EFFECTS OF TRAUMATIC BRAIN INJURY ON THE INTESTINAL TRACT AND GUT MICROBIOME

DISSERTATION

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ABSTRACT OF DISSERTATION

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Traumatic brain injury (TBI) initiates not only complex neurovascular and glial changes within the brain but also pathophysiological responses that extend beyond the central nervous system. The peripheral response to TBI has become an intensive area of research, as these systemic perturbations can induce dysfunction in multiple organ systems. As there are no approved therapeutics for TBI, it is imperative that we investigate the peripheral response to TBI to identify targets for future intervention. Of particular interest is the gastrointestinal (GI) system. Even in the absence of polytrauma, brain-injured individuals are at increased risk of suffering from GI-related morbidity and mortality. Symptoms such as intestinal dysmotility, inflammation, ulceration, and fecal incontinence can drastically diminish quality of life. The GI tract is inhabited by trillions of microbes that have been implicated as modulators of many neurological disorders. Clinical and preclinical studies implicate gut dysbiosis, a pathological imbalance in the normally symbiotic microbiota, as both a consequence of TBI as well as a contributing factor to brain damage. However, our understanding of this interplay is still limited. While relatively little is known about the effects of TBI on the structure and function of the GI tract, prior studies report that experimental TBI induces intestinal barrier dysfunction and morphological changes. To confirm these findings, male C57BL/6J mice underwent a sham control or a controlled cortical impact (CCI) procedure to induce a contusive brain injury, and intestinal permeability was assessed at 4 h, 8 h, 1 d, and 3 d post-injury. An acute, transient increase in permeability was observed at 4 h after CCI. Histological analyses of the ileum and colon at multiple time points from 4 h to 4 wks revealed no overt morphological changes, suggesting that CCI induced a short-lived physiologic dysfunction without major structural alterations to the GI tract. As the microbiome is a modulator of GI physiology, we performed 16s gene sequencing on fecal samples collected prior to and over the first month after CCI or sham injury. Microbial community diversity was assessed using common metrics of alpha and beta diversity. Alpha diversity was lower in the CCI injury group and beta diversity differed among groups, although these effects were not observed in all metrics. Subsequent differential abundance analysis revealed that the phylum *Verrucomicrobiota* was increased in CCI mice at 1, 2, and 3 d post-injury when compared to sham mice. Subsequent qPCR identified the *Verrucomicrobiota* species as *Akkermansia muciniphila*, an obligate anaerobe that resides in and helps regulate the intestinal mucus layer and barrier. To determine whether TBI promotes changes to the GI tract favorable

for the proliferation of A. muciniphila, mucus-producing goblet cells and the level of GI hypoxia were evaluated. Goblet cell density in the medial colon was significantly increased at 1 d, while colon hypoxia was significantly increased at 3 d. Taken together, these studies show that CCI induces transient intestinal barrier dysfunction followed by increased goblet cell density and hypoxia in the colon with a concomitant increase in A. muciniphila that may suggest a compensatory response to systemic stress after TBI.

KEYWORDS: Traumatic Brain Injury, TBI, Gastrointestinal Tract, Neuroenteric Axis, Microbiome, Gut-Brain Axis

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To my family and friends for their unwavering support in everything I have done.

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CHAPTER 1: Introduction

1.1. Traumatic brain injury epidemiology and clinical assessments

Traumatic brain injury (TBI) is any external insult that results in alteration of normal brain function. This is most often described as a bump, blow, jolt, or penetrating injury to the head, but a TBI can also occur in the absence of a direct blow to the head/body as evidenced by blast-related brain injuries (CDC, 2006; Peterson, Xu, & Daugherty, 2019). The Center for Disease Control's most recent TBI surveillance report estimates that 2.87 million people suffer a TBI each year (Peterson et al., 2019). This number does not account for the numerous mild TBIs (mTBI) that are sustained where the individual never seeks medical care (Lumba-Brown et al., 2018). A large number of people live beyond their initial injury, as only a relatively small fraction of TBIs result in death in a given year (~60,000 or 2%) (Peterson et al., 2019). With an estimated $40.6 billion spent yearly, TBI is a major source of financial stress for the individual as well as their family and friends (Miller, DePadilla, & Xu, 2021). The most common causes of injury are unintentional falls, motor vehicle crashes, unintentionally being struck by/against an object, intentional self-harm, and assault (Peterson, Zhou, Thomas, & Daugherty, 2021).

With the wide variety of mechanisms that can lead to a TBI, it is of no surprise that there is a high level of heterogeneity in the types of brain damage that occur. Because each head injury is unique, as are the injured patients, the timing and extent of recovery can vary greatly. Clinically, head injury severity is commonly assessed using the Glasgow coma scale (GCS) (Teasdale & Jennett, 1974). The GCS is a 15-point scale based on a patient's eye, verbal, and motor responses to stimuli. Scores range from 3-15 with the lowest score of 3 representing a patient that is unable to open their eyes or provide verbal or motor

responses. A score of 15, in contrast, would be assigned to an individual who opens their eyes spontaneously, provides oriented verbal responses, and can provide a motor response to commands (Jain & Iverson, 2023). The scale is often stratified to differentiate mild (GSC 13-15), moderate (GSC 9-12), and severe (GSC 3-8) injuries. However, these classifications are based on symptomology rather than injury mechanism. GCS scores are most informative as an assessment of consciousness while also being informative as a prognostic for mortality after severe TBI, as unconscious severe TBI patients are 3 times more likely to die than those that are conscious (Grote, Böcker, Mutschler, Bouillon, & Lefering, 2011). While the GCS has prognostic value, it provides limited insight into the pathoanatomical features of TBI or the progression of damage (Saatman et al., 2008). As such, reliance on the GCS has been widely criticized as lacking appropriate nuance, especially as an inclusion criterion for clinical trials. Therefore, it is best utilized in concert with imaging techniques as well as other assessments (Demetriades et al., 2006; Fulkerson et al., 2015).

Clinically, imaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI) provide insights into the nature of brain injury including the location and extent of pathologies such as hemorrhage, edema, contusion and white matter damage. Given that CT scans are relatively quick compared to MRI, most patients undergo a CT scan to provide an initial assessment. Specifically, hemorrhage is a major concern immediately after TBI, as it is a predictor of mortality and may be cause for surgical intervention (Perel et al., 2009). Serial imaging provides information on the early progression of damage as well as on chronic pathologies such as white matter thinning, regional atrophy including that of the hippocampus (Maxwell et al., 2003), and ventricular

dilation (Bigler & Maxwell, 2011). Importantly, neuroimaging findings correspond well with post-mortem tissue pathology, although they lack the resolution to observe cellular changes within the brain (Bigler & Maxwell, 2011; Maxwell et al., 2003).

While some individuals make a full recovery, approximately 43-66% of brain injury survivors report that they live with long-term changes, deficits, and dysfunction resulting from their injury (Ruet et al., 2019; Selassie et al., 2008a). Brain-injured individuals often live with cognitive impairments associated with memory, attention, processing speed, and executive dysfunction (Rabinowitz & Levin, 2014) and are at increased likelihood of developing conditions like epilepsy (Annegers, Hauser, Coan, & Rocca, 1998), Alzheimer's disease, and other neurodegenerative diseases (Lye & Shores, 2000). It is important to note that there are numerous socioeconomic considerations for how well individuals recover from TBI, but this is beyond the scope of the presented research (Daugherty, Sarmiento, Waltzman, & Xu, 2022).

1.2. Systemic complications associated with TBI

It has long been recognized clinically that multiple organ systems are altered after brain injury. Appreciation for the peripheral effects of TBI has been slower to develop in basic science studies. Given the overarching input that the brain provides to the rest of the body, it is not surprising that TBI leads to far reaching effects that extend beyond the central nervous system (CNS). Brain-injured individuals that survive the acute period after TBI have, on average, a 6-7 yr lower life expectancy than the standard population (Harrison-Felix, Whiteneck, DeVivo, Hammond, & Jha, 2004; Ventura et al., 2010). Indeed, those living with a TBI have an increased likelihood of developing serious medical comorbidities irrespective of the severity of the TBI (Izzy et al., 2022; Izzy et al., 2021). The development

of neurological and psychiatric conditions, such as depression, anxiety, and post-traumatic stress disorder, after TBI is well established (Whelan-Goodinson, Ponsford, Johnston, & Grant, 2009), but TBI survivors are also at increased risk of death due to seemingly unrelated conditions such as cancer, sepsis, and cardiovascular, respiratory and digestive system diseases even years after their initial injury (Harrison-Felix, Whiteneck, Devivo, Hammond, & Jha, 2006; Ventura et al., 2010). The median onset of these comorbidities is approximately 3.5 years after injury, with people that sustained their TBI between 18-40 years old being at the highest risk of developing post-TBI comorbidities (Izzy et al., 2022). Notably, this study found that brain-injured individuals were at increased risk for conditions that have multi-system consequences including diabetes, hypertension, and other neuroendocrine diseases. This suggests that brain injury serves as a major stressor across systems and its pathogenesis is not limited to the CNS.

One system that is of particular interest is the gastrointestinal (GI) tract because it mediates the intake of nutrients that are vitally needed after brain injury (Chapple et al., 2016; Chiang et al., 2012; Wang et al., 2013) while also being a major immune interface with the outside world. Brain-injured individuals are 2.5-2.82 times more likely to die due to digestive system diseases than people without TBI (Harrison-Felix et al., 2006; Ventura et al., 2010). The most common GI symptoms that TBI survivors face are feeding intolerance and dysmotility (Kao, ChangLai, Chieng, & Yen, 1998; Norton et al., 1988). Enteral feeding intolerance, or reflux of gastric contents, is most common during the acute phase following a moderate or severe TBI and accidental aspiration of gastric contents is a contributor to the prevalence of ventilator-associated pneumonia after TBI (Norton et al., 1988; Saxe, Ledgerwood, Lucas, & Lucas, 1994). Brain-injured individuals tend to have

reduced transit time (i.e. diarrhea) early after their injury followed by delayed transit time later on (Ott et al., 1991; Vieira, Pedrosa, Souza, Paula, & Rocha, 2018). Additionally, GI permeability is increased following TBI in a severity dependent manner (Faries, Simon, Martella, Lee, & Machiedo, 1998). This is especially problematic given the trillions of microbes that live within the GI tract, collectively known as the gut microbiota (Huttenhower et al., 2012). Normally, these microbes are sequestered within the GI tract and live in a symbiotic manner with their host. When barrier function is impaired, gut microbes and related pro-inflammatory factors (e.g. lipopolysaccharide; LPS) from these microbes are able to enter the bloodstream and can lead to a massive immune response and sepsis (Assimakopoulos et al., 2018; Miller, Keskey, & Alverdy, 2021). Gut leakiness is therefore believed to contribute to systemic and CNS inflammation after TBI (Sundman, Chen, Subbian, & Chou, 2017) and may underlie the increased incidence of sepsis after TBI (Selassie, Fakhry, & Ford, 2011).

Despite the multi-organ dysfunction that occurs after TBI, the vast majority of preclinical research has taken a brain-centric approach to understanding the cellular and molecular mechanisms of TBI and testing therapeutic interventions with the goal of mitigating secondary damage to the brain. To provide context for subsequent discussion of the scientific literature focused on the gut-brain axis in TBI, major mechanisms of secondary injury within the brain will be reviewed and the common experimental TBI models will be introduced.

1.3. Primary and secondary injury mechanisms in the brain

Brain injury is often thought of as occurring in two different phases: a primary injury due to the initial insult and a secondary injury process that evolves at a cellular and

molecular level as a result of the primary injury. These secondary injury cascades are triggered by the primary insult but can develop over time out to years after injury (McKee & Daneshvar, 2015).

The primary insult causes rapid deformation of the cells within the brain, leading to cell membrane disruption and loss of membrane potential. This rapid neuronal depolarization leads to uncontrolled release of the neurotransmitter glutamate. In a process termed excitotoxicity, excessive glutamate receptor activation in post-synaptic cells allows calcium influx and overload in the cells near the site of injury. Calcium overload contributes to cytoskeletal degradation, mitochondrial dysfunction and increased reactive oxygen species (ROS) that can cause rapid lipid peroxidation that further damages the cell membrane, ultimately leading to cell death.

Uncontrolled cell death leads to the release of damage-associated molecular patterns (DAMPs; Ransohoff & Brown, 2012; Sheikh et al., 2009; Uchida, 2013). Recognition of these DAMPs by nearby cells sets off a response that leads to the release of inflammatory cytokines and chemokines which promote blood-brain barrier (BBB) permeabilization. Breakdown of the BBB, together with chemokine gradients, promotes the infiltration of peripheral neutrophils at the site of injury (Clark, Schiding, Kaczorowski, Marion, & Kochanek, 1994). Neutrophils have historically been associated with debris clearance and an increase in ROS production that triggers apoptosis in cells surrounding the site of injury, thereby contributing to secondary injury progression. Recent research suggests that their role after TBI is more complicated than previously believed (Jin et al., 2023). Additional immune cell populations enter the brain within days of the injury as part of the adaptive immune response, including T-cells, natural killer cells, dendritic cells, and

peripheral macrophages. While their infiltration may promote wound healing, these cells can persist for long periods of time after the injury and contribute to secondary injury (Simon et al., 2017).

While this complex immune response is occurring, immunocompetent cells within the brain, namely astrocytes and microglia, also respond to the injury. Astrocyte and microglial activation are well established histological hallmarks of TBI. Both glial cell types release cytokines acutely to promote immune cell trafficking to the site of injury. Astrocyte reactivity is marked by graduated hypertrophy and upregulation of glial fibrillary acidic protein (GFAP) in cells around the site of injury (Burda & Sofroniew, 2014). In focal TBI, astrocytes can proliferate and form an astroglial scar around the injured area that sequesters the wounded area to limit the spread of toxic neurochemicals but is also inhibitory to axonal regrowth (Villapol, Byrnes, & Symes, 2014). GFAP detected in the blood is considered to be a potential biomarker of TBI severity and prognosis (Abdelhak et al., 2022).

Microglia are the brain's resident macrophage population. Under healthy conditions they survey the brain parenchyma for evidence of damage, infection, or other inflammatory mediators. Following injury, microglia switch from a relatively passive role to an active one. In response to injury, they alter their morphology allowing them to move and phagocytose debris to clear the injury site. This is a necessary part of injury resolution, but these microglia exist across a gradient of activation states, mediating anti-inflammatory or pro-inflammatory roles (Woodburn, Bollinger, & Wohleb, 2021). Pro-inflammatory microglia can remain chronically activated after TBI, maintaining neuroinflammation for years after injury (Simon et al., 2017).

DAMPs released during primary and secondary injury processes are able to enter into systemic circulation after TBI and mediate immunosuppression and peripheral organ dysfunction (Vourc'h, Roquilly, & Asehnoune, 2018). Indeed, brain injury can lead to widespread peripheral dysfunction involving the peripheral nervous, endocrine, cardiovascular, pulmonary, and gastrointestinal (GI) systems. This will be further discussed in Section 1.5.

1.4. Overview of experimental models

While human TBI is heterogeneous, this level of variability does not lend itself well to scientific manipulation. As such, experimental models are often developed to recapitulate selected aspects of human TBI pathology, but all share a goal of providing reliable and reproducible injuries. Ideally, a model that produces clinically relevant pathology will provide a platform for testing therapeutic manipulations yielding results that are translationally relevant to human TBI. While reductionist models, such as cell culture, have been utilized to demonstrate a variety of cellular and molecular mechanisms of trauma, they are unable to account for multi-system responses to a CNS insult. This is especially important in the current context, as the purpose of the present dissertation is to identify changes to the GI tract after TBI. Within the following section, rodent models of TBI will be reviewed to provide a brief overview of the models commonly used in the field that have been utilized to study the effects of TBI on the GI tract or microbiome. Each model will be introduced with some background information followed by a generalized procedure. All TBI research presented within this dissertation was conducted using a controlled cortical impact (CCI) model of TBI and, as such, this model will be addressed first.

1.4.1. Controlled cortical impact

CCI produces a TBI using a pneumatically (Figure 1.1) or electromagnetically (Figure 1.2) driven piston to impact the exposed cortical tissue of the brain to produce a contusion injury (Brody et al., 2007). This model was first developed for use in ferrets (Lighthall, 1988) before being adapted for use in rats (Dixon, Clifton, Lighthall, Yaghmai, & Hayes, 1991) and mice (Smith et al., 1995). The CCI model allows for many different aspects of the primary injury to be manipulated. For example, the severity of injury can be modulated by altering the velocity and depth of impact allowing for mild, moderate, and severe contusion injuries (Washington et al., 2012; Yu et al., 2009). This is important because severity of injury corresponds with the likelihood of GI complications after TBI in people (Faries et al., 1998). Other factors that can be controlled include location of impact, dwell time at extension, and impactor tip size and shape (Osier, Carlson, DeSana, & Dixon, 2015; Pleasant et al., 2011).

CCI replicates many histological aspects of brain injury that have also been identified in post-mortem samples from brain-injured humans. CCI results in a cortical contusion at the site of impact with progressive tissue loss associated with cell death due to necrosis and apoptosis at and beyond the site of injury (Dixon et al., 1999). Additionally, CCI results in BBB disruption, intraparenchymal hemorrhage, axonal injury, glial reactivity, inflammation, and oxidative stress (Osier et al., 2015). Motor changes are present after CCI, but often require specific, sensitive outcome measures to assess, especially in milder versions of CCI. The CCI model also recapitulates neurocognitive changes similar to those seen in humans, including impaired memory and learning (Dixon et al., 1999; Zhao, Loane, Murray, Stoica, & Faden, 2012).

Because of the flexibility of the CCI device, additional variations of the model have been developed to alter pathology. One of the more common modifications is the use of bilateral craniotomies to accentuate axonal injury (Meaney et al., 1994). Others have altered the location of the craniotomy to impact the brain at different sites as a means of modeling TBI to different brain regions, such as the forebrain. Deficits will vary based on the area of the brain injured (Mao et al., 2010).

1.4.1.1. Generalized procedure: CCI

The animal is anesthetized, and a scalp incision is performed. The skin is retracted to expose the skull. A craniotomy is then performed to remove a portion of the skull to expose the intact dura of the brain. Traditionally, the craniotomy is performed laterally between the bregma and lambda sutures, but this can be altered depending upon the researchers' desired impact site. The impactor is then lowered onto the brain until it contacts the dura to provide a "zero point." The impactor tip is then retracted, and the depth adjusted so that when the impactor tip strikes the brain it will rapidly depress the brain to the desired depth. The craniotomy may be left open or covered in some manner before closing the incision.

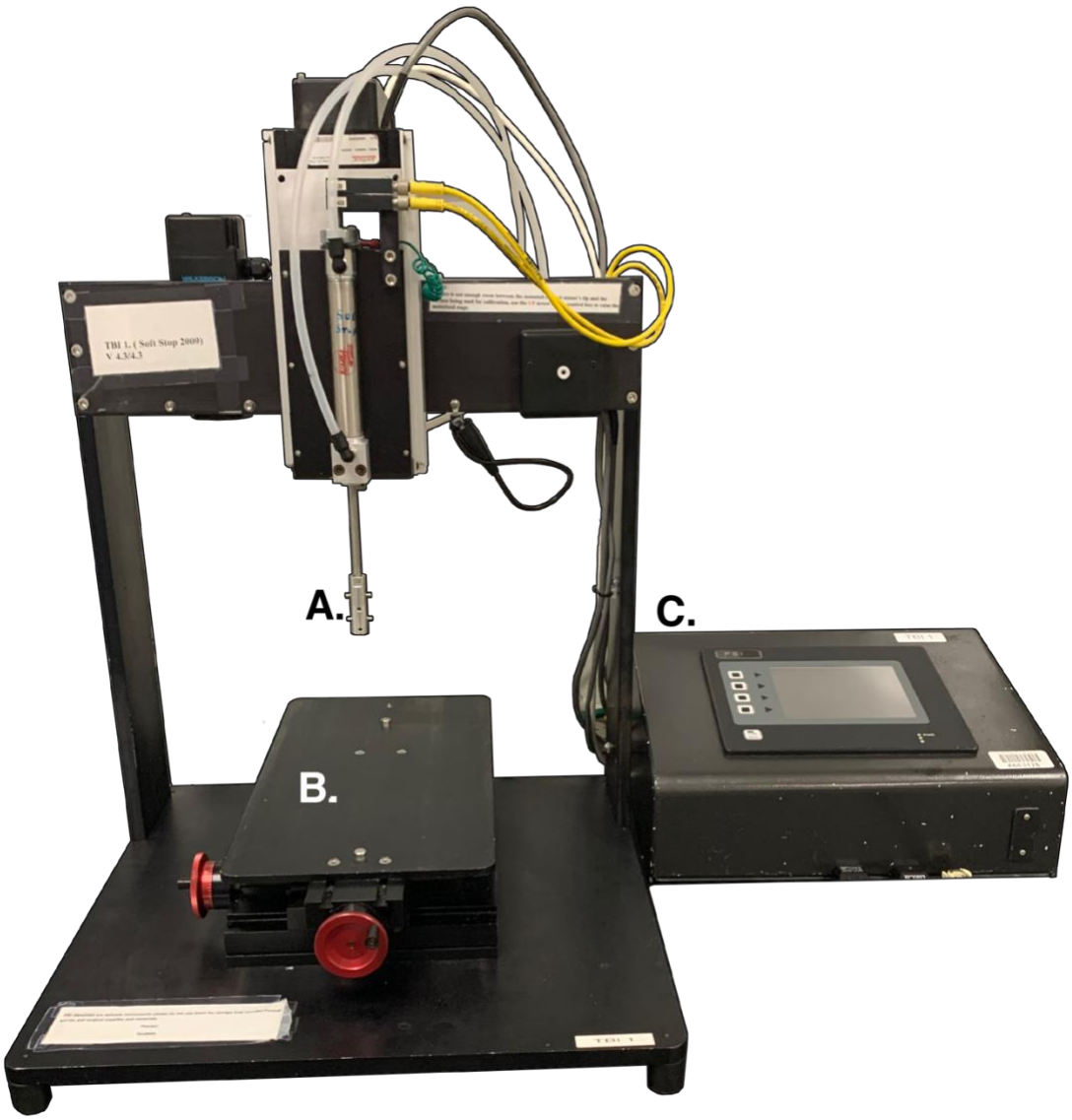

Figure 1.1: Pneumatic Controlled Cortical Impact Device

An impactor tip is attached to **(A)** the tip holder, and a stereotactic frame containing the anesthetized animal is placed upon **(B)** the stereotactic frame mount allowing for adjustments in the X and Y planes. **(C)** The controller box allows the researcher to set the impactor's speed, depth, and dwell-time. Not pictured: The air compressor unit that drives the piston.

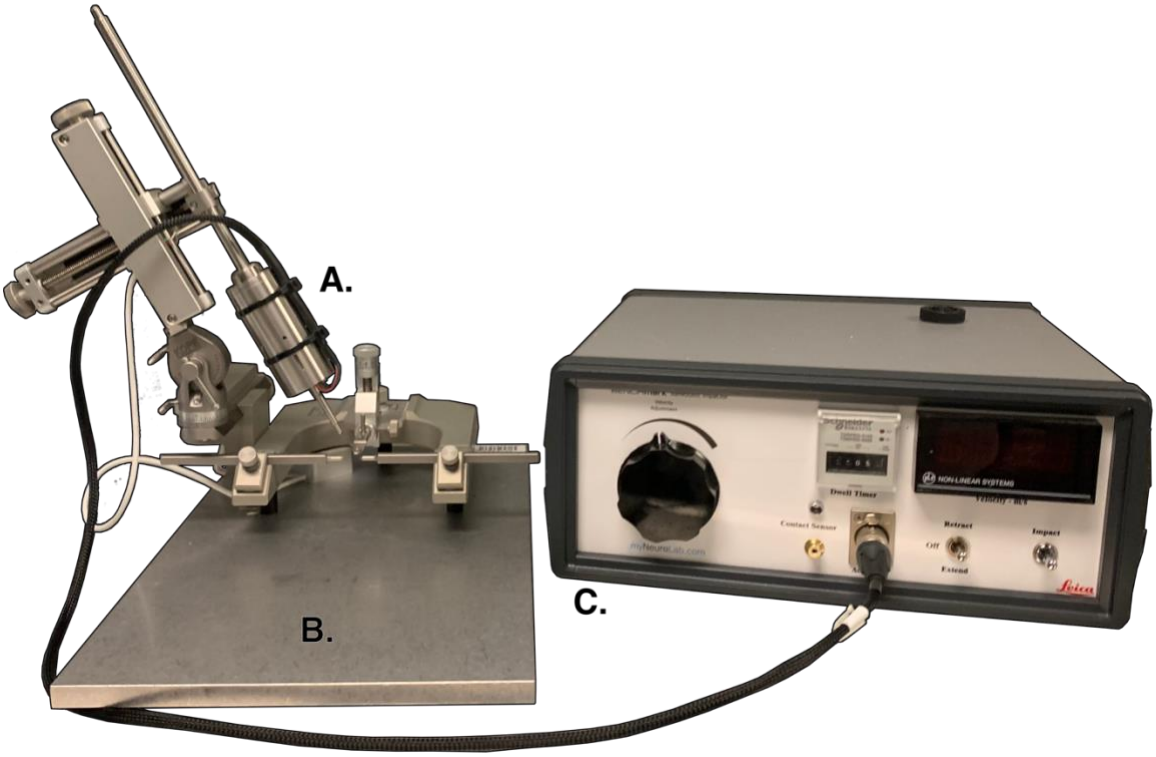

Figure 1.2: Electromagnetic CCI Device

(A) The electromagnetically driven impactor arm allows for adjustment of the X, Y, and Z planes and impact angle when attached to **(B)** the stereotactic frame where the anesthetized animal's head is affixed within the ear bars. **(C)** The controller box allows the researcher to set the impactor's speed, depth, and dwell-time.

