced levels of c-peptide, TNF, and ghrelin in circulation (Fig. 3.7.D-G) in recipients of SCI-derived microbiomes. Abundances of many, but not all, of these circulating factors mirror those found in the microbiome donor animals, including reduced levels of c-peptide and TNF in mice with SCI (Fig. 3.7.H-M), providing evidence for microbiome-dependent contributions to these aspects of metabolic and immune function. The microbiome that arises post-SCI therefore is sufficient to differentially modulate physiologic pathways which may affect other

pathophysiologies post-injury, despite not being sufficient to modulate intestinal transit in injury-naïve, germ-free mice.

Since we observe that the early SCI-associated microbiome itself does not trigger intestinal dysmotility in injury-naïve gnotobiotic mice, the loss of certain beneficial organisms may instead result in increased susceptibility to slowed intestinal transit post-injury. We therefore sought to determine whether those microbes decreased following injury could prevent SCI-induced NBD. We tested a panel of organisms consistently depleted following SCI (Fig. 3.2), in a mono-colonized gnotobiotic setting to establish their sufficiency to modulate intestinal physiological parameters central to SCI-induced NBD (Fig. 3.3.C). We found that mono-colonization of germ-free mice with *Bacteroides thetaiotaomicron* (closely related to *Bacteroides sp.12288*) significantly improved total intestinal transit time compared to the germ-free controls (with impaired intestinal transit due to an underdeveloped ENS (159, 262)) (Fig. 3.3.D), but did not fully recapitulate the transit time of conventionally-colonized mice. Mono-colonization with *Lactobacillus johnsonii* or *Clostridium celatum* showed no significant improvement (Fig. 3.3.D). Notably, western blot analysis of colonic tissue revealed that *B. thetaiotaomicron* substantially increased those ENS markers also impacted by inulin supplementation, PGP9.5 and nNOS, compared to germ-free controls (Fig. 3.3.E-G). *C. celatum* increased both ChAT and PGP9.5, while *L. johnsonii* had no impact on any tested marker (Fig. 3.3.E-G). Thus, some organisms depleted after SCI are sufficient to modulate the production of ENS markers involved in intestinal motility.

Given our observation that *B. thetaiotaomicron* was sufficient to both improve intestinal transit and increase molecular markers of the ENS, including those lost following SCI, we next set out to

test whether this organism could differentially prevent the onset of NBD following SCI, similar to the action of dietary inulin. Following SCI, mice were orally gavaged with one of the three select taxa, *B. thetaiotaomicron, L. johnsonii*, or *C. celatum* (Fig. 3.3.H), which each displayed differential ENS outcomes in the mono-colonized system (Fig. 3.3.C-G). While other probiotic species (specifically a cocktail of *Lactobacillus sp.* and *Bifidobacterium sp.*) have been observed to improve locomotor recovery in a similar SCI model (60), supplementation with any of these three organisms, that we noted to be specifically depleted following SCI, did not result in improved locomotor outcomes (Fig. 3.3.I). Despite its ability to modulate intestinal physiology in a mono-colonized paradigm, *B. thetaiotaomicron* exposure did not improve transit time in injured mice compared to vehicle-treated controls (Fig. 3.3.J). In addition, neither *L. johnsonii* nor *C. celatum* exposure modulated intestinal transit in mice with SCI (Fig. 3.3.J). Overall, these data suggest that while some members of the gut microbiome are sufficient to modulate aspects of the ENS and intestinal motility in a mono-colonized and injury-naïve setting, SCI- and inulin-associated microbial communities are not themselves sufficient to recapitulate, or reduce, NBD in the absence of injury. Furthermore, gut exposure to individual taxa that are preferentially depleted post-SCI did not prevent intestinal dysmotility, suggesting a diet-microbiome interaction in the facilitation of inulin's beneficial effects.

3.3.4 IL-10 signaling is necessary to limit intestinal dysmotility post-SCI.

Certain cytokines can promote neurogenesis and limit neurodegenerative processes (263, 264). We therefore comprehensively assessed a panel of intestinal cytokines post-SCI via multi-plex ELISA. At 14-DPI, we did not observe any significant differences in colonic cytokine levels between sham and injured mice (Fig. 3.8.A). Inulin intervention in mice with SCI, however, resulted in a

substantial increase in the pleiotropic cytokine IL-10 in the colon relative to vehicle-treated, SCI mice (Fig. 3.4.A), as well as decreased levels of the pro-inflammatory cytokine IL-1β (Fig. 3.8.B). This observation appears highly specific to IL-10, with limited diet-induced effects on other cytokines post-SCI (Fig. 3.8.A). We therefore hypothesized that IL-10 signaling may be a necessary signaling component to mediate inulin-induced enteric resilience following SCI.

We thus directly tested the contribution of IL-10 signaling to resilience post-SCI using IL10rb KO mice, which lack a key subunit in the IL-10 receptor (Fig. 3.4.B). Unlike WT mice (Fig. 3.1), inulin supplementation did not improve any post-SCI locomotor or enteric outcomes in IL10rb KO mice (Fig. 3.4.C-G). Inulin intervention could not limit SCI-induced deficits in intestinal transit with both diet and vehicle groups displaying impaired transit post-injury (Fig. 3.4.D). In addition, colonic PGP9.5 remained unchanged despite inulin supplementation in IL10rb KO mice post-SCI (Fig. 3.4.E), with subtle alterations to other colonic ENS markers following injury (Fig. 3.4.F, G). Thus, the IL-10 signaling pathway is central to both intestinal behaviors and molecular resilience to SCI-induced enteric pathologies and is activated by inulin intervention.

3.3.5 SCFA signaling is sufficient to improve SCI-induced dysmotility.

Inulin is fermented by the gut microbiome to SCFAs (265), which have broad roles in host physiology, including the ability to promote a beneficial anti-inflammatory intestinal environment (75). We observed that the injury-associated microbes themselves were not solely responsible for either the pathogenesis of SCI-induced NBD nor its inulin-mediated prevention (Fig. 3.3). We therefore sought to determine whether the microbiome-derived SCFA metabolites of inulin were mediating the limitation of SCI-triggered intestinal dysmotility. Fecal SCFA abundances between

treatment groups were indeed altered in a diet-dependent manner (Fig. 3.5.A-C). Concurrently, we also noted a significant increase in the SCFA receptor FFAR2 in colonic tissue of inulin-treated mice with SCI, indicating an increased physiological response to SCFAs (Fig. 3.5.D). This colonic FFAR2 response appeared specific, since we observed no alteration to other SCFA receptors, including FFAR3 and GPR109 (Fig. 3.5.E, F). We therefore hypothesized that microbiome-derived SCFA signaling is involved in limiting intestinal pathologies post-SCI, rather than the microbiome composition *per se*.

To determine the role of SCFA signaling in protection against SCI-triggered NBD, we assessed two triglycerides – tripropionin and tributyrin. These molecules are absorbed in the small intestine and metabolized to the SCFAs propionate and butyrate, respectively, by host metabolic processes, rather than the gut microbiome (266). Immediately following T9/10 contusion SCI, and daily thereafter, injured mice were provided with daily oral gavages containing ~20mg of tripropionin (TRP) or tributyrin (TRB) diluted in corn oil, or corn oil vehicle alone (Veh) (Fig. 3.5.G). Tributyrin-treated mice displayed significantly improved locomotor outcomes and intestinal transit, when compared to vehicle-treated mice (Fig. 3.5.H, I). Western blot analysis revealed a significant increase in colonic PGP9.5 and nNOS in mice treated with TRB, but no effect on ChAT abundance (Fig. 3.5.J-L), phenocopying inulin-intervention (Fig. 3.1) in its ability to limit ENS deterioration and intestinal dysmotility. TRP did not have any effect on nNOS or PGP9.5 levels, but did significantly increase colonic ChAT, relative to vehicle treated controls (Fig. 3.5.J-L).

We additionally note an increase in colonic FFAR2 in TRB-treated mice (Fig. 3.5.M), as we also observe after inulin treatment. This corresponds with an increase in circulating IL-10 (Fig. 3.5.N

and Fig. 3.8.C), implicating this pathway in protection from SCI-induced intestinal dysmotility, along with an increase in the pleiotropic cytokine IL-6 in TRB-treated mice (Fig. 3.8.D). Therefore, SCFA signaling is sufficient to limit the effects of SCI on intestinal transit and suggests that the therapeutic activity of inulin is through its fermentation to these microbially-dependent products. Overall, these data indicate that supplementation with inulin reduces the SCI-associated loss of neuronal processes in the ENS, through an SCFA-mediated and IL-10-dependent pathway, but largely independent of the microbiome composition as a whole, leading to the prevention of SCI-triggered neurogenic bowel.

3.4 Discussion.

The panoply of gastrointestinal dysfunctions that arise following SCI are a significant burden for those with injury and their care partners. Despite the fact that the healthy ENS has the capacity to recover after perturbation, those with injury-induced NBD often show chronic pathophysiologies. In this work, we establish that SCI-triggered enteric pathologies are able to be modified by the intrinsic intestinal environment. Slowed intestinal transit, gut microbial dysbiosis, and enteric neuropathy are each prevented following the addition of the dietary fiber inulin into the intestinal environment. Although SCI modifies the gut microbiome composition, our gnotobiotic and probiotic approaches suggest that the presence or absence of specific SCI- or fiber-associated microbes does not contribute to these intestinal pathologies. We provide evidence that inulin and its microbially-derived SCFAs, through an ability to promote IL-10 signaling, are capable of reducing the deterioration of myenteric neuronal processes and promoting intestinal transit following SCI. Together these data highlight a central diet-microbiome interaction that can be modulated to limit SCI-induced NBD in mice.

Some probiotic treatments (*e.g.* VSL#3/Visbiome), have been demonstrated to promote locomotor recovery following SCI (60). In our approach, we observe that SCI-sensitive microbes are not sufficient to restore colonic motility nor trigger intestinal dysmotility in gnotobiotic models. We conclude that the composition of the microbiome is therefore not solely responsible for chronic enteric pathologies following SCI. It is possible that injury-associated microbes require an intestinal environment shaped by SCI to impart an effect on the host. For instance, increased intestinal permeability post-SCI (60) may result in translocation of specific microbial molecules more readily. It may also be that the under-developed ENS of germ-free mice lacks the ability to respond to the microbes we introduced. However, we do observe improvements in intestinal transit in certain mono-colonized conditions, suggesting the capacity of some SCI-associated microbes to restore intestinal transit of germ-free mice. Nonetheless, our data support the notion that diet-microbe interactions or microbe-microbe interactions (*i.e.* microbial metabolism or cross-feeding, *etc.*) have the capacity to restore colonic motility after SCI and should be considered in conjunction with probiotic therapy. It is important to note that our study relied on male animals. Since ~79% of all SCI incidences in the United States occur in males, this is a relevant experimental group (80). While our tests of microbiome impacts were performed in mice across both sexes, we do not note any observable sex-dependent effects in microbiome-dependent transit in those studies. Future studies, powered to compare male and female responses, would be necessary to understand whether these interventions have similar effects regardless of biological sex.

Emerging evidence indicates that the ENS undergoes homeostatic neurogenesis (29), and can acutely recover following perturbation, such as infection (267). Intestinal signaling pathways,

including serotonergic signaling from enterochromaffin cells, microbial stimulation of neuronal TLR2, and SCFAs have each been shown independently to modulate enteric neurogenesis (159-161). Enteric nitrergic neurons appear more sensitive to perturbation than other cells in the ENS (268), which we also observe following SCI, leading to decreased propulsion of luminal contents. Our data suggest that diet-induced SCFAs regulate resilience of enteric nitrergic neurons by broadly creating an anti-inflammatory/neuroprotective intestinal environment, rich in IL-10. Our data provide evidence that modulation of SCFA- and IL-10-dependent processes can promote enteric neuron resilience and limit the effects of SCI on intestinal dysmotility.

Through the study of dietary fiber and the microbiome after SCI, we have identified a previously unexplored signaling pathway able to be readily modulated to prevent injury-associated intestinal dysmotility and loss of ENS neuronal processes. While intestinal serotonergic signaling has been used to treat SCI-induced NBD (269), FFAR2 activity and IL-10 treatment have not. IL-10, however, has been shown to have therapeutic value in locomotor recovery during systemic or spinal cord application post-injury (264, 270). Our observations suggest that inulin and TRB increase local and systemic IL-10 and therefore may prevent not only the onset of enteric pathologies, but also improve systemic outcomes. In line with this, we observed significant locomotor recovery in mice treated with inulin or TRB post-SCI (Fig. 3.1.B and Fig. 3.5.H), similar to prior work with SCFAs themselves (116). Despite the ease of administration, few studies to date have investigated a defined dietary fiber or its metabolites in persons living with SCI (112, 113). Our data here provide pre-clinical justification for the continued investigation of diet-microbe interactions as therapeutic targets, to improve bowel function in those with traumatic spinal cord injury.

3.5 Methods.

3.5.1 Animals.

Wild-type C57Bl6/J, IL10rb$^{-/-}$, and DBA/2J mice were obtained from Jackson Laboratory (#000664, 005027, & 000671). Mice were housed within a central vivarium in sterile microisolator cages on static racks, with autoclaved food (Teklad Autoclavable Diet, Cat 2019S) and water provided *ad libitum* with a 12:12hr light-dark cycle for the duration of the studies. All handling and cage changes occurred under a sterilized biosafety cabinet. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Emory University (Protocols 201700855, 201900030, and 201900145). Germ-free DBA/2NTac mice were obtained from Taconic Biosciences (#DBA2) following embryonic rederivation and bred within the Emory Gnotobiotic Animal Core (EGAC). Prior to colonization mice were assessed for sterility by plating fecal pellets under aerobic and anaerobic conditions on non-selective tryptic soy agar with 5% sheep's blood (Hardy #A10).

Mice were humanely euthanized via open-drop isoflurane overdose in an induction chamber followed by cardiac puncture and exsanguination. Mice were then perfused with ice-cold sterile phosphate buffered saline (PBS) prior to tissue collection. The entirety of the GI tract was removed, and the colon length measured. Approx. one cm portions of tissue were taken from the proximal colon, flash-frozen in liquid nitrogen, and stored at -80°C until future analysis. The remaining intestinal tissue was fixed in 4% paraformaldehyde (PFA) overnight at 4°C, then transferred to PBS with 0.01% sodium azide for long-term storage.

3.5.2 Spinal cord injury.

Male mice were deeply anesthetized with 3% isoflurane, using oxygen as a carrier gas, and were maintained on 3% isoflurane for the duration of the surgery. Immediately prior to surgery mice received a subcutaneous injection of meloxicam (5 mg/kg). Under sterile conditions, a dorsal laminectomy (vertebrae T7-T8) was performed to expose the underlying T9/T10 segment of the thoracic spinal cord (261). Following laminectomy, mice received a 70 kdyne impact onto the dorsal surface of the spinal cord with an Infinite Horizon impactor device (Precision Systems and Instrumentation, Fairfax Station, VA), as performed previously (271). Displacement was recorded at 1,554±198μm, indicating a severe contusion for each animal (272). Care was taken to ensure that dorsal roots were not damaged by the laminectomy or impact, and on-target bilateral bruising of the dorsal spinal cord was verified by examination under a dissecting microscope.

Following surgery, the wound was closed using sterile reflex #7 wound clips, and 0.5ml of 0.9% sterile saline was administered subcutaneously. Sham surgery control mice underwent an identical surgical procedure, including laminectomy, but did not receive a contusive SCI. Mice recovered in sterile cages on a heating pad. Subsequent subcutaneous injections of meloxicam (5mg/kg) were administered each day for 2 days following surgery for all sham and injured animals. Experimenters manually expressed the bladders of injured mice twice daily, ~12 hours apart. Mice were assessed for impairment of locomotor function at one day post-injury (DPI) using the Basso Mouse Scale (BMS) (217), to ensure effectiveness of the injury. SCI mice were excluded if they recorded a BMS score of 2 or greater 1-DPI. Sham animals were excluded if they had a BMS below 9 at 1-DPI. Following surgical procedures animals were transferred to cages containing sterile absorbent bedding (ALPHA-dri®, Shepherd Specialty Papers) to prevent rashes and

abrasions. Additionally, portion cups (Dart Solo SCC100S 1 oz. Squat White Paper Portion Cup) containing sterile moistened chow were changed daily to provide all mice with easy access to food and water. For mice receiving inulin intervention, a 1% inulin (Sigma, 12255) weight/volume solution in water was provided immediately following surgical procedures, replacing standard water, *ad lib*. All handling was performed under a sterile biosafety cabinet.

Mice receiving SCFA triglycerides were given a once daily 200µl oral gavage consisting of diluted tripropionin (Thermo, AC275101000) or tributyrin (Sigma, T8626). Liquid triglycerides were diluted 1:10 in corn oil (Veh) with a single 200µl gavage containing 20µl of triglyceride, corresponding to a dose of 21.6mg (415.7mM) of tripropionin or 20.6mg (341.3mM) of tributyrin per gavage. Initial gavage was provided on the day of surgery with subsequent doses provided daily thereafter.

3.5.3 Total gastrointestinal transit.

Total gastrointestinal transit was performed via carmine red dye elution. Mice were acclimated to an isolated behavior space for 1hr prior to gavage with 100µl of sterile carmine red dye (6% w/v) (Sigma, C1022) dissolved in 0.5% methylcellulose (Sigma, M7027). Following gavage mice remained in their home cages for 2hrs and were then transferred to individual, sterile cages devoid of bedding and food/water. Every 15min, cages were checked for the presence of a red fecal pellet. Immediately upon discovery of a red pellet the time was recorded, and the mice were returned to their home cages, with *ad lib* access to food and water. Any mice that did not produce a red fecal pellet were returned to their cages at 8hrs post-gavage and the time recorded was set to a maximum value of 8.5hrs for their transit time.

3.5.4 Ex vivo colonic contractility recordings.

Tests were performed at 2 weeks post laminectomy or spinal cord injury in independent cohorts selected prior to their injury. Mice were anesthetized via brief isoflurane inhalation followed by euthanasia with urethane (1.5g/kg) and tissue collection. Whole colon together with cecum was removed and transferred to a dissection chamber containing ice-cold Krebs solution saturated with carbogen (95% O_2, 5% CO_2). Cecum and attached adipose tissue then were carefully dissected away from the colon, and the colon was transferred to a recording chamber containing carbogen saturated Krebs solution and maintained at 34°C, with a flow rate of ~6ml/min. After 1h of incubation, for whole colon motility recording, a force transducer (dual force and length controller, 300C-LR, Aurora Scientific, Inc) was attached to the middle of the colon with both colon oral and distal end fixed to the recording chamber. For distal colon motility recording, the distal colon was longitudinally mounted in the bath, with the distal end fixed and the oral end attached to the force transducer.