1.4.2. Lateral fluid percussion injury

The fluid percussion injury (FPI; Figure 1.3) model of TBI was originally developed to study head injury in rabbits (Lindgren & Rinder, 1966) before eventually being translated for use in rats (Dixon et al., 1987; McIntosh et al., 1989) and mice (Carbonell, Maris, McCall, & Grady, 1998). The FPI device consists of a swinging hammer arm that impacts the end of a saline-filled tube in order to transmit a pulse wave along the length of the tube and into the cranial cavity of the animal attached to the other end of the tube. The pressurized saline bolus is rapidly injected into the cranial cavity through a sealed Luer-Loc mechanism that is glued to a craniotomy on the skull at the desired site of injury. Rapid introduction of the saline bolus increases intracranial pressure and displaces and deforms the brain. Severity is controlled by the height from which the hammer arm is released and thus by the pressure within the tube which is measured by a pressure transducer. Lateral FPI results in a foci of cortical neuron death that progresses to a necrotic cavity. The cortical contusion is accompanied by selective and hippocampal neuronal death, axonal injury in multiple white matter tracts, BBB disruption and glial reactivity. Chronically, progressive atrophy of the cortex, hippocampus and white matter structures mimics pathology seen in humans (Bigler & Maxwell, 2011; Bramlett & Dietrich, 2002; McIntosh et al., 1989; Smith et al., 1997). Lateral FPI is associated with deficits in motor and cognitive function and the extent of neurobehavioral impairment and histopathology scales with injury severity (Dixon et al., 1987; Lifshitz, Witgen, & Grady, 2007; McIntosh et al., 1989)

1.4.2.1. Generalized procedure: FPI

The animal is anesthetized, and a scalp incision is performed to expose the skull. A craniotomy is performed and a female Luer-Loc connector is affixed over the craniotomy site. The craniotomy can be placed anywhere on the skull, but it is most often placed in a lateral position above the parietal cortex (McIntosh et al., 1989). The Luer-Loc connector is then connected at the end of the plexiglass tube and the hammer pendulum is released to initiate the pressure wave that results in the FPI. The animal is removed from the device and monitored for apnea and righting reflex time (Alder, Fujioka, Lifshitz, Crockett, & Thakker-Varia, 2011). Once the righting response has returned, the animal is placed back under anesthesia so that the Luer-Loc connector can be removed. The animal is then allowed to recover from injury.

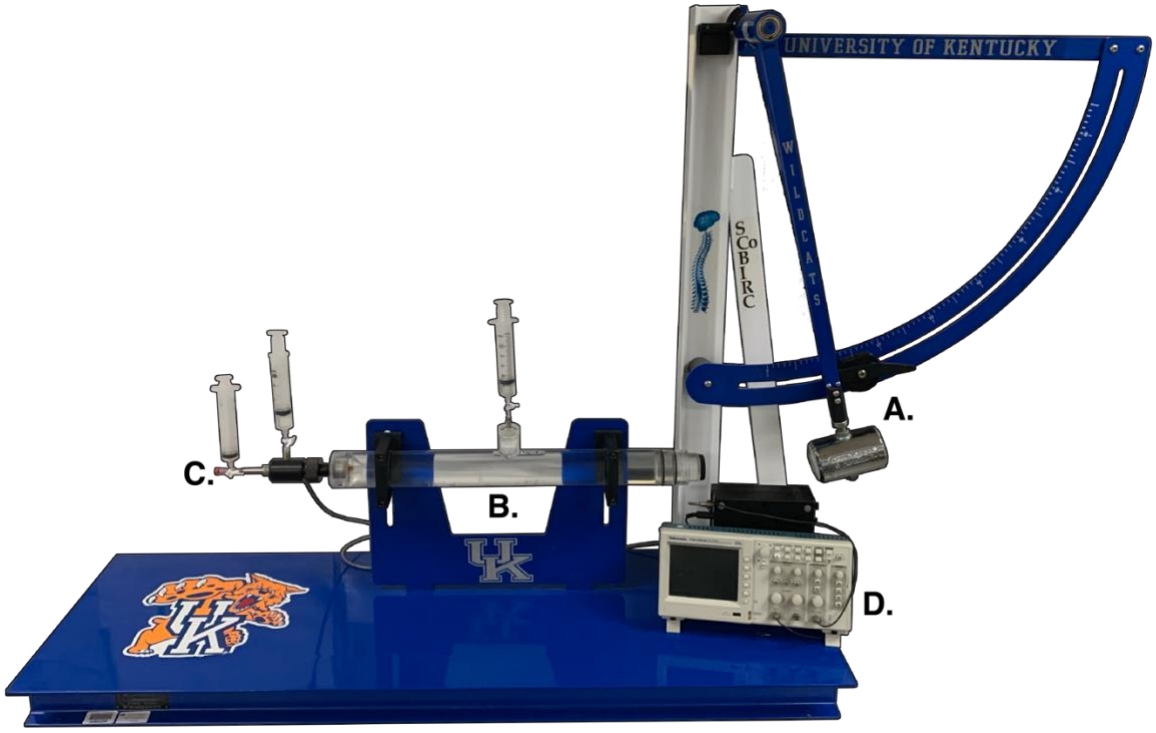

Figure 1.3: Fluid Percussion Injury Device

(A) Injury severity is determined by the height at which the hammer arm is raised to and then released from. The hammer arm impacts the plunger on the end of **(B)** the plexiglass tube and fluid within the tube transmits the pressure wave through the **(C)** Leur-Loc connector affixed to an anesthetized animal. **(D)** Pressure is monitored using an oscilloscope.

1.4.3. Weight drop injury

Weight drop injury (WDI; Figure 1.4) was first described by Feeney, Boyeson, Linn, Murray, and Dail (1981) as a focal contusion injury model in rats. A weight is dropped through an upright guide tube onto a footplate that is in contact with the exposed dura through a craniotomy. This footplate is held within a sleeve that limits the maximum distance that the impactor can travel into the brain. This model leads to similar pathology to that observed and described in CCI models. The WDI model was later adapted into a closed head model of diffuse brain injury where a metal disk, or "helmet", is affixed to the head to prevent skull fractures while also distributing the force of the impact more evenly across the head (Marmarou et al., 1994). With all varieties of the weight drop model, the severity of the injury is modulated by the mass of the weight being dropped and the height from which it is dropped. However, there can be a large level of variability in WDI models due to rebound of the weight after the initial impact (Ma, Aravind, Pfister, Chandra, & Haorah, 2019a).

Closed head models of WDI can be associated with skull fracture, necessitating exclusion of animals, and may require intubation and ventilation as a result of brainstem damage (Marmarou et al., 1994). Mortality is more common with closed head WDI than with CCI or open skull WDI. The complications and increased mortality in closed skull WDI limits most studies to the mild end of the TBI severity spectrum. Recently steps have been taken to increase the level of control over WDI models through the use of electronic monitoring and automation to better titrate the injury to prevent mortality (Clay & To, 2022). Other labs have utilized breakaway surfaces that allow the animal to drop on to a padded surface after the impact. This breakaway design prevents the rebound effect, minimizes mortality, and allows for repeated injuries (Kane et al., 2012; Khuman et al.,

2011). This also has the added benefit of mimicking aspects of acceleration/deceleration injury, but injury severity is still limited to mild TBI. Utilization of these breakaway WDI models has allowed for repetitive injury paradigms to be assessed and models some of the changes observed post-mortem tissue from athletes in contact sports (Kane et al., 2012).

1.4.3.1. Generalized procedure: WDI

WDI models induce a TBI by dropping a weight within a plexiglass guide tube that directs the weight to land on a prescribed location on an anesthetized animal's head. This can be done directly onto the scalp, the skull, onto a "helmet" or some other guard, or directly onto the exposed dura. Depending upon the WDI model being used, there may be a preparatory procedure to expose the dura through a craniotomy or to attach a guard to the animal's head. The anesthetized animal is placed on either a firm, soft, or breakaway surface underneath of the guide tube. With the animal's head either being free or in a fixed position, the guide tube is aligned over the intended site of impact. The weight is then positioned within the guide tube and dropped from the intended height. Upon reaching the end of the tube, the weight makes either direct or indirect contact with the animal. Frequently animals are assessed for their apnea time and righting reflex time. If needed, the animal's injury site is closed, and the animal is then allowed to regain consciousness.

Figure 1.4: Weight Drop Injury Device

Example of a closed head WDI device where an anesthetized animal would be **(A)** placed upon the foam pad in a prone position with its head positioned under the guide tube. **(B)** A weight within the guide tube is dropped from a prescribed height onto the animal's skull, scalp, or a helmet to produce a TBI.

1.4.4. Blast TBI models

Blast models are used to model concussive injuries caused by the pressure waves created by explosions such as those experienced by military personnel. Blast TBI can produce neurological deficits even without a loss of consciousness (Rosenfeld et al., 2013). These types of injuries are most problematic when people are exposed to multiple blasts over relatively short periods of time (Bryden, Tilghman, & Hinds, 2019). Determining an appropriate amount of recovery time between blast exposures has been of particular interest to the field (Bryden et al., 2019). Because the entire body is exposed to a blast wave, blast injury can be considered as polytrauma. Indeed, gas-containing organs such as the lungs and GI tract are especially vulnerable to primary injury due to explosive blast waves, but damage typically occurs when the explosion is in close vicinity to the individual (Ritenour et al., 2010). Animal models of blast TBI have been developed to study effects of whole-body blast exposure or of exposure isolated to the head. The latter is most useful to investigate the effects of blast-induced brain damage on the function of the GI system, without the direct effects of the blast wave on the gut, but little work has been done in this area (Baskin et al., 2023). Therefore, a detailed description of blast TBI model methodology will not be provided here.

1.4.5. Mild TBI models

Numerous variants of closed head models exist, adapted from CCI or WDI models, to produce injury by impacting the scalp or exposed, intact skull. Generally, these models are designed to replicate diffuse, mild TBI. Increasingly these are used to investigate multiple impacts to replicate the subconcussive blows experienced by athletes in contact sports (Bolton-Hall, Hubbard, & Saatman, 2019). There will not be a generalized procedure

provided for this section as mild TBI is most often modeled using variants of models already described above. For in depth descriptions and comparisons of closed head injury models, please refer to published reviews by Bodnar, Roberts, Higgins, and Bachstetter (2019) and Bolton-Hall et al. (2019).

1.4.6. Injury control groups

Commonly the control group for TBI models, referred to as a sham, consists of all parts of the procedure aside from the impact. As such, mice undergo the same anesthesia, preparation, craniotomy (if applicable), wound closure, and post-operative care procedures. Doing so helps to attribute any findings to the actual traumatic insult rather than other aspects of the surgical procedure. Some researchers have opted for the use of alternative control groups including, but not limited to, naïve mice, anesthesia controls, and the use of self-referential pre-injury baselines. One common criticism is that the craniotomy procedure in and of itself produces a mild brain injury. This inadvertent injury may lead to a low level of physiological response that could in turn make it more difficult identify between group differences (Cole et al., 2011). Naïve control mice can be useful as an additional control group especially when assessing behavioral paradigms (Cole et al., 2011). Anesthesia controls are of vital importance as different anesthetics have been shown to alter outcomes after brain injury (Statler et al., 2000; Thal et al., 2014).

1.5. Impact of TBI on peripheral systems

Peripheral dysfunction after TBI is posited to occur via the peripheral nervous system (PNS), through circulating (neuro)endocrine factors, and/or through immune

signaling. Given that all three are tightly interconnected, animal models of TBI allow researchers to identify mechanisms of multi-organ dysfunction as well as identify potential therapeutic targets in the periphery. In the following section, the effects of experimental TBI on these three systems will be discussed. This is intended as a broad overview to convey the far-reaching effects that TBI has on the body with special attention paid to the system of interest, the GI tract. This section is not intended to be an exhaustive review of the effects of TBI on the periphery and will focus on aspects that are interconnected with GI function.

1.5.1. Peripheral nervous system and neuroendocrine system

The PNS can broadly be split into two arms, the somatic nervous system and the autonomic nervous system (ANS). The somatic nervous system is involved in voluntary movement, reflex arcs that affect muscles, and sensation. The ANS regulates involuntary processes and can be further split into three branches: the sympathetic nervous system, the parasympathetic nervous system, and the enteric nervous system (ENS).

1.5.1.1. Somatic nervous system

Both afferent and efferent signaling through the PNS can be altered after TBI with severity and location of injury playing a role. Changes in somatic control over the skeletal musculature after TBI is most often associated with damage to regions of the brain associated with control over those muscles. Inability to exercise and/or walk normally can indirectly alter one's bowel activity (Iovino et al., 2013) but in regard to the GI tract, only the proximal and distal ends of the GI tract are under somatic control allowing for voluntary control of eating and defecation. Issues with swallowing (dysphagia) and fecal incontinence are especially problematic after severe brain injury (Foxx-Orenstein et al.,

2003; Lee et al., 2016). However, if dysphagia and fecal incontinence occur after experimental TBI, they have not been assessed to our knowledge. Dysphagia can lead to aspiration pneumonia which is a common consequence after clinical TBI. Aspiration pneumonia does not naturally occur after experimental TBI in rodents and needs to be induced to study preclinically (Doran et al., 2020). Rodents experience dysmotility after experimental TBI (Olsen et al., 2013; Wang, Liu, & Yang, 2011), but the involvement of the rectal sphincter has not been investigated.

1.5.1.2. Autonomic nervous system and hypothalamic-pituitary-adrenal axis

TBI is a major systemic stressor that leads to a rapid response from the sympathetic nervous system in coordination with the hypothalamic-pituitary-adrenal (HPA) axis. In response to a TBI, or any stressor, sympathetic neurons from the splanchnic nerve signal to the adrenal medulla to release the catecholamines norepinephrine and epinephrine into the blood stream. TBI is associated with a massive surge in circulating catecholamines that triggers the body's "fight-or-flight" response. Catecholamine levels and sympathetic tone remain elevated for up to 2 wks after human TBI (Schroeppel et al., 2019). Plasma epinephrine levels have been found to be elevated in rodents as early as 3 h post injury (Lang, Fu, Sun, Xi, & Chen, 2015), and norepinephrine levels after TBI are positively correlated with increasing severity as determined by GCS (Clifton, Ziegler, & Grossman, 1981). The hypothalamus responds to incoming catecholamine signaling by releasing corticotropin-releasing hormone (CRH) which in turn leads to the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland into the blood stream. ACTH stimulates the adrenal cortex to increase cortisol levels in the blood. Acutely after TBI, cortisol levels are increased in a severity-dependent manner and are an indicator

of prognosis, with persistent cortisol elevation associated with increased likelihood of death (Srivastava et al., 2022).

Sympathetic activation leads to increased blood pressure, heart rate and cardiac output while also reducing blood flow to "non-critical" organ systems such as the viscera, skin, and GI tract (Gregory & Smith, 2012; Shepherd & Riedel, 1988). Elevated sympathetic tone is associated with reduced blood flow to the intestinal tract as well as reduced motility (Jackson & Davidoff, 1989; Kao et al., 1998), and both reduced motility and blood flow have been observed after experimental TBI in rats (Olsen et al., 2013; Wang et al., 2011). Wang et al. (2011) reported increased intestinal transit time and reduced jejunal blood flow, as measured by laser doppler, at 12 h, 1 d, and 2 d after contusive WDI, and Olsen et al. (2013) found a downward trend in ileal contractility at 1 and 3 d after CCI with a significant reduction at 7 d post-injury compared to sham controls. Intestinal mucosal blood flow is resistant to sympathetic stimulation, allowing maintenance of normal blood flow longer than the muscle layer of the GI (Shepherd & Riedel, 1988).

One of the major neural links between the brain, the ANS/ENS and the GI tract is the vagus nerve. The vagus nerve originates in the brainstem and innervates several organs throughout the body including the lungs, heart, liver, stomach, pancreas, and intestinal tract (Prescott & Liberles, 2022). It innervates both the small and large intestine (Altschuler, Escardo, Lynn, & Miselis, 1993; Mei, 1983) and is primarily responsible for parasympathetic actions associated with "rest and digest" functions that counterbalance sympathetic signaling from spinal nerves. Vagal sensory afferents provide the CNS with vital information about the status of peripheral organ systems. TBI impairs several processes that are mediated by the vagus nerve including but not limited to: swallowing

and coughing, increasing the likelihood of ventilator-associated pneumonia (Li, Liu, Xiao, Song, & Wang, 2020); baroreceptor function, resulting in impaired control of blood pressure and cardiac function (Levy, Ng, Lipman, Zieske, & Nelson, 1966); and upper intestinal tract signaling, altering gastric emptying (Kao et al., 1998). Sensory vagal afferents innervate the small and large intestine terminating on sensory cells within the gut that are able to interact with the luminal microbiota and signal to the brain (Wang & Powley, 2007). However, it is not known if vagus-mediated dysfunction after TBI is due to direct vagal dysfunction or due to improper integration at the level of the brain.

The vagus nerve also indirectly modulates other systems by providing inhibitory signaling to sympathetic nerve ganglia. The vagus nerve innervates the celiac ganglion which contains the cell bodies for sympathetic neurons that innervate organs such as the upper small intestine, liver, and spleen. Removal of this indirect modulation via vagotomy prior to FPI in mice leads to an altered immune response after TBI (Newell-Rogers et al., 2022). Because of the number of vagal associated symptoms that present after TBI and are linked to loss of vagal tone, researchers have explored vagal stimulation as a potential treatment after TBI. This has shown promise in preclinical models of TBI in improving both central and peripheral outcomes (Bansal et al., 2012; Smith et al., 2005; Smith et al., 2006), but clinical efficacy has yet to be shown (Shi, Flanagan, & Samadani, 2013). Newer transcutaneous vagal stimulation techniques show potential, but require greater testing (Hakon et al., 2020).

1.5.1.3. Enteric nervous system

The third arm of the ANS, the ENS, is also altered after TBI, but our understanding of this is extremely limited (Hanscom, Loane, & Shea-Donohue, 2021b). The ENS is

indirectly connected to the CNS via the vagus nerve (Berthoud, Carlson, & Powley, 1991). While still under central control, the ENS is not dependent upon CNS input to maintain proper motility, mucosal blood flow, and mucosal barrier function, and it even maintains many of its reflex responses when isolated from the rest of the body (Furness, Johnson, Pompolo, & Bornstein, 1995). A major role of the ENS is to coordinate sensory input and motor outputs in order to propagate the luminal contents through the intestinal tract. Neurons of the ENS are enmeshed with peripheral neurons and form two major plexuses: the myenteric plexus and submucosal plexus (Hansen, 2003). The myenteric plexus is found between the circular and longitudinal smooth muscle layers of the GI tract and coordinates peristaltic movements indirectly through interstitial cells of Cajal which serve as pacemaker cells for the gut (Berthoud, Blackshaw, Brookes, & Grundy, 2004). The submucosal plexus, as its name suggests, innervates the submucosa and coordinates secretion and absorption. Within each plexus are both excitatory and inhibitory neurons that form ganglia that resemble beads on a string with each node being connected to pass information down the length of the GI tract (Brookes, Song, Ramsay, & Costa, 1995). Neuronal signaling is supported by cells similar to CNS astrocytes known as enteric glial cells (Seguella & Gulbransen, 2021). Enteric neurons also interface with local immune cells that allow for rapid responses to changes in luminal contents (Barajon et al., 2009). These interfaces allow the body to respond to the microbiota within GI tract.

It is not known whether altered gut motility following TBI is due to altered PNS input, changes to the ENS, or both. The number of interstitial cells of Cajal present is reduced within the ileum at 3 d after CCI in rats (An et al., 2023). Additionally, hypertrophy of enteric glial cells has been reported following CCI in mice (Ma et al., 2017). If these

astrocyte-like cells react in a similar fashion to those of the CNS then this would suggest damage to the enteric neurons that they support or, at the very least, a local pro-inflammatory environment.

1.5.2. Immune system

Beyond the localized immune response to TBI, there is a profound and long-term depression of global immune function. Systemic inflammation is sustained for months after mild, moderate, and severe TBI (Chaban et al., 2020; Di Battista, Churchill, Rhind, Richards, & Hutchison, 2019; Vijapur et al., 2021). This sustained inflammation can influence the function of other systems as well as cause robust impairment of the body's response to secondary insults such as infection (Sharma et al., 2019).

The GI tract is a large immune interface with the outside world. As such, the immune system continually monitors the luminal contents through dendritic and other antigen presenting cells found within the GI tract (Zheng, Liwinski, & Elinav, 2020). Virtually all cell types that interface with the lumen play some role in gut immunity. Several cell types communicate with nearby enteric and peripheral neurons to convey important information about the luminal contents to the CNS via peripheral nervous tissue (Barajon et al., 2009; Lai et al., 2020). This includes signaling coming from the gut microbiota, which are recognized as a major modulator of several inflammatory conditions both within the GI tract and beyond it. In fact, in stroke, which presents with similar pathology to TBI, there is evidence of direct trafficking of a gamma-delta T-cells from the intestinal tract to the meninges (Benakis et al., 2016). Hence, the extensive cross-talk between the gut microbiota, intestinal epithelium, immune cells, PNS and CNS is likely to influence both GI and brain pathology after TBI.

1.5.3. Gastrointestinal system

1.5.3.1. Intestinal permeability

Clinically, TBI has been found to cause changes in GI tract function, and many of these findings have been recapitulated in animal models. Through the use of preclinical models, researchers have been able to further investigate the pathophysiology of the GI tract after TBI. It is well established that models of moderate-severe TBI induce intestinal permeability, but the timing and persistence of this appears to vary by study as well as the means of assessing permeability (Table 1.1). Microbial components can pass through the intestinal tract's epithelium either transcellularly, paracellularly, or through gaps created by cell death or damage. Transcytosis is an active process that requires luminal contents to bind to receptors and interact with lipid rafts or vesicle-forming pits to enter the epithelial cell and be passed through (Ménard, Cerf-Bensussan, & Heyman, 2010). While there are specific pathogens that enter the blood stream in this manner, they are not generally what is assessed when determining intestinal permeability (Camilleri, 2019). Functional assays of intestinal permeability typically assess the ability of an inert molecule of a known molecular weight to pass through the epithelial layer by paracellular movement or uncontrolled passage through regions of damage (Camilleri, 2019). This type of "leakiness" is of particular importance as it can be indicative of inflamed or damaged tissue that may allow for the passage of pathogens and inflammatory molecules from the intestinal lumen to interact with sub-epithelial tissue and potentially pass into the blood stream (Turner, 2009). One common inflammatory molecule that is of concern is LPS, a component of the outer membrane of Gram-negative bacteria (Płóciennikowska, Hromada-

Judycka, Borzęcka, & Kwiatkowska, 2015). In fact, introduction of this molecule into an animal is often used as a general inflammatory challenge (Fenn et al., 2014).

Researchers have utilized several means of assessing intestinal permeability (Table 1.1 and 1.2). The lactulose and mannitol assay is a common means of assessing permeability *in vivo* in both experimental and clinical settings. Both sugars are administered orally and the ratio of lactulose to mannitol excreted in the urine provides information about how readily the two molecules were able to pass through the intestinal tract. Mannitol is a smaller molecule (182 Da) believed to be able to readily pass through the gut barrier while the larger lactulose (342 Da) was believed to only be able to pass through sites of damage or tight junction disintegrity. While its validity and role as a prognostic have recently come into question, it is still utilized (Camilleri, 2019). Another common means of assessing "gut leakiness" is through the administration of fluorescein isothiocyanate (FITC) labeled dextran (typically 4 kDa) and measurement of how much passes through the intestinal tract and enters the blood stream (Gupta & Nebreda, 2014). FITC dextran can be either administered orally by gavage or directly injected to an isolated segment of the intestinal tract. To our knowledge this assessment is only utilized in a preclinical setting. Permeability can also be assessed directly using *ex vivo* sections of the intestinal tract. Most commonly this is done by measuring transepithelial electrical resistance (TEER) or the flux of FITC dextran or mannitol across the epithelium.