Data were acquired using AxoClamp 900A (Axon Instruments). Each tissue segment was recorded for at least 30min in 5min gap-free files. 15min of stable recordings were selected from the middle of each recording session for analysis. Using Clampfit software (Molecular Devices, RRID:SCR_011323), data files were filtered at 300Hz (Bessel 8-pole) and reduced by a factor of 100. Files were concatenated and the baseline was adjusted based on overall slope. Amplitude was calculated by subtracting the minimum value of the whole trace from the value of the peak being assessed.

Krebs solution was used in *ex vivo* colon motility experiments. It was composed of (in mM) 117 NaCl, 4.6 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1 $NaHPO_4$, 11 D-glucose and 25 NaH_2CO_3. It was saturated with carbogen (95% O_2, 5% CO_2) and maintained at pH 7.4.

3.5.5 Microbiome sequencing.

Mice were placed into sterile 1000ml plastic cups in a biosafety cabinet. Fecal pellets were collected with sterilized forceps and placed into sterile 1.5ml plastic tubes. Tubes were immediately frozen over dry ice and placed into a -80°C freezer until they were shipped, on dry ice, for DNA extraction and 16S sequencing.

Full service 16S sequencing and computational analysis was performed via Zymo Inc (Irvine, CA) through the commercial ZymoBIOMICS® Targeted Sequencing Service pipeline (Zymo Research, Irvine, CA). Briefly, DNA was extracted using ZymoBIOMICS®-96 MagBead DNA Kit. Bacterial 16S ribosomal RNA gene targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA). Vendor-designed primers for bacterial 16S amplified the V3-V4 region, pooled for equal molarity, cleaned with the Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA), then quantified with TapeStation® (Agilent Technologies, Santa Clara, CA) and Qubit® (Thermo Fisher Scientific, Waltham, WA). The ZymoBIOMICS® Microbial Community Standard (Zymo Research, Irvine, CA) was used as a positive control for each DNA extraction and the ZymoBIOMICS® Microbial Community DNA Standard (Zymo Research, Irvine, CA) was used as a positive control for each targeted library preparation. Negative controls (*i.e.* blank extraction control, blank library preparation control) were included by the vendor to assess the level of bioburden carried by the wet-lab process. The

final library was sequenced on Illumina® MiSeq™ with a V3 reagent kit (600 cycles). The sequencing was performed with 10% PhiX spike-in. Unique amplicon sequences variants were inferred from raw reads using the DADA2 pipeline (273). Potential sequencing errors and chimeric sequences were also removed with the DADA2 pipeline. Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 with the Zymo Research Database. Composition visualization, alpha-diversity, and beta-diversity analyses were performed with Qiime v.1.9.1 (274) and visualized using GraphPad PRISM software.

3.5.6 SCFA analysis.

Fecal samples were collected into 1.5ml plastic tubes with 500µL of methanol, over dry ice, and stored at -80°C until analysis. Samples were homogenized in 50% acetonitrile with disruptor beads, then centrifuged at 4000xg for 10 min at 4°C. 40µL of supernatant from each sample was further derivatized with 20µL 200mM 3-Nitrophenylhydrzine, 20µL 120mM N-(3-dimethylaminopropyl)-N'-ethylcarodimmide, and 20µL 6% pyridine for 30 min at 40°C. 1.5mL of 10% acetonitrile was added to stop the reaction. The derivatized solution was then filtered and injected into LCMS for further data analysis. 10µL of each sample was injected into a mass spectrometer (Sciex 5500) to generate data. Lipid samples were passed over an Accucore C18 (4.6 x 100mm, 2.6µm) analytical column at 40°C for separation with aqueous mobile phase consisting of 0.1% formic acid in water and organic phase consisting of 0.1% formic acid in acetonitrile. The SCFA were analyzed with multiple reaction monitoring scans. A pool QC and a standard curve were run after every 10 samples to ensure the quality of the sample analysis as well as the instrument performance. Standards consisted of Acetate, Formate, Propionate, Butyrate, Valerate, Stearic, Palmitic, Oleic, Linoleic, Arachidonic, and Linolenic fatty acids. The concentration of the

detected SCFA species were determined based on 6 points calibration curves using external standards with R square value greater than 0.95.

3.5.7 Western blots.

500µl of ice-cold Meso Scale Discovery (MSD) homogenization buffer (1L of buffer: 125ml of 1M Tris, 30ml 0.5M MgCl, 25ml of 0.1M EDTA, 10ml Triton X 100, 810ml deionized water [addition of 1 tablet of protease inhibitor (Thermo, A32961) per 10ml of buffer]) was added to frozen tissue, which was then quickly dissolved over ice using a probe sonicator. Lysed samples were then centrifuged for 10 min at 20,000x *g* at 4°C and the soluble, protein-rich supernatant was moved into a fresh 1.5ml tube. Protein levels were quantified using a standard Pierce BCA protein assay kit (Thermo Scientific). Samples were normalized to 1.5µg/µl with Novex Tris-glycine SDS sample buffer with 10% BME. 10ug of each sample were denatured by boiling for 15 minutes and separated on a 15-well Novex WedgeWell 4-20% Tris-Glycine Gel (Thermo Scientific) before transfer to a 0.22µm PVDF membrane overnight at 4°C. Membranes were then blocked for 2hrs in a 5% bovine serum albumin (BSA) solution in Tris-buffered saline with 0.1% Tween-20 (TBST). Following blocking, primary antibodies diluted in blocking solution (Table 3.6), were incubated on membrane overnight at 4°C. Membranes were washed in TBST and incubated at RT with indicated diluted secondary antibodies (Table 3.6), conjugated to horseradish peroxidase enzyme. Chemiluminescence images were taken using an Azure Biosystems c400 imaging system and Cell Signaling SignalFire ECL reagent. Images were then quantified using FIJI, and normalized to a loading control, either GAPDH, beta actin, or total protein (quantified via Coomassie Brilliant Blue staining), as indicated in each figure.

3.5.8 Multiplexed ELISAs.

Tissue was prepared via sonication in MSD homogenization buffer, detailed above. 50µl of each sample, at a concentration of 1.6µg/µl for tissue lysate, or undiluted serum, was analyzed using the V-PLEX proinflammatory panel 1 mouse kit (MSD, K15048D-2) and the U-PLEX metabolic hormone mouse panel combo 1 (MSD, K15306K-1). Assays were performed following manufacture's protocol and analyzed on the MSD QuickPlex SQ120 instrument and evaluated on the MSD discovery workbench 4.0 platform.

3.5.9 Immunofluorescence imaging.

Following tissue fixation (described above) mouse colon segments of ~1cm in length were blocked for 2 hrs in 1.5ml tubes containing 1 drop of Mouse-on-Mouse blocking reagent (MOM; Vector Labs) in 1ml of PBS with 0.5% Triton-X 100. Tissue was then added to 1ml of primary antibody mixture containing primary antibodies (Table 3.6) diluted in 3% normal goat serum (NGS) and 0.1% Triton X 100 in PBS, for ~72hrs with gentle agitation at 4°C. The tissue was then washed 5 times for 1hr each with gentle agitation at room temperature. Tissue was then placed into amber 1.5ml tubes containing secondary antibodies (Table 3.6) diluted in in 3% NGS and 0.1% Triton X 100 in PBS, overnight with gentle agitation at room temperature. The tissue was then washed 3 times for 1 hr each, protected from light. The tissue was then stained with DAPI (1:300 in PBS) in 1.5ml tubes for 1hr at RT with gentle shaking, followed by 3 more 1hr washes in PBS. Tissue segments were then placed in 1ml of refractive index matching solution (RIMS) (275) buffer (88% Histodenz [Sigma, D2158] in 0.02M phosphate buffer with 0.1% Tween-20 and 0.01% sodium azide) overnight at room temp with gentle agitation. Following tissue clearing, samples were

mounted in 100µl of fresh RIMS, luminal side down, on glass slides with 0.25mm tissue spacers (SunJin labs). Glass cover slips were sealed to the spacers with clear nail polish.

Slides were imaged with a Leica SP8 multiphoton confocal microscope with Leica Application Suite software and analyzed by blinded manual analysis using FIJI software. Images were collected using Z-stacks of the myenteric plexus, and a 1-frame image of the underlying crypts for standardization to correct for tissue stretching during tissue fixation and mounting. Cells, ganglia, and crypts were manually quantified using FIJI Image J. 2-7 colonic regions were assessed for each mouse, with 7-8 mice in each group.

3.5.10 Bacterial manipulations and colonization.

Bacteria were obtained from the American Type Culture Collection (ATCC) and cultured in the indicated conditions in Table 3.6. For mono-colonization, overnight cultures were pelleted and resuspended in 50% glycerol:PBS and a single 100µl gavage provided to GF mice. Colonization was confirmed via fecal culture on indicated selective media under aerobic and anaerobic conditions (5% H_2, 10% CO_2, 85% N_2). Probiotic bacterial administration occurred daily. For whole microbiome reconstitution, fecal pellets were resuspended in sterile PBS with 5% sodium bicarbonate and a single 100µl gavage was administered to GF mice.

3.6 Author contributions.

Adam M. Hamilton (AMH) and Timothy R. Sampson (TRS) conceptualized the study and research plan. AMH performed animal surgeries. AMH, Jianjun Chang (JC), and Lisa Blackmer-Raynolds (LBR) performed gnotobiotic experiments. Yaqing Li (YL) and AMH performed and analyzed the

ex vivo recordings. AMH performed and analyzed all behavioral, molecular, and microbiome assessments. AMH, Sean Kelly (SK), Nardos Kebede (NK), and Anna Williams (AW) performed and analyzed intestinal histology. Shanthi Srinivasan (SS) and Sandra M. Garraway (SG) provided critical expertise and conceptual discussion. AMH and TRS wrote the manuscript. All authors revised the manuscript. TRS supervised the study.

3.7 Acknowledgements.

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3.8 Figures.

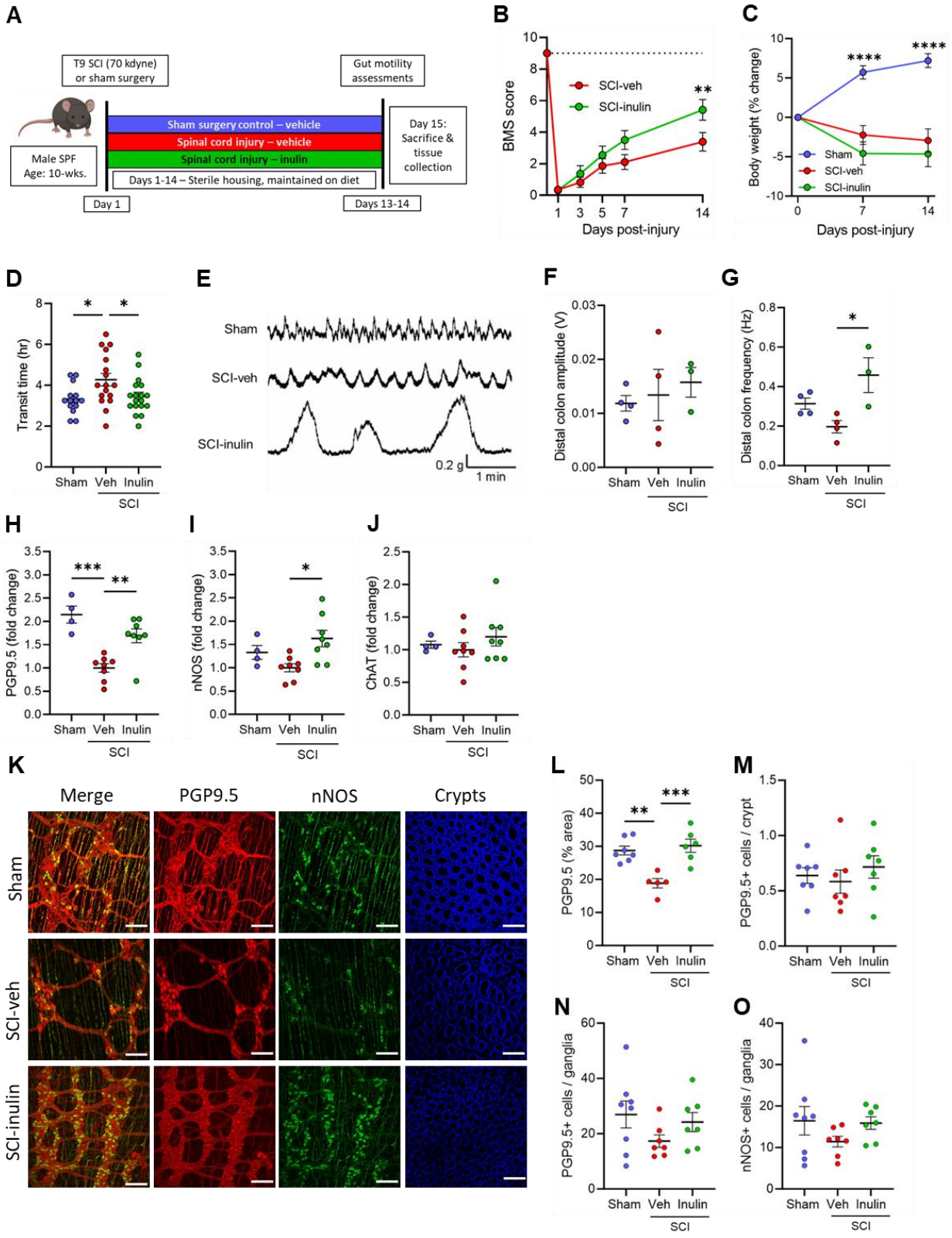
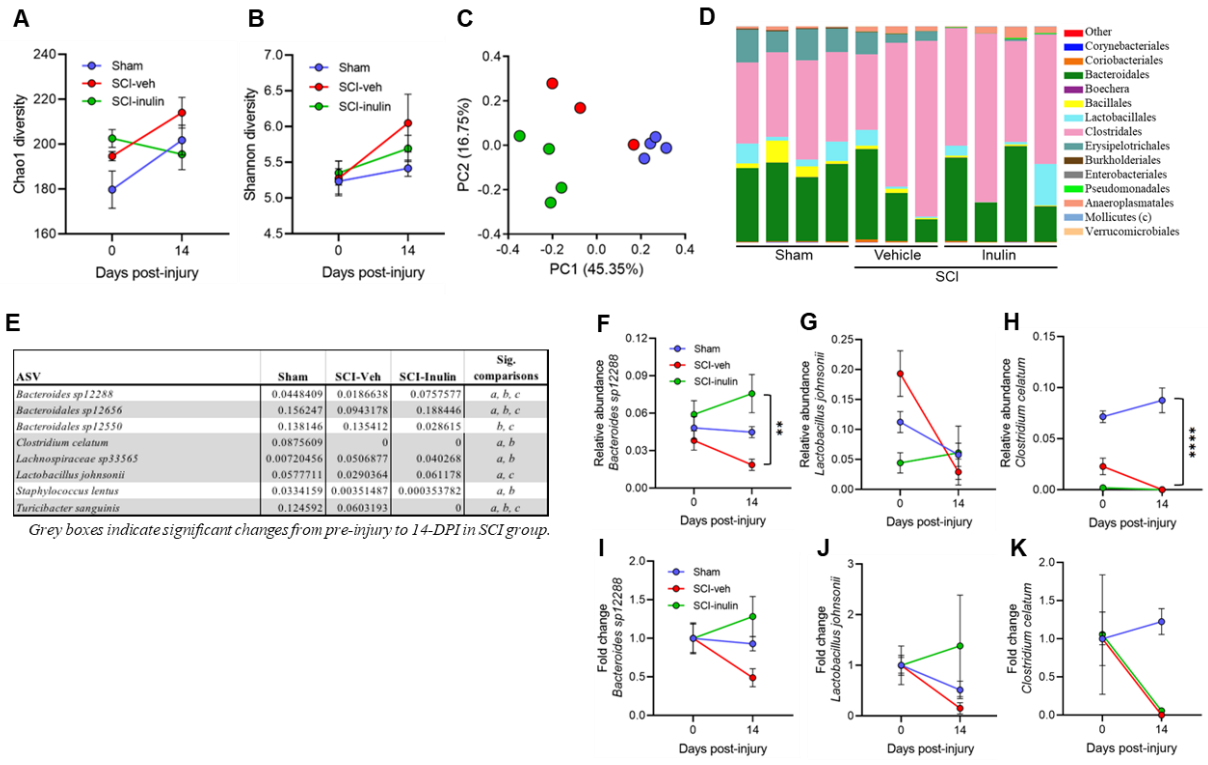

A
T9 SCI (70 kdyne) or sham surgery
Gut motility assessments
Male SPF Age: 10-wks.
Sham surgery control – vehicle
Spinal cord injury – vehicle
Spinal cord injury – inulin
Days 1-14 – Sterile housing, maintained on diet
Day 15: Sacrifice & tissue collection
Day 1
Days 13-14
B
BMS score
SCI-veh
SCI-inulin
Days post-injury
C
Body weight (% change)
Sham
SCI-veh
SCI-inulin
Days post-injury
D
Transit time (hr)
Sham
Veh
Inulin
SCI
E
Sham
SCI-veh
SCI-inulin
0.2 g
1 min
F
Distal colon amplitude (V)
G
Distal colon frequency (Hz)
H
PGP9.5 (fold change)
I
nNOS (fold change)
J
ChAT (fold change)
K
Merge
PGP9.5
nNOS
Crypts
Sham
SCI-veh
SCI-inulin
L
PGP9.5 (% area)
M
PGP9.5+ cells / crypt
N
PGP9.5+ cells / ganglia
O
nNOS+ cells / ganglia

Figure 3.1. Dietary inulin rescues SCI-induced enteric neuropathy and neurogenic bowel.

A, Experimental overview: male wild-type mice receive T9 laminectomy (Sham) or laminectomy with 70kilodyne contusive spinal cord injury (SCI), with either a standard diet (Veh) or inulin (Inulin). **B,** Hindlimb locomotor score, as assessed by Basso mouse scale (BMS), with dashed line at 9 representing no deficits, indicative or sham or uninjured locomotor function. **C,** Percent changes in body weight relative to pre-injury weight. **D,** Quantification of total intestinal transit time. **E**, Representative traces of *ex vivo* colonic contractility. **F-G**, Average amplitude (**F**) and frequency (**G**) recorded from distal colon. H-J, Colonic protein levels measured via western blot for protein gene product 9.5 (PGP9.5) (**H**), neuronal nitric oxide synthase (nNOS) (**I**), and choline acetyltransferase (ChAT) (**J**). **K**, Representative images of myenteric plexus where red=PGP9.5, green=nNOS, and representative blue=DAPI-stained intestinal crypts. Scale bar is 100 microns. **L-O**, IF quantification showing percentage of PGP+ area (**L**), PGP9.5+ cells per crypt (**M**), PGP9.5+ cells per ganglia (**N**), and nNOS+ cells per ganglia (**O**). Asterisks in (**C**) indicate differences between sham and both SCI groups. Each circle represents individuals excluding (**B, C**), where each circle is the mean within that experimental group. IF data points are the averaged values of 2-7 images per mouse with N=7-8 mice per group. N=11-14 (**B,**), N=18-19 (**C**) N=14-18 (**D**), N=3-4 (**F, G**), N=4-8 (**H-J**), N=5-8 (**L-O**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Data are shown as mean ± SEM and compared by repeated measures 2-way ANOVA with post-hoc Tukey's test (for **B, C**) and by one-way ANOVA with post-hoc Tukey's test (for **D, F-J, L-O**). Dashed line in (**B**) indicates maximum possible score for BMS of 9.



ASV	Sham	SCI-Veh	SCI-Inulin	Sig. comparisons
Bacteroides sp12288	0.0448409	0.0186638	0.0757577	*a, b, c*
Bacteroidales sp12656	0.156247	0.0943178	0.188446	*a, b, c*
Bacteroidales sp12550	0.138146	0.135412	0.028615	*b, c*
Clostridium celatum	0.0875609	0	0	*a, b*
Lachnospiraceae sp33565	0.00720456	0.0506877	0.040268	*a, b*
Lactobacillus johnsonii	0.0577711	0.0290364	0.061178	*a, c*
Staphylococcus lentus	0.0334159	0.00351487	0.000353782	*a, b*
Turicibacter sanguinis	0.124592	0.0603193	0	*a, b, c*

Grey boxes indicate significant changes from pre-injury to 14-DPI in SCI group.

Figure 3.2. SCI-triggered dysbiosis is prevented by inulin.

A, B, Pre-surgery to 14-DPI changes in fecal microbiome in male mice. Chao1(**A**) and Shannon (**B**) alpha diversity measures. **C**, Bray-Curtis principal component analysis (PCA) plot. **D**, Order level microbiome composition plot at 14-DPI. **E**, Table highlighting the mean relative abundance of bacterial taxa that differ between experimental groups at 1% FDR, where grey boxes indicate significant changes from pre-injury to 14-DPI in SCI-veh group, and "a, b, & c" indicate significant differences between groups at endpoint. **F-K**, Progressive changes in select taxa represented as relative abundance (**F-H**) and as proportion of pre-injury abundance (**I-K**) for *Bacteroides sp12288* (**F, I**), *Lactobacillus johnsonii* (**G, J**), and *Clostridium celatum* (**H, K**). Asterisks in (**F**) indicate significant difference between SCI-veh and SCI-inulin groups at 14-DPI. Asterisks in (**H**) indicate significant difference between sham and both SCI groups. Each circle represents within group mean, excluding **C**, where each circle represents individuals. N=3-4. ** $P < 0.01$, **** $P < 0.0001$.

Data are shown as mean ± SEM and compared by repeated measures 2-way ANOVA with post-hoc Sidak's test (**A, B**) or Tukey's test (**F-K**) and by 1% FDR (for **E**).

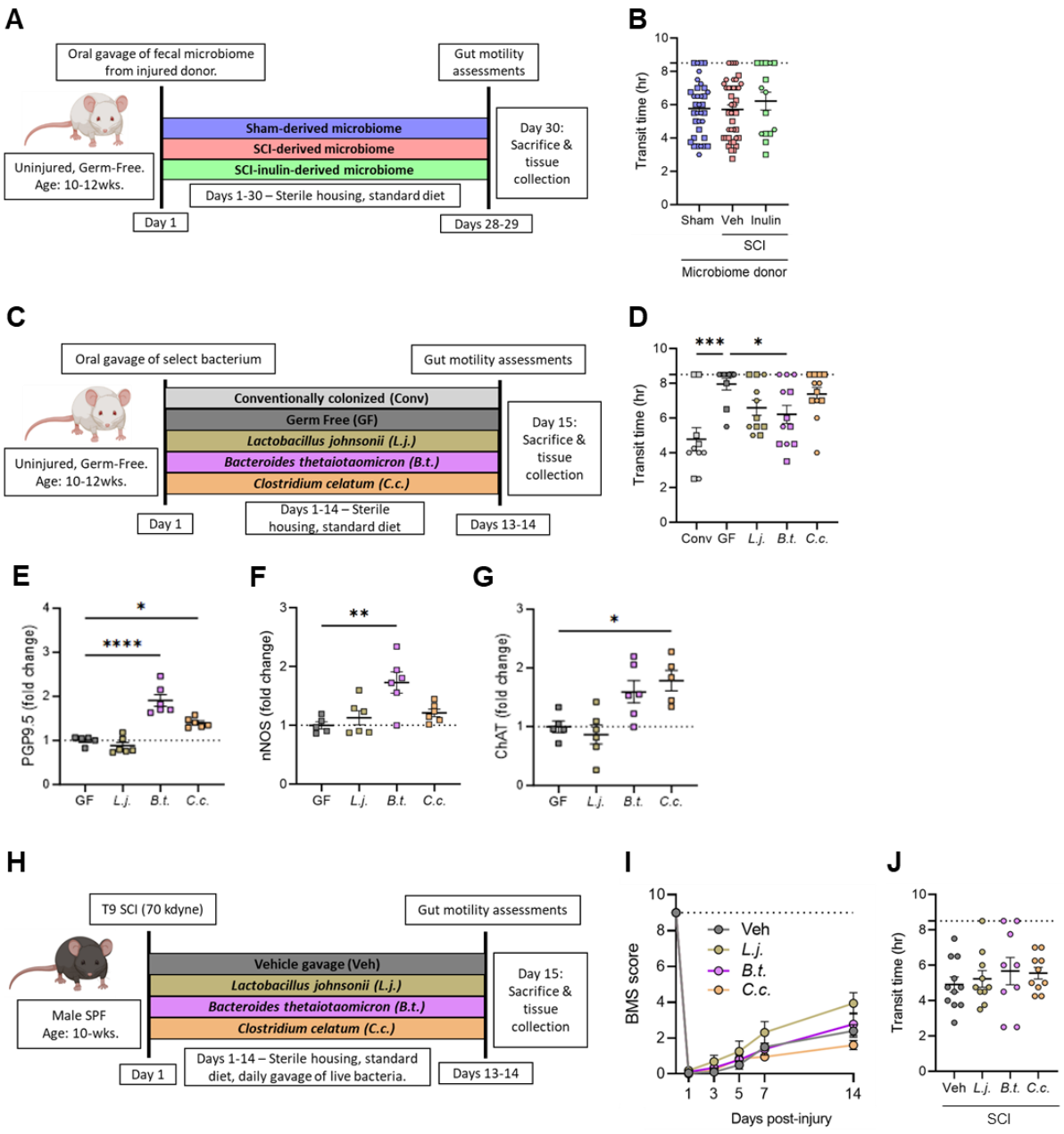


Figure 3.3. Post-injury and diet-induced microbes differentially impact the ENS.

A, Experimental overview: male and female, uninjured, wild-type, germ-free mice received fecal microbiomes of donors with laminectomy (sham), SCI (veh) or SCI with inulin (inulin). **B,** Quantification of total intestinal transit time. **C**, Experimental overview: male and female, uninjured, wild-type, germ-free mice are mono-colonized with *Lactobacillus johnsonii* (*L.j.*),

Bacteroides thetaiotaomicron (*B.t.*), or *Clostridium celatum* (*C.c.*), alongside conventionally colonized (Conv) and germ-free (GF) controls. **D**, intestinal transit time at experimental endpoint. **E-G**, Quantification of proximal colon, from male mice, by western blots for protein gene product 9.5 (PGP9.5) (**E**), neuronal nitric oxide synthase (nNOS) (**F**), and choline acetyltransferase (ChAT) (**G**). **H**, Experimental overview: male, wild-type mice received laminectomy with 70kilodyne contusive spinal cord injury (SCI), followed by daily oral gavage of *L.j., B.t., C.c.*, or vehicle (veh). I, Progressive Basso mouse scale (BMS) scores of mice provided daily probiotic gavage of veh, *L.j., B.t., or C.c.,* with dashed line at 9 representing uninjured locomotor function. **J**, Endpoint intestinal transit time. Each point represents individuals for all but (**I**), where each circle is the average of all mice within that experimental group. (**A-D**) Squares represent males, hexagons represent females. N=15-41 (**B**), N=10-12 (**D**), N=5-6 (**E-G**), N=9-11 (**I-J**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Data are shown as mean ± SEM and compared by ordinary one-way ANOVA with post-hoc Tukey's (**B**) or Dunnett's (**D-G, J**) tests. Dashed line in (**I**) indicates maximum possible score for BMS of 9.

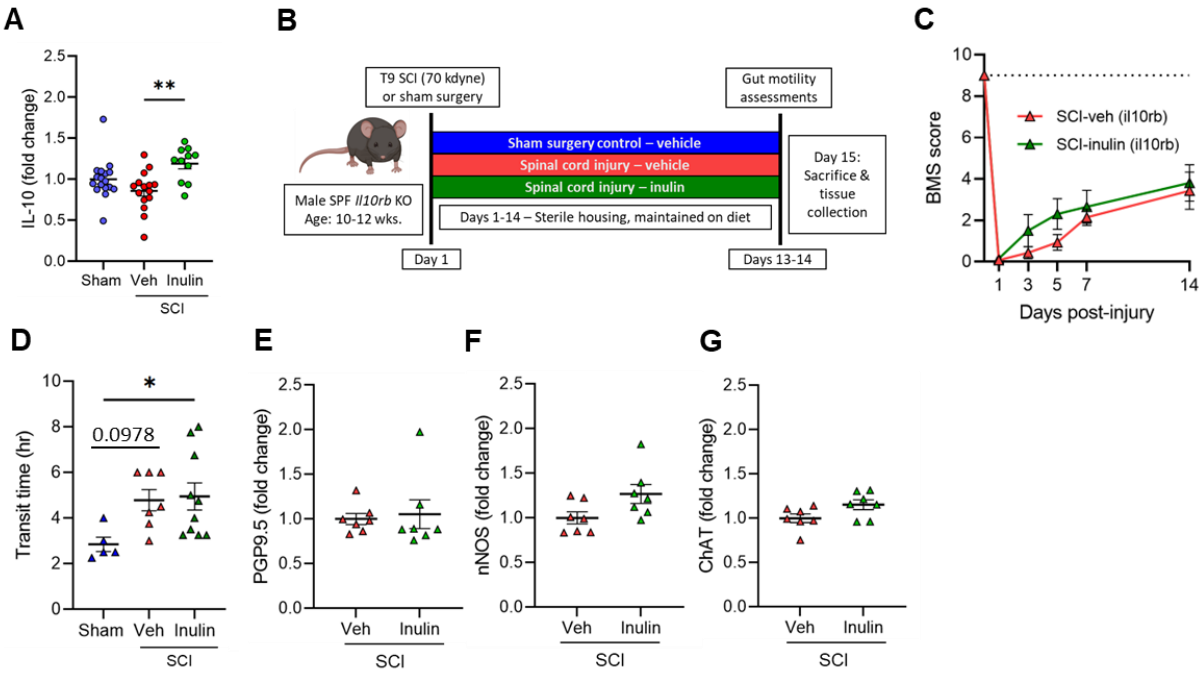


Figure 3.4. IL-10 signaling is necessary for inulin-mediated resilience to NBD.

A, Concentration of colonic IL-10 by ELISA from male mice with laminectomy (sham), SCI (veh), or SCI with inulin-supplemented diet (inulin). **B**, Experimental overview: male *IL10rb* KO mice undergo laminectomy at T9 (Sham) or laminectomy with 70kilodyne contusive spinal cord injury (SCI) and receive either standard diet (Veh) or inulin (Inulin). **C**, Progressive Basso mouse scale (BMS) scores with dashed line at 9 representing uninjured locomotor function. **D**, intestinal transit time at endpoint. **E-G**, Quantification of colonic protein gene product 9.5 (PGP9.5) (**E**), neuronal nitric oxide synthase (nNOS) (**F**), and choline acetyltransferase (ChAT) (**G**) by western blot. Each point represents individuals for all but (**C**), where each circle is the average of all mice within that experimental group. N=11-17 (**A**), N= 5-10 (**C-G**) * $P < 0.05$, ** $P < 0.01$. Data are shown as mean ± SEM and compared by ordinary one-way ANOVA with post-hoc Tukey's (**A, D**), 2-way ANOVA (**C**) or two-tailed unpaired t-test (**E-G**). Dashed line in (**C**) indicates maximum possible score for BMS of 9.

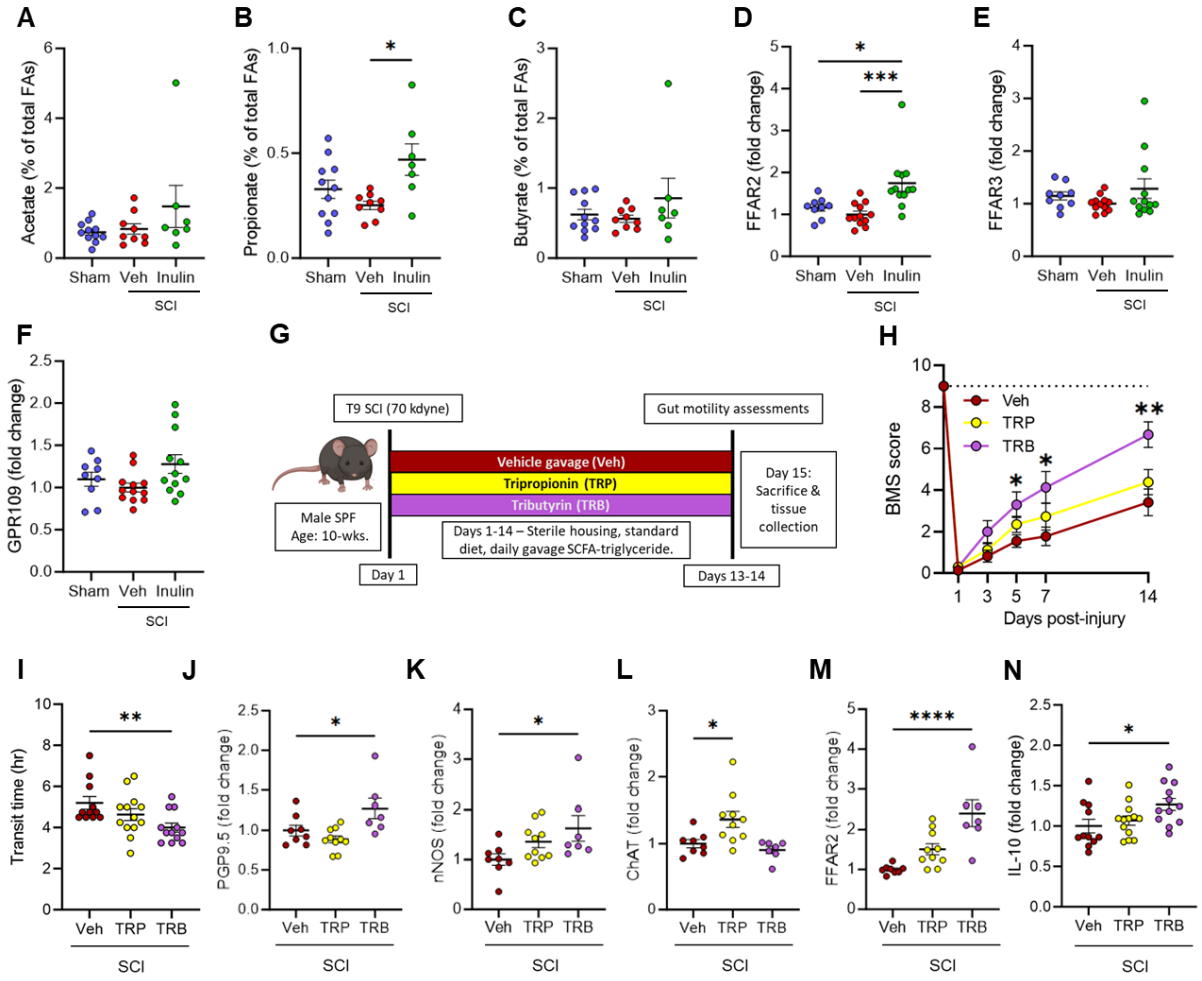


Figure 3.5. Intestinal SCFA signaling is impacted post-SCI and promotes rescue of NBD.

A-C, Quantification of indicated short-chain fatty-acids at endpoint fecal pellets from male mice with sham laminectomy (sham), or with SCI (SCI-veh) or inulin-supplemented diet (SCI-inulin). **D-F**, Quantification of colonic free fatty acid receptor 2 (FFAR2) (**D**), free fatty acid receptor 3 (FFAR3) (**E**), and G-protein-coupled receptor 109 (GPR109) (**F**) by western blot. **G**, Experimental overview: male wild-type mice receive laminectomy with 70kilodyne contusive spinal cord injury (SCI) followed by daily gavage of vehicle (veh), tripropionin (TRP), or tributyrin (TRB). **H**, Progressive Basso mouse scale (BMS) scores, with dashed line at 9 uninjured locomotor function. **I**, intestinal transit time at experimental endpoint. **J-M**, Quantification of colonic protein gene

product 9.5 (PGP9.5) (**J**), neuronal nitric oxide synthase (nNOS) (**K**), choline acetyltransferase (ChAT) (**L**), and free fatty acid 2 (FFAR2) (**M**) by western blot. **N**, Concentration of serum IL-10 by ELISA. Each point represents individuals for all but (**H**), where each circle is the average of all mice within that experimental group. N=7-11 (**A-C**), N=9-12 (**D-F**), N=11-13 (**H, I, N**), N=7-10 (**J-M**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Data are shown as mean ± SEM and compared by ordinary one-way ANOVA with post-hoc Tukey's (**A-F**) or Dunnett's (**I-N**) tests, and by 2-way ANOVA with post-hoc Dunnett's multiple comparison test (**H**). Dashed line in (**H**) indicates maximum possible score for BMS of 9.