In rats, contusive WDI induces intestinal permeability, as tested by lactulose and mannitol assay, at 12 h, 1 d, 3 d, and 1 wk post-injury (Hang, Shi, Li, Wu, & Yin, 2003; Zhang & Jiang, 2015). Increased permeability as early as 3 h after WDI was confirmed by FITC dextran injected directly into the ileal lumen (Lang et al., 2015). This suggests that

in WDI models of severe TBI, intestinal permeability presents within hours after injury and is sustained out to at least 1 wk post-injury. Adding to this, in an *ex vivo* prep, Feighery et al. (2008) found increased mannitol flux across the ileum but not the colon at 6-8 h after a moderate frontal lobe CCI but did not find evidence of altered TEER in the same tissue, suggesting different permeability assays may have varying levels of sensitivity. Notably, Mazarati, Medel-Matus, Shin, Jacobs, and Sankar (2021) found that not all rats that underwent a lateral FPI presented with increased intestinal permeability, but that if intestinal permeability presents after TBI, the timing of when it happens may make a difference. Rats with increased permeability at 1 wk post-injury were less likely to develop post-traumatic epilepsy than rats that had elevated intestinal permeability at 7 mo post-injury, suggesting that intestinal permeability may play a role in the development of chronic sequelae to TBI. In three separate studies from the same group, mice subjected to a contusive WDI showed increased permeability at 6 h post-injury following the direct injection of FITC dextran into the isolated ileum (Bansal et al., 2009; Bansal et al., 2010a; Bansal et al., 2010c). However, when assessing permeability in *ex vivo* colon preps after moderate-severe CCI, Ma et al. (2017) found no evidence of increased TEER or paracellular flux at 1 d but did find elevated paracellular flux at 4 wks post-injury. Given that most of the rat and mouse studies that identified early permeability assessed it in the ileum suggests that the distal small intestine may be more vulnerable to TBI-induced permeability while barrier dysfunction in the colon may take longer to develop.

Several other groups have either indirectly assessed permeability using biomarkers and/or tissue assays assessing the tight junctions that mediate paracellular permeability (Table 1.2). Of the studies that utilized blood biomarkers of intestinal permeability, most

assays suggest increased permeability as early as 3 h post-injury and persisting up to 2 wk post-injury (Hang et al., 2003; Li et al., 2018; Ma et al., 2019b; Yang et al., 2022). These biomarkers are useful, but do not provide any information on what part of the GI tract may be affected. Assessment of tight junctions in specific tissue allows for regional determinations of likely barrier breakdown; however, without additional measurements of permeability it is difficult to confirm permeability solely on tight junction protein levels (Camilleri, 2019). Using western blotting, tight junction proteins were shown to be reduced in the ileum from 3-12 h after contusive WDI (Lang et al., 2015), while electron microscopy revealed no tight junction changes at 12 h or 1 d, but delayed disruption at 3 d and 1 wk post-injury (Hang et al., 2003). In single time point studies, tight junction immunolabeling was reduced in the cecum at 3 d after moderate-severe CCI (Simon et al., 2020) and in the proximal colon a decrease was observed by western blotting and immunohistochemistry at 2 wks after lateral contusive WDI (Li et al., 2018).

Intestinal permeability commonly coincides with increased inflammation within the GI tract. This has been observed in multiple studies, primarily assessing the small intestine. The most commonly assessed inflammatory cytokine is tumor necrosis factor-α (TNFα) which is elevated in the ileum as early as 2-6 h in mice, 3-12 h in rats, and as late as 5 d in rats after TBI (Bansal et al., 2010c; Feng et al., 2007; Lang et al., 2015). TNFα is of particular interest as it is a key mediator of tight junction permeability via the nuclear factor-κB (NF-κB) signaling cascade to induce transcription of myosin light chain kinase (MLCK) in epithelial cells (Al-Sadi, Guo, Ye, Rawat, & Ma, 2016). Cytokines interleukin (IL-) 1β and IL-6 have also been identified as contributors to barrier disruption. All three of these cytokines were found to be increased in the ileum of rats at 5 d post-WDI (Feng et

al., 2007) while IL-6 was also found to be elevated in the proximal colon at 2 wk post-WDI in mice (Li et al., 2018)

Citation	Species (Origin)	TBI Model	TBI Model Parameters	Time Post-Injury	Method of Assessment	Findings
Bansal et al. (2009); (2010a; 2010c)	BALB/c (Jackson)	Lateral WDI w/ craniotomy	250g rod dropped from 2cm	6h	Assessment of isolated distal ileum where 4kDa FITC-dextran was injected into distal lumen and then returned to the abdominal cavity. 30min post-injection cardiac blood was collected and plasma fluorescence measured	↑ [FITC dextran] in plasma at 6h post TBI (30min post FITC injection into terminal ileum
Ma et al. (2017)	C57BL/6t mice (Taconic)	Lateral CCI (pneumatic)	3.5mm diameter tip, 2mm depth, 6m/s velocity	1d, 4wks	*Ex vivo* prep of mounted colonic mucosa: TEER of colon tissue stripped of muscle	No ∆TEER from Sham at either timepoint
					Ex vivo prep of mounted colonic mucosa: movement of 3kDa dextran across colon	No ∆Paracellular flux at 24h but ~250% increase at 28d compared to sham
Hang et al. (2003)	Wistar Rats (ACCAS)	Feeney's Lateral WDI w/ craniotomy	4mm diameter, 5mm depth	12h, 1d, 3d, 7d (shams only from 3d)	Lactulose/mannitol permeability (urine lactulose/mannitol ratio)	↑ permeability as per increased lactulose/mannitol ratio at 12h, 1d, 3d, and 1wk vs control (3d sham) w/ peak at 3dpi
Lang et al. (2015)	Wistar Rats (ACCAS)	Lateral WDI w/ craniotomy	40g weight dropped from 25cm	3h, 6h, 12h	Assessment of isolated jejunum where 4kDa FITC-dextran was injected into distal lumen and then returned to the abdominal cavity. 30min post injection cardiac blood was collected and plasma fluorescence measured	↑ [FITC dextran] at 3h, 6h, and 12h compared to sham
Feighery et al. (2008)	Sprague-Dawley Rats (Harlan Labs)	Terminal Frontal lobe, lateral CCI	3.5mm round tip, 2.62mm depth, 1.2m/s velocity	6-8h	*Ex vivo* prep of mounted ileum and colonic mucosa: TEER, potential difference, and short circuit current of colon tissue stripped of muscle	No ∆ in paracellular flux across the ileum or colon, but there was a 30% reduction in short circuit current in the ileum when measured in the presence of Veratridine
					Ex vivo prep of mounted ileum and colonic mucosa: mannitol flux across mucosa	↑ mannitol flux across ileum (not colon) compared to sham mice
Zhang and Jiang (2015)	Sprague-Dawley Rats (SLAC)	Lateral WDI w/ craniotomy	20g weight dropped from 30cm	1wk	Lactulose/mannitol permeability (urine lactulose/mannitol ratio)	↑ permeability at 1wk vs naïve control
Mazarati et al. (2021)	Sprague-Dawley Rats (Charles River)	Lateral FPI	2atm pressure	1wk Pre-FPI, 1wk, & 7mo	*In vivo* 4kDa FITC Dextran gavage 4hr prior to euthanasia + measure plasma fluorescence from tail vein blood (non-terminal)	Plasma [FITC dextran] negatively correlated with neuroscore at 1wk post LFPI, but did not correlate with recorded impact pressure (atm), apnea time, time to toe pinch response, or righting reflex
Hou et al. (2021)	Sprague-Dawley Rats (EACAMMS)	Surgical resection model	2x3mm area of brain tissue resected from the right frontal lobe	3d, 1wk	*In vivo* 4kDa FITC Dextran gavage 1hr prior to tail vein blood draw + measure plasma fluorescence	↑ [FITC dextran] at 3d and 1wk compared to sham

Table 1.1: Functional Assessments of GI permeability after TBI

Table summarizing the parameters and findings from studies that have assessed intestinal permeability using functional permeability assays in various rodent models of TBI. The information presented does not include treatment or intervention. If a treatment or intervention was utilized in the respective study, only information pertaining to the control groups that received vehicle/ no treatment is included. Up (↑) and down (↓) arrows represent an increase and decrease respectively, ∆ corresponds with "change in", and [ ] corresponds with concentration of the molecule between the brackets. Abbreviations not contained elsewhere in the text: Animal Center

of the Chinese Academy of Sciences (ACCAS); The Institute of the Chinese Academy of Sciences, Shanghai Laboratory Animal Center (SLAC); Experimental Animal Center of Academy of Military Medical Sciences (EACAMMS)

Citation	Species (Origin)	Sex	Age at Injury	Weight (g)	TBI-model	Reported model parameters	Timepoint post-injury	Tissue assessed	Method of assessment	Findings
Hang et al. (2003)	Wistar Rats (ACCAS)	M	NR	220-250	Lateral WDI w/ craniotomy	4mm diameter, 5mm depth	12h, 1d, 3d, 7d (shams only from 3d timepoint)	Plasma	Chromogenic limulus amebocyte lysate test to assess [LPS] in plasma	↑ [LPS] in plasma at all timepoints compared to control (3d sham) w/ peaks at 3h and 3dpi
								Ileum	Electron Microscopy	3d & 1wk: disruption in TJs and widened distance between epithelial cells.
Ma et al. (2019b)	C57BL/6 mice (NR)	M	8-9wk	18-24	Lateral WDI w/ Craniotomy	3mm diameter, 18g rod dropped from 16cm	3d, 1wk	Serum	ELISA to assess [D-lac] in serum	No ∆[D-lac] in serum
									limulus amebocyte lysate test to assess [LPS] in serum	↑ [LPS] in serum of TBI+Veh at 3d and 1wk compared to Sham+Veh
								Ileum	WB for TJ protein Occludin	↓ Occludin in the ileum of TBI+Veh compared to Sham+Veh
Mazarati et al. (2021)	Sprague-Dawley Rats (Charles River)	M	50d	NR	Lateral FPI	2atm pressure	1wk Pre-FPI, 1wk, & 7mo	Plasma	ELISA to assess [LPS] in plasma	[LPS] negatively correlated with neuroscore at 1wk post LFPI, but did not correlate with recorded impact pressure (atm), apnea time, time to toe pinch response, or righting reflex
Simon et al. (2020)	C57BL/6J mice (Jackson)	M	Adult (specific NR)	NR	Moderate/ Severe Lateral CCI vs Naïve	1.2/ 1.8mm depth, 5/ 6m/s velocity, 0.05s dwell time	3d	Cecum	IHC for TJ protein ZO-1	↓ density of ZO-1 labeling in the cecum compared to naïve mice
Bansal et al. (2010c)	BALB/c (Jackson)	M	NR	20-24	Lateral WDI w/ craniotomy	250g rod dropped from 2cm	6h	Terminal Ileum	Western Blot for MLCK	73% increase in intestinal MLCK at 6h post TBI compared to sham mice
Li et al. (2018)	C57BL/6 mice (WMU)	M	6-8wk	18-22	Lateral WDI w/ craniotomy	20g weight dropped from 20cm	2wk	Serum	ELISA to assess [D-lac] in serum	↑ [D-lac] in serum
								Proximal Colon	WB for TJ protein Occludin	↓ Occludin in the colon compared to sham mice
									Qualitative assessment of IHC	↓ Occludin staining (not quantified)
Lang et al. (2015)	Wistar Rats (ACCAS)	M	Adult (specific NR)	200-250	Lateral WDI w/ craniotomy	40g weight dropped from 25cm	3h, 6h, 12h	Distal ileum	WB for TJ protein ZO-1	↓ ZO-1 in the distal ileum compared to sham
									qPCR for ZO-1 mRNA	↓ ZO-1 mRNA in the distal ileum compared to sham

Table continued on next page

Citation	Species (Origin)	Sex	Age at Injury	Weight (g)	TBI-model	Reported TBI-model parameters	Timepoint(s) post-injury	Tissue assessed	Method of assessment	Findings
Yang et al. (2022)	C57BL/6 mice (SLBC)	M	6-8wk	NR	Lateral CCI vs anesthesia only control	3mm tip, 3mm depth, 1.5m/s, 0.1s dwell time	3h, 1d, 3d, 1wk, 2wk	Plasma	ELISA to assess [LPS-binding protein]	↑ [LPSbp] in plasma at 1d, 3d, 1wk, and 2wk post-TBI, but was not significantly elevated at 3h post TBI.
									ELISA to assess [Zonulin]	↑ [Zonulin] in plasma at 3d, 1wk, and 2wk post-TBI, but was not significantly elevated at 3h or 1d post TBI.
Feighery et al. (2008)	Sprague- Dawley Rats (Harlan Labs)	M	NR	300-350	Terminal Frontal lobe, lateral CCI	3.5mm rounded tip, 2.62mm depth, 1.2m/s velocity	6h	Plasma	Chromogenic limulus amebocyte lysate test to assess [LPS] in plasma	No Δ [LPS] in serum

Table 1.2: Indirect Assessments of GI permeability after TBI

The information presented does not include treatment or intervention. If a treatment or intervention was utilized in the respective study, only information pertaining to the control groups that received vehicle/ no treatment is included. Up (↑) and down (↓) arrows represent an increase and decrease respectively, Δ corresponds with "change in", [ ] corresponds with concentration of the molecule between the brackets, and parameters that were not reported are denoted with NR. Abbreviations not contained elsewhere in the text: Western Blot (WB); Tight junction (TJ); Myosin Light Chain Kinase (MLCK); Wenzhou Medical University (WMU)

1.5.3.2. Histopathology of the GI tract

Beyond evidence of increasing intestinal permeability, many groups have shown some level of TBI-related morphological changes/damage of the intestinal tract using histology and immunohistochemistry. Most studies have focused on the small intestine with limited assessment of the colon. Changes in ileal morphology at early timepoints (3-12 h) include evidence of lacteal congestion at villous tips associated with the epithelial layer lifting away from the underlying mucosal layer (Feighery et al., 2008; Hang et al., 2003; Lang et al., 2015). Villus shortening/blunting is also observed from 1 d to 1 wk post-injury (Feng et al., 2007; Hang et al., 2003; Ma et al., 2019b; Sun et al., 2015; Zhang & Jiang, 2015). This is posited to be due to an initial sloughing of epithelial cells followed by a regrowth of malformed villi.

In the colon, Feighery et al. (2008) observed edema at 6 h in roughly half of the rats that received a frontal lobe CCI. Ma et al. (2017) noted no changes to the colon at 1 d post-CCI, but found evidence of mucosal hyperplasia and thickening of the muscle layer at 4 wks. Based on these findings it is possible that this thickening is an adaptive response to progressive damage not yet detectable at 1 d or is due to sustained inflammation/ sympathetic signaling as observed in other bowel conditions (Tappenden, 2014). The authors also found evidence of chronic enteric glial activation which would suggest sustained immune and sympathetic activation (Ma et al., 2017; Seguella & Gulbransen, 2021).

1.6. Effects of TBI on the gut microbiome

Intestinal physiology is highly influenced by the gut microbiota. Under healthy conditions, there is a high level of communication between the luminal microbes and the host epithelial cells. Microbial metabolites such as the short chain fatty acid (SCFA) butyrate serve as a major energy source for epithelial cells (Ahmad et al., 2000), while other metabolites influence barrier function and epithelial proliferation and differentiation (Kim et al., 2021). Maintenance of the intestinal barrier is influenced by microbial signaling that promotes epithelial tight junction expression (Karczewski et al., 2010; Madsen et al., 2001) and mucus production (Johansson et al., 2015). Due to the cross-talk between the gut microbiome and intestinal epithelium, GI dysfunction frequently coincides with changes to the microbiome and vice versa (Hinrichsen et al., 2021; Kayama, Okumura, & Takeda, 2020; Yang & Chen, 2022). Perturbation of the microbiome leads to a pathological state referred to as gut dysbiosis.

The influence of the microbiome extends beyond the GI tract and has been shown to play an important role in the normal development of the immune system and CNS. Studies using germ-free mice provide strong evidence that the microbiota are imperative for education of the naïve immune system (Lubin et al., 2023; Macpherson, de Agüero, & Ganal-Vonarburg, 2017) and that in the absence of a microbiota brain development is impaired (Thion et al., 2018), resulting in behavioral deficits (Heijtz et al., 2011; Luczynski et al., 2016).

The microbiome has shown to be a modulating factor in numerous disease states including those of the CNS. Neurodegenerative diseases such as Alzheimer's disease (Tiihonen, Ouwehand, & Rautonen, 2010) and Parkinson's disease (Sampson et al., 2016), have been shown to have microbiome alterations that can begin to manifest before the onset

of symptoms (Ferreiro et al., 2023; Huang et al., 2023). Even as an acquired injury/disease state, TBI is associated with changes to the GI microbiome and GI dysfunction, as detailed above, both in the clinic and in preclinical models.

1.6.1. Common methods of analyzing the gut microbiome

The gut microbiota consist of various types of microorganisms including bacteria, fungi, and viruses, but research has generally focused only on bacteria due to the wide variety of sequencing information and databases that have already been accrued. The microbiome refers to the genetic sequencing information associated with the microbiota. The term gut microbiome is typically a collective term for sequencing information from samples taken from the lower intestinal tract or feces. While each region of the GI tract has differences in the microbes present there, they all tend to be analyzed using similar techniques. Sequencing information has primarily been gathered from the bacterial 16s rRNA gene's hypervariable regions. This is a marker gene that allows for the identification of various bacterial taxa from a phylum to even a species level depending upon the sequencing depth. Sequencing of the gut microbiome provides an abundance of data for analysis. Common means of analyzing this data have been developed and are used to assess changes to the microbiome. The following sections will provide a brief overview of some of the microbiome analysis techniques that have been utilized in TBI studies. The most common first-pass metrics of assessing the microbiome are alpha and beta diversity which provide a broader perspective on overall bacterial composition. Differential abundance analyses are also commonly utilized to identify specific bacterial taxa that are increased or reduced compared to their control.

1.6.1.1. Alpha diversity

In microbiome studies, alpha diversity refers to within group differences in bacterial communities associated with the ideas of richness and evenness of a bacterial population. Richness refers to the number of taxonomic groups, and evenness refers to the distribution of those groups (Kers & Saccenti, 2021). These two parameters can be addressed separately, together, or in relation to some other factor such as phylogenetic distance. Common alpha diversity metrics that assess richness include the number of observed taxa (Callahan, McMurdie, & Holmes, 2017) and Chao1 index (Chao, 1984). Faith's phylogenetic distance also accounts for richness while also accounting for how far apart taxa are from one another on a phylogenetic tree (Faith, 2006). Evenness is commonly assessed using Pielou's E (Pielou, 1966), but can also be assessed using a number of other metrics (Jost, 2010). Some of the most commonly reported alpha diversity measures are those that consider both richness and evenness such as Shannon's Diversity Index (Lemos, Fulthorpe, Triplett, & Roesch, 2011) and Simpson's Diversity Index (Simpson, 1949).

1.6.1.2. Beta diversity

Beta diversity refers to between sample differences in regard to the abundance or presence of taxa while considering all samples as they relate to one another (Kers & Saccenti, 2021). This results in a high level of multidimensionality that is often portrayed using principal component/coordinate analysis (PCoA/PCA) plots where more similar samples are grouped closer to each other while dissimilar groups are distributed further apart. Common beta diversity metrics include Bray-Curtis dissimilarity (Bray & Curtis, 1957a), Jaccard distance (Jaccard, 1912), unweighted UniFrac distance (Lozupone & Knight, 2005a), and weighted UniFrac distance (Lozupone, Hamady, Kelley, & Knight,

2007). For a more in depth overview of beta diversity analyses please refer to the recent published review from IMPACTT-investigators (2022).

1.6.1.3. Differential abundance analysis

Differential abundance analysis is utilized to determine if the experimental group has a greater amount/representation of a given bacterial taxa compared to the control group. This comparison can be done in various different ways with the most simple being a direct comparison of absolute or relative abundance numbers by multiple t-tests with corrections for multiple testing. Direct comparison of these values is typically insufficient as it does not account for the complexity of the data. As such, various statistical tests have been developed to provide a determination if any given bacterial taxa is greater in an experimental or control group. Many of these tests have been adapted from analyses utilized to determine differential expression of genes via microarray or RNAseq datasets. Common differential abundance analysis techniques that have been used include LEfSe (linear discriminant analysis effect size; Segata et al., 2011), ANCOM (Analysis of Composition of Microbiomes; Mandal et al., 2015), ANCOM-BC (ANCOM with Bias Correction; Lin and Peddada, 2020a), DESeq2 (Differential Expression Analysis for Sequence Count Data 2; Love, Huber, and Anders, 2014), and many more. For a more in depth comparison of each method please refer to the recent published reviews by Lin and Peddada (2020b) or Yang and Chen (2022).

1.6.2. Clinical studies assessing changes to the microbiome after TBI

To date, only four clinical studies, all published in the last six years, have assessed changes in the microbiome at acute timepoints after TBI, demonstrating the infancy of this field. In a study of 24 patients with moderate or severe TBI, TBI was associated with altered

beta diversity (Weighted UniFrac), increased alpha diversity (Chao1 and Observed OTUs), increased *Enteroccoccus*, *Parabacteroides*, *Akkermansia*, and *Lachnoclostridium*, and decreased *Bifidobacterium* and *Faecalibacterium* compared to healthy controls (Hou et al., 2021). In a pediatric TBI study conducted by Rogers et al. (2022), 16s rRNA gene sequencing was performed on rectal swab samples from 23 children (2-18 yrs old) with a severe TBI and 35 healthy volunteers. A significant difference in beta-diversity between groups in the initial rectal sample suggested that the gut microbiome is rapidly changed after TBI, and reductions were noted in the bacterial families *Ruminococcaceae*, *Bacteroidaceae*, and *Lachnospiraceae*.

Two additional clinical studies focused on early microbiome changes, but they have major limitations that make it difficult to generalize their results to head-injured individuals more broadly. Howard et al. (2017) conducted a study on a cohort of 12 severe trauma patients, but patients with isolated TBI were excluded from the study and 4 of the 12 participants did not suffer a TBI as part of their trauma. Mahajan et al. (2023) assessed the microbiota of moderate-to-severe TBI patients that lacked major polytrauma, but this study did not perform any sequencing analysis to identify bacterial taxa and instead utilized bacterial culturing which massively limits the variety of bacteria that can be identified. A large number of gut microbes are currently unable to be cultured while others require anaerobic conditions or very specific culturing techniques that were not utilized in the study (Renwick et al., 2021). Without the use of anaerobic culturing, any conclusions from this study are limited, as many "beneficial bacteria" are obligate anaerobes while many "pathogenic bacteria" are either facultative anaerobes or aerobic thus skewing the findings towards identification of deleterious bacteria (André, Debande, & Marteyn, 2021).

Only two studies were identified that assessed microbiome changes in humans at chronic timepoints after injury, and they had conflicting results. Brenner et al. (2020) found no difference in alpha or beta diversity in veterans with a TBI compared to veterans without a TBI, while Urban et al. (2020) identified microbiome differences in assisted living patients that had incurred a TBI between 2.3 - 41.8 yrs prior compared to a control group composed of workers from the facility. The latter study found that alpha diversity was elevated in TBI survivors compared to controls, with increased levels of the bacterial phyla *Actinobacteria*, *Firmicutes*, and *Verrucomicrobia* and decreased levels of *Bacteroidetes*. There is a great need for additional clinical research on the effects of TBI on the gut microbiome as they have been greatly outpaced by the number of preclinical studies on the same topic. The following section will provide an overview of pertinent findings from microbiome studies conducted in rodent models of TBI.

1.6.3. Preclinical studies assessing microbiome changes after TBI

The vast majority of our knowledge on how the microbiota interact with their host comes from preclinical studies. Several groups have assessed the microbiome after experimental TBI, under the shared hypothesis that TBI induces gut dysbiosis that can feed back and alter the outcomes within the brain. However, there has yet to be a consensus on how and when the microbiome is altered after brain injury. Despite this, experimental evidence suggests that the microbiome does modulate outcomes after TBI. Studies from the Faden lab (University of Maryland) and from the Friess lab (Washington University in St. Louis) have shown that disrupting the microbiome after TBI results in an exacerbation

of brain injury pathology. These studies are a vital proof of concept that the microbiome can modify neurological outcomes after TBI through effects on the brain.

Ma et al. (2017) showed that introduction of the pathogenic bacteria *C. rodentium* at 28 d after CCI in mice led to worsened behavioral outcomes and increased contusion volume. Using a similar paradigm, Hanscom et al. (2021a) showed that induction of colitis using the detergent dextran sodium sulfate (DSS) was capable of exacerbating CCI pathology and even inducing damage in sham mice such as hippocampal neurodegeneration and increased numbers of reactive microglia. Due to the commonality of administering antibiotics after TBI in a clinical setting, Celorrio et al. (2021) induced gut dysbiosis through antibiotic administration prior to and/or after severe CCI and showed increased hippocampal cell loss, BBB breakdown, and neuroinflammation.