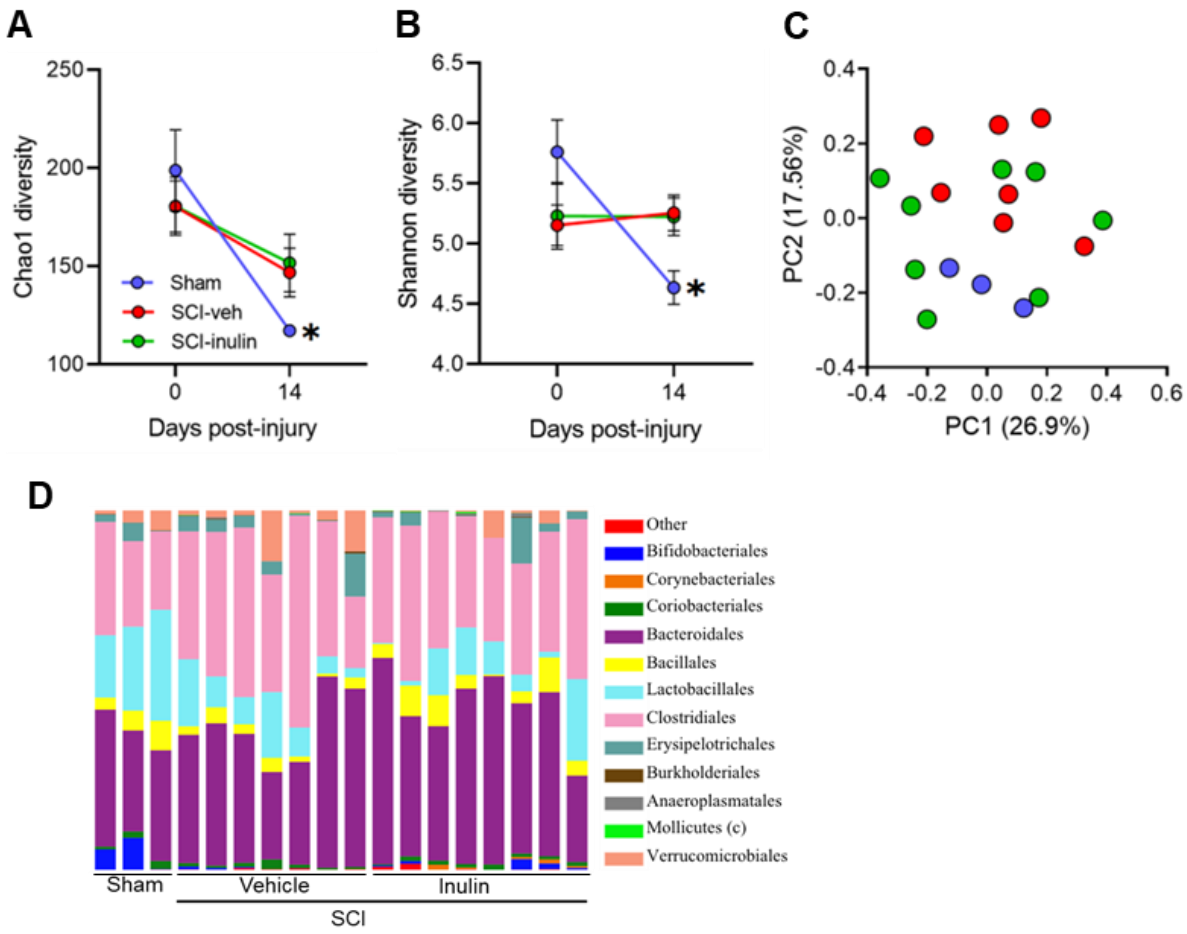


Figure 3.6. Cohort-dependent impacts on SCI-triggered dysbiosis.

Supplement to Fig. 3.2. 16S sequencing results from an independent cohort of male mice. **A, B,** Pre-surgery to end point (14-DPI) changes in fecal microbiome 16S rRNA alpha diversity represented by Chao1(**A**) and Shannon (**B**) diversity measures. **C**, Principal component analysis (PCA) plot representing changes in overall community structure of fecal microbiomes. **D**, Microbial composition barplot, order level, of fecal microbiomes at 14-DPI. Asterisks in (**A**) and (**B**) represent a significant decrease in alpha diversity from 0-DPI to 14-DPI in the sham group. Each circle represents the average of all mice in a group for all but (**C**), where each circle represents an individual mouse. N=3-8. * $P < 0.05$. Data are shown as mean ± SEM. Statistically significant

differences were determined by repeated measures 2-way ANOVA with post-hoc Sidak's multiple comparison test to compare temporal changes within groups.

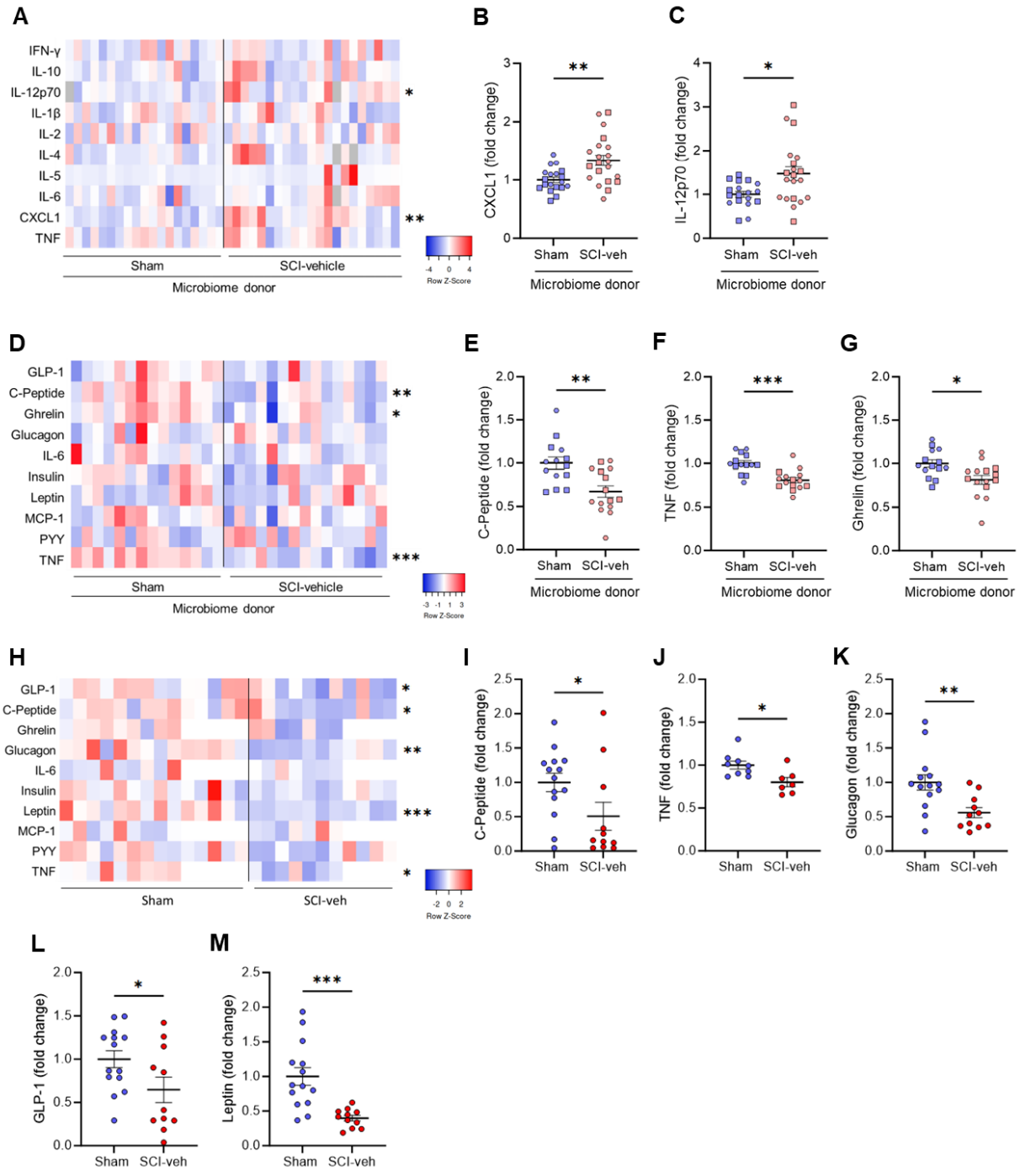


Figure 3.7. Injury and diet-associated microbes are not sufficient to trigger NBD.

Supplement to Fig. 3.3. **A-C**, Heatmap of cytokines present in proximal colon tissue lysate from male and female uninjured germ-free mice that were colonized with either sham-derived or SCI-

veh-derived fecal microbiomes (**A**), as assessed by multiplexed ELISA, with significant increases in CXCL1 (**B**) and IL-12p70 (**C**). **D-G**, Heatmap of serum metabolic markers, as assessed by multiplexed ELISA (**D**), with significant decreases in C-peptide (**E**), TNF (**F**), and ghrelin (**G**). **H-M**, Heatmap of serum metabolic markers in male mice with laminectomy (Sham) or injury (SCI-veh), as assessed by multiplexed ELISA (**H**), with significant decreases in C-peptide (**I**), TNF (**J**), glucagon (**K**), GLP-1 (**L**), and leptin (**M**). Each data point represents one mouse. Squares represent ex-GF males, hexagons represent ex-GF females, and circles represent SPF males. N=19-21 (**A-C**), N=14-15 (**D-G),** N=11-14 (**H-M**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Data are shown as mean ± SEM. Statistically significant differences were determined by two-tailed unpaired t-test.

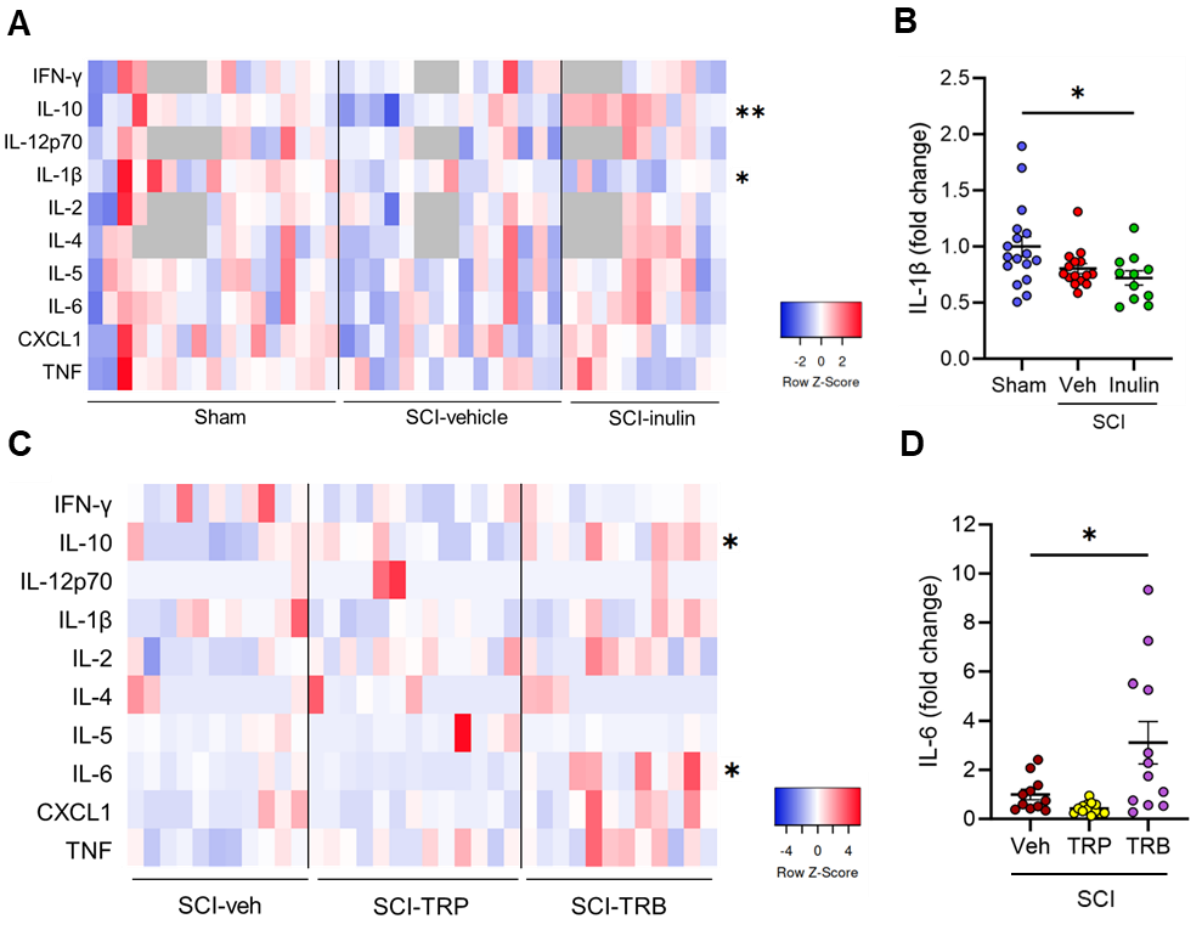


Figure 3.8. SCFA-induced IL-10 signaling is necessary for SCI recovery.

Supplement to Fig. 3.4 & Fig. 3.5. **A**, Heatmap of cytokines, assessed by multiplexed ELISA, in proximal colon tissue lysate from male wild-type mice with laminectomy (Sham), SCI with standard diet (SCI-vehicle), and SCI with inulin-supplemented diet (SCI-inulin). **B**, Concentration of colonic IL-1β by ELISA. **C**, Heatmap of cytokines, assessed by multiplexed ELISA, in serum from male wild-type mice with SCI treated with either vehicle (SCI-veh), tripropionin (SCI-TRP), or tributyrin (SCI-TRB). **D**, Concentration of serum IL-6 by ELISA. Each data point represents one mouse. N=11-17 (**A**), N=11-13 (**B**), * $P < 0.05$, ** $P < 0.01$. Statistically significant differences were determined by ordinary one-way ANOVA with post-hoc Tukey's (**A, B**) or Dunnett's (**C, D**) multiple comparison test.

3.9 Tables.

Table 3.1. SCI-induced microbiome compositional differences. ASV-level taxonomic changes from stool microbiomes at 2 weeks post-SCI or sham, with 1% FDR.

Endpoint			
Sham vs SCI-Vehicle			
ASV	**Sham**	**SCI-Veh**	**Adjusted *p* value (1% FDR)**
Bacteroides sp12288 (thetaiotaomicron)	0.0448409	0.0186638	6.95E-07
Bacteroidales sp12656	0.156247	0.0943178	1.16E-30
Staphylococcus lentus	0.0334159	0.00351487	1.50E-08
Lactobacillus johnsonii	0.0577711	0.0290364	5.24E-08
Clostridium celatum	0.0875609	0	0.00E+00
Lachnospiraceae sp33565	0.00720456	0.0506877	2.83E-16
Turicibacter sanguinis	0.124592	0.0603193	8.76E-33
Sham vs SCI-Inulin			
ASV	**Sham**	**SCI-Inulin**	**Adjusted *p* value (1% FDR)**
Bacteroides sp12288 (thetaiotaomicron)	0.0448409	0.0757577	5.81E-08
Bacteroidales sp12550	0.138146	0.028615	0.00E+00
Bacteroidales sp12656	0.156247	0.188446	1.63E-08
Staphylococcus lentus	0.0334159	0.000353782	6.74E-09
Clostridium celatum	0.0875609	0	0.00E+00
Lachnospiraceae sp33565	0.00720456	0.040268	6.73E-09
Turicibacter sanguinis	0.124592	0	0.00E+00
SCI-Vehicle vs SCI-Inulin			
ASV	**SCI-Veh**	**SCI-Inulin**	**Adjusted *p* value (1% FDR)**
Bacteroides sp12288 (thetaiotaomicron)	0.0186638	0.0757577	1.16E-15
Bacteroidales sp12550	0.135412	0.028615	0.00E+00
Bacteroidales sp12656	0.0943178	0.188446	2.24E-38
Lactobacillus johnsonii	0.0290364	0.061178	5.50E-06
Turicibacter sanguinis	0.0603193	0	2.92E-17
Pre-SCI vs Post-SCI			
ASV	**SCI-Pre**	**SCI-2wks**	**Adjusted *p* value (1% FDR)**
Bacteroidales sp12656	0.158599	0.0943178	2.07E-30
Lactobacillus johnsonii	0.193283	0.0290364	0.00E+00
Clostridium celatum	0.022866	0	2.93E-05
Lachnospiraceae sp33565	0.0128364	0.0506877	6.27E-12
Turicibacter sanguinis	0.00972012	0.0603193	7.71E-20

Table 3.2. LEfSE identified taxonomic changes from stool microbiomes at 14-DPI.