With the knowledge that the microbiome is capable of modulating outcomes after TBI, it is important to characterize how the native microbiota are altered after brain injury as well. Multiple labs have assessed the microbiome after TBI using various different models, injury parameters, and timepoints. Because model of TBI and animals used are potential variables that may alter how the gut microbiome responds after injury, two different summary tables have been compiled below. The first, Table 1.3, only includes findings from CCI studies conducted in C57BL/6 mice and is designed to provide context for the findings presented in Chapter 2. The second, Table 1.4, includes studies that utilized mice in non-CCI models of TBI and rats in various models of TBI. These studies and how they relate to the data within this dissertation will be addressed further in Chapter 2 and 3.

Citation	Mouse Strain	Sex	Age at Injury	CCI Severity	Non-injury Control group	Timepoint post injury	α-diversity		β-diversity		Differential Abundance	
							Metric	Findings	Metric	Findings	Timepoint	Findings
Bao, Sun, Lin, Yang, and Chen (2023)	C57BL/6 (ZEAC)	M	8wk	Severe: 2mm depth, 3m/s, 200μsec dwell	Sham	1wk	Observed OTUs Shannon Index Simpson Index Chao1 Good's Coverage	No Δα-diversity vs Sham	NS	No Δβ-diversity vs Sham	1wk	LEfSE: ↑ in several taxa within Actinobacteria phylum, including specific species of *Bifidobacterium* and *Lactobacillus*. LEfSE: ↓*Parvibacter* genus, *streptococcus danieliae* species and unclassified species of *Bacteroides* and *Parvibacter*
Davis et al. (2022)	C57BL/6 (Jackson)	M	14wk	Severe: 2mm depth, 2.5m/s, 0.1sec dwell	Anesthesia + incision (Referred to as Sham in paper)	Pre-injury and 59d	Observed OTUs Simpson Index	↓α-diversity in TBI vs Control	NS	PCoA suggests separation between TBI+Veh and anesthesia control[Q]	Pre-injury and 59dpi	From heatmap[Q]: ↓Akkermansia, ↑Faecalbaculum
Opeyemi et al. (2021)	C57BL/6J (Jackson)	M	Adult (NS)	Severe: 2mm depth, 6m/s, 50msec dwell	Pre-injury	Pre-injury, 3h, 1d, 3d, 2wk, and 4wk	Observed species Shannon Entropy	↓α-diversity after 3d and continuing downward trend out to 4wks post-injury[Q]	Jaccard	Separation of 4wk post-injury group and all other timepoints	Pre-injury, 3h, 1d, 3d, 2wk, and 4wk	LEfSE: ↑Verrucomicrobiaceae and Erysipelotrichaceae with time post injury while ↓Lachnospiraceae, Ruminococcaceae, and Bacteroidaceae -- post-hoc comparisons of specific timepoints not reported
Simon et al. (2020)	C57BL/6J (Jackson)	M	Adult (NS)	Moderate: 1.2mm depth, 5m/s, 50msec dwell	Pre-injury	Pre-injury and 3d	Observed species Shannon Entropy	↓Observed species No ΔShannon	W. UniFrac	Pre-injury and 3d post CCI group together on NMDS[Q]	Pre-injury and 3d	LEfSE: no difference between pre-injury and 3d CCI mice reported
Treangen, Wagner, Burns, and Villapol (2018)	C57BL/6J (Jackson)	M	9wk	Moderate-Severe: 1.5mm depth, 5.25m/s, 0.1sec dwell	Sham	Pre-injury and 1d	States that QIIME2 basic diversity metrics performed, but no α or β-diversity reported				Pre-injury and 1d	Genus level: ↑Marvinbryantia and Clostridiales family XII Species level: ↓Lactobacillus gasseri, Ruminococcus flavefaciens, and Eubacterium ventriosum. ↑Eubacterium sulci and Marvinbryantia formatexigens

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Citation	Mouse Strain	Sex	Age at Injury	CCI Severity	Non-Injury Control group	Timepoint post-injury	α-diversity		β-diversity		Differential Abundance	
							Metric	Findings	Metric	Findings	Timepoint	Finding
Zheng et al. (2022)	C57Bl/6 (SLAC)	M	6-8wks	Moderate: 1mm depth, 3m/s, 0.1sec dwell, 2.5mm diameter tip	Sham	1wk and 4wk	Chao1, ACE, Shannon, Simpson	No Δα-diversity at 1wk vs Sham ↓Chao1 and ACE at 4wk vs Sham	W. UniFrac	PCoA suggests separation between TBI and sham at 1wk and 4wk. Presented on different plots possibly using different calculations	1wk and 4wk	Relative abundance comparison at phylum level: ↓*Verrucamicrobiota* at 1wk and 4wk ↑Proteobacteria, Actinobacteria, and TM7 at 4wk only ↓*Bacteroidetes, Cyanobacteria*, and Deferribacteres at 4wk only
												LEfSE (genus level): ↓*Akkermansia* at 1wk and 4wk ↑*Streptococcus* at 1wk and 4wk

Table 1.3: Summary of gut microbiome findings in CCI injured mice

Table summarizing microbiome findings and experimental parameters from studies conducted in mice using a CCI model. The information presented does not include treatment or intervention. If a treatment or intervention was utilized in the respective study, only information pertaining to the control groups that received vehicle/ no treatment is included. Up (↑) and down (↓) arrows represent an increase and decrease respectively, Δ corresponds with "change in", ^{Q} denotes a qualitative conclusion made by the author without formal statistical analysis, and parameters that were not specified are denoted with NS. Abbreviations and notations not contained elsewhere in the text: Zhejing Experimental Animal Center (ZEAC); Non-metric multi-dimensional scaling (NMDS).

Citation	Species (Origin)	Sex	Age at Injury	Weight (g)	Model	Model information	Non-injury Control group	Timepoint post injury	α-diversity		β-diversity		Differential Abundance	
									Metric	Findings	Metric	Findings	Timepoint	Findings
Angoa-Pérez et al. (2020)	C57BL/6J mice (Envigo)	M	8wk	NR	WDI rmTBI w/ rotation acceleration	20 impacts total (1mTBI/day for 5d/week)	Baseline and Anesthesia control (Sham)	Baseline (0d), 45d, 90d	Chao1, Shannon, Simpson	↓Shannon at 45dpi vs baseline	Jaccard, Bray-Curtis	No ΔBeta Diversity between injury groups	Baseline (0d), 45d, 90d	LEfSe: Inconclusive results, showed significantly different groups for each Timepoint & Injury group, but comparisons were made across all groups rather than rmTBI vs Sham/ Baseline control
Ma et al. (2019b)	C57BL/6 mice (NR)	M	8-9wk	NR	Lateral WDI w/ craniotomy	Severe Injury (3mm diameter, 18g rod dropped from 16cm)	Sham+ Veh	3d	Chao1	↓Chao1 vs sham	Unweighted UniFrac	PCoA suggests separation between TBI (+Veh) and Sham (+Veh)[Q]	3d	Kruskal-Wallis w/ Bonferroni post-hoc: ↓*Epsilonproteobacteria* ↑Alphaproteobacteria, Actinoproteobacteria, and Gammaproteobacteria vs Sham (+Veh)
Yanckello et al. (2022)	C57BL/6 mice (NR)	M	4mo	NR	Closed head mTBI using emCCI device	Mild Injury (5.0m/s, 100ms dwell, 1.0mm depth onto skull)	Baseline and Sham	Baseline (1d before mTBI1), 1d, 1.5mo, 3mo, 5mo	No comparison between Sham and CCI without treatment	NR	Bray-Curtis	ΔBeta diversity between baseline sham and 1d sham timepoint	Sham: Baseline VS 1d	SHAP: ↓Akkermansia muciniphila; Dorea sp. 5-2, Bacteroides sp. 1_1_14 at 1d post-Sham ↑*Firmicutes* bacterium ASF500; *Anaerotruncus* sp. G3(2012); *Clostridiales*_u_s; *Lachnospiraceae* bacterium 3-1; *Erysipelotrichaceae* bacterium 6_1_45; *Lachnospiraceae* bacterium 28-4; *Lachnospiraceae* bacterium COE1 at 1d post-Sham
												ΔBeta diversity between 1d CHI and 1.5mo post-injury	CHI: 1d VS 1.5mo	SHAP: ↓Lachnospiraceae bacterium A2 and Bacteroides sp. 1_1_14 at 1.5mo compared to 1dpi ↑Lactobacillus johnsonii; Lachnospiraceae bacterium 10-1; Clostridiales_u_s; Lachnospiraceae bacterium COE1; Eubacterium sp. 14-2; Lacnospiracease bacterium 28-4; Lachnospiraceae bacterium A4; and Enterococcus faecalis at 1.5mo compared to 1dpi
Hou et al. (2021)	Sprague-Dawley Rats (EACAMMS)	M	NR	220-250	Surgical Brain Injury (surgical resection model)	2x3mm area of brain tissue resected from the right frontal lobe	Sham	3d, 1wk	Chao1, Shannon, Faith's PD, Whole Tree Index, Observed	↓Chao1 at 3d vs sham that returns to sham levels at 1wk	Unweighted UniFrac	PCoA suggests separation between sham, 3d TBI, and 1wk TBI groups	3d, 1wk	LEfSe: ↓Lactobacillus at 3d and 1wk ↓Parabacteroides at 1wk ↑Akkermansia at 3d

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Citation	Species (Origin)	Sex	Age at Injury	Weight (g)	Model	Model information	Non-injury Control group	Timepoint post injury	α-diversity		β-diversity		Differential Abundance	
									Metric	Findings	Metric	Findings	Timepoint	Findings
Sgro et al. (2022)	Sprague-Dawley Rats (MARP)	M & F	Adolescent and Adult (specific NR)	NR	Repeated mTBI	Lateral impact rotation acceleration injury every other day for 3 d	Sham	Baseline (1d before mTBI1), 1d after mTBI1, 11d after final mTBI	Shannon	No difference between injury group or age group at 11d after final rmTBI	Weighted UniFrac	No difference between injury group or age group at 11d after final rmTBI	Baseline (1d before mTBI1), 1d after mTBI1, 11d after final mTBI	Limma: ↓*Muribaculaceae* family in adolescent mice at baseline ↓*Lachnospiraceae* ASVin adolescent mice at all timepoints Limma or MaAsLin2 (NS): When comparing adolescent sham to adolescent rmTBI, ↑*Faecalibaculum* and *Lachnospiraceae* at 1d (1 mTBI)
Nicholson et al. (2019)	Sprague-Dawley Rats (NR)	M	NR	225-250	Pneumatic CCI	Lateral CCI, 5mm craniotomy, 5.0m/s, 0.25ms dwell, 2mm depth)	Baseline	Baseline, 2hr, 1d, 3d, 7d	Whole Tree Index, Chao1, Observed, Shannon	↓Chao1 and Observed at 3d vs baseline	Weighted and Unweighted UniFrac (Only 1 presented as PCA, but unclear which)	PERMANOVA showed difference between animals but not timepoint	Baseline, 2hr, 1d, 3d, 7d	1way ANOVA of relative abundances: Phylum level: ↑Bacteroidetes and Defferibacteres at 1d Family level: ↓*Lachnospiraceae* at 2h, 1d, and 3d ↓*Mogibactereiaceae* at 1d and 3d ↓Ruminococcaceae at 1d ↓Anaeroplasmataceae at 2h-7d ↓Deferribacteraceae at 1d and 3d ↑Bacteroidaceae & Verrucomicrobia at 1d
Baskin et al. (2023)	C57Bl/6J	M & F	9-11 wks	NS	Repeated (x3) mild blast TBI	Condensed helium to create a blast over pressure wave (19.1 psi)	Sham	1d after final blast exposure	Shannon	No difference between injury groups	Bray-Curtis	ANOSIM showed difference across groups	1d after final blast exposure	LEfSe (Males) Order Level: ↑*Bacillales*, *Lactobacillales* ↓Clostridiales, Erysipelotrichales LEfSe (Females) Order Level: ↑Bacillales, Streptomyceltales, Streptosporangiales, Verrucomicrobiales ↓Bifidobacterriales

Table 1.4: Other mouse and rat TBI models utilized to assess the microbiome

Table summarizing microbiome findings and experimental parameters from studies conducted in mice using a non-CCI TBI model and in rats using several different TBI models. The information presented does not include treatment or intervention. If a treatment or intervention was utilized in the respective study, only information pertaining to the control groups that received vehicle/ no treatment is included. Up (↑) and down (↓) arrows represent an increase and decrease respectively, Δ corresponds with "change in", ^{Q} denotes a Multivariate Association with Linear Models 2 (MaAsLin2); Analysis of Similarities (ANOSIM)

CHAPTER 2: TBI induces transient intestinal permeability, increased colon hypoxia and goblet cell density, and subacute microbiome changes in mice

2.1. Abstract

Pathophysiological responses to traumatic brain injury (TBI) extend beyond the central nervous system. Systemic perturbations can induce gastrointestinal (GI) dysfunction and changes in microbial diversity, but our understanding of these events in the context of TBI is limited. Brain-injured individuals are at increased risk of suffering from GI-related morbidity and mortality. Symptoms such as intestinal dysmotility, inflammation, ulceration, and fecal incontinence can drastically diminish quality of life. Clinical and preclinical studies have implicated gut dysbiosis as a consequence of TBI as well as a potential amplifier of brain damage. However, little is known about the effects of an isolated TBI on the structure and function of the GI tract and the relationship of such changes to gut dysbiosis. To assess GI dysfunction after TBI, mice received a controlled cortical impact (CCI) or sham injury and permeability was assessed at 4 hr, 8 hr, 1 d, and 3 d after injury by oral administration of 4 kDa FITC-Dextran prior to euthanasia (n_{sham}= 9, n_{CCI}=6-8/timepoint). Quantification of serum fluorescence revealed an acute, short-lived increase in permeability at 4 hr after CCI. Despite transient intestinal dysfunction, no overt morphological changes were evident in the ileum or colon across timepoints from 4 hr to 4 wks post-injury (n_{sham}= 3-6/timepoint, n_{CCI} = 6-10/timepoint). To elucidate the timeline of changes to the microbiome after TBI, 16s gene sequencing was performed on fecal samples collected prior to and over the first month after CCI or sham injury (n = 6-7/group) for 16s gene sequencing. Differential abundance analysis revealed that the phylum *Verrucomicrobiota* was increased in CCI mice at 1, 2, and 3 d post-injury when compared

to sham mice. Subsequent qPCR identified the *Verrucomicrobiota* species as *Akkermansia Muciniphila*, an obligate anaerobe that resides in and helps regulate the intestinal mucus layer and barrier. To determine whether TBI promotes changes to the GI tract favorable for the proliferation of *A. muciniphila*, mucus-producing goblet cells and the level of GI hypoxia were evaluated. Consistent with this premise, the relative area of goblet cells in the proximal region of the colon was significantly increased at 1 d post-injury, while colon hypoxia was significantly increased at 3 d post-injury (n_{sham}= 11, n_{CCI} = 6-7/timepoint). Taken together, CCI induces transient intestinal barrier dysfunction which may support increased goblet cell density and hypoxia in the colon followed by a subacute increase in *A. muciniphila.* Our findings suggest an acute GI disturbance and an increase of beneficial bacteria suggesting a potential compensatory response to systemic stress after TBI.

2.2. Introduction

Traumatic brain injury (TBI) is a major source of death and disability with approximately 2.8 million people suffering a TBI annually in the United States alone (Rivara et al., 2012; Selassie et al., 2008b; Taylor, Bell, Breiding, & Xu, 2017). The initial brain insult and ensuing secondary injury cascade results in complex damage and dysfunction to not only the brain but also to other organ systems (Kemp, Cotton, Johnson, & Weaver, 2006; Krishnamoorthy et al., 2021). Increasing evidence suggests that the neuroenteric axis is altered following TBI, compromising bidirectional coordination of external and internal stimuli by the central and enteric nervous systems (Bansal et al., 2009; Ma et al., 2017). Gastrointestinal (GI) dysfunction, including dysmotility, fecal incontinence and sepsis, is well documented in the TBI population (Carlos Dourado, Nascimento de Miranda Engler, & Barbosa de Oliveira, 2012; Falcão de Arruda & de

Aguilar-Nascimento, 2004; Harrison-Felix et al., 2006). Indeed, at one or more years after injury, TBI is associated with a 2.5-fold increase in the incidence of digestive system conditions and 12-fold increase in death due to septicemia (Harrison-Felix et al., 2006; Harrison-Felix et al., 2009). Rodent models of TBI recapitulate aspects of GI dysfunction seen in humans, including altered transit time (Olsen et al., 2013) and increased intestinal permeability (Bansal et al., 2009; Feighery et al., 2008). Such perturbations to normal GI function may result in disrupted nutrient absorption and bacterial translocation outside of the GI tract, potentially contributing to worsened outcomes after brain injury (Bansal et al., 2009; Bansal et al., 2010c; Ma et al., 2017; Santos, Gonçalves, Araújo, & Martel, 2008).

The microbiota residing in the intestinal tract help mediate signaling along the neuroenteric axis (Margolis, Cryan, & Mayer, 2021). The cells of the small and large intestine interact with microbes and microbial metabolites to effect direct and indirect signaling to the central nervous system. The gut's native bacterial populations are critical for maintaining GI health by contributing to intestinal metabolism and digestion, and influence many systems critical for normal development of the brain and immune system (Erny et al., 2015; Sharon, Sampson, Geschwind, & Mazmanian, 2016; Thaiss, Zmora, Levy, & Elinav, 2016; Thion et al., 2018). Changes to the gut microbiome have been reported after TBI, although the specific findings vary (reviewed in Hanscom et al., 2021b). Experimental perturbation of the microbiome worsens outcomes after experimental TBI (Celorrio et al., 2021; Hanscom et al., 2021a; Ma et al., 2017). More specifically, TBI-associated pathology and neurologic deficits are exacerbated by the induction of colitis early after injury (Hanscom et al., 2021a) and with the introduction of intestinal pathogens at chronic timepoints (Ma et al., 2017). Antibiotic-induced dysbiosis after experimental

TBI amplifies neuroinflammation and decreases hippocampal neurogenesis, compromising potentially reparative neuroplasticity (Celorrio et al., 2021). Given that gut-to-brain signaling can affect multiple outcomes, it is important to understand the brain-to-gut changes that are initiated after TBI.

TBI results in changes not only in GI function and the gut microbiome, but also in the structural integrity of the GI tract. Acute small intestinal villi blunting and chronic thickening of the muscularis mucosae have been noted after experimental brain injury (Bansal et al., 2009; Bansal et al., 2010b; Ma et al., 2017). Moreover, due to the close association between the gut microbiota and the intestinal epithelium, deleterious changes to the gut microbiome are often associated with damage to the intestinal tract and vice versa (Hinrichsen et al., 2021; Kayama et al., 2020; Yang et al., 2018). The intestinal epithelium is protected by a layer of mucus that is secreted by goblet cells that line the GI tract. The outer mucus layers provide important niches for symbiotic bacteria; thus, diminished or excessive mucus production can alter the composition of the microbiome.

The microbiome and GI tract have been posited as a potential therapeutic target after TBI (Zhu, Grandhi, Patterson, & Nicholson, 2018), but how the GI tract is altered by TBI is still poorly understood. To test the hypothesis that TBI initiates intestinal dysfunction and damage in concert with gut dysbiosis, we induced a controlled cortical impact (CCI) brain injury in mice whose microbiome had been normalized prior to injury. Damage to the GI tract was assessed at multiple timepoints over a 4 wk post-injury period by quantifying morphological features of crypt-villi and crypt structures and goblet cells in the small and large intestine. The fecal microbiome was profiled at corresponding timepoints by performing 16s rRNA gene sequencing. We show that TBI induces an

increase in intestinal permeability in the absence of overt histological damage in the small or large intestine. We identify a potentially beneficial bacteria, *Akkermansia Muciniphila*, that is increased after experimental TBI as well as changes to the GI tract that may promote the growth of this bacteria. Our findings provide novel information that suggests that TBI leads to a transient intestinal dysfunction which may trigger changes in the GI tract supporting a compensatory response within the gut microbiome. Further understanding of these changes may lead to potential therapeutic manipulation of the microbiome as a means of promoting endogenous reparative mechanisms that limit GI dysfunction after TBI.

2.3. Methodology

2.3.1. Mice and housing information / food

C57BL/6J male mice were obtained from Jackson Laboratories at 4-8 wks of age. Mice were allowed to acclimate within the University of Kentucky Medical Center animal vivarium for at least one week prior to any procedures. Mice were housed within the vivarium maintained at a constant temperature with a 14/10 hr light/dark cycle. Unless otherwise stated, mice were provided food and water *ad libitum*. All procedures were approved by the University of Kentucky's Institutional Animal Care and Use Committee, under IACUC protocol 2019-3170.

For microbiome studies, mice were co-housed by injury condition following inoculation with a common microbiome to minimize inter-individual variation (see pre-injury microbiome normalization methods for additional information). The mice utilized for microbiome studies were euthanized at 4 wks post-CCI, and their GI tissue collected as

the 4 wk timepoint for GI histology studies. All other mice were co-housed regardless of injury condition with their cage-mates as determined upon arrival to the vivarium.

2.3.2. CCI surgery, group randomization

Adult mice (8-10 wks of age) were randomized into CCI or sham injury groups based on the desired group size. CCI mice received a moderate brain injury (1.1 mm depth, 3.5 m/s speed) as previously described (Littlejohn et al., 2021). In short, mice were initially anesthetized using 3% isoflurane and placed in a stereotaxic frame (David Kopf Instruments, CA). Once the head was stabilized, anesthesia was maintained at 2.5% isoflurane for the duration of the surgery. The shaved scalp was prepped with alcohol and betadine prior to analgesic administration via subcutaneous injection (0.5% bupivacaine with 1:200,000 epinephrine in sterile saline; 2 mg/kg bodyweight). A midline incision was made and the scalp retracted. A 5 mm diameter craniotomy was made over the left parietal cortex midway between Bregma and lambda. The 3 mm diameter, rounded impactor tip was centered within the craniotomy and a cortical contusion was produced using an electromagnetically driven impactor device (Leica Biosystems). The craniotomy site was then closed by affixing a cranioplasty made of dental acrylic to the skull using a non-toxic cyanoacrylate adhesive to protect the brain from further damage. Following surgery, mice were placed on a heated pad to maintain body temperature until they were ambulatory. Sham-injured mice underwent all procedures except the impact.

2.3.3. Intestinal permeability assay

Intestinal permeability was assessed as previously published (Gupta & Nebreda, 2014) in mice that survived 4 hr, 8 hr, 1 d, or 3 d after sham injury or CCI (n_{sham}= 2-3/timepoint; n_{CCI} = 6-9/timepoint). All mice were fasted approximately 10-13 hr prior to

euthanasia to allow for complete clearance of the GI tract. Mice designated for the 4 hr survival group were fasted overnight prior to surgery. Mice surviving 8 hr were fasted beginning 2 hr prior to surgery. To assess permeability, 4 kDa FITC dextran (100 mg/ml FD4, Sigma Aldrich, CAS: 60842-46-8) was administered 4 hr prior to euthanasia by oral gavage at a dose of 44 mg/100 g mouse body weight. Mice designated for a 4 hr survival were gavaged directly after they regained consciousness from surgery.

Mice were euthanized by overdose of sodium pentobarbital (Fatal Plus, 150mg/kg i.p.). A midline laparotomy was then immediately performed, followed by a thoracotomy to expose the heart. Blood was drawn from the left atria using a blunt tip 18g syringe. If insufficient blood was collected, the right auricle was clipped, and blood was collected using a syringe as it pooled in the thoracic cavity. Blood was placed in a light-shielded sterile tube and allowed to clot at room temperature. Blood was spun down in a centrifuge (4°C; 1500xG for 15-20 min), and serum supernatant was collected and stored at -20°C until all samples could be analyzed together. After blood collection, mice were perfused with heparinized saline and tissue collection proceeded as outlined below. Serum was diluted 1:1 in 1x PBS, and fluorescence was measured on a plate reader (Synergy | HTX multi-mode reader; Gen5 Image+). Serum concentration of FITC dextran was determined based upon a standard curve generated using known concentrations of FITC dextran.

2.3.4. Pimonidazole-HCl administration

To assess intestinal hypoxia after TBI (n_{sham} = 11, n_{CCI} = 6-7/ timepoint), mice received an intraperitoneal injection of 60 mg/kg pimonidazole-HCl (Hypoxyprobe-1 | Hypoxyprobe, Inc., Burlington, MA) 1 hr prior to euthanasia as per manufacturer

instructions. Under hypoxic conditions, HP-1 is reduced and forms protein adducts that can then be detected using antibodies against this protein adduct (Aguilera & Brekken, 2014).