Bacterial taxa	Group	Effect size	*p*-value
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33760	SCI.Inulin	3.280	0.030
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__NA.g__NA.s__sp31072	SCI.Inulin	2.989	0.048
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Pseudomonadales.f__Moraxellaceae.g__Acinetobacter.s__radioresistens	SCI.Inulin	3.180	0.013
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32706	SCI.Inulin	3.829	0.013
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32638	SCI.Inulin	3.785	0.023
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33456	SCI.Inulin	3.173	0.027
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33694	SCI.Inulin	3.760	0.020
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__NA.s__sp34867	SCI.Inulin	3.300	0.027
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Anaerotruncus.s__sp34475	SCI.Inulin	2.995	0.046
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Pseudomonadales	SCI.Inulin	3.256	0.016
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Pseudomonadales.f__Moraxellaceae.g__Acinetobacter	SCI.Inulin	3.229	0.016
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33417	SCI.Inulin	3.759	0.012
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__sp12288_thetaiotaomicron	SCI.Inulin	4.438	0.028
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33525	SCI.Inulin	3.270	0.048
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32701	SCI.Inulin	3.965	0.016
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33616_sp33639	SCI.Inulin	3.428	0.029
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Enterococcaceae.g__Enterococcus.s__faecalis	SCI.Inulin	3.057	0.025
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Pseudomonadales.f__Moraxellaceae.g__Acinetobacter.s__calcoaceticus_radioresistens_variabilis	SCI.Inulin	3.200	0.041
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__NA.s__sp34731	SCI.Inulin	3.432	0.027
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Enterococcaceae	SCI.Inulin	3.073	0.025
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae	SCI.Inulin	4.417	0.028
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides	SCI.Inulin	4.451	0.028
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32758	SCI.Inulin	3.973	0.009
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32623	SCI.Inulin	3.562	0.041
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__NA.g__NA.s__sp31116	SCI.Inulin	3.056	0.044
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Pseudomonadales.f__Moraxellaceae	SCI.Inulin	3.233	0.016
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Enterococcaceae.g__Enterococcus	SCI.Inulin	3.064	0.025
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33721	SCI.Inulin	3.089	0.009
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__NA.s__sp35841	SCI	3.081	0.030
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33658	SCI	3.422	0.024
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32702	SCI	3.853	0.025

Table 3.2. Continued.

k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Lachnoclostridium.s__sp32402	SCI	3.694	0.035
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33739	SCI	3.156	0.046
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae	SCI	5.057	0.049
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33565	SCI	4.290	0.027
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32872	SCI	3.092	0.028
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Oscillibacter.s__sp34648	SCI	3.047	0.029
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33718	SCI	3.353	0.024
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32146	SCI	3.279	0.027
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__NA.s__sp35077	Sham	3.083	0.027
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae.g__Staphylococcus.s__lentus	Sham	4.224	0.016
k__Bacteria.p__Firmicutes.c__Erysipelotrichia.o__Erysipelotrichales	Sham	4.773	0.016
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Clostridiaceae	Sham	4.650	0.025
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Clostridiaceae.g__Clostridium.s__celatum	Sham	4.630	0.009
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__NA.s__sp35361	Sham	3.598	0.020
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__NA.s__sp35360	Sham	3.089	0.041
k__Bacteria.p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales.f__Alcaligenaceae	Sham	3.438	0.011
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae.g__Staphylococcus	Sham	4.279	0.038
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33419	Sham	3.323	0.016
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA.g__NA.s__sp12550	Sham	4.729	0.048
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Clostridiaceae.g__Clostridium	Sham	4.652	0.025
k__Bacteria.p__Firmicutes.c__Erysipelotrichia.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Turicibacter.s__sanguinis	Sham	4.779	0.013
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae.g__Jeotgalicoccus	Sham	3.427	0.014
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae	Sham	4.300	0.038
k__Bacteria.p__Firmicutes.c__Erysipelotrichia	Sham	4.768	0.016
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33537	Sham	3.083	0.041
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__NA.g__NA.s__sp31125	Sham	3.078	0.041
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__NA.g__NA.s__sp31122	Sham	3.426	0.034
k__Bacteria.p__Firmicutes.c__Erysipelotrichia.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Turicibacter	Sham	4.802	0.013
k__Bacteria.p__Firmicutes.c__Erysipelotrichia.o__Erysipelotrichales.f__Erysipelotrichaceae	Sham	4.779	0.016
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__NA.s__sp35498	Sham	3.087	0.041
k__Bacteria.p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales	Sham	3.417	0.011
k__Bacteria.p__Proteobacteria.c__Betaproteobacteria	Sham	3.404	0.011
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales	Sham	4.309	0.038
k__Bacteria.p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales.f__Alcaligenaceae.g__Parasutterella	Sham	3.414	0.011
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae.g__Jeotgalicoccus.s__halophilus_halotolerans_nanhaiensis	Sham	3.406	0.014
k__Bacteria.p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales.f__Alcaligenaceae.g__Parasutterella.s__excrementihominis	Sham	3.426	0.011

Table 3.3. Bacterial taxa significantly different following SCI in an independent cohort. With 1% FDR correction, alterations between Sham and SCI (a), sham and SCI-inulin (b), or SCI and SCI-inulin (c). Grey boxes indicate significant comparisons (1% FDR) between pre- and post-SCI groups at 2wks post injury.

ASV	Sham	SCI-Veh	SCI-Inulin	Significant comparisons	Identified in independent cohort (Fig 2)
Akkermansia muciniphila	0.032851	0.04787	0.016194	c	
Alistipes sp14336	0.029102	0.067027	0.06584	a, b	
Allobaculum sp36555	0.000152	0.021406	0.006325	a	
Bacteroidales sp12473-sp12526-sp12633	0.074217	0.084304	0.042379	b, c	
Bacteroidales sp12610	0	0.030116	0.013937	a	
Bacteroidales sp12645	0	0.019732	0.058406	b, c	
Bacteroidales sp12656	0.105666	0.052678	0.156417	a, b, c	Yes
Bacteroidales sp12768	0.033087	0.05611	0.04598	a	
Bacteroides sp12288 (thetaiotaomicron)	0.080995	0.071137	0.050251	b, c	Yes
Bifidobacterium choerinum-pseudolongum	0.049002	0.002335	0.007556	a, b	
Lachnoclostridium sp32402	0.021026	0.040619	0.01123	c	
Lachnospiraceae sp32778	0.009123	0.043795	0.014431	a, c	
Lactobacillus johnsonii	0.237838	0.079619	0.078155	a, b	Yes
Staphylococcus lentus	0.027879	0.005809	0.019373	a	Yes

Table 3.4. Independent SCI-induced microbiome compositional differences. ASV-level taxonomic changes from stool microbiomes at 2 weeks post-SCI or sham, with 1% FDR, from independent cohort.

Endpoint			
Sham vs SCI-Vehicle			
ASV	**Sham**	**SCI-Veh**	**Adjusted *p* value (1% FDR)**
Bifidobacterium choerinum-pseudolongum	0.0490019	0.00233502	1.39E-15
Bacteroidales sp12610	0	0.0301156	2.28E-07
Bacteroidales sp12656	0.105666	0.0526781	1.42E-19
Bacteroidales sp12768	0.0330871	0.05611	7.46E-05
Alistipes sp14336	0.0291018	0.0670273	7.72E-11
Staphylococcus lentus	0.0278791	0.00580894	1.46E-04
Lactobacillus johnsonii	0.237838	0.0796191	0.00E+00
Lachnospiraceae sp32778	0.00912332	0.0437946	2.65E-09
Allobaculum p36555	0.00015199	0.0214064	2.55E-04
Sham vs SCI-Inulin			
ASV	**Sham**	**SCI-Inulin**	**Adjusted *p* value (1% FDR)**
Bifidobacterium choerinum-pseudolongum	0.0490019	0.00755608	4.74E-08
Bacteroides sp12288 (thetaiotaomicron)	0.0809949	0.0502512	4.99E-05
Bacteroidales sp12473-sp12526-sp12633	0.0742173	0.0423789	2.67E-05
Bacteroidales sp12645	0	0.0584062	1.67E-14
Bacteroidales sp12656	0.105666	0.156417	2.43E-11
Alistipes sp14336	0.0291018	0.0658396	1.28E-06
Lactobacillus johnsonii	0.237838	0.0781547	0.00E+00
SCI-Vehicle vs SCI-Inulin			
ASV	**SCI-Veh**	**SCI-Inulin**	**Adjusted *p* value (1% FDR)**
Bacteroides sp12288 (thetaiotaomicron)	0.0711372	0.0502512	1.39E-04
Bacteroidales sp12473-sp12526-sp12633	0.0843043	0.0423789	2.45E-14
Bacteroidales sp12645	0.0197316	0.0584062	1.96E-12
Bacteroidales sp12656	0.0526781	0.156417	0.00E+00
Lachnoclostridium sp32402	0.040619	0.0112297	8.54E-08
Lachnospiraceae sp32778	0.0437946	0.0144314	8.77E-08
Akkermansia muciniphila	0.0478695	0.0161938	7.92E-09
Pre SCI vs Post SCI			
ASV	**SCI-Pre**	**SCI-2wks**	**Adjusted *p* value (1% FDR)**
Other	0.0400304	0.00230997	3.01E-20
Bifidobacterium choerinum-pseudolongum	0.0387627	0.00233502	5.36E-19
Bacteroidales sp12473-sp12526-sp12633	0.0566063	0.0843043	1.20E-11
Bacteroidales sp12768	0.0328508	0.05611	1.22E-08
Enterococcus faecalis	0.00133197	0.0180171	4.33E-05
Lactobacillus johnsonii	0.130668	0.0796191	1.58E-35
Lachnoclostridium sp32402	0.00983318	0.040619	4.98E-14
Lachnospiraceae sp32778	0.00608032	0.0437946	3.06E-20
Ruminococcaceae sp35297	0.0262177	0.0028927	1.11E-08
Allobaculum sp36555	0.0653365	0.0214064	7.95E-27

Table 3.5. LEfSE identified taxonomic changes from stool microbiomes at 14-DPI, from an independent cohort.

Bacterial taxa	Group	Effect size	*p*-value
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33761	SCI	3.207	0.039
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Anaerotruncus.s__sp34475	SCI	3.144	0.015
k__Bacteria.p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__NA.g__NA.s__sp12473_sp12526_sp12633	SCI	4.290	0.033
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32862	SCI	3.497	0.033
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32735	SCI	3.603	0.027
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32721	SCI	3.475	0.027
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__NA.s__sp33140	SCI	2.966	0.025
k__Bacteria.p__Actinobacteria.c__Actinobacteria.o__Corynebacteriales.f__Corynebacteriaceae	SCI.Inulin	3.518	0.019
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33731	SCI.Inulin	3.210	0.014
k__Bacteria.p__Actinobacteria.c__Actinobacteria.o__Corynebacteriales.f__Corynebacteriaceae.g__Corynebacterium	SCI.Inulin	3.518	0.019
k__Bacteria.p__Actinobacteria.c__Actinobacteria.o__Corynebacteriales	SCI.Inulin	3.518	0.019
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32635_sp32668	SCI.Inulin	3.091	0.019
k__Bacteria.p__Actinobacteria.c__Actinobacteria.o__Corynebacteriales.f__Corynebacteriaceae.g__Corynebacterium.s__amycolatum	SCI.Inulin	3.518	0.019
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33432	SCI.Inulin	3.160	0.018
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Acetatifactor	SCI.Inulin	3.087	0.047
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33421_sp33679	SCI.Inulin	3.694	0.001
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Planococcaceae.g__Sporosarcina.s__luteola_pasteurii	Sham	3.154	0.042
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Lactobacillaceae.g__Lactobacillus	Sham	4.945	0.048
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Lactobacillaceae	Sham	4.934	0.048

Table 3.5. Continued.

Bacterial taxa	Group	Effect size	*p*-value
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae.g__Staphylococcus.s__lentus	Sham	4.052	0.023
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Planococcaceae	Sham	3.135	0.042
k__Bacteria.p__Firmicutes.c__Erysipelotrichia.o__Erysipelotrichales.f__Erysipelotrichaceae.g__NA	Sham	3.046	0.009
k__Bacteria.p__Firmicutes.c__Erysipelotrichia.o__Erysipelotrichales.f__Erysipelotrichaceae.g__NA.s__sp36786	Sham	3.285	0.001
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33694	Sham	3.518	0.0196
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33673	Sham	3.201	0.0243
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae.g__Staphylococcus	Sham	4.232	0.0446
k__Bacteria.p__Actinobacteria	Sham	4.407	0.028
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33636	Sham	3.500	0.008
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Marvinbryantia.s__sp32979	Sham	3.517	0.003
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae	Sham	4.221	0.045
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Blautia.s__sp32038	Sham	3.558	0.026
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp33374	Sham	3.353	0.027
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__NA.g__NA.s__sp31125	Sham	3.024	0.005
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Planococcaceae.g__Sporosarcina	Sham	3.143	0.042
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32622	Sham	3.842	0.038
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__NA.s__sp32156	Sham	3.134	0.039
k__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Marvinbryantia	Sham	3.637	0.009

Table 3.6. Reagents & Resources.

Antibodies used				
Primary antibodies for Western Blot				
Antibody	**Concentration**	**Host**	**Supplier**	**Cat Number**
Neuronal nitric oxide synthase (nNOS, C7D7)	1:1000	Rabbit	Cell Signaling	4231S
Choline acetyltransferase (ChAT)	1:1000	Goat	Sigma	AB144P
Protein gene product 9.5 (PGP9.5)	1:1000	Rabbit	Millipore	AB1761-I
Free fatty acid receptor 2 (FFAR2 / GPR43)	1:1000	Rabbit	Thermo	PA5-111780
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, D16H11)	1:1000	Rabbit	Cell Signaling	5174S
β-Actin (D6A8)	1:1000	Rabbit	Cell Signaling	8457S
Secondary antibodies for Western Blot				
Antibody	**Concentration**	**Host**	**Supplier**	**Cat Number**
Anti-rabbit (HRP-linked)	1:1000	Goat	Cell Signaling	7074S
Anti-goat (HRP-linked)	1:1000	Donkey	Thermo	A16005
Primary antibodies for Immunohistochemistry				
Antibody	**Concentration**	**Host**	**Supplier**	**Cat Number**
Neuronal nitric oxide synthase (nNOS, C7D7)	1:100	Rabbit	Cell Signaling	4231S
Protein gene product 9.5 (PGP9.5)	1:200	Mouse	Abcam	AB72911
Secondary antibodies for Immunohistochemistry				
Antibody	**Concentration**	**Host**	**Supplier**	**Cat Number**
Anti-mouse (Alexa Fluor™ 594)	1:200	Goat	Thermo	A-11005
Anti-rabbit (Alexa Fluor™ 488)	1:200	Goat	Thermo	A-11008
ATCC Strains and Growth Conditions				
Bacterium	**Medium**	**Environment**	**CFU per gavage**	**ATCC Number**
Lactobacillus johnsonii	de Man-Rogosa-Sharpe	Aerobic	10^16	33200
Bacteroides thetaiotaomicron	Brain-heart infusion	Anaerobic	10^14	29148
Clostridium celatum	Chopped Meat Carbohydrate	Anaerobic	10^10	27791

Chapter 4: Discussion.

4.1 SCFA-producing bacteria are diminished after SCI.

In Chapter 2 we investigated the existing data on microbiome composition after spinal cord injury. Our investigation highlighted findings from human and animal studies, and correlated taxanomic changes in the gut microbiome to aspects of injury severity and recovery. Our investigation revealed that microbiome composition is highly correlated with other aspects of injury, including level and severity of the SCI, inflammatory and metabolic profile, and bowel dysfunction. This indicates that the microbiome composition post-injury could serve as a biomarker to uncover additional details about post-injury autonomic function or be used to predict outcomes of injury-associated sequelea. Our key findings from this analysis (Tables 2.1 & 2.2), were the notable decrease in SCFA-producing bacterial taxa after injury. These groups of microbes have been shown to be diminished in other injuries and disease states, especially those involving negative neurological outcomes (251, 253), highlighting a potentially shared pathway through which the microbiome exacerbates disease or injury by influencing the host immune system. Based on the knowledge that genetically unrelated microbes can fill similar niches and produce identical metabolites (276), we decided to focus our experimental approach on therapeutics that increased microbial production of SCFAs, rather than target specific microbial communities. We selected inulin fiber, based on its easy solubility in water, its ability to be readily fermented into SCFAs, and its proven ability to reduce risk of infection and improve gut health in other disease states (69, 277, 278).

4.2 Inulin fiber increases SCFAs and improves outcomes after SCI.

Our data presented in Chapter 3 provide compelling evidence that inulin fiber improves GI outcomes after SCI. We demonstrated that two weeks after a severe contusive T9 SCI, mice

developed substantial GI dysfunction, characterized by a significantly slower total GI transit time, a qualitative decrease in frequency of spontaneous colonic contractions, and atrophy of colonic myenteric neurons (Fig. 3.1). Mice provided 1% inulin in their drinking water had substantially less GI dysfunction as compared to vehicle treated mice with SCI, with statistically significant improvements in all of the aforementioned outcomes. Intriguingly, supplementation of drinking water with 1% inulin only increases total fiber intake of mice by around 7%, and soluble fiber intake by 38%, based on rodent diet (Teklad Sterilizable Diet 2019S), a target reasonably attainable in human populations. The majority of fiber found in the sterilizable Teklad diet is insoluble fiber, including cellulose, hemicellulose, and lignin, which contribute to stool bulk, but are less readily fermentable by the gut microbiome and do not influence SCFA levels (279, 280). The soluble fiber found in this standard mouse diet is derived from wheat and corn, which is primarily fermented into the SCFA acetate, while inulin most abundantly increases levels of the SCFA butyrate (281). There were no significant differences in body weight between SCI-veh and SCI-inulin mice, suggesting that total caloric input was similar between groups (Fig. 3.1.C). Based on the minor 7% increase in total fiber, it is unlikely that the supplemented inulin had much of a stool-bulking effect, indicating instead that microbial fermentation products were responsible for the notable GI improvements in the inulin-treated mice.

In line with this, our data revealed a significant increase in the SCFA propionate in the fecal pellets of inulin-treated mice after injury (Fig. 3.5.B). Intriguingly, we did not see a significant increase in any other fecal SCFAs after inulin supplementation, nor did we see a significant decrease in any fecal SCFAs when comparing sham to SCI-veh (Fig. 3.5). Our understanding is that this lack of significant difference is due to the absorption and metabolism of the bulk of the SCFAs in the

colon prior to collection of fecal pellets for analysis (282-284). The majority of fiber fermentation in mice occurs in the cecum, the bacteria-filled pouch at the junction of the small and large intestines (284). The SCFAs are then absorbed by the host as stool travels down the colon, with only a fraction remaining in fecal pellets after defecation (282, 285). Previous studies have found that butyrate is the most significantly increased SCFA in cecal contents of mice fed inulin (286), and human studies have shown that serum levels of all SCFAs are increased after consuming inulin (278). This collectively helps explain why TRB but not TRP improved GI and locomotor function post-SCI (Fig. 3.5), despite our fecal SCFA data showing increased propionate but not butyrate after inulin treatment.

Based on our literature review we anticipated that SCI would decrease SCFAs, but there were no fecal SCFA differences between sham and SCI-veh groups. However, analysis of SCFA composition in serum revealed that mice had significantly lower levels of propionate, butyrate, and valerate after SCI, as compared to sham (Fig. 4.1). While this confirms that SCI did lead to decreased levels of SCFAs, we were surprised to find that serum SCFA levels were not significantly increased in mice following inulin treatment (Fig. 4.1). However, only butyrate (not propionate or valerate) was significantly decreased in inulin-treated mice when compared to sham levels, indicating that inulin may be protective against the detrimental SCFA-diminishing effect of SCI.

4.3 The role of IL-10 in recovery from SCI.

Based on the known association between SCFAs and immune system regulation (287) we investigated immune changes in injured mice treated with inulin or SCFA-triglycerides. Serum

markers of inflammation were not different between SCI-veh and SCI-inulin groups (Fig. 4.2), despite the significant increase in IL-10 in gut tissue of inulin-fed mice (Fig. 3.4.A & Fig. 3.8.A). Intriguingly the opposite was true for TRB-treated mice, which displayed increased serum IL-10 (Fig. 3.5.N & Fig. 3.8.C) but no changes in gut cytokine levels (Fig. 4.3). These data suggest that 1% inulin has a more gut-local effect, while SCFA-triglycerides have a more pronounced systemic effect.