2.3.5. Euthanasia, tissue collection and processing

Animals were euthanized with an overdose of sodium pentobarbital (Fatal Plus, 150mg/kg i.p.), and were transcardially perfused with heparinized saline. Following the clearance of blood, the small and large intestine were dissected out together for processing (described below) before resuming perfusion with 10% buffered formalin. As previously described (Littlejohn et al., 2021), brains were removed from the skull after an overnight post-fix in 10% buffered formalin, and further post-fixed for 24 hr prior to cryoprotection with 30% sucrose solution. After water displacement, brains were snap frozen in a beaker of cold isopentanes (-25 ± 5°C) surrounded by dry ice and stored at -80°C until cutting. Brains were cut coronally at 40 μm thickness using a freezing, sliding microtome and stained with Cresyl Echt Violet to assess gross histology. After evaluation by two investigators, one sham mouse was excluded from the study due to substantial damage to the brain along the perimeter of the craniotomy. One mouse surviving 4 hr after CCI was excluded due to insufficient tissue damage. Reported groups sizes account for these exclusions.

Following removal of the intact small intestine and colon, the ileum was identified as the distal third of the small intestine and separated. For the colon, the cecum was excluded and the medial and distal portions of the colon were dissected out together for further processing. The dissected ileum and colon were Swiss rolled onto syringe plungers (Moolenbeek & Ruitenberg, 1981) before overnight post-fixation in 10% buffered formalin. Swiss-rolled tissue was then pinned and transferred to 70% ethanol for paraffin

embedding by the University of Kentucky Pathology Research Core. Paraffin-embedded tissue was cut on a rotary microtome at a 4 μm thickness and mounted on positively charged slides using a warm water bath. Tissue was allowed to air dry onto the slide overnight prior to baking overnight in an incubator at 50°C to improve tissue adherence to the slide.

2.3.6. Histology, immunohistochemistry, and image analysis

GI tissue utilized for image analysis was collected from animals generated for several experiments within the present study (n_{Sham}= 3-6/ timepoint; n_{CCI} = 6-10/ timepoint). Animals euthanized at 4 hr, 8 hr, 1 d, and 3 d after injury were also utilized for the FITC dextran permeability assay described above, while the 1 wk and 2 wk mice were generated for the specific purpose of GI tissue analysis studies. Tissue for the 4 wk survival timepoint was collected from the animals that were utilized for the microbiome studies outlined below. All measurements were taken by a researcher blinded to brain injury status and timepoint.

2.3.6.1. Alcian blue staining

Swiss-rolled intestinal tissue sections were deparaffinized in xylenes and rehydrated through an ethanol gradient into ddH_2O before staining with Alcian Blue (VECTOR labs | cat no: H-3501), as per manufacturer instructions, to label acidic mucins with a pH < 2.5. Tissue was then counterstained with nuclear fast red and dehydrated through an ethanol gradient into xylenes before coverslipping with cytoseal (Thermo Shandon Limited, Cheshire, Washington). Slides were scanned at 20x with a Zeiss Axio Scan Z.1 digital slide scanner allowing for a single high-resolution, zoomable image of

each slide. These images were analyzed as detailed below using the HALO image analysis platform (version 3.2; Indica Labs) or Adobe Photoshop (version 4 for iPad; Adobe).

2.3.6.2. Quantification of intestinal morphological parameters

Intestinal tissue stained with Alcian Blue and nuclear fast red was used to assess alterations to the morphology of both the small and large intestine. The functional unit within the ileum is the crypt-villi structure, which consists of a villus surrounded by invaginations known as crypts. The colon is comprised of three regions: the cecum, medial region and distal region. The medial and distal portions of the colon were analyzed. The medial region, also known as the ascending colon, was differentiated from the distal region, or descending colon, based on the presence of transverse folds. As crypt structures within the medial portion of the colon are shorter than those of distal colon, crypt depth was measured separately for these regions. Either increased or decreased ileum crypt-villi distance or colon crypt depth is indicative of damage to the GI tract and is associated with altered nutrient absorption, damage, and inflammation (DeRoche, Xiao, & Liu, 2014; Owens & Greenson, 2007).

Within the HALO software, the length from the tip of the villus to the base of that villi's crypts was measured in the ileum (minimum = 20 well oriented crypt-villi structures/animal). In the colon, the distance from the luminal edge of the crypt to its base was measured (minimum = 10 well oriented crypts/region/animal). All well-oriented ileum crypt-villi and colon crypt structures were measured along the length of the Swiss roll and lengths were averaged for each animal.

2.3.6.3. Alcian blue staining quantification

For the ileum, Alcian Blue-labeled goblet cells were manually counted on a subset of well-oriented crypt-villi structures within five square fields (perimeter = 5 mm; area = 1.5 mm^2) that were placed over the ileum Swiss roll within the HALO software. Images of each villus within the field were exported from HALO using the "Figure Maker" tool at a resolution of 5000 pixels/in for manual counting within Adobe Photoshop. Goblet cells were counted if they had positive staining and a clearly identifiable nucleus.

Within the colon, individual well-oriented crypts within the medial and distal colon were manually outlined using HALO software. Due to the tight packing of goblet cells, colonic goblet cells were quantified using the Area Quantification v1.0 algorithm within HALO to determine the area of Alcian Blue-positive tissue relative to the individual crypt area. This algorithm was adjusted per staining run to account for lighter or darker Alcian Blue labeling. The percent Alcian blue-positive staining was averaged across crypts within the medial and the distal colon per animal.

2.3.6.4. Colon hypoxia immunohistochemistry

Swiss-rolled intestinal tissues were deparaffinized using xylenes and rehydrated through an ethanol gradient into ddH_2O. Antigen retrieval was performed using a pressure cooker (Instant Pot LUX; Instant Pot Company, Ontario, Canada) with slides in 10 mM Citrate Buffer (pH 6.0) for 4 min. After pressure was released, slides were removed from the pressure cooker and allowed to cool to room temperature before washing in 1x TBS. Tissue was incubated in a blocking solution of 5% normal horse serum (NHS) in 0.1% Triton X-100 in 1x TBS (TBS-T) for 1 hr. Tissue was incubated in biotinylated anti-HP1 mAb (1:50 in 5% NHS in 0.1% TBS-T | Hypoxyprobe, inc, Burlington, MA) overnight

within a humidified chamber at 4°C. After washing in 1x TBS, the tissue was incubated in streptavidin Alexa 594 (1:1000 in 0.1% TBS-T | Invitrogen, Carlsbad, CA). Omission of primary antibody served as a negative control. Slides were scanned on a Zeiss Axio Scan Z.1 digital slide scanner at 20x. Image files were imported into HALO software.

2.3.6.5. Colon hypoxia quantification

Healthy colon tissue is more hypoxic at the luminal edge compared to the crypt base (Singhal & Shah, 2020). To determine the effects of CCI on this hypoxic gradient, three well-oriented visual fields with at least three well-oriented crypts were identified within the distal colon and exported from HALO using the "Figure Maker" tool. These images were imported into FIJI (ImageJ version 1.53k, Schindelin et al., 2012) to plot the pixel intensity profile from luminal edge to crypt base as a means of assessing both the degree (intensity) and depth of hypoxia. The image scale was set based on the 100 μm scale bar embedded into each image. Using the line tool, two lines were drawn at the edges of each crypt from the luminal edge to the base of the crypt. After all lines were drawn, a gaussian blur was applied (σ radius = 3 μm) to minimize the effect that unlabeled goblet cells had on the pixel intensity profile. The pixel intensity gradient was plotted for the red channel using the "plot profile" tool. Because each intensity profile consisted of differing numbers of X, Y coordinates, points were interpolated within R to provide Y-values along an X-axis normalized to a percent distance (0 – 100% with 1% increments). The two interpolated hypoxia profiles for each crypt were then averaged to provide an overall pixel intensity profile of hypoxia from luminal edge to crypt base. Crypt hypoxia profiles were averaged within a field. Field hypoxia profiles were then averaged for each animal. Hypoxia intensity and depth profiles were imported from R to GraphPad Prism 9

(GraphPad Software, San Diego California, USA) to calculate the area under the curve and peak intensity after subtracting for the baseline of each curve as determined by the point at which the curve leveled out (average of values from the 51-100% distance from the lumen).

2.3.7. Pre-injury microbiome normalization and fecal collection

Because preliminary work suggested pre-injury microbiome variation among mice (data not shown), we normalized the microbiome of all mice prior to injury by flushing the GI tract of its contents with the osmotic laxative polyethylene glycol (PEG; Macrogol 4000, 425 g/L | Fortrans, Ipsen Pharma, France). The mice were then inoculated with cecal contents from age and sex matched mice (Wrzosek et al., 2018) as described below.

2.3.7.1. Cecal content collection

Conventionally raised, male mice were used as cecal content donors (n = 10). To allow for stabilization of their microbiome within our facilities, mice were acclimated to the vivarium with normal bedding and cage changes for 3 wks after their arrival. Following euthanasia at 8-9 wks of age, the cecum was rapidly removed, and its contents extruded into a sterile, DNase-free, polypropylene tube. Samples were flash frozen and stored at -80°C until thawing. Equal parts, by weight, from each animal were mixed and vortexed to a homogenate that was aliquoted and stored at -80°C until reconstitution for administration.

2.3.7.2. Whole bowel irrigation and microbiome transplant

Five-week-old mice were allowed to acclimate for one week prior to the three-week whole bowel irrigation procedure (outlined below). This allowed for mice to be injured within our target age range of 8-10 wks of age. One hour prior to whole bowel irrigation, mice were placed in a clean, bedding-free cage to prevent coprophagy. Animals were

allowed to acclimate for 1 hr with free access only to water. Mice were gavaged four times at 20 min intervals with 200 µl of PEG solution (Wrzosek et al., 2018). This procedure was effective in reducing overall DNA content within the colon's luminal contents while also visibly clearing the contents from the large intestine (Supplemental Figure S1). Cecal content homogenates were reconstituted immediately prior to gavage by thawing the homogenates in a warm water bath and rapidly diluting 100-fold in sterile PBS (Ubeda et al., 2013). Four hours after the final PEG gavage, animals were gavaged with reconstituted cecal material, and then gavaged again weekly for the next 3 wks (Figure 2.1A). Throughout the inoculation period, additional steps were taken to minimize microbiome differences prior to injury by using a mixed bedding approach (Miyoshi et al., 2018). At cage change, soiled bedding from all cages were mixed and redistributed in equal parts with fresh bedding. Weight of animals was monitored with each inoculation, 1 and 2 d prior to injury, on the date of surgery, and then again at each fecal collection timepoint after injury (Supplemental Figure S2). Body weight changes across time were equivalent for mice randomized into sham and CCI groups.

The week after the final microbiota inoculation, animals underwent sham or CCI injury as described above. Following surgery, additional steps were taken to minimize microbiome drift between cages of the same injury group by mixing equal parts soiled bedding from each cage of the same injury group with fresh bedding. The bedding from each injury group was then redistributed to all cages within that injury group.

Despite these efforts to normalize the microbiome in all mice prior to CCI, our subsequent analysis detected a difference in the CCI and sham groups prior to injury. At the order level, *Erysipelotrichales* was more abundant at pre-injury baseline in the CCI

group compared to the sham group (Supplemental Figure S3; $q = 0.02$). This suggests that our approach to microbiome normalization was largely effective but could not extinguish all pre-existing microbiome variation among the mice.

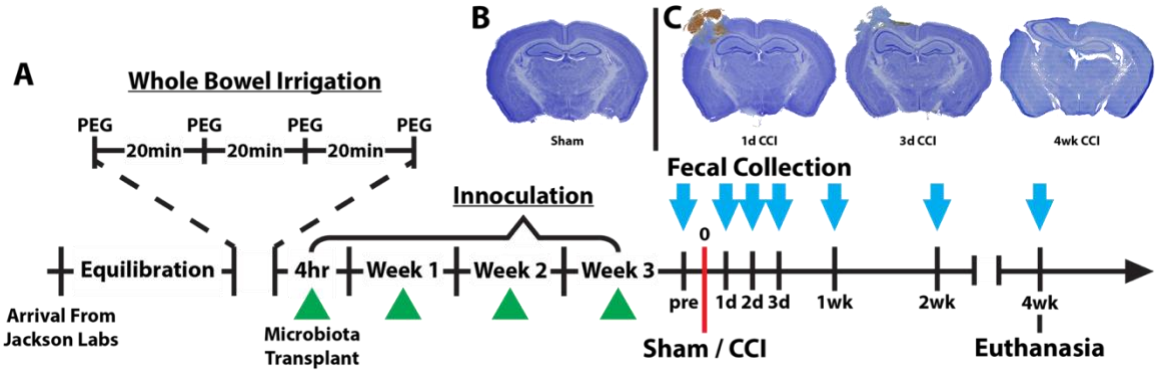


Figure 2.1: Study timeline of microbiome normalization, TBI and fecal collection

(A) Prior to fecal collection, the microbiome was normalized by performing a whole bowel irrigation followed by repeated inoculations with a cecal slurry (green triangles) collected from age and sex-matched mice. Fecal samples were collected (light blue arrow) prior to injury and at several timepoints out to 4 wks post-injury. Examples of Nissl stain from **(B)** a sham control and **(C)** controlled cortical impact (CCI) mice at early and chronic timepoints illustrate injury progression from a hemorrhagic contusion to cortical cavitation.

2.3.8. Fecal collection, DNA extraction, and DNA sequencing

Mice were placed in a clean cage without bedding and allowed to move around freely. Fresh fecal pellets were collected using a sterile syringe tip or sterile lancet and placed in a sterile DNase-free, polypropylene tube. Samples were flash frozen and stored at -80°C. DNA was purified from feces with a QIAamp PowerFecal Pro DNA Kit (Qiagen, Cat. No. 51804) as per manufacturer instructions. In brief, fecal samples were dissociated by vortexing with the provided beads and solutions. Cells were then lysed and inhibitors removed. DNA was purified, eluted, and stored at -80°C until sequencing.

Sequencing of the 16s rRNA gene was conducted by the University of Kentucky Genomics Core using the Illumina MiSeq platform. Sequencing libraries were prepared as per Illumina documentation for the V3-V4 region using the suggested primers (Fwd: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; Rev: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTA TCTAATCC), quantified, and normalized prior to sequencing (Illumina, 2013; Klindworth et al., 2013).

2.3.9. Microbiome analyses

2.3.9.1. Read processing and alpha and beta diversity

Adapter sequences were trimmed within the Illumina Miseq platform and the resultant raw reads were imported into R (version 3.6.3 – 4.1.2 as released). The raw and processed 16s rRNA sequencing data discussed within this document have been deposited in the NCBI Gene Expression Omnibus (Barrett et al., 2013) and are accessible through the accession number GSE239472 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE239472). All pre-processing and processing was conducted in R using RStudio

(RStudio Team, 2020), based on the workflow outlined by Callahan, Sankaran, Fukuyama, McMurdie, and Holmes (2016b). Low quality sequences were filtered out and the remaining sequences were trimmed to a consistent length by trimming the first 13 bases and truncating the final 10 bases. Forward and reverse reads were joined, and sequence variants inferred using Divisive Amplicon Denoising Algorithm (DADA2) to generate an amplicon sequence variant (ASV) table (Callahan et al., 2016a). Chimeric sequences were removed and samples containing fewer than 10,000 high quality reads were excluded from further downstream analysis. Two samples from a single sham animal (3 d and 2 wk) were excluded for having low read counts prior to filtering. Prior to filtering there was an average of 137413 reads per sample (minimum = 23253 | maximum = 320550) with an average of 29% of reads being discarded as low quality. After filtering for low-quality reads there was average of 98351 reads per sample with a minimum number of reads of 11691 and a maximum of 320550. Taxonomy was assigned using the SILVA reference database (v132) and a phylogenetic tree was constructed using DECIPHER (Wright, 2016). Within the Phyloseq package (McMurdie & Holmes, 2013), the ASV table, sample metadata, taxonomy information, and phylogenetic tree were merged into a Phyloseq object for diversity analysis.

Diversity metrics were calculated in R primarily utilizing the Phyloseq and Microbiome packages (Lahti, 2017; McMurdie & Holmes, 2013). Due to the high level of variability in read depth, alpha diversity metrics were calculated using data rarefied to a read depth of 10,000. To assess beta diversity, ASVs were transformed into relative abundance values and “distance” values were generated for three commonly used beta

diversity metrics: Bray Curtis Dissimilarity, UniFrac distance, and Weighted UniFrac distance. Diversity metrics are reviewed in Kers and Saccenti (2021).

2.3.9.2. Differential abundance analysis (ANCOM-BC)

Differential abundance analysis was conducted using Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC v1.2) within R (Lin & Peddada, 2020a). Importantly, this analysis accounts for the compositionality that is inherent to microbiome data sets (Gloor, Macklaim, Pawlowsky-Glahn, & Egozcue, 2017). Additionally, this analysis controls for the false discovery rate (FDR) and reports adjusted p-values (q-values). This analysis was conducted at different taxonomic levels by collapsing the ASV table by taxonomic rank (Phylum, Class, Order, and Family) to compare sham and CCI injury groups at each timepoint. Genus and species level comparisons were not conducted due to increasing uncertainty for assigning a read to a genus or species with each progressive taxonomic rank.

2.3.9.3. qPCR to assess Akkermansia muciniphila

Utilizing the same DNA isolated for 16s rDNA gene sequencing, qPCR was conducted using a custom TaqMan probe for *A. muciniphila* (Fwd: 5' CGGTGGAGTATGTGGCTTAAT; Rev: 5' CCATGCAGCACCTGTGTAA; Probe: 5' CGCCTCCGAAGAGTTCGCATG; [20x]) and a 16s pan-bacterial primer/probe set (Assay ID: Ba04930791_s1, ThermoFisher Scientific Catalog# 4331182, [20x]). A 10µl reaction was conducted with sample [DNA] = 5 ng/µl diluted in DNase/RNase free water, and TaqMan Universal PCR Master Mix [2x] in the proportions recommended by the manufacturer. Samples were run in triplicate on MicroAmp Fast Optical 96-well reaction

plates (0.1 ml; Catalog #: 4346907) and within-plate sample position was randomized. Reactions were carried out on an Applied Biosystems QuantStudio 7 Flex under the default parameters for use with Universal Master Mix. The initial holding stage was carried out from 25°C to 50°C with a ramp of 1.6°C/s where it was held for 2 min and then raised to 95°C with a 1.6°C/s ramp followed by 40 cycles each of separation at 95°C for 15 s and annealing at 60°C for 1 min.

Data output from qPCR were analyzed using the comparative CT ($2^{-\Delta\Delta Ct}$) method to compare sham and CCI mice at 1, 2, and 3 d post-injury normalized to pre-injury (Schmittgen & Livak, 2008). The ΔCt value was derived by subtracting the mean Ct from the *A. muciniphila* probe from the mean Ct of the universal 16s probe for each sample ($\Delta Ct = Ct_{A.\ muciniphila} - Ct_{16s}$), and the ΔΔCt was derived by subtracting the $\Delta Ct_{post\text{-}injury}$ from $\Delta Ct_{pre\text{-}injury}$ ($\Delta\Delta Ct = \Delta Ct_{post\text{-}injury} - \Delta Ct_{pre\text{-}injury}$). Data were then linearized using a log10 transform prior to statistical comparisons.

2.3.10. Statistical analyses

All analyses were performed in GraphPad Prism 9 unless otherwise specified. For statistical analysis of GI permeability data, sham mice from all timepoints were binned into a single sham group (n = 9) and compared against CCI mice that survived 4 hr (n = 7), 8 hr (n = 8), 1 d (n = 7), or 3 d (n = 6) by one-way ANOVA ($\alpha = 0.05$). Post-hoc analyses were conducted using Holm-Šídák's multiple comparisons test for each timepoint against sham controls ($\alpha = 0.05$). These group sizes reflect the exclusion of any mice for which an insufficient volume of blood was collected at euthanasia to perform the FITC Dextran assay. Ileum crypt-villi, colon crypt, and Alcian Blue measurement data were assessed by

two-way ANOVA ($\alpha = 0.05$) followed by post-hoc Holm-Šídák's multiple comparisons tests ($\alpha = 0.05$). For hypoxia parameters, sham animals from both timepoints were binned for data analysis and a one-way ANOVA was performed followed by post-hoc Holm-Šídák's multiple comparisons tests ($\alpha = 0.05$).

Statistical comparisons for alpha diversity were conducted by mixed-effects ANOVA. Statistical analysis and graphing of beta diversity was conducted within R. Beta diversity ordination distances were compared by PERMANOVA ($n_{perm} = 999$) using the "adonis" function within the vegan package [2.6-2 as described previously (Callahan et al., 2016b)]. Post-hoc, pairwise comparisons were conducted using the "pairwise.adonis" function contained within the RVAideMemoire package (version 0.9-81-2) (Herve, 2023), and p-values were adjusted using the False Discovery Rate ($n_{perm} = 999$; FDR adj $\alpha = 0.05$) (Martinez Arbizu, 2020). Comparative CT data generated from qPCR output (described above) were analyzed by pairwise one-tailed t-tests to test for an increase in *A. muciniphila* in CCI mice compared to sham mice.

2.4. Results

2.4.1. Intestinal permeability

Although several studies have examined parameters related to intestinal permeability in rodent models of TBI, the majority have relied on *ex vivo* or invasive *in situ* permeability assays, measurement of permeability biomarkers in blood, and inferences drawn from measurements of tight junction protein levels. We chose to employ an established, noninvasive method for assessing intestinal permeability in mice (Gupta & Nebreda, 2014) which has not yet been widely employed in neurotrauma models. We

administered FITC dextran (4 kDa) by oral gavage 4 hr prior to euthanasia at 4 hr, 8 hr, 1 d or 3 d post-injury. At euthanasia blood was collected and serum fluorescence was measured (Gupta & Nebreda, 2014). Under healthy conditions, paracellular permeability to FITC dextran within the GI tract should be minimal, leading to low serum fluorescence levels.

We chose to assess GI permeability *in vivo* to ensure that our CCI model induced gut barrier dysfunction as previously shown in a weight-drop model of TBI (Bansal et al., 2009; Bansal et al., 2010a; Bansal et al., 2010c; Lang et al., 2015). These prior studies assessed permeability by injecting FITC dextran directly into the jejunum or terminal ileum of the small intestine at sub-acute timepoints after WDI while others have utilized urinary lactulose: mannitol ratio after WDI (Hang et al., 2003; Zhang & Jiang, 2015)

Permeability differed significantly among groups (Figure 2.2; $F(4,32)=3.094$; $p = 0.03$). Subsequent post-hoc comparisons of injured groups to the sham control group revealed that CCI resulted in increased GI permeability at 4 hr after injury (adj $p = 0.0098$), but not at 8 hr, 1 d or 3 d post-CCI. These data demonstrate transient intestinal barrier dysfunction after brain injury.

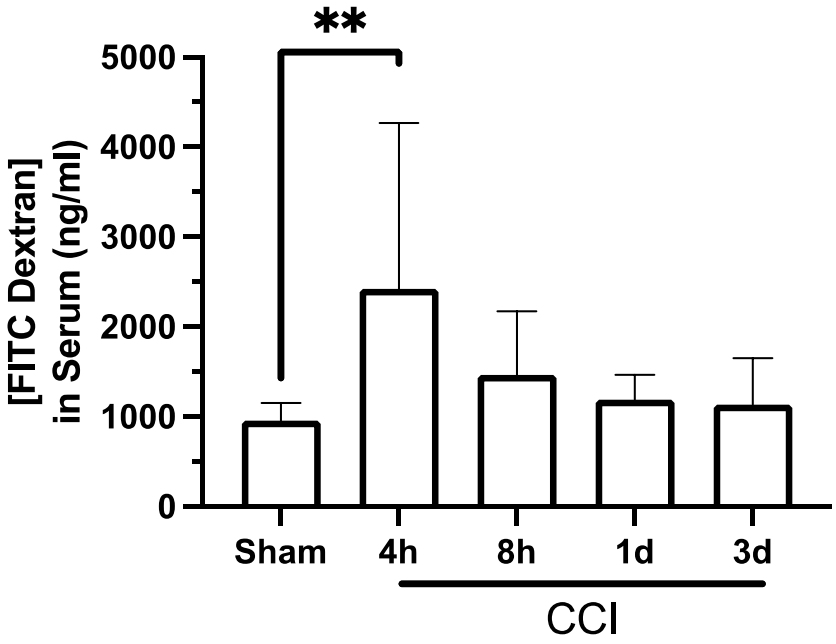


Figure 2.2: CCI results in a transient increase in intestinal permeability

Intestinal permeability was increased at 4 hr following controlled cortical impact (CCI) when compared to sham animals binned from all timepoints. Permeability returned to sham levels beginning at 8 h and remained there at 1 and 3 d post-injury. Data represent mean and standard deviation (**$p < 0.01$; n_{sham}= 2-3/timepoint, n_{CCI} = 6-9/timepoint).