To further evaluate the effect of inulin on SCI outcomes we conducted a pilot study in which we provided a cohort of injured mice with 10% inulin. This led to significantly increased levels of IL-10 in serum (Fig. 4.4.A), similar to our findings in TRB-treated mice (Fig. 3.5.N), but not achieved at our standard 1% dose of inulin (Fig. 4.2). This finding suggests that additional systemic anti-inflammatory effects could be achieved by optimizing the dose of fiber used. Mice treated with 10% inulin presented with colonic FFAR2 levels higher than those of mice treated with vehicle or 1% inulin, confirming a greater physiological response to increased SCFAs (Fig. 4.4.B). Together these data provide additional evidence that inulin, SCFAs, and IL-10 are linked in a microbiome-immune pathway with the potential to mediate GI recovery after SCI.

In line with our findings that inulin and SCFAs increase IL-10, and that IL-10 is a necessary component of the pathway through which inulin improves post-SCI outcomes (Fig. 3.4), recent studies have shown that increasing IL-10 in the spinal cord improves locomotor outcomes after SCI (288-290). These studies demonstrate that systemic or CNS-specific increases in IL-10 improve locomotor function, consistent with our finding that TRB led to the most rapid and

substantial improvement in BMS (Fig. 3.5.H), along with significantly elevated serum IL-10 (Fig. 3.5.N).

It is thought that IL-10 may improve SCI outcomes by diminishing the production of inflammatory cytokines and chemokines that are responsible for recruitment of inflammatory cells (291). IL-10 reduces the activation and proliferation of most T cells, with the exception of inflammation-suppressing regulatory T cells, which are activated by IL-10 (291). However, these effects are likely to be highly dose and time dependent, as IP injection of IL-10 in the initial 24hrs of SCI improves outcomes, but later treatment and higher doses do not (292, 293). Future studies will need to investigate if inulin or SCFA-based treatments improve locomotor outcomes when administered at more chronic timepoints. Our experiments all provided treatment immediately after SCI, raising the possibility that the beneficial outcomes we see are entirely due to physiological changes that occur within the first day after injury.

However, it is possible that IL-10 based therapeutics could lead to lasting improvements if the IL-10 was generated endogenously, rather than systemically administered, because IL-10 cannot cross an intact blood spinal cord barrier (BSCB) (294). Immediately following SCI the BSCB is compromised, but the eventual re-establishment of the BSCB (295) may negate the benefits of additional IL-10 administration. SCFAs, however, can cross the BSCB, where they have the potential to continually induce the production of IL-10 at the lesion site, even after the re-establishment of an intact BSCB (296). This suggests that treatment with inulin or SCFAs could improve SCI outcomes even after the re-establishment of the BSCB, providing benefit beyond that initial 24hr window.

By repeating our standard inulin treatment in mice deficient in the IL10rb subunit, we confirmed the necessity of IL-10 signaling, as these mice did not show any GI or locomotor improvements when treated with inulin (Fig. 3.4). However, it is important to note that the beta subunit of the IL-10 receptor is also a subunit of other type-two cytokine receptors, including IL-22 (297). IL-22 also increases in response to inulin consumption and has been shown to improve colonic and metabolic health (68). Intriguingly these IL-22 increases, and subsequent changes in physiology, were found to occur independent of SCFA signaling (68), while our research provides compelling evidence that treatment with SCFA-triglycerides closely recapitulates the beneficial GI and locomotor effects seen in inulin-treated mice (Fig. 3.5). This opens the possibility that inulin exerts its beneficial effects though multiple independent immune-based pathways; a concept that will need to be investigated in future studies.

Despite the stark increase in colonic and systemic anti-inflammatory markers in inulin-treated mice, we saw only minor changes in pro-inflammatory cytokine levels in SCI-veh mice when compared to sham controls (Fig. 3.8.A & Fig. 4.2). One explanation for this is that a sham injury alone is a major surgery and is expected to have significant effects on inflammation and other aspects of physiology. Another factor is that inflammatory cytokine levels tend to decrease significantly by the 2-week timepoint that we evaluated in this study (298). Because of this, we decided to evaluate a more chronic indicator of GI inflammation, colon length. Colon length is a common marker of GI inflammation, often used in mouse studies of inflammatory bowel disease, where a shorter colon is considered indicative of inflammation (299). Using this method we found that mice with SCI had significantly shorter colon lengths than sham controls, but mice treated

with inulin were no different that shams (Fig. 4.5.A). Similarly, mice treated with TRB or *B. thetaiotaomicron* saw significant increases to colon length when compared to vehicle treated mice. (Fig. 4.5.B, C). Although this is likely due to inflammation, a handful of other SCI-associated changes in physiology may also play a role in shortening of colons such as the thickening of colonic muscles due to increased collagen deposition (100, 101) or the over-contraction of GI muscles – either due to the loss of inhibitory motor neurons or due to dysregulated autonomic function of extrinsic neurons – all of which have been shown to occur after SCI (see Chapter 1, Section 1.9). Additional studies are necessary to evaluate how fiber or postbiotics impact each of the above processes.

4.4 Inulin and SCFA-triglycerides prevent atrophy of ENS neurons.

To get a better idea of how increases in SCFAs and IL-10 might be improving GI transit we took a deeper look at the enteric nervous system. We found that after SCI there is a significant loss of neuronal projections from enteric neurons, which is rescued by treatment with inulin (Fig. 3.1.K, L). Our findings indicate that the number of myenteric neurons themselves remain unchanged after injury, but that the number of neuronal projections and the area they cover within the gut is reduced significantly. Previous studies in more severe upper thoracic transections have noted the loss of nNOS+ enteric neurons, a marker specific to inhibitory interneurons and inhibitory motor neurons of the gut (102-104). Inhibitory nitrergic neurons have been shown to be more susceptible to damage than other types of enteric neurons (268). We found that inulin treatment (Fig. 3.1.I) and TRB treatment (Fig. 3.5.K), significantly increased the protein levels of colonic nNOS. However, inulin had no effect on the number of nNOS+ neurons or neuronal projections (Fig. 3.1.I-O). A possible interpretation of this data is that these inhibitory neurons are not dying, nor their processes

atrophying, but they are instead losing the ability to produce sufficient amounts of neurotransmitter. A significant decrease in nNOS production could mean less efficient muscle contractions and reduced peristalsis. This is in line with other studies in which researchers have noted a diminished inhibitory junction potential in the colons of mice post-SCI (102, 104).

4.5 The influence of inulin outside of the GI tract.

As mentioned in Chapter 1, the gut functions semi autonomously, but is still influenced by numerous sources of extrinsic neuronal innervation. Many of these non-enteric neurons have receptors for SCFAs and are likely changing in response to inulin or SCFA pro-drug treatment (300). Our *ex vivo* gut contractility data shows significantly increased spontaneous contractions in colons of inulin-treated mice when compared to vehicle-treated mice (Fig. 3.1.G). These contractions occur despite the removal of all extrinsic neuronal connections, indicating that those connections are not necessary for increased contractility at end point, but it does not rule out the possibility that extrinsic neuronal input to the gut throughout the 2-week experimental timeline played a role in strengthening the colon and preventing ENS atrophy, with lasting effects that remain in the *ex vivo* prep. Additional experimentation is necessary to test the involvement of extrinsic innervation to the gut in inulin/SCFA-mediated recovery from SCI. Surgical, pharmacological, or viral/genetic intervention to ablate specific neuronal types could provide insight into the necessity of certain peripheral connections, while electrophysiological and molecular assessment could provide insight into physiological changes of peripheral neurons after SCI.

We started our investigation of microbiome-targeting therapeutics with the premise that improving the composition of the gut microbiome would have the most pronounced effect in the part of the body most closely associated with the microbiome, the gut itself. Upon deeper investigation we found that both inulin and tributyrin supplementation also led to improved BMS scores (Fig. 3.1.B & Fig. 3.5.H), confirming that these treatments are acting on targets outside of the gut, including the CNS. This led us to investigate how the spinal cord changed in response to inulin administration after SCI. When evaluating inflammatory markers in the cord we found that some markers of inflammation, assessed by qPCR, were increased after SCI, but were notably improved after inulin treatment (Fig. 4.6). However, similar inflammatory markers assessed by multiplexed ELISA revealed increased inflammation in the spinal cords of injured mice, regardless of treatment group, with no improvements in inulin-treated mice (Fig. 4.7). Collectively these data might suggest that inulin is leading to reduced transcription of inflammatory genes, but that inflammatory protein levels are unchanged due to recruitment of inflammatory proteins from cells in other parts of the body, as observed in other studies of SCI (290). It is also possible that our temporal snapshot captured a moment in which transcription had been downregulated, but protein levels had not yet reached their terminal elimination half-lives. Importantly, both protein and mRNA levels of cytokines have been shown to return to basal levels in the spinal cord within 1-2 weeks of SCI in rodents (298). Our data suggest that inulin treatment returned mRNA levels back to baseline at 2-weeks post-SCI, but vehicle treatment did not, perhaps indicating a more rapid recovery from inflammation in inulin-treated mice. However, it is also possible that the most significant inulin-derived reductions in spinal cord inflammation occurred within the first week after injury and were less pronounced at our experimental endpoint. Future studies will need to evaluate the effects of inulin on spinal cord inflammation at earlier and later timepoints.

4.6 The influence of the SCI-associated microbiome on host health.

Once we determined that microbiome-altering inulin treatment improved GI and locomotor outcomes we next sought to determine if the SCI-associated microbiome itself could lead to negative GI outcomes. We evaluated this question by transplanting SCI-associated microbiomes into injury-naïve germ free (GF) mice, where we could study the effects of SCI-associated microbiomes on host physiology, outside the context of injury (Fig. 3.3.A, B). Our study did not reveal any differences in GI transit of mice colonized with microbiomes from sham, SCI-veh, or SCI-inulin mice. However, we did see changes in metabolic and inflammatory markers, some of which recapitulated data observed in the donor animals (Fig. 3.7). Serum markers of dysregulated metabolic and immune function have also been observed in humans after SCI, and often are correlated with microbiome changes (167, 301-304). It is possible that some of this dysfunction is directly caused by the dysbiosis associated with SCI, and that improving the microbiome in people with SCI could improve some of this metabolic and inflammatory dysfunction.

It is also possible that the injury-associated microbiome directly influences neuronal function. A recent study found that fecal supernatants from injured, but not from injury-naïve, mice induced hypersensitivity in cultured sensory afferent neurons (305). Importantly this occurred in neurons derived from both injured and injury-naïve mice, indicating that the neurons had not been conditioned to injury-associated stimuli, and that the microbiome alone increased sensitivity to pain signals. In line with this, injuries associated with increased pain also have a greater impact on microbiome composition (118), which is being investigated for its potentially contributory role to SCI-associated pain (131). Collectively these studies suggest that metabolites of the SCI-

associated microbiome directly influence neuronal signaling as well as metabolic and inflammatory processes.

A potential explanation for the lack of GI transit differences between gnotobiotic groups is the model itself. Temporary depletion of the gut microbiome with antibiotics during early postnatal development leads to impaired GI transit and a loss of enteric neurons (306). In GF mice, completely devoid of a microbiome during development, the effects on the ENS (307) and immune system are even more significant (184). Colonization of GF mice has been shown to rapidly promote ENS neurogenesis and improve gastrointestinal transit (159), as well as restore intrinsic and extrinsic nerve function (308). However, the rapid exposure to a complex microbiome is thought to influence the ENS differently than the concurrent development of the ENS and microbiome that occurs in specific pathogen free (SPF) mice (309). It is possible that colonization of GF mice with any microbiome leads to improvements in GI transit and ENS function, masking any differences between experimental groups that may occur in mice with already-developed enteric nervous systems. One additional consideration is that the maintenance of the microbiome composition induced by SCI may be dependent on the environment of the gut, which is shaped by injury, resulting in the inability to recapitulate the composition and spatial distribution of the injured microbiome in a previously GF mouse.

Numerous recent studies have demonstrated that FMT from a healthy donor increases SCFA levels, improves gastrointestinal and locomotor function, decreases inflammation, and reduces anxiety-like behavior in rodents with SCI (120, 148, 310). These studies confirm that microbiome transplantation can significantly improve host physiology in injured mice, while our data show

similar FMT does not improve GI transit in injury-naïve ex-GF mice. Future studies will have to evaluate the influence of the SCI-associated microbiome in other injury-naïve models, such as SPF mice treated with broad spectrum antibiotics (311).

A recent study of FMT administered to people with SCI proved to be safe and effective at eradicating multidrug-resistant bacterial infections (312). This pivitol study suggests that FMT may be a safe and feasable treatment for other types of infection, or gastrointestinal dysfunction, after SCI. This is a particularly important finding for the SCI field, as the most common causes of rehospitilizaton and death after injury are related to infection (257, 258). Antibiotics are often the first line of defense provided by medical doctors to combat infection, but animal studies have shown detrimental locomotor outcomes in mice treated with antibiotics around the time of SCI (60). FMT or other microbiome-based theraputics to combat infection post-SCI are critical to reduce antibiotic use in people with SCI, as antibiotic treatments may exacerbate negative injury outcomes.

4.7 Comparing microbiome-based therapeutic options.

In our experimentation to understand if, and then how, microbial manipulation could improve GI outcomes after SCI, we essentially tested the efficacy of a handful of potential therapeutics including a prebiotic (inulin fiber), probiotics (*Bacteroides thetaiotaomicron*, *Lactobacillus johnsonii*, & *Clostridium celatum*), and postbiotics (tripropionin & tributyrin).

Probiotic administration was the least effective of the treatments, with no significant improvement in GI or locomotor outcomes for any of the three evaluated bacterial species. *B. thetaiotaomicron*

showed promise based on improved GI transit and increased ENS markers in monocolonization studies (Fig. 3.3.C-G), and increased colon length observed in mice with SCI (Fig. 4.5.C). However, these beneficial outcomes were minimal compared to prebiotics and postbiotics, as probiotic treatments did not lead to any significant improvements in GI or locomotor outcomes when given to mice after SCI (Fig. 3.3.H-J).

One explanation for this lack of notable benefit is the absence of successful colonization of the mice with the administered probiotics. 16S sequencing of fecal samples taken from probiotic-treated mice revealed no changes in relative abundance of any of the administered bacterial taxa despite daily oral gavage. This lack of successful colonization suggests that the selected taxa were faced with an environment unfit for survival, due either to the environment itself (stomach acid, or SCI-associated changes in the luminal environment, such as increased dissolved oxygen) or due to competition with native bacteria that already filled all available niches. Future studies could evaluate other routes of administration that bypass the stomach and small intestine to increase the number of viable bacteria making it to the colon. Alternatively, it is possible that *B. thetaiotaomicron* may be more beneficial if used in conjunction with fiber or antibiotics, which could lead to a less hostile gut environment or less competition between species, respectively, providing opportunity for colonization.

When considering postbiotics, we found that tributyrin was much more successful at improving GI and locomotor outcomes than a similar dose of tripropionin (Fig. 3.5.G-N). Among the handful of extensively studied SCFAs, butyrate is considered to be the most influential to immune system function, acting through FFARs and HDAC inhibition (313), some aspects of which are IL-10

independent (314). Butyrate also serves as the primary source of energy for colonocytes, with 90-95% of microbially produced butyrate used locally for ATP production in gut epithelial cells (283, 313, 315). Butyrate also directly improves the health of the gut by increasing tight junction proteins, reducing gut permeability, and increasing mucin production, on which propionate has no effect (316, 317). Considering our GI and locomotor results and the current literature on butyrate we feel that tributyrin is the best candidate postbiotic evaluated in this study.

We only evaluated one prebiotic therapeutic in this project, the soluble fiber inulin. We did however compare outcomes in a pilot study between 1% and 10% inulin. As mentioned previously, we found that higher dose inulin increased serum levels of IL-10, and likely increased production of SCFAs, based on high colonic levels of the SCFA receptor FFAR2 (Fig. 4.4). However, a concerning finding was how much the additional fiber increased cecum size in mice (Fig. 4.8.A). Although some increase in size is expected with increased consumption of fiber, this drastic increase in cecum weight in mice may correspond to increased colonic distention in humans, based on the anatomical differences in location of fiber fermentation between species (284). GI distention is uncomfortable for most people but is of particular concern for people with T6 or higher SCI, as GI distention is one of the most common triggers of autonomic dysreflexia (318). In addition, a common symptom of SCI is visceral pain (319), which can be exacerbated by intestinal distention (320).

Beyond this, we also noted that mice that received 10% inulin had a qualitatively (though non-significant) lower BMS score than mice receiving 1% inulin (Fig. 4.8.B). This detrimental impact on locomotor outcomes could be due to the higher level of anti-inflammatory IL-10 (Fig. 4.4.A),

which might influence the formation of a glial scar in the acute recovery phase after SCI (321). This is less likely to be a consideration if higher doses of inulin are used as a therapeutic in more chronic cases of injury after the acute period of scar formation has passed. Importantly, this small pilot study also found no significant differences in BMS between SCI-veh and the 1% inulin group, unlike our findings in a better powered experiment (Fig. 3.1.B). Indicating that a larger experimental sample size is necessary to confirm the effect of 10% inulin on locomotor outcomes.

4.8 Potential risks associated with inulin and SCFAs.

Despite the well documented beneficial effects of inulin and SCFAs in multiple disease states, recent studies have shown that diets supplemented with soluble fiber (inulin, FOS, and pectin) can lead to hepatocellular carcinoma (HCC) in mice (286, 322). This increased incidence of liver cancer was found to be dependent on bile acid metabolism and microbial fermentation products, such as SCFAs. Intriguingly, HCC only occurred in wild-type mice when they received a high-fat and highly processed compositionally defined diet. HCC did not occur in mice that ate an inulin-supplemented grain-based diet, even in mice genetically predisposed to developing HCC. These studies suggest that inulin may be detrimental when used to supplement a high-fat and highly processed western diet.

In addition to liver damage, inulin-supplemented diets have also been shown to exacerbate colonic inflammation in a DSS colitis mouse model (323). These effects are likely due to SCFAs, which play contrasting roles in cell proliferation and autophagy in the gut (324). They are essential nutrients for colonocyte growth and metabolism but also inhibit proliferation of intestinal stem cells (325). They are widely known for their anti-inflammatory properties, but they have also been

shown to promote inflammation in certain contexts (326). Chronically elevated SCFAs have been implicated in kidney damage (327) and metabolic syndrome (328). Collectively these studies show that the beneficial effects of fiber and microbial metabolites on host health are highly context and dose dependent, warranting further investigation into the appropriate dose and delivery of inulin or SCFAs in the context of SCI.