2.4.2. Ileum and colon morphology

Changes in GI morphology have been reported in both the short and long term in various models of TBI (Bansal et al., 2009; Bansal et al., 2010c; Hang et al., 2003; Ma et al., 2017). Therefore, we assessed GI morphology in the ileum and colon over a comprehensive time course including 4 hr, 8 hr, 1 d, 3 d, 1 wk, 2 wk, and 4 wk after CCI or sham injury. Because alterations in the small intestine's crypt-villi structure are indicative of damage associated with altered nutrient absorption (Owens & Greenson, 2007), villi morphology was assessed by measuring the crypt-villi distance in well-oriented regions of both sham (Figure 2.3A) and CCI mice (Figure 2.3B). Within the ileum, crypt-villi distance was not significantly altered by CCI (main effect of injury, $F(1, 66) = 2.59$, $p = 0.11$) and did not vary as a function of survival time ($F(6, 66) = 0.21$, $p = 0.97$; Figure 2.3C). Further, no interaction was observed between injury and survival timepoint ($F(6, 66)=1.33$, $p = 0.26$).

Within the colon, shortened crypt depth is associated with acute inflammation while increased depth is associated with latent inflammation (DeRoche et al., 2014; Rubin & Levin, 2016). To assess whether CCI alters colon crypt morphology, the crypt depth was measured in the medial and distal regions of the colon for sham (Figure 2.3D) and CCI mice (Figure 2.3E). These two regions were quantified separately due to regional differences in function and crypt depth between the medial and distal portions of the colon. CCI had no overall effect on medial colon crypt depth ($F(1,66)=0.05$, $p = 0.82$; Figure 2.3F) or distal colon crypt depth ($F(1,66)=3.64$, $p = 0.11$; Figure 2.3G). No effect of survival timepoint was observed in the medial colon ($F(6,66)=1.18$, $p = 0.33$), but a main effect was present in the distal colon ($F(6,66)=5.87$, $p < 0.0001$). However, no interaction was observed between injury and time in the medial colon ($F(6,66)=1.28$, $p = 0.28$) or distal

colon ($F(6,66)=1.29$, $p = 0.27$), suggesting no overt morphological changes in the large intestine due to injury.

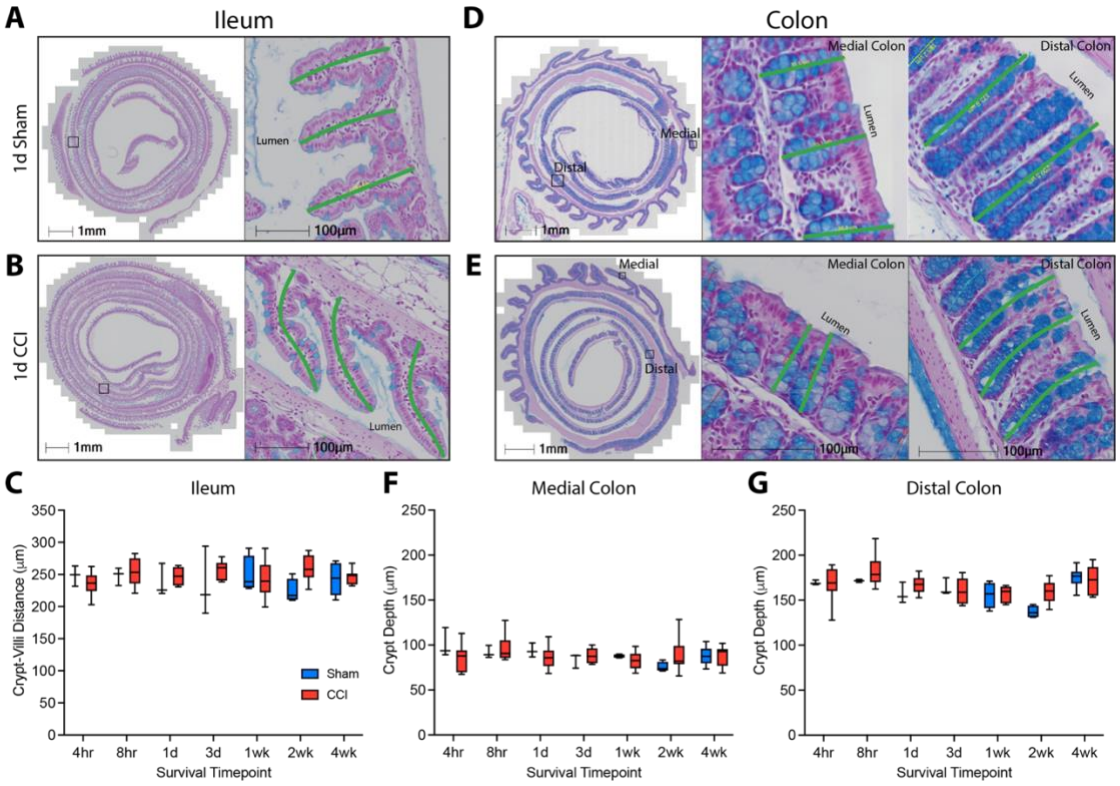


Figure 2.3: CCI does not alter morphology of the ileum or colon

Representative images of the crypt-villi structure from **(A)** Sham and **(B)** CCI mice. Ileal crypt-villi distance and colon-crypt depth was measured in small and large intestinal tissues. Green lines have been overlaid on top of the software's measurement annotations to accentuate the distance measurements acquired. **(C)** Quantification of average crypt-villi depth measurements for each survival timepoint shows that CCI did not alter crypt-villi distance in the ileum. Representative images illustrating crypt depth measurements in the colon (green lines) from **(D)** Sham and **(E)** CCI mice. Quantification of the average crypt depth suggests that CCI did not alter crypt morphology in the **(F)** medial or **(G)** distal colon. In the distal colon, a main effect of time after injury was present. Whiskers represent minimum and maximum, box represents 25th-75th percentile, and line represents median value (n_{Sham}= 3-6/ timepoint, n_{CCI} = 6-10/ timepoint).

2.4.3. Gut microbiome

2.4.3.1. Alpha diversity

To evaluate the hypothesis that TBI alters the microbiome, fecal samples were collected at multiple timepoints in mice surviving 4 wks following sham or CCI injury. Microbial DNA was isolated from fecal pellets and the microbiome was analyzed using 16s rDNA gene sequencing. Alpha diversity, a measure of within sample diversity, was assessed by using Shannon Diversity Index and Faith's phylogenetic diversity (PD). Shannon Diversity Index assesses within sample diversity while accounting for richness (the number of observed taxa present) and evenness (the distribution of each taxon). Faith's PD accounts for richness and evenness, as well as for phylogenetic distance between bacteria. Shannon diversity (Figure 2.4A) was not altered with collection timepoint ($F(3.32, 41.50) = 0.87$; $p = 0.47$), but was reduced in the CCI group ($F(1,75) = 8.03$; $p = 0.006$) independent of time ($F(1,75) = 1.08$; $p = 0.38$). Faith's PD (Figure 2.4B) was not altered with collection timepoint ($F(3.24, 34.53) = 0.44$; $p = 0.74$), injury condition ($F(1,11) = 3.20$; $p = 0.10$), or their interaction ($F(6,64) = 1.37$; $p = 0.24$).

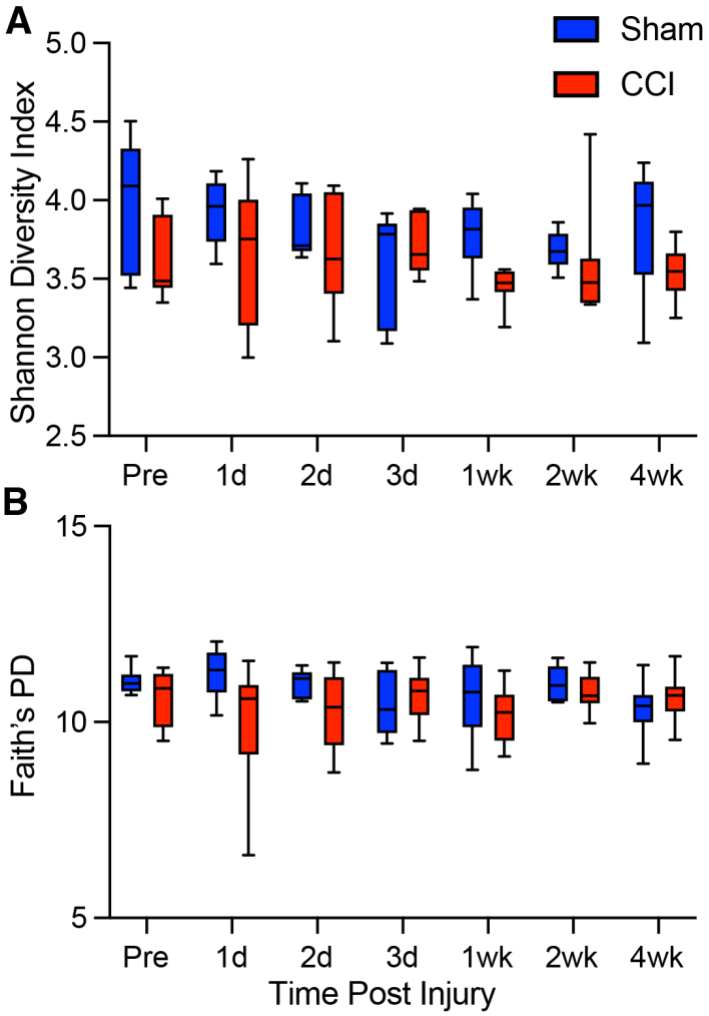


Figure 2.4: CCI results in decreased alpha diversity as measured by Shannon diversity index

(A) The Shannon Diversity Index was significantly reduced in mice with CCI as compared to sham controls, but this injury effect did not depend on survival time point. **(B)** Faith's phylogenetic diversity was not altered by injury or time point. Whiskers represent minimum and maximum, box represents 25th-75th percentile, and line represents median value (n_{Sham}= 6, n_{CCI} = 7).

2.4.3.2. Beta diversity

To continue to investigate community level changes in the microbiome, we analyzed beta diversity, a between sample comparison method used to determine the extent that bacterial profiles differ from each other. Beta diversity was assessed by three common measures: Bray-Curtis Dissimilarity (Bray & Curtis, 1957b), UniFrac Distance, and weighted UniFrac Distance (Lozupone & Knight, 2005b). Beta diversity metrics are reviewed by IMPACTT-investigators (2022). CCI induced a change in beta diversity when comparing groups for Bray-Curtis Dissimilarity (p = 0.002; Figure 2.5A), a well-established ecological calculation that provides a quantitative metric of how dissimilar two groups are from one another (Bray & Curtis, 1957b). Additionally, CCI altered weighted UniFrac distance (p = 0.008; Figure 2.5C) and there was a trend present with UniFrac distance (p = 0.053; Figure 2.5B). UniFrac distance, weighted or unweighted, is a newer method of calculating differences between communities created specifically for 16s rRNA gene sequencing data and in which differences between groups are determined by shared taxa while accounting for phylogenetic distance (Lozupone & Knight, 2005b). Notably, post-hoc pairwise PERMANOVA for both Bray-Curtis and UniFrac revealed a significant difference that developed at 4 wks following CCI compared to baseline and many early timepoints in the sham controls. Full pairwise comparison matrices for Bray-Curtis dissimilarity are presented within Supplemental Table S1 (unadjusted p-values) and Supplemental Table S2 (FDR-adjusted p-values). Full pairwise comparison matrices for weighted UniFrac are found within Supplemental Table S3 (unadjusted p-values) and Supplemental Table S4 (FDR-adjusted p-values).

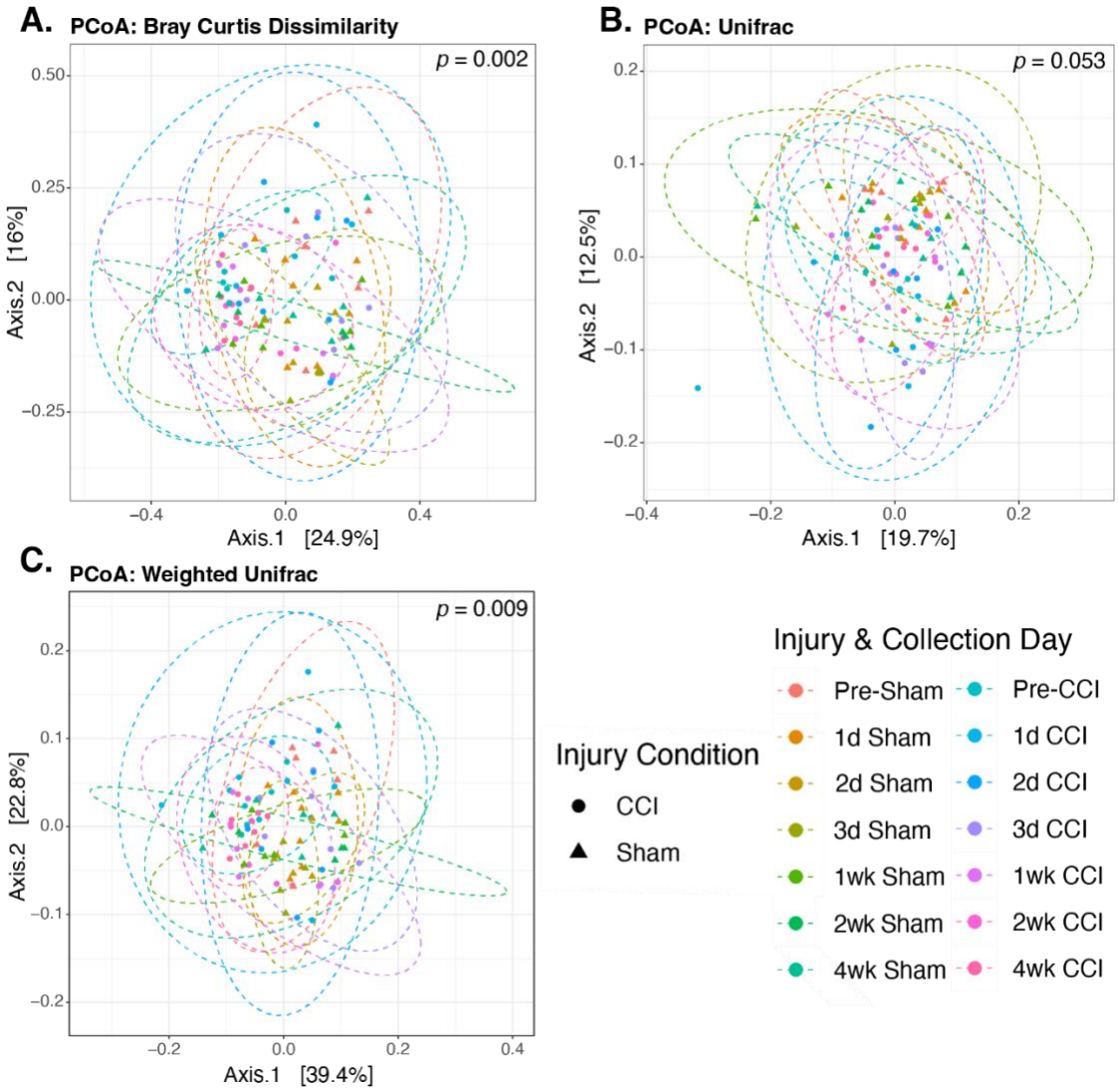


Figure 2.5: Altered Bray-Curtis dissimilarity and weighted UniFrac distance following CCI

Principal coordinate analysis visualizations for various beta-diversity metrics: Statistical comparison by PERMANOVA: **(A)** Bray-Curtis Dissimilarity (p = 0.002), **(B)** UniFrac distance (p = 0.053), and **(C)** weighted UniFrac distance (p = 0.009; n_{Sham}= 6, n_{CCI} = 7).

2.4.3.3. Differential abundance analysis

We next sought to identify differences in bacterial taxa between the CCI and sham-injured animals. To visualize these results, the relative abundance of each taxa at phylum and family levels was represented as a function of time point within each injury group (Figure 2.6). Consistent with prior studies, large changes between injury and sham groups were not seen (Angoa-Pérez et al., 2020; Nicholson et al., 2019). The primary visual impression is that the effect of CCI on the microbiome is subtle with a potential difference between the phylum *Verrucomicrobiota* between sham and CCI mice (Figure 2.6A). Within the *Verrucomicrobiota* phylum, the family *Akkermansiaeceae* shows a similar trend (Figure 2.6B).

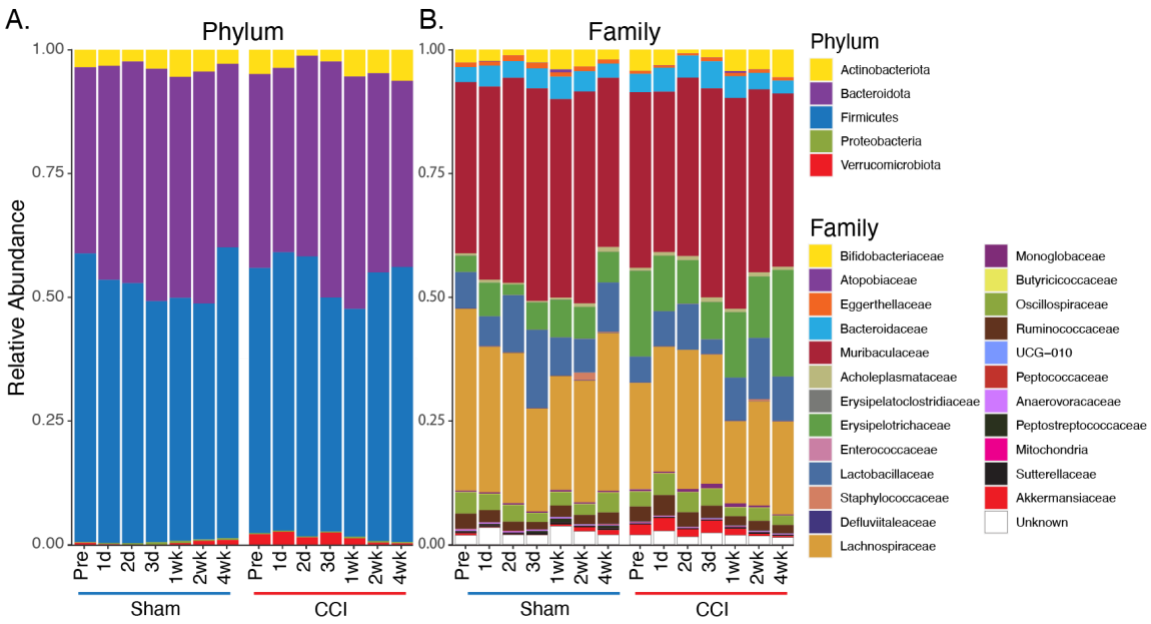


Figure 2.6: Relative abundance taxa distribution

Representation of relative abundance by injury group and collection timepoint at (**A**) phylum level, and (**B**) family level. Complete relative and absolute abundance information can be found in the Supplemental Figure S4.

To identify statistically significant differences in taxa between sham and CCI mice, we performed differential abundance analysis (ANCOM-BC) at a phylum, class, order, and family taxonomic levels. The primary taxa altered after CCI was the phylum *Verrucomicrobiota*, which showed an early increase in CCI mice which was statistically significant at 1, 2, and 3 d post-injury when compared to time-matched sham animals (Figure 2.7A). This increase was also statistically significant at the class level at these same time points (*Verrucomicrobiae;* data not shown) and at the order level at 2 and 3 d post-injury (*Verrucomicrobiales*; Figure 2.7B).

The *Verrucomicrobiota* species typically residing in the mammalian GI tract is *Akkermansia Muciniphila*. Because 16s sequencing does not provide sufficient resolution to consistently quantify at the species level, we performed qPCR to directly assess whether *A. muciniphila* changed with CCI. We found that *A. muciniphila* was increased within the microbiome of injured mice at 2 d ($p = 0.0361$) and 3 d ($p = 0.0366$), with a trend at 1 d ($p = 0.0982$) (Figure 2.7C). These findings confirm that the increase in *Verrucomicrobiota* with CCI is due to an increase in *A. muciniphila*.

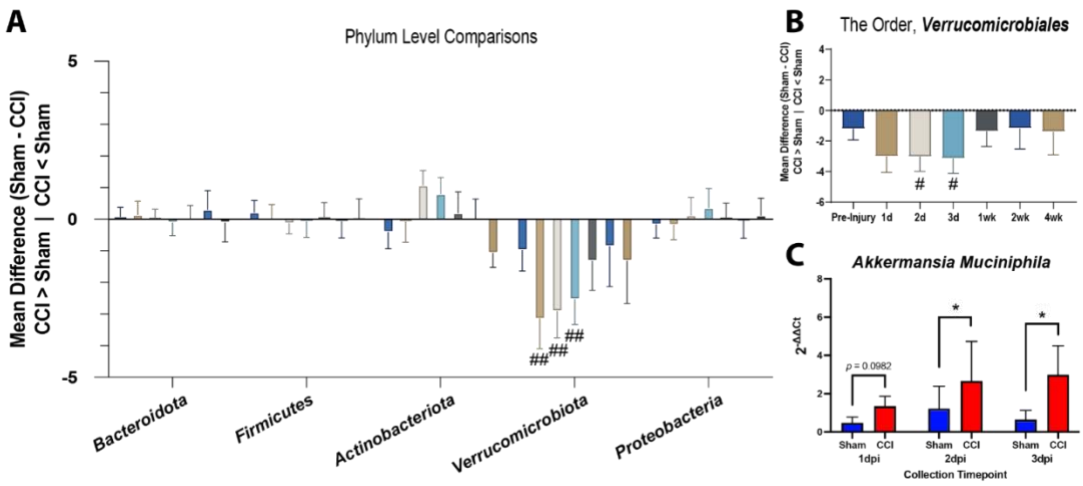


Figure 2.7: Early increase in abundance of taxa *Verrucomicrobiota* following CCI

(**A**) The phylum *Verrucomicrobiota* is differentially abundant in CCI animals compared to time-matched sham animals at 1, 2, and 3 d post-injury using ANCOM-BC. A mean difference value (y-axis) of 0 indicates equivalent taxa abundance between sham and CCI injured mice, above 0 indicates increased abundance in sham mice compared to time matched CCI animals, and a mean difference value below 0 indicates that a taxa is more abundant in CCI animals. (**B**) This taxa is differentially abundant down to an order level (*Verrucomicrobiales*) in CCI animals compared to time-matched sham animals at 2 and 3 d post-injury. Data are represented as mean difference and standard error (# $q \leq 0.05$, ## $q \leq 0.01$). (**C**) The *Verrucomicrobiota* species *Akkermansia muciniphila* is increased at 2 and 3 d post-injury as determined by qPCR. Data are represented as mean and standard deviation (* $p \leq 0.05$).

2.4.4. Intestinal goblet cell quantification

Given that *A. muciniphila* lives within the mucus layer and is increased after CCI, we investigated the effect of CCI on the ileal and colonic goblet cells. Mucus produced by goblet cells primarily acts as a lubricant while forming a selective barrier that limits epithelial interaction with luminal contents and bacteria (Gustafsson & Johansson, 2022). Mucus contained within goblet cells was labeled using the muciniphilic dye, Alcian blue.

CCI did not alter the number of goblet cells within the ileum (main effect: $F(1,66) = 0.10$; $p = 0.76$) (Figure 2.8A-C). Goblet cell number varied as a function of survival timepoint ($F(6,66) = 4.92$; $p = 0.0003$), but this effect did not depend on injury status ($F(6,66) = 1.05$; $p = 0.40$). Goblet cell density was assessed in the medial (Figure 2.8D-F) and distal (Figure 2.7G-I) regions of the colon. Within the medial colon a significant interaction between injury condition and survival timepoint was observed ($F(6,66)=2.65$; $p = 0.02$) with a significant main effect of survival timepoint ($F(6,66)=8.94$; $p < 0.0001$), but no main effect of injury ($F(1,66)=0.01$; $p = 0.92$). Post-hoc comparisons revealed a significant increase in goblet cell density at 1 d in CCI mice compared to sham mice (Figure 2.8F; $p = 0.03$). Within the distal colon, goblet cell density was not altered by injury condition ($F(1,66)=0.82$; $p = 0.38$), but varied across survival timepoints ($F(6,66)=12.93$; $p < 0.0001$) without a significant interaction (Figure 2.8I; $F(6,66)=1.67$; $p = 0.14$).