4.9 Direct comparison of inulin and tributyrin.

Although similar GI and locomotor outcomes were achieved in our experiments using fiber and TRB, there are some differences that need to be compared and contrasted when considering these options as candidate therapeutics. As mentioned earlier in the chapter, inulin has a substantial effect on GI transit, ENS health, and colonic inflammatory status (Fig. 3.1), but less significant improvements systemically (Fig. 4.2) and in the CNS (Fig. 3.1.B & Fig. 4.7), when compared to the locomotor improvements associated with TRB treatment (Fig. 3.5.H). This gut-local effect is likely due to the microbial fermentation of inulin into SCFAs in the colon, which are used locally, evident by increased colonic SCFA receptors (Fig. 3.5.D), but are produced in too small a quantity to contribute to any notable increases of SCFAs in circulation (Fig. 4.1).

On the other hand, TRB is primarily absorbed in the small intestine, where it is broken down into butyrate, and quickly enters circulation (329). Once in circulation butyrate can readily cross into the CNS and influence inflammatory cells and gene transcription in the spinal cord (296). Intriguingly, TRB administration resulted in increased levels of colonic FFARs and ENS proteins (Fig. 3.5.I-M), as well as significantly increased colon length as compared to veh-treated mice

(Fig. 4.5.B), indicating that despite the rapid absorption in the small intestine, the SCFAs still had a substantial effect on the colon, likely through circulation.

TRB is not only rapidly absorbed, but also short lived. A once-daily oral gavage of TRB delivers a large dose of SCFAs over a short period of time, reaching a maximum concentration in serum within 1 hr, and rapidly returning to baseline within a few hours (330). Inulin on the other hand, provides a constant low-level influx of SCFAs as the gut microbiome endogenously generates SCFAs by fermentation of each inulin-containing bolus that enters the cecum. The pros and cons of the slow and steady vs rapid influx of SCFAs will need to be evaluated in future studies.

The major focus of our study was the large intestine, which appears to be the most effected component of the GI tract in this model of SCI (305). However, dysmotility after SCI is also known to impact the stomach and small intestine (130). One potential advantage of treatment with TRB is that the stomach and small intestine, not just the colon, are exposed to SCFAs. This could potentially contribute to a healthier upper GI tract that would not be replicated by treatment with inulin, which is devoid of SCFAs until fermented by the colonic microbiome. One caveat to this is that most mice, but exceptionally few humans, intentionally consume their own feces. It is thought that small mammals do this to absorb any residual nutrients that were not absorbed in the colon, including inulin-derived SCFAs (331). An additional consideration regarding regions of the gut and SCFA distribution, is that while mice have the highest concentration of SCFAs in their cecum and proximal colon, while humans have similar amounts of SCFAs in proximal and distal colon (284).

As mentioned in the previous section, inulin, and other fibers, not only increase SCFAs, but also contribute to stool bulk. Increased stool bulk can have positive effects such as increased mucus production and more responsive enteric reflex circuitry (200), and negative effects such as bloating, gas, and increased risk of visceral pain and autonomic dysreflexia (318, 320). When administered alongside antibiotics, which are frequently prescribed to people with SCI, fiber runs the risk of not being adequately fermented in the gut due to the depletion of inulin-fermenting genera such as *Bacteroides* & *Faecalibacterium* (332). Not only would this result in a decreased production of SCFAs, but it would also further increase stool bulk, potentially leading to negative side effects.

On the other hand, SCFA-triglycerides do not increase stool bulk, they don't interact with antibiotics, and they don't require the microbiome to function. However, the lack of interaction with the microbiome could be a disadvantage in certain circumstances. Recent evidence has shown that nodose, DRG, and ENS sensory afferents become hyperresponsive to pain when cultured with microbial metabolites from mice with SCI (305, 333, 334). Inulin requires the gut microbiome to function, but it also improves the microbiome composition in the process, potentially leading to reductions of harmful metabolites, and therefore reductions in visceral pain sensitivity. TRB is not likely to directly influence the microbiome composition, meaning pathogenic taxa would not be outcompeted. However, TRB could indirectly change the microbiome by improving inflammation and GI motility, leading to a healthier gut environment. There are numerous pros and cons when comparing the two treatments, which will need to be evaluated in additional pre-clinical and clinical studies, and then again on the individual patient level, before recommendations can be made.

4.10 Future directions & concluding remarks.

This research project has ultimately uncovered a novel therapeutic microbiome-associated pathway for improving gut and locomotor function after SCI. However, the extensive body of knowledge outlined in the chapters above only just begins to scratch the surface of the work required to fully understand GI dysfunction after SCI. Of note these experiments were all evaluated under a single injury paradigm: a severe T9 contusion in male mice, evaluated at a sub-chronic endpoint of 14 days post-injury, with therapeutic treatment started immediately following injury. A pilot study we conducted suggests that the therapeutic GI benefit of inulin seen at 2-weeks persists out to 3-weeks after injury (Fig. 4.9), with trends suggesting GI improvement out to 4-weeks and qualitative but non-significant improvements to BMS out to 4-weeks. A larger sample size is necessary to properly evaluate more chronic timepoints. It is still unclear at what point during the injury timeline the administration of inulin confers the most benefit. As mentioned previously, it's possible that the key to improving injury outcomes is providing treatment immediately after injury. Additional studies will need to investigate the efficacy of inulin or SCFA-based treatments provided at more chronic stages of injury. Future pre-clinical studies will also need to investigate how fiber and SCFAs effect GI and locomotor function in other animal models and under different injury conditions. The location and severity of the injury, as well as the timing and duration of treatment are likely to bring about new considerations for treatment of neurogenic bowel in the context of traumatic SCI.

Beyond traumatic SCI, neurogenic bowel is prevalent in people with spina bifida, Parkinson's disease, traumatic brain injury (TBI), stroke, and multiple sclerosis, among others (335). Many of

these diseases and disorders also present with microbiome changes, leading researchers to question the involvement of the microbiome in the etiology of these conditions and raising the possibility that targeting the microbiome in these conditions may improve secondary complications of the diseases, including neurogenic bowel, or even influence disease progression (251). In fact, inulin fiber was recently shown to improve both dysbiosis and neurological outcomes in mice with TBI (70, 71) and in mice with stroke (336, 337). These neurological injuries occur regardless of the starting microbiome composition, but the resulting changes in microbiome composition can influence recovery, similar to SCI. It's possible that many of these conditions that lead to gut dysbiosis and neurogenic bowel share a common microbiome-based pathway that can be targeted to improve disease outcomes.

4.11 Figures.

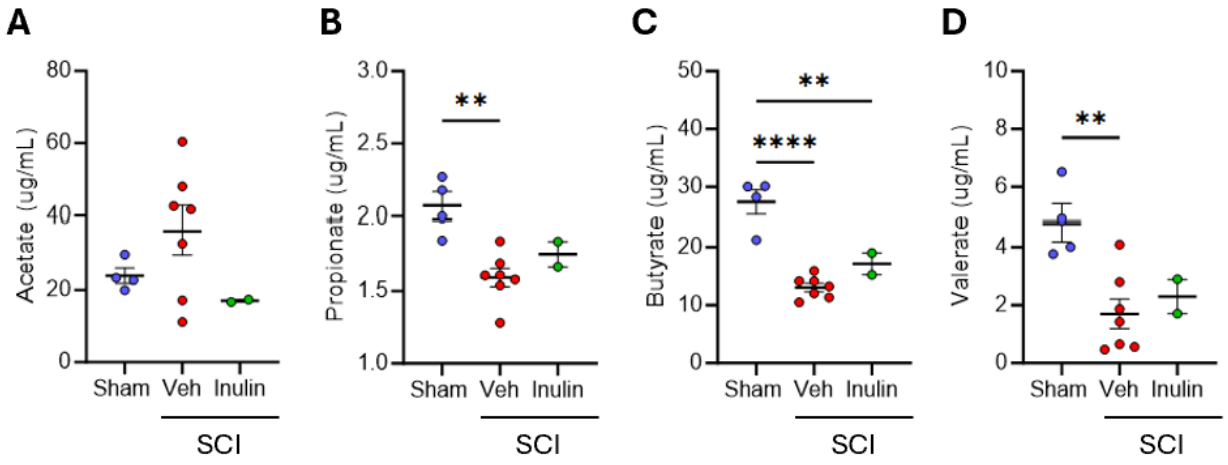


Figure 4.1. SCI leads to reduced levels of SCFAs in serum.

A-D, Levels of the SCFAs acetate (**A**), propionate (**B**), butyrate (**C**), and valerate (**D**) in serum of sham, SCI-veh, and SCI-inulin mice, two weeks after surgery. N=2-7. Each circle represents an individual mouse. ** $P < 0.01$, **** $P < 0.0001$. Data are shown as mean ± SEM and compared by one-way ANOVA with post-hoc Tukey's test.

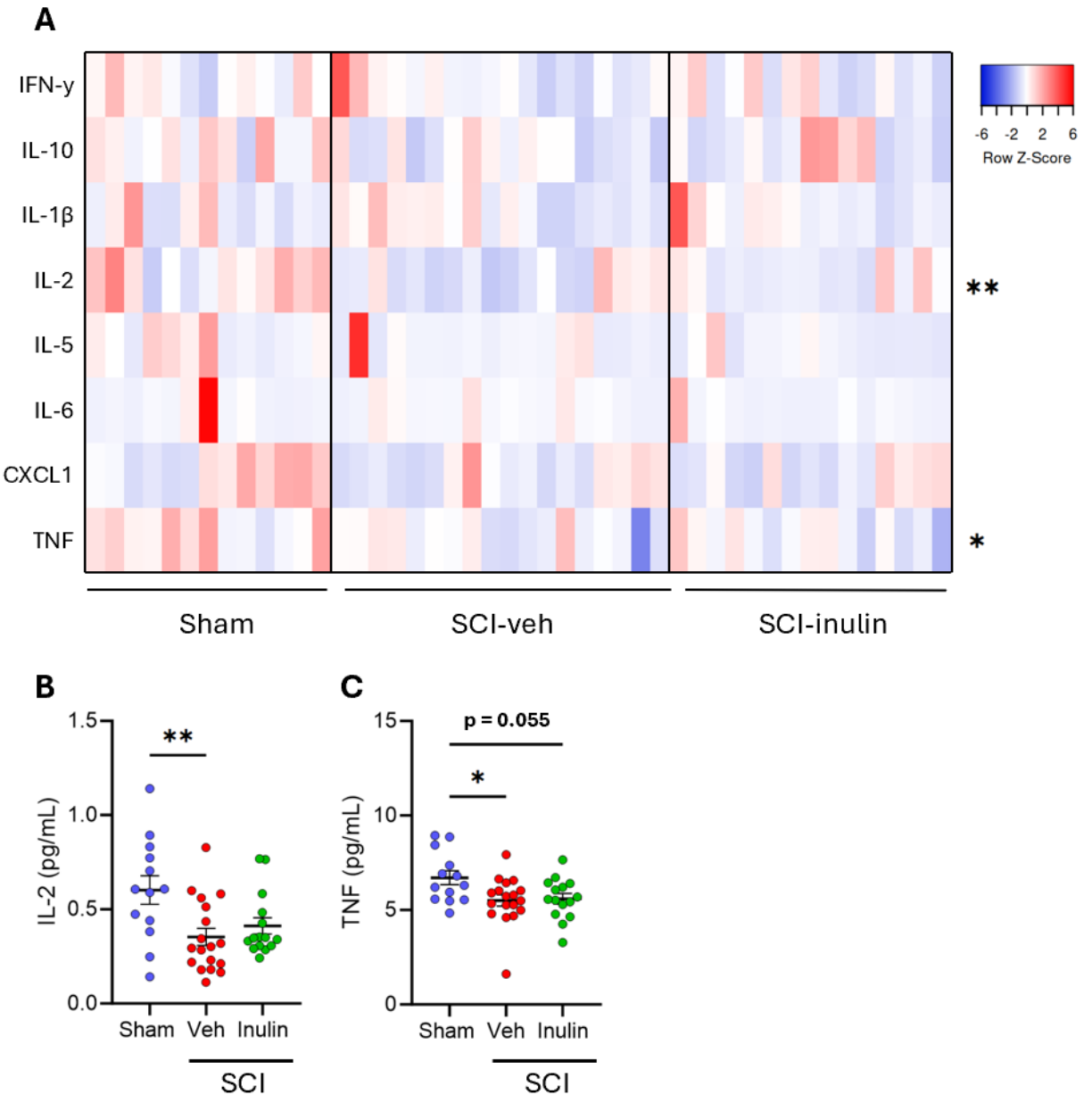


Figure 4.2. SCI has minimal impact on serum inflammatory markers at 14-DPI.

A, Heatmap of inflammatory markers in serum from mice two weeks after surgery. **B, C**, Significant findings from inflammatory panel include a decrease in IL-2 in mice with SCI-veh (**B**), and a decrease in TNF in both injury groups (**C**), as compared to sham. N=13-18. Each circle represents an individual mouse. * $P < 0.05$, ** $P < 0.01$. Data are shown as mean ± SEM and compared by one-way ANOVA with post-hoc Tukey's test.

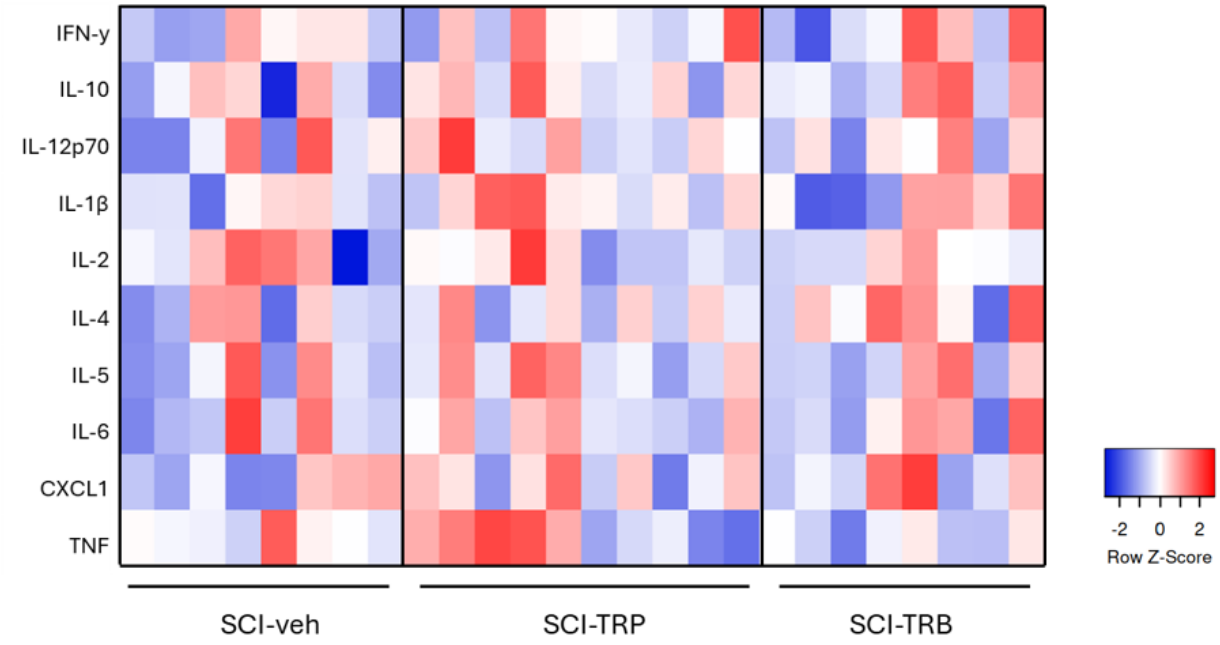


Figure 4.3. SCFA pro-drug treatment does not influence colonic inflammation at 14-DPI.

Heatmap of colonic inflammatory markers of injured mice treated with vehicle, TRP, or TRB, two weeks after injury. N=8-10. Each marker was compared between groups by one-way ANOVA with post-hoc Dunnett's test, but there were no significant findings.

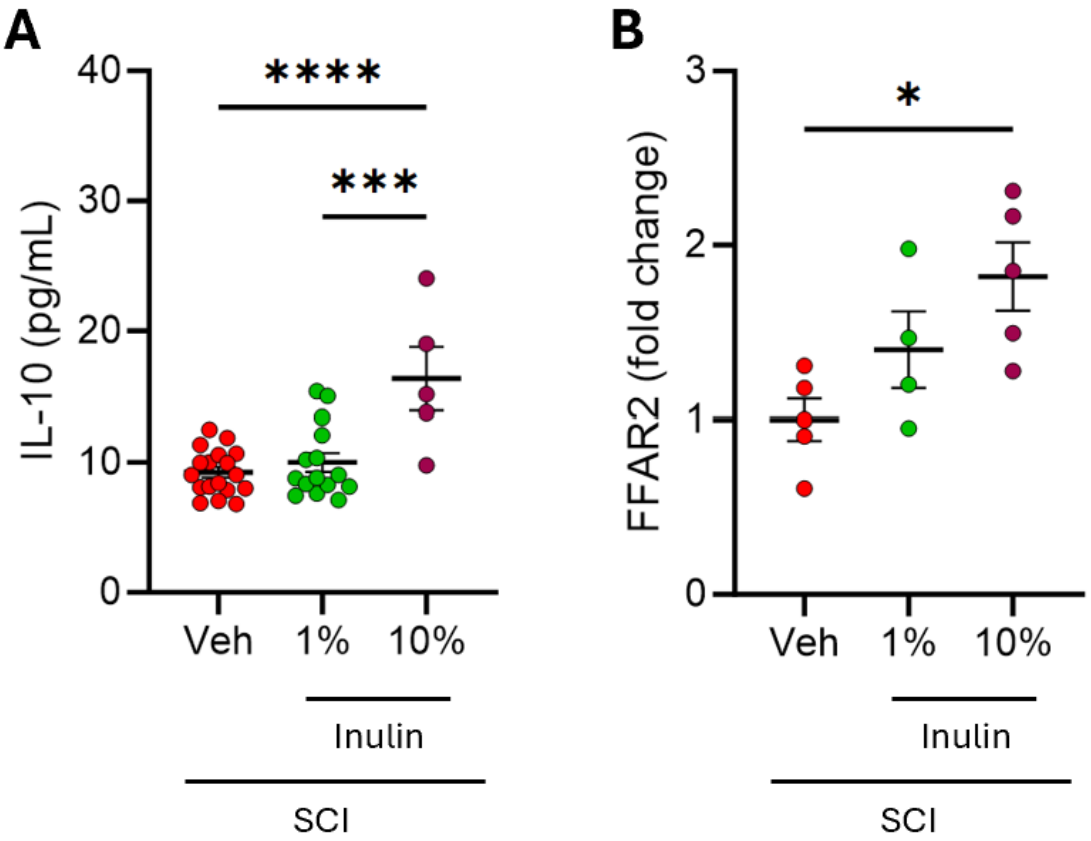


Figure 4.4. Higher dose of inulin increases serum IL-10 and colonic FFAR2.