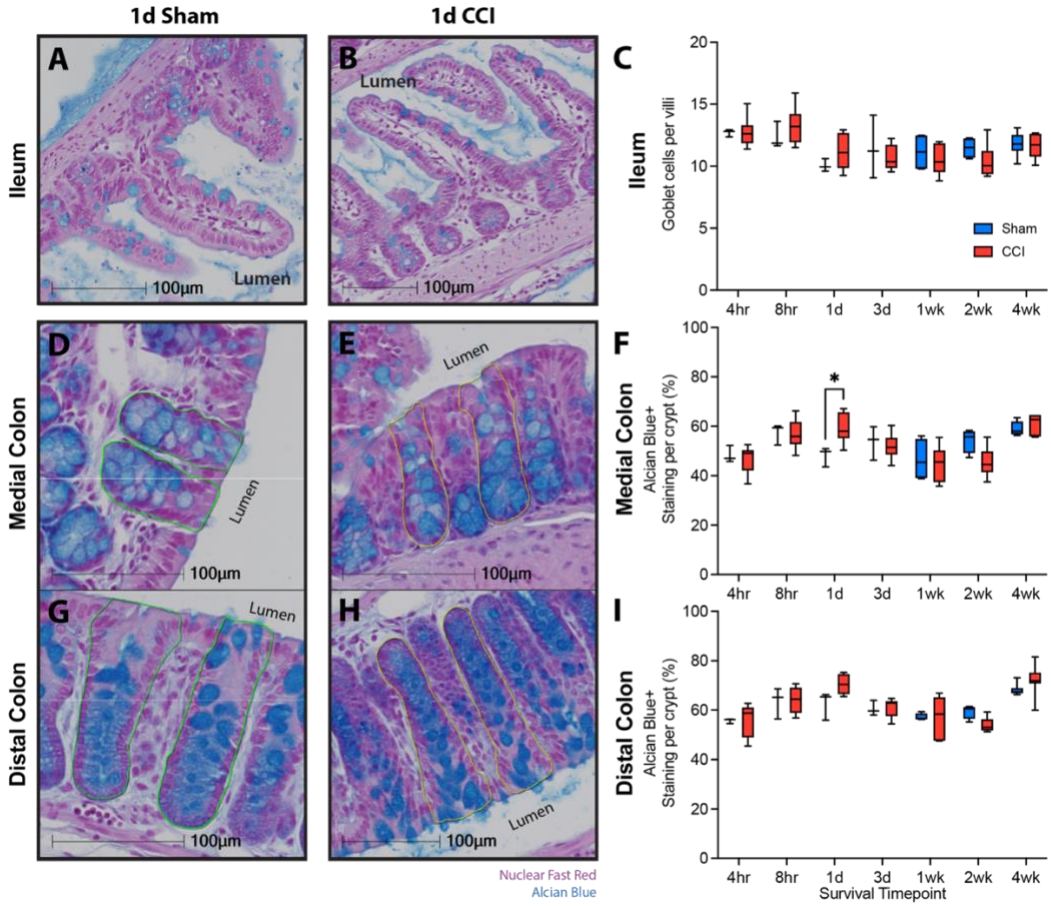


Figure 2.8: CCI increases goblet cell density in the medial colon at 1 d post-injury while goblet cells in the ileum and distal colon are not altered

Intestinal tissue was stained with Alcian Blue to label mucins within goblet cells and counterstained with nuclear fast red. Representative images of the distal third of the small intestine, the ileum, from **(A)** 1 d sham and **(B)** 1 d CCI mice. **(C)** Counts of goblet cells per villi were equivalent for Sham and CCI groups but varied across survival timepoints. Representative images of colon tissue from both the **(D, E)** medial and **(G, H)** distal colon of 1 d sham and 1 d CCI mice, respectively. **(F)** CCI increased goblet cell density in the medial colon at 1 d, **(G)** but no effect of injury was observed in the distal colon. A main effect of survival timepoint was present in both regions of the colon. Whiskers represent minimum and maximum, box represents 25th-75th percentile, and line represents median value (*$p \leq 0.05$; n_{Sham}= 3-6/ timepoint; n_{CCI} = 6-10/ timepoint).

2.4.5. Colon hypoxia

To further investigate the means by which *A. muciniphila* may be increasing after CCI we assessed colon hypoxia using pimonidazole-HCl in sham (Figure 2.9A) and CCI mice (Figure 2.9B). Because *A. muciniphila* is an obligate anaerobe and lives in relatively close association with the host epithelium, its abundance has been shown to increase under hypoxic conditions. For example, Alam et al. (2016) showed that wound-induced hypoxia results in a preferential increase of *A. muciniphila* at the site of hypoxia. These microbes then in turn helped to promote epithelial outgrowth and wound healing (Alam et al., 2016).

Under healthy circumstances, the colon exists under a state of physiologic hypoxia. As cells migrate out of the crypt stem-cell niche toward the lumen there is a reduction in the partial pressure of oxygen. As such, luminal facing epithelial cells exist in a state of hypoxia (Singhal & Shah, 2020). Disturbances in oxygen distribution can alter the level of hypoxia as well as the depth of penetration of this hypoxic gradient. Immunolabeling for Pimonidazole-HCl protein adducts formed under hypoxic conditions allows for visualization and quantification of the hypoxic gradient within the colon via analysis of staining intensity as a function of distance along the luminal-basal axis (Figure 2.9C). Following CCI, hypoxia appeared more intense with deeper penetration into crypt structures. Indeed, both the area under the curve (1-way ANOVA: $F(2,21) = 3.64$; $p = 0.04$) and peak intensity (1-way ANOVA: $F(2,21) = 4.1$; $p = 0.03$) of hypoxyprobe-1 labeling revealed progressive increases over the first days after CCI. Peak hypoxic intensity was significant elevated at 3 d post-injury ($p = 0.03$; Figure 2.9D), while the area under the curve showed a trend toward an increase at 1 d ($p = 0.06$) and a significant increase at 3 d ($p = 0.03$; Figure 2.9).

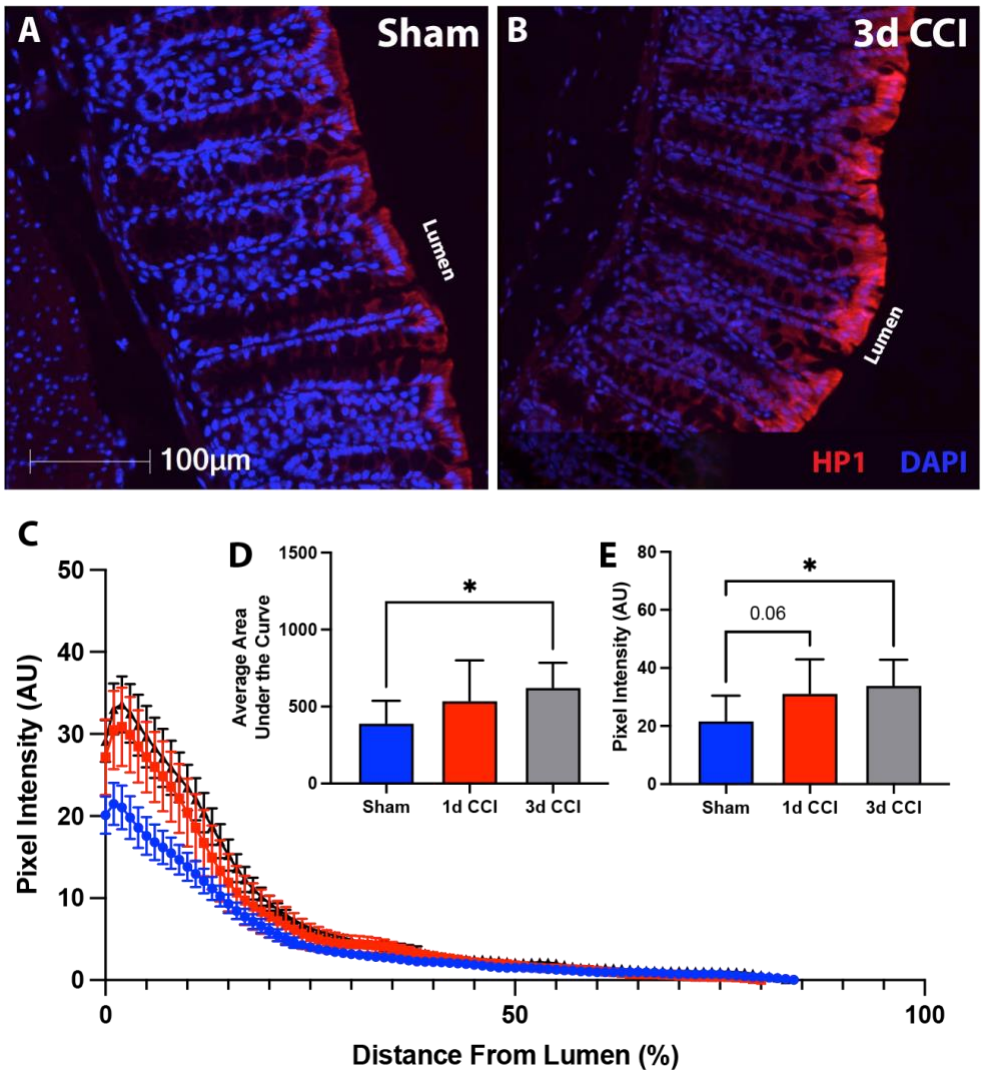


Figure 2.9: CCI induces colon hypoxia at 3 d post-injury

Representative images of hypoxyprobe-1 staining (HP1, red) in the colon of **(A)** Sham and **(B)** 3 d post-CCI mice. **(C)** Traces of hypoxia intensity as a function of distance from the lumen, averaged from three crypts within three randomly selected fields of view from Sham (blue), 1 d CCI (red), and 3 d CCI (black). **(D)** Area under the hypoxia intensity curve is increased at 3 d post-CCI compared to sham mice. **(E)** Peak hypoxia is also increased at 3 d post-CCI. Data is presented as mean and standard deviation (*$p \leq 0.05$; $n_{sham} = 11$, $n_{CCI} = 6\text{-}7$/ timepoint).

2.5. Discussion

In this study, we assessed effects of a moderate severity contusive brain injury on GI tract barrier function and morphology, the fecal microbiome, and colon hypoxia. Using a noninvasive FITC-dextran permeability assay, intestinal permeability was found to increase at 4 hr after CCI injury, returning fully to the level of sham controls by 1 d. To determine if this early change in permeability contributed to tissue damage along the GI tract, we evaluated small and large intestinal morphology. CCI did not alter ileal crypt-villi distance or colon crypt-depth at the time of the permeability increase or at subsequent timepoints spanning a month after the injury. Microbiome analyses revealed a reduction in Shannon diversity in the CCI group and alterations in beta diversity between groups. Differential abundance analysis identified increases in the phylum Verrucomicrobiota at 1, 2, and 3 d post-injury. The *Verrucomicrobiota* species, *A. muciniphila*, was confirmed by qPCR to increase over the same timeperiod. To assess physiological factors known to either coincide with or propagate growth of this obligate anaerobe, mucus-producing goblet cells and colonic hypoxia were evaluated. CCI led to increased goblet cell density in the medial colon at 1 d post-injury but did not affect goblet cells within the ileum or distal colon. In addition, CCI promoted an early increase in colon hypoxia that was statistically significant by 3 d post-injury.

Several previous studies have provided evidence that TBI results in increased permeability of the GI tract. Clinically, increased permeability of the small intestine can be detected by an increased lactulose: mannitol urine concentration after oral administration of these two non-metabolized sugars (Sequeira, Lentle, Kruger, & Hurst, 2014). This approach has also been used to infer TBI-related increases in gut permeability in rats from 3 hr to 1 wk after injury (Hang et al., 2003; Zhang & Jiang, 2015), but its interpretation

may not be straightforward, as an elevation lactulose: mannitol concentration can result from either increased paracellular transport of lactulose (i.e. increased permeability) or a decrease in mannitol due to loss of absorptive area in the gut consequent to damage to ileal villi structures (Hang et al., 2003). Increased paracellular permeability after brain injury is believed to be due to loss or dysfunction of tight junction proteins that regulate paracellular flux. Reductions in several tight junction proteins have been demonstrated following rodent experimental TBI in the ileum at 3-12 hr (Bansal et al., 2009; Lang et al., 2015) and 3 d (Ma et al., 2019b), in the cecum at 3 d (Simon et al., 2020), and in the proximal colon at 2 wk post-injury (Li et al., 2018).

Here, we found only a transient increase in GI permeability at 4 hr using a noninvasive FITC-dextran intestinal permeability assay to measure paracellular movement of FITC-dextran from the intestinal lumen into the bloodstream. The brief nature of the alteration in permeability in our study as compared more sustained dysfunction reported in other studies may be due, in part, to differences in injury severity, injury model, or permeability assay. Notably, prior studies that identified increased FITC-dextran permeability up to 12 hr after weight drop injury in mice (Bansal et al., 2009; Bansal et al., 2010a; Bansal et al., 2010c) or rats (Lang et al., 2015) used an invasive approach in which the intestinal tract is removed from the abdominal cavity, ligated and injected with FITC-dextran before being returned to the body cavity. These manipulations may contribute to a more protracted permeability disruption.

Changes in intestinal barrier function often correspond with changes to the specialized crypt-villi and crypt structures that line the small and large intestine, respectively. Contusion TBI in rodents has been reported to induce damage in the ileum.

However, many prior studies have focused on a single region of the GI tract and single survival timepoints, utilizing qualitative or semi-quantitative assessments. Here, we performed a quantitative histological examination of ileal crypt-villi distance and colon crypt depth over an extended time course. We found that CCI did not induce changes to the crypt-villus length in the small intestine or crypt depth in the colon at any timepoint. These data contrast findings of epithelial lifting (Hang et al., 2003; Lang et al., 2015) and either villus lengthening (Feighery et al., 2008) or shortening (Bansal et al., 2010c) within the first several hours, followed by decreases in villus length and crypt depth over the period from 1d to 7 d post-injury (Hang et al., 2003; Ma et al., 2019b; Zhang & Jiang, 2015). A potential caveat to several of these studies is that postmortem histological examinations were performed on segments of the ileum that had previously been externalized from the mouse or rat, ligated and injected with FITC-dextran to assay GI permeability (Bansal et al., 2009; Bansal et al., 2010a; Bansal et al., 2010c; Lang et al., 2015). Thus, tissue ischemia, hypoxia or inflammation resulting from this procedure may have exaggerated effects of the TBI. As the majority of studies noting GI tissue pathology employed injury parameters described as creating a severe TBI (Feighery et al., 2008; Hang et al., 2003; Lang et al., 2015; Ma et al., 2019b), overt GI histological damage may be a consequence of severe, rather than moderate, experimental TBI. For example, images of brain histology provided in Ma et al. (2019b) demonstrate loss of not only of cortex but also the underlying hippocampus, while the moderate CCI employed here results in a contusion localized to the cortex, leaving the hippocampus grossly intact (see Figure 2.1). Few studies have examined histopathology within the colon after TBI. Following severe CCI, edema was reported in some rats at 6 hr (Feighery et al., 2008), but no damage was

detected at 3 d (Simon et al., 2020). While Ma et al. (2017) found no histological damage in the colon at 1 d consistent with our findings, they noted increased crypt depth and smooth muscle layer thickness at 28 d, suggestive of delayed or progressive damage. Our data argue that moderate CCI is not associated with overt histological damage to the small or large intestine over the first 4 wk after injury. It is possible, however, that small, localized regions of shortening/lengthening may exist but were not detected due to the large number of crypt-villi and crypt structures we assessed along the longitudinal axis of the Swiss-rolled tissue preparations.

Mice with CCI had reduced alpha diversity compared with sham controls, as measured by Shannon Diversity. This effect was not dependent upon time point post-injury, but is consistent with observations of reduced alpha diversity in previous studies (Opeyemi et al., 2021; Simon et al., 2020) Microbiome composition differed among experimental groups with respect to beta diversity, with most notable differences in the 4 wk post-injury group, in line with late changes in beta diversity reported in other studies of CCI (Opeyemi et al., 2021).

Changes in the compositional abundance of the gut microbiome after TBI are complex and vary across studies, as recently reviewed by Hanscom et al. (2021b). Here we show that moderate CCI resulted in relatively limited alterations in the microbiome, with significant increases in taxa within the phylum *Verrucomicrobiota* at 1, 2 and 3 d. The lack of widespread changes may be due in part to stringent efforts to minimize variation in pre-injury microbiome composition, where we first cleared the microbiome with PEG before inoculating them with a common microbiome collected from the cecal contents of age and sex matched mice and then used a mixed bedding approach to maintain similar

microbiomes prior to injury. As mentioned above, the use of moderate, rather than severe, injury may also have resulted in more limited changes to the microbiome than previously reported. Increases in the phylum *Verrucomicrobiota* have been observed in mice (Opeyemi et al., 2021; Yanckello et al., 2022), in rats (Ma et al., 2019b; Nicholson et al., 2019) and humans (Urban et al., 2020) after brain injury, affirming the robustness of this observation across species and injury model.

Although the major gut microbe within this phylum is *A. muciniphila*, our study is the first to our knowledge to utilize qPCR to confirm that the *Verrucomicrobiota* species increased after TBI is *A. muciniphila*. This species is a gram-negative bacteria that is most prevalent in the colon where it resides in the intestinal mucus layer (Geerlings, Kostopoulos, de Vos, & Belzer, 2018). *A. muciniphila* is critical to maintaining normal gut health (Rodrigues et al., 2022), with alterations in abundance influencing intestinal epithelial growth, differentiation (Kim et al., 2021), integrity (Reunanen et al., 2015), and wound healing (Alam et al., 2016). An early increase in *A. muciniphila* may be part of a compensatory response to systemic damage initiated by TBI. It should be noted that a recent paper in CCI injured mice of a similar severity to the present study showed a reduction in in *Akkermansia* at 1 wk and 4 wk following CCI compared to sham (Zheng et al., 2022), suggesting that there may be changes at more chronic timepoints that may be based on housing or other factors, but those are not readily apparent based on their report.

A. muciniphila feeds off of the intestinal mucus layer and, by doing so, signals to the host to increase and maintain mucus production (Belzer & de Vos, 2012), while also influencing intestinal stem cell differentiation and subsequent outgrowth (Kim et al., 2021). Based on the important role that *A. muciniphila* plays in maintaining the intestinal mucus

layer, we investigated changes to the mucus-producing goblet cells within the ileum, medial colon, and distal colon. Numbers of goblet cells in the ileum were unchanged with moderate CCI. However, goblet cell density increased within the medial colon at 1 d post-injury, with no change in the distal colon. The medial colon may be the site for this increase as it contains transverse folds that provide a more stable mucosal niche for bacteria than in the distal region of the colon (Donaldson, Lee, & Mazmanian, 2016). An increase in density could reflect increases in goblet cell number or size. The goblet cell response to inflammation is complicated in that under most inflammatory conditions, goblet cell differentiation markers generally increase leading to an increase in numbers of goblet cells. Some inflammatory conditions such as ulcerative colitis, however, are associated with a reduction in the number and size of goblet cells (Gersemann et al., 2009; Smillie et al., 2019). Only one other study has assessed goblet cells after brain injury, finding no difference in the cecum at 3 d after a mild closed head injury (Houlden et al., 2016).

Epithelial metabolism can alter the oxygen availability within the intestinal lumen (Singhal & Shah, 2020). Due to the relatively close association of *A. muciniphila* with the epithelium, hypoxic conditions within the epithelium and lumen can promote the growth of this obligate anaerobe (Alam et al., 2016). Our findings show that CCI leads to a progressive increase in colon hypoxia over 3 d post-injury. Together with the increase in goblet cell density at 1 d, posttraumatic hypoxia leads to conditions within the colon that promote *A. muciniphila* proliferation.

Others have shown beneficial effects of direct administration of *A. muciniphila* as a means of treatment for neurological conditions in rodent models of depression (chronic restraint stress) (Ding et al., 2021), Alzheimer's disease (APP/PS1) (Ou et al., 2020), and

amyotrophic lateral sclerosis (ALS | *Sod1*-Tg) (Blacher et al., 2019). Additionally, the fermentable fiber inulin, which increases *A. muciniphila*, was found to increase thalamic and hippocampal blood flow after closed head TBI (Yanckello et al., 2022), suggesting that inulin and perhaps *A. muciniphila* are beneficial after TBI. More broadly, fecal *A. muciniphila* levels have been proposed as a marker for discriminating between healthy control patients and patients with both intestinal and extra-intestinal conditions (Lopetuso et al., 2020).

Overall, our findings add to the field's understanding of the complexity of the GI tract's response to TBI. Our current working hypothesis is visually summarized in Figure 2.10. Our primary findings are that TBI induces early intestinal permeability and an increase in *A. muciniphila* that temporally corresponds with increased medial colon goblet cell density and increased colon hypoxia in the absence of altered GI tract morphology. Since this bacterium has been associated with improved barrier integrity and general gut health, the increase in *A. muciniphila* with TBI may reflect a beneficial compensatory alteration in the gut microbiome sufficient to limit histological damage to the GI tract after moderate TBI. Because of the unique role of *A. muciniphila*, potential interventions that prolong or promote the growth of this bacteria warrant further investigation as novel therapeutics after TBI.

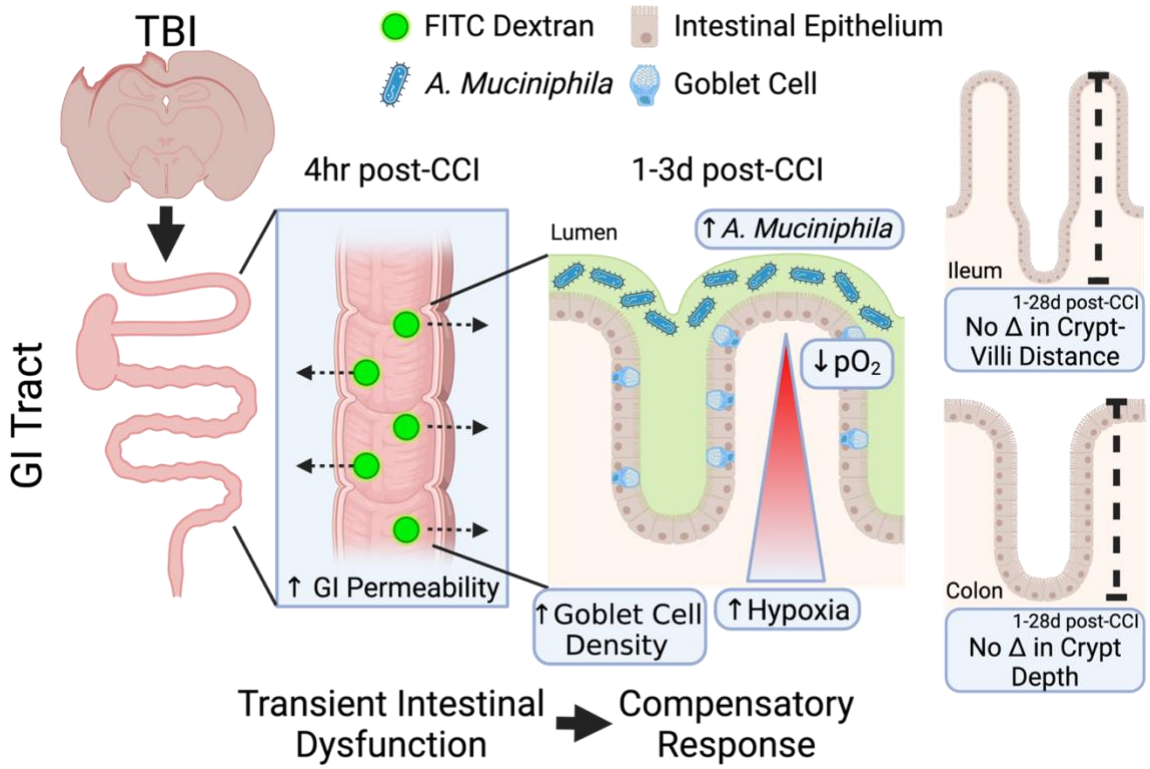
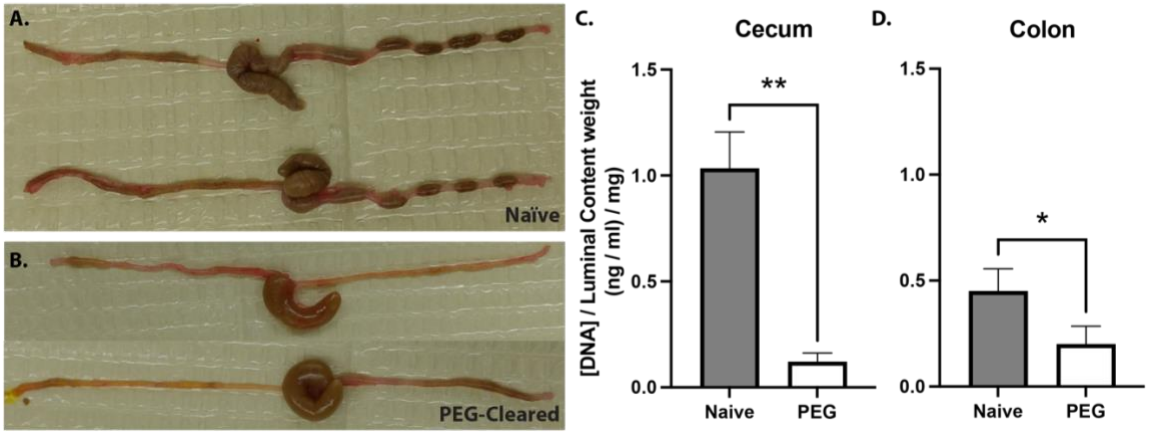


Figure 2.10: Visual abstract

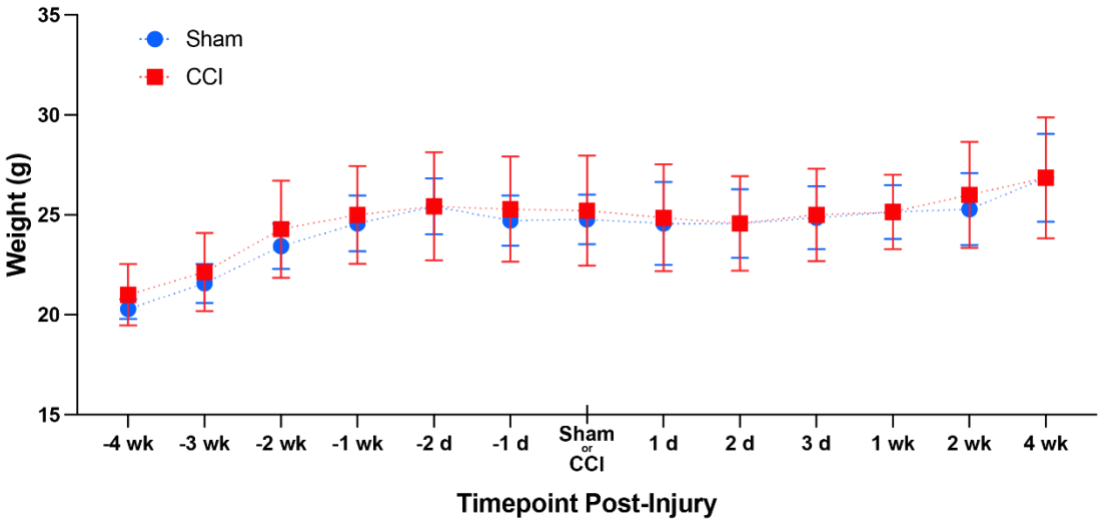
We hypothesize that TBI leads to a transient disruption of the intestinal barrier that initiates a colonic response characterized by increased goblet cell density and hypoxia, facilitating an increase in *A. muciniphila* as a beneficial compensatory response to limit damage to the GI tract. Figure created with BioRender.com.