A, Serum levels of IL-10 are significantly increased, two weeks after injury, in SCI mice treated with 10% inulin, relative to injured mice treated with vehicle or 1% inulin. **B**, Colonic FFAR2 is significantly increased, two weeks after injury, in SCI mice treated with 10% inulin, as evaluated by western blot. SCI-veh and SCI-inulin (1%) data used in (**A**) are the same data from Fig. 4.2A. N=4-18 (**A**), N=4-5 (**B**). Each circle represents an individual mouse. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. Data are shown as mean ± SEM and compared by one-way ANOVA with post-hoc Tukey's test.

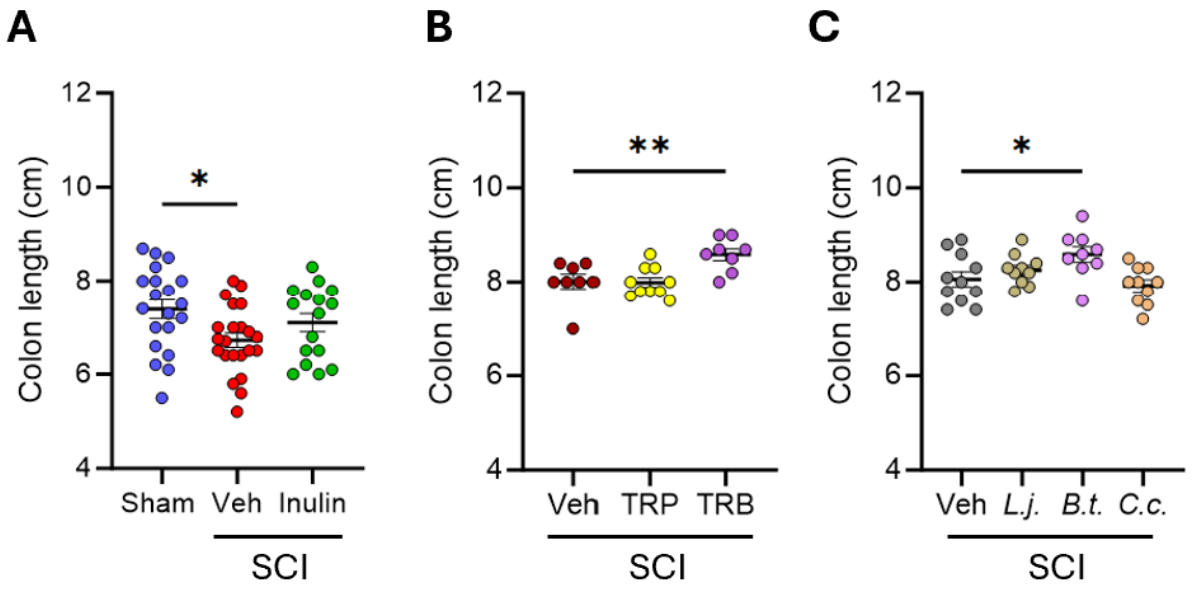


Figure 4.5. SCI decreases colon length, which is ameliorated by microbe-based treatments.

A, SCI decreases colon length relative to sham controls, but not when mice receive inuin treatment. **B**, TRB treatment leads to increased colon length relative to SCI-veh controls. **C**, Treatment with *B. thetaiotaomicron* leads to increased colon length relative to SCI-veh controls. N=16-22 (**A**), N=8-10 (**B**), N=9-11 (**C**). Each circle represents an individual mouse. All measurements were taken two weeks after surgery. * $P < 0.05$, ** $P < 0.01$. Data are shown as mean ± SEM and compared by one-way ANOVA with post-hoc Tukey's test (**A**) or post-hoc Dunnett's test (**B, C**).

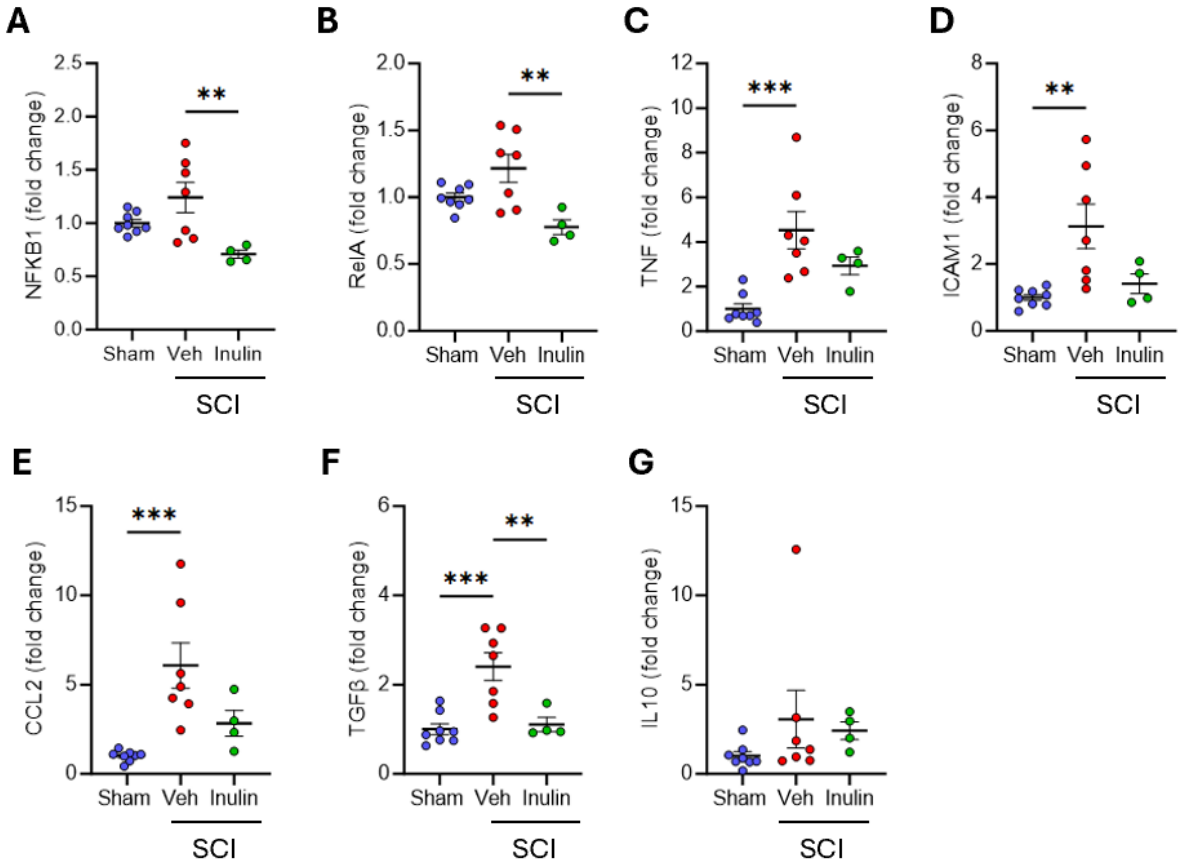


Figure 4.6. Inulin reduces transcription of SCI-induced inflammatory genes in spinal cord.

Inflammation associated transcriptional changes in the spinal cords of mice, as evaluated by qPCR. **A-G**, Genes evaluated include NFKB1 (**A**), RelA (**B**), TNF (**C**), ICAM1 (**D**), CCL2 (**E**), TGFβ (**F**), and IL10 (**G**). There were no significant changes in expression of IL10 in the spinal cord (**G**). Data displayed as fold change relative to sham, using the 2-ΔΔCT method (338), with GAPDH as a housekeeping gene. N=4-8. Each circle represents an individual mouse. All measurements were taken two weeks after surgery. ** $P < 0.01$, *** $P < 0.001$. Data are shown as mean ± SEM and compared by one-way ANOVA with post-hoc Tukey's test.

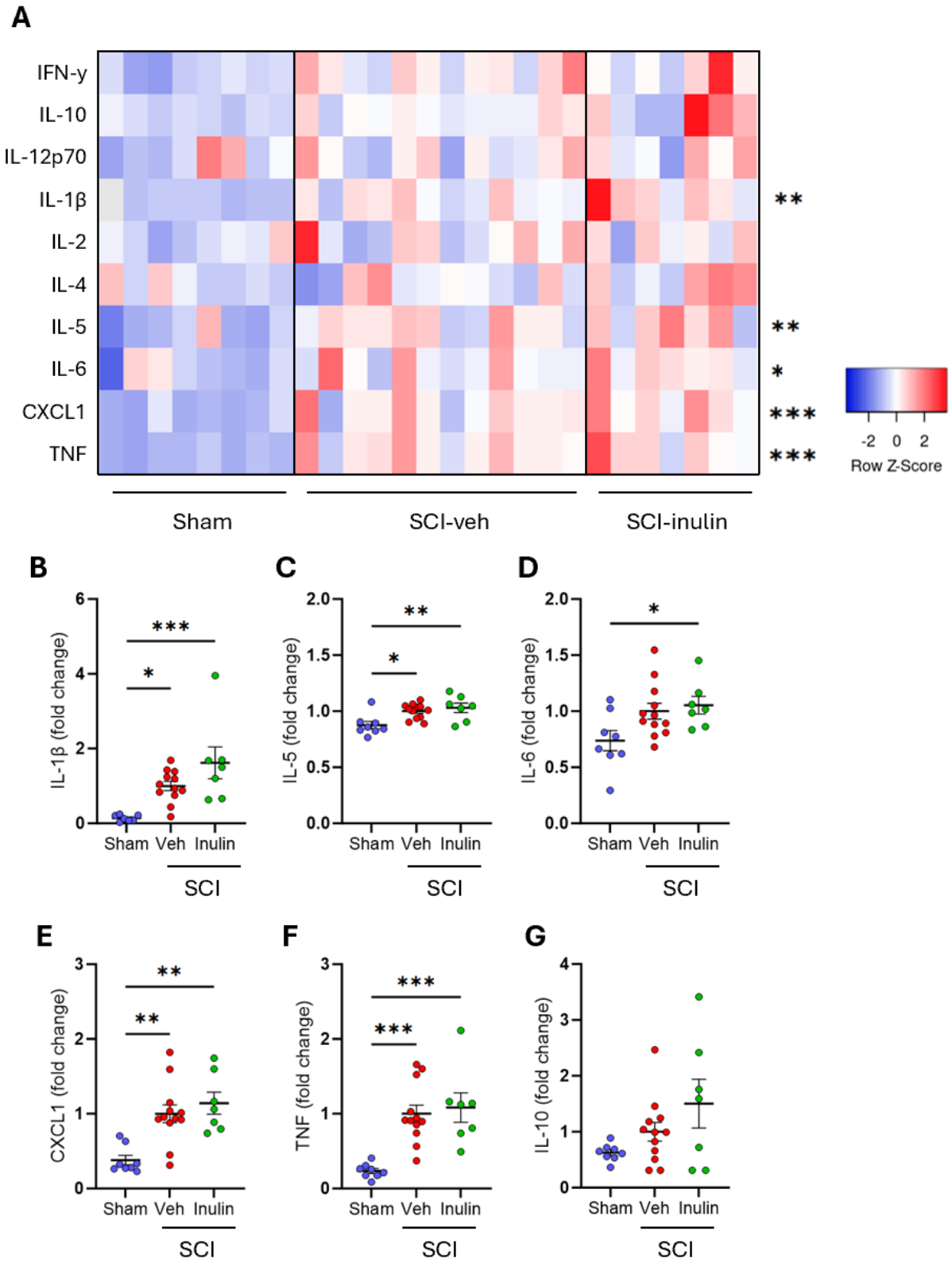


Figure 4.7. SCI increases inflammatory markers in the spinal cord regardless of treatment.

A, Heatmap of inflammatory markers, evaluated by multiplexed ELISA, in spinal cord tissue lysate of mice, two weeks after surgery. **B-F**, Regardless of treatment – vehicle or inulin – mice with SCI showed increased levels of IL-1β (**B**), IL-5 (**C**), IL-6 (**D**), CXCL1 (**E**), and TNF (**F**). IL-10 was not different between groups (**G**). N=7-12. Each circle represents an individual mouse. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are shown as mean ± SEM and compared by one-way ANOVA with post-hoc Tukey's test.

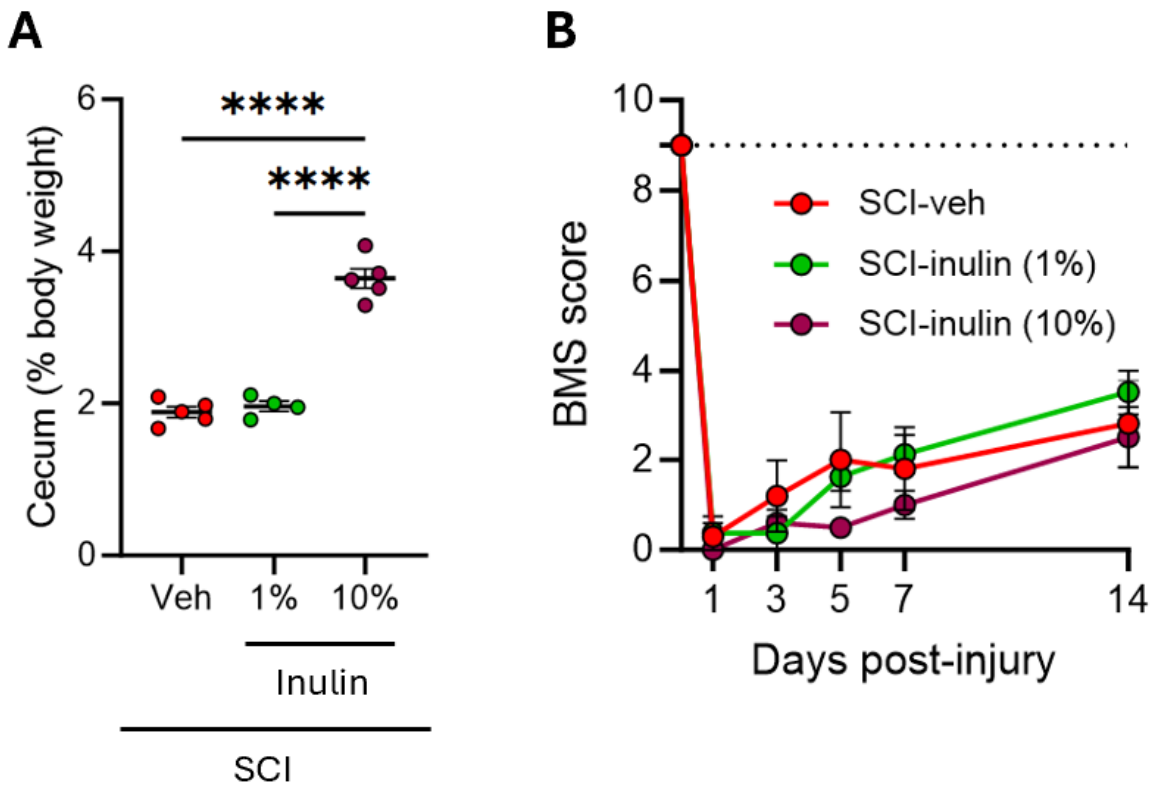


Figure 4.8. Higher dose of inulin increases cecum weight but does not improve BMS.

A, Cecum weights, as percent of total body weight, of vehicle-treated and inulin-treated mice two weeks after injury. **B**, Hindlimb locomotor score, as assessed by Basso mouse scale (BMS), with dashed line representing no deficits. N=4-5. Each circle represents an individual mouse in (**A**), or the average of all mice in a given treatment group in (**B**). **** $P < 0.0001$. Data shown as mean ± SEM and compared by one-way ANOVA with post-hoc Tukey's test for (**A**), or 2-way ANOVA with post-hoc Tukey's test for (**B**). Dashed line in (**B**) indicates maximum possible score for BMS of 9.

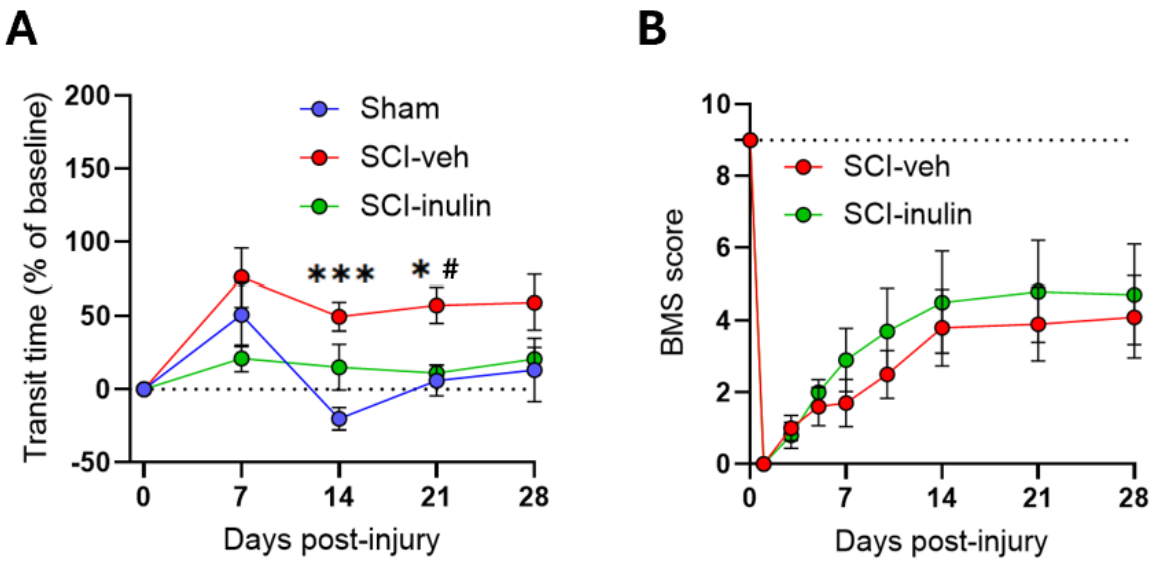


Figure 4.9. Beneficial outcomes associated with inulin-treatment may persist at more chronic stages of SCI.

A, Temporal changes in total gastrointestinal transit time, as percentage of pre-surgery baseline. **B**, Hindlimb locomotor score, as assessed by Basso mouse scale (BMS), with dashed line representing no deficits. N=5-7. Each circle represents the average of all mice in a given treatment group. Significant differences between sham and SCI-veh groups denoted by (*). Significant differences between SCI-veh and SCI-inulin denoted by (#). */# $P < 0.05$, *** $P < 0.001$. Data are shown as mean ± SEM and compared by 2-way ANOVA with post-hoc Tukey's test. Dashed line in (**A**) indicates baseline transit time. Dashed line in (**B**) indicates maximum possible score for BMS of 9.

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