2.6. Supplemental figures



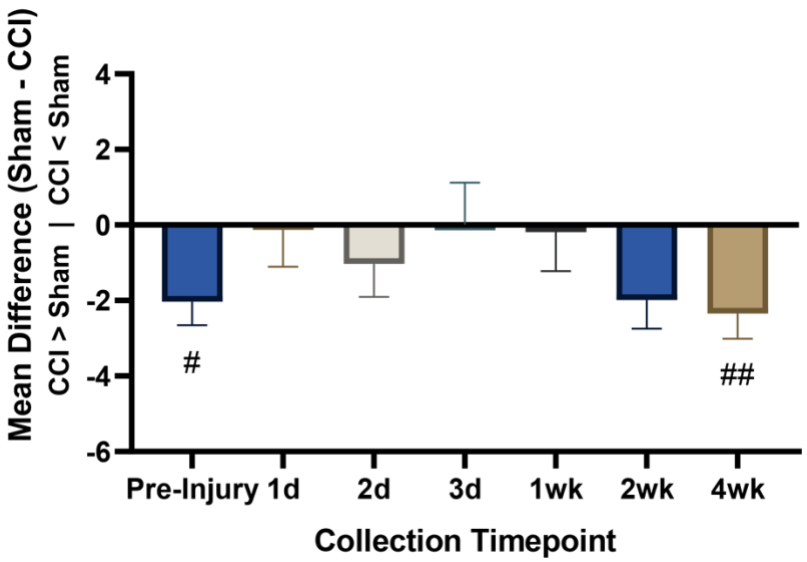
Supplemental Figure S1: Whole bowel irrigation with PEG effectively reduces the concentration of DNA in the luminal contents of the large bowel

Image of the terminal segment of the small intestine, cecum, and colon excised from **(A)** a naïve animal and **(B)** a mouse that underwent whole bowel irrigation with polyethylene glycol. The concentration of DNA within the luminal contents is significantly reduced in the **(C)** cecum and **(D)** colon. Data represent mean and standard deviation (one-tailed T-test; $*p \leq 0.05$, $**p \leq 0.01$; $n_{naive} = 2$, $n_{PEG} = 3$)



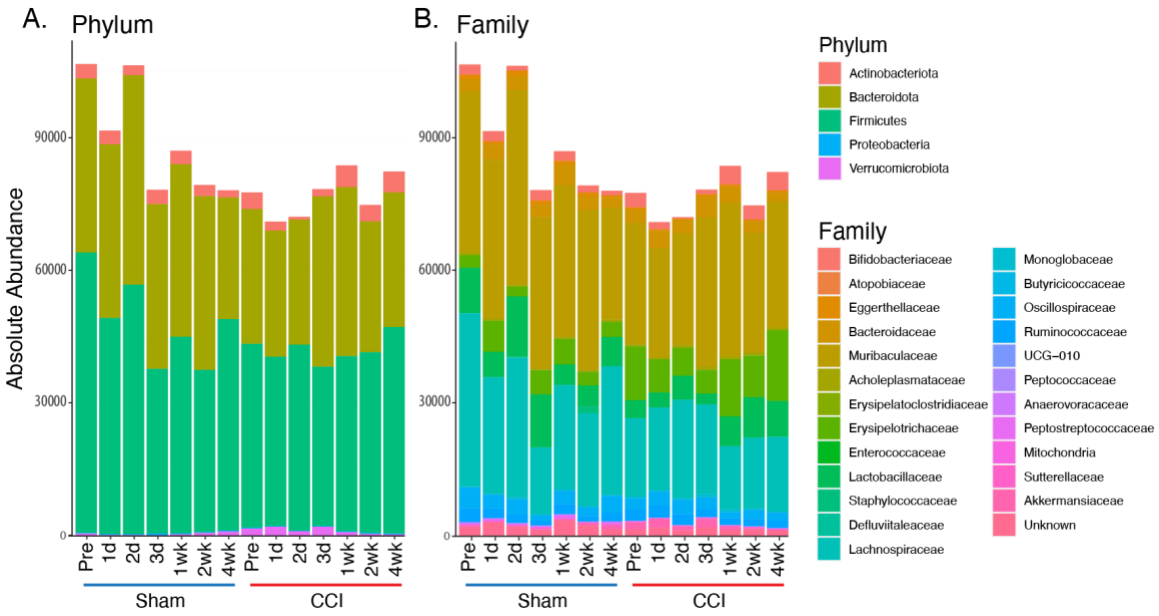
Supplemental Figure S2: Weight did not differ between injury groups prior to or after injury

Mice were weighed at each inoculation prior to injury, at 1 and 2 d prior to injury, on the day of Sham/ CCI surgery, and then again with each fecal collection out to 4 wk post-injury. There was no significant effect of injury condition ($F(1, 12) = 0.13$; $p = 0.73$), and a main effect of time was observed ($F(3.84, 46.12) = 74.40$; $p < 0.0001$) without an interaction (rmANOVA; $F(12, 144) = 0.70$; $p = 0.75$). Data represent mean and standard deviation ($n_{Sham} = 6$, $n_{CCI} = 7$).



Supplemental Figure S3: The order *Erysipelotrichales*

Prior to injury the order *Erysipelotrichales* is differentially abundant in mice that would receive a CCI, and this difference returns at 4wks post-injury. Data are represented as mean difference and standard error (ANCOM-BC; # $q \leq 0.05$, ## $q \leq 0.01$; $n_{Sham} = 6$, $n_{CCI} = 7$).



Supplemental Figure S4: Absolute abundance taxa distribution

Representation of absolute abundance by injury group and collection timepoint at a phylum and family level ($n_{Sham} = 6$, $n_{CCI} = 7$).

	Pre-Sham	1d Sham	2d Sham	3d Sham	1wk Sham	2wk Sham	4wk Sham	Pre-CCI	1d CCI	2d CCI	3d CCI	1wk CCI	2wk CCI	4wk CCI
Pre- Sham	x	0.730	0.660	0.123	0.281	0.284	0.828	0.015	0.176	0.341	0.096	0.020	0.060	0.001
1d Sham	0.730	x	0.759	0.362	0.914	0.657	0.728	0.036	0.264	0.223	0.311	0.136	0.132	0.002
2d Sham	0.660	0.759	x	0.768	0.537	0.510	0.791	0.002	0.012	0.104	0.011	0.043	0.044	0.001
3d Sham	0.123	0.362	0.768	x	0.777	0.763	0.637	0.008	0.037	0.06	0.024	0.190	0.205	0.001
1wk Sham	0.281	0.914	0.537	0.777	x	0.722	0.760	0.046	0.183	0.157	0.183	0.440	0.361	0.002
2wk Sham	0.284	0.657	0.51	0.763	0.722	x	0.601	0.070	0.170	0.267	0.324	0.423	0.247	0.039
4wk Sham	0.828	0.728	0.791	0.637	0.760	0.601	x	0.035	0.272	0.566	0.127	0.134	0.286	0.014
Pre- CCI	0.015	0.036	0.002	0.008	0.046	0.070	0.035	x	0.746	0.260	0.123	0.245	0.149	0.098
1d CCI	0.176	0.264	0.012	0.037	0.183	0.170	0.272	0.746	x	0.884	0.490	0.208	0.272	0.024
2d CCI	0.341	0.223	0.104	0.060	0.157	0.267	0.566	0.260	0.884	x	0.692	0.194	0.230	0.003
3d CCI	0.096	0.311	0.011	0.024	0.183	0.324	0.127	0.123	0.490	0.692	x	0.166	0.022	0.002
1wk CCI	0.020	0.136	0.043	0.190	0.440	0.423	0.134	0.245	0.208	0.194	0.166	x	0.741	0.149
2wk CCI	0.060	0.132	0.044	0.205	0.361	0.247	0.286	0.149	0.272	0.230	0.022	0.741	x	0.185
4wk CCI	0.001	0.002	0.001	0.001	0.002	0.039	0.014	0.098	0.024	0.003	0.002	0.149	0.185	x

Supplemental Table S1: Significance matrix of unadjusted p-values from post-hoc pairwise comparisons of Bray-Curtis Dissimilarity

Each cell represents a p-value for comparison between the corresponding row and column titles. Row and column titles are matched to show comparison of each timepoint and injury condition compared against all others. An "x" has been placed at matched group comparisons. Values above the diagonal line duplicate those below, and, as such are in a lighter font ($n_{Sham} = 6$, $n_{CCI} = 7$).

	Pre-Sham	1d Sham	2d Sham	3d Sham	1wk Sham	2wk Sham	4wk Sham	Pre-CCI	1d CCI	2d CCI	3d CCI	1wk CCI	2wk CCI	4wk CCI
Pre- Sham	x	0.81	0.79	0.34	0.43	0.43	0.85	0.11	0.37	0.48	0.31	0.13	0.21	0.03
1d Sham	0.81	x	0.81	0.50	0.91	0.79	0.81	0.17	0.43	0.41	0.46	0.34	0.34	0.03
2d Sham	0.79	0.81	x	0.81	0.69	0.66	0.82	0.03	0.10	0.32	0.10	0.17	0.17	0.03
3d Sham	0.34	0.50	0.81	x	0.81	0.81	0.78	0.08	0.17	0.21	0.13	0.38	0.39	0.03
1wk Sham	0.43	0.91	0.69	0.81	x	0.81	0.81	0.17	0.37	0.37	0.37	0.59	0.50	0.03
2wk Sham	0.43	0.79	0.66	0.81	0.81	x	0.75	0.24	0.37	0.43	0.47	0.57	0.42	0.17
4wk Sham	0.85	0.81	0.82	0.78	0.81	0.75	x	0.17	0.43	0.72	0.34	0.34	0.43	0.11
Pre- CCI	0.11	0.17	0.03	0.08	0.17	0.24	0.17	x	0.81	0.43	0.34	0.42	0.36	0.31
1d CCI	0.37	0.43	0.10	0.17	0.37	0.37	0.43	0.81	x	0.89	0.65	0.39	0.43	0.13
2d CCI	0.48	0.41	0.32	0.21	0.37	0.43	0.72	0.43	0.89	x	0.81	0.38	0.41	0.03
3d CCI	0.31	0.46	0.10	0.13	0.37	0.47	0.34	0.34	0.65	0.81	x	0.37	0.13	0.03
1wk CCI	0.13	0.34	0.17	0.38	0.59	0.57	0.34	0.42	0.39	0.38	0.37	x	0.81	0.36
2wk CCI	0.21	0.34	0.17	0.39	0.50	0.42	0.43	0.36	0.43	0.41	0.13	0.81	x	0.37
4wk CCI	0.03	0.03	0.03	0.03	0.03	0.17	0.11	0.31	0.13	0.03	0.03	0.36	0.37	x

Supplemental Table S2: Significance Matrix of FDR-adjusted p-values from post-hoc pairwise comparisons of Bray-Curtis Dissimilarity

Each cell represents an adjusted p-value for comparison between the corresponding row and column titles. Row and column titles are matched to show comparison of each timepoint and injury condition compared against each other. An "x" has been placed at matched group comparisons. Values above the diagonal line of x's are the same as those below that line, and as such, are in a lighter font. Highlighted cells represent those that are considered to be significant comparisons after multiple testing correction ($n_{Sham} = 6$, $n_{CCI} = 7$).

	Pre-Sham	1d Sham	2d Sham	3d Sham	1wk Sham	2wk Sham	4wk Sham	Pre-CCI	1d CCI	2d CCI	3d CCI	1wk CCI	2wk CCI	4wk CCI
Pre- Sham	x	0.544	0.281	0.059	0.22	0.412	0.953	0.034	0.387	0.471	0.126	0.045	0.081	0.002
1d Sham	0.544	x	0.382	0.136	0.925	0.774	0.616	0.048	0.41	0.471	0.592	0.199	0.093	0.001
2d Sham	0.281	0.382	x	0.39	0.367	0.498	0.497	0.007	0.043	0.228	0.066	0.104	0.041	0.003
3d Sham	0.059	0.136	0.39	x	0.675	0.755	0.288	0.007	0.122	0.136	0.035	0.443	0.151	0.002
1wk Sham	0.22	0.925	0.367	0.675	x	0.652	0.448	0.095	0.339	0.355	0.251	0.639	0.318	0.013
2wk Sham	0.412	0.774	0.498	0.755	0.652	x	0.414	0.181	0.444	0.614	0.456	0.745	0.314	0.059
4wk Sham	0.953	0.616	0.497	0.288	0.448	0.414	x	0.094	0.492	0.755	0.12	0.159	0.431	0.033
Pre- CCI	0.034	0.048	0.007	0.007	0.095	0.181	0.094	x	0.729	0.162	0.025	0.185	0.132	0.048
1d CCI	0.387	0.41	0.043	0.122	0.339	0.444	0.492	0.729	x	0.815	0.179	0.236	0.445	0.109
2d CCI	0.471	0.471	0.228	0.136	0.355	0.614	0.755	0.162	0.815	x	0.387	0.269	0.242	0.003
3d CCI	0.126	0.592	0.066	0.035	0.251	0.456	0.12	0.025	0.179	0.387	x	0.156	0.027	0.002
1wk CCI	0.045	0.199	0.104	0.443	0.639	0.745	0.159	0.185	0.236	0.269	0.156	x	0.413	0.116
2wk CCI	0.081	0.093	0.041	0.151	0.318	0.314	0.431	0.132	0.445	0.242	0.027	0.413	x	0.189
4wk CCI	0.002	0.001	0.003	0.002	0.013	0.059	0.033	0.048	0.109	0.003	0.002	0.116	0.189	x

Supplemental Table S3: Significance matrix of unadjusted p-values from post-hoc pairwise comparisons of Weighted UniFrac Distance

Each cell represents an adjusted p-value for comparison between the corresponding row and column titles. Row and column titles are matched to show comparison of each timepoint and injury condition compared against each other. An "x" has been placed at matched group comparisons. Values above the diagonal line of x's are the same as those below that line, and as such, are in a lighter font ($n_{Sham} = 6$, $n_{CCI} = 7$).

	Pre-Sham	1d Sham	2d Sham	3d Sham	1wk Sham	2wk Sham	4wk Sham	Pre-CCI	1d CCI	2d CCI	3d CCI	1wk CCI	2wk CCI	4wk CCI
Pre- Sham	x	0.64	0.50	0.26	0.44	0.58	0.95	0.23	0.58	0.59	0.35	0.23	0.32	0.05
1d Sham	0.64	x	0.58	0.35	0.94	0.80	0.70	0.23	0.58	0.59	0.69	0.41	0.33	0.05
2d Sham	0.50	0.58	x	0.58	0.58	0.60	0.60	0.08	0.23	0.45	0.27	0.35	0.23	0.05
3d Sham	0.26	0.35	0.58	x	0.74	0.79	0.50	0.08	0.35	0.35	0.23	0.58	0.38	0.05
1wk Sham	0.44	0.94	0.58	0.74	x	0.72	0.58	0.33	0.56	0.58	0.47	0.72	0.54	0.13
2wk Sham	0.58	0.80	0.60	0.79	0.72	x	0.58	0.40	0.58	0.70	0.58	0.79	0.54	0.26
4wk Sham	0.95	0.70	0.60	0.50	0.58	0.58	x	0.33	0.60	0.79	0.35	0.38	0.58	0.23
Pre- CCI	0.23	0.23	0.08	0.08	0.33	0.40	0.33	x	0.79	0.38	0.22	0.40	0.35	0.23
1d CCI	0.58	0.58	0.23	0.35	0.56	0.58	0.60	0.79	x	0.83	0.40	0.46	0.58	0.35
2d CCI	0.59	0.59	0.45	0.35	0.58	0.70	0.79	0.38	0.83	x	0.58	0.49	0.46	0.05
3d CCI	0.35	0.69	0.27	0.23	0.47	0.58	0.35	0.22	0.40	0.58	x	0.38	0.22	0.05
1wk CCI	0.23	0.41	0.35	0.58	0.72	0.79	0.38	0.40	0.46	0.49	0.38	x	0.58	0.35
2wk CCI	0.32	0.33	0.23	0.38	0.54	0.54	0.58	0.35	0.58	0.46	0.22	0.58	x	0.40
4wk CCI	0.05	0.05	0.05	0.05	0.13	0.26	0.23	0.23	0.35	0.05	0.05	0.35	0.40	x

Supplemental Table S4: Significance Matrix of FDR-adjusted p-values from post-hoc pairwise comparisons of Weighted UniFrac Distance

Each cell represents an adjusted p-value for comparison between the corresponding row and column titles. Row and column titles are matched to show comparison of each timepoint and injury condition compared against each other. An "x" has been placed at matched group comparisons. Values above the diagonal line of x's are the same as those below that line, and as such, are in a lighter font. Highlighted cells represent those that are considered to be significant comparisons after multiple testing correction ($n_{Sham} = 6$, $n_{CCI} = 7$).

CHAPTER 3: Discussion

3.1. Review of major findings

Following a TBI, many individuals exhibit GI symptoms that can contribute to impaired quality of life and even mortality (Harrison-Felix et al., 2006; Ventura et al., 2010). However, our understanding of the etiology of these symptoms is still very limited. Within this dissertation, novel data are presented that further characterize the effects that TBI has on the GI tract and point towards a potential compensatory mechanism to minimize GI-related deficits following head injury. Using a contusive injury model of moderate TBI, we found a transient increase in GI permeability without overt morphological changes to the ileal crypt-villus unit or the colonic crypt structures at the time of permeability or out to a month after injury. We believe that this intestinal dysfunction leads to a compensatory response within the colon wherein the density of goblet cells is increased and the tissue becomes more hypoxic creating an environment that promotes the expansion of the beneficial microbe, *A. muciniphila*. These findings are important because they suggest an endogenous response to TBI-induced GI dysfunction which may mitigate further secondary damage in cases of mild-to-moderate TBI. We posit that this natural intestinal response to brain injury may be insufficient to prevent structural damage following severe TBI. This would be consistent with the much greater incidence of GI-related pathology in individuals with severe TBI compared to mild or moderate TBI. Strategies designed to amplify these innate compensatory responses may have therapeutic benefit in people with severe TBI. The following sections will provide a brief overview of how we interpret each of our findings followed by speculation on the mechanisms through which TBI alters the GI tract and gut microbiome, and potential next steps for related research.

3.2. Intestinal Permeability after TBI

Clinically, GI permeability is known to present after TBI with the extent of barrier dysfunction determined by the severity of injury (Faries et al., 1998). We assessed intestinal permeability at 4 hr, 8 hr, 1 d, and 3 d following a moderate CCI using orally delivered FITC dextran and found that CCI induced transient intestinal permeability at the 4 hr timepoint. Previously, others have shown evidence of increased permeability within hours of TBI and out to more chronic timepoints after injury. While many of these studies utilized models of contusion injury, those in which permeability persisted used injury parameters that were intended to produce severe TBI (Hang et al., 2003; Li et al., 2018; Yang et al., 2022; Zhang & Jiang, 2015). Given that barrier dysfunction is related to TBI severity in humans, it is possible that increased injury severity accounts for prolonged intestinal permeability in experimental models as well.

Our study is the first to identify acute permeability by oral gavage of FITC dextran in a mouse CCI model. Prior studies that evaluated permeability after TBI used different injury models and/or different species (i.e. rats). Additionally, several previous studies utilized a more invasive means of assessing GI permeability whereby the intestinal tract was removed from the body and FITC dextran was injected directly into an isolated, ligated section of jejunum (Lang et al., 2015) or ileum (Bansal et al., 2009) which was then returned to the abdominal cavity. While this approach allows the researcher to attribute permeability to a specific intestinal segment, it also involves a second major surgery and ligation of the intestinal tract that likely leads to decreased blood flow and in turn greater pathology. Ligation of the GI tract is utilized to model intestinal strangulation and obstruction in mice, and it results in intestinal permeability and bacterial translocation that can be appreciated in the lymph nodes within 6 hr (Deitch et al., 1990). Intestinal ligation

also leads to notable changes in villous morphology associated with mucosal edema at the tips of villi (Hartmann et al., 2019; Yuan et al., 2013) similar to that observed acutely after TBI by Lang et al. (2015). While we do not dismiss these findings, we believe that intestinal ligation may represent a second insult that contributes to more prolonged or extensive permeability changes than we observed with TBI alone. While assessing permeability using oral gavage avoids potentially confounding secondary surgical manipulations, it prohibits us from identifying regional permeability of the GI tract. *Ex vivo* preparations are one method for examining permeability in localized segments of the GI tract and have been used following TBI to demonstrate increased permeability in both the small and large intestine (Feighery et al., 2008; Ma et al., 2017).

Typically, a posttraumatic increase in gut permeability has been assumed to be due to loss of tight junction proteins within the GI tract, but reduction of these proteins does not necessarily correlate with functional permeability assays (Galipeau & Verdu, 2016). To more directly implicate tight junction protein content or makeup as a causative factor, TBI studies which manipulate specific intestinal tight junction proteins are required (Horowitz, Chanez-Paredes, Haest, & Turner, 2023). Nonetheless, findings presented in this dissertation could be expanded through the assessment of tight junction proteins following injury. Regional assessment of tight junction proteins by Western blot or immunohistochemistry could provide information on the mechanism of barrier dysfunction and about the section of the GI tract that is most affected in our CCI model. Future research should also utilize assays that provide more nuanced information on the mechanisms of GI permeability. For example, oral gavage of three different molecules of varying sizes to

differentiate the extent of barrier disruption could provide insight into the mechanism by which gut barrier function is occurring (Chanez-Paredes, Abtahi, Kuo, & Turner, 2021).

In addition to a better understanding of the mechanism underlying post-TBI gut permeability and the regional vulnerability, more detailed examination of the time course of permeability changes is important. In a study by Mazarati et al. (2021), late onset of permeability after TBI was associated with increased incidence of post-traumatic epilepsy in rats. Thus, the timing of post-TBI intestinal permeability may predict long-term outcomes. It is unclear whether acute changes in permeability are mediated by similar or different mechanisms than permeability that arises at chronic timepoints. Other long-term sequelae of TBI including depression and anxiety are also associated with changes in GI permeability (Safadi, Quinton, Lennox, Burnet, & Minichino, 2022; Stevens et al., 2018), but there is no current evidence to suggest that TBI-induced permeability is directly related to the development of such psychological conditions. As such, GI permeability following brain injury, and the timing at which it occurs, is an important consideration future research, but the means of assessing permeability should be carefully considered.

3.3. Morphological assessment of the GI tract

Despite conducting an extensive examination of histological features of both the ileum and colon in Swiss-rolled tissue preparations obtained at multiple survival time points in the first month, we found no morphological differences between mice with a moderate CCI and their sham counterparts. Multiple prior studies have reported morphological changes to the intestinal tract within 1 d after experimental severe TBI (Bansal et al., 2009; Feighery et al., 2008; Hang et al., 2003; Lang et al., 2015; Yang et al., 2022). The vast majority of these studies only assessed changes in morphology within the

small intestine where several identified similar features of damage including formation of edema at villus tips and villus blunting.

To our knowledge, all studies that have assessed intestinal morphology have utilized transverse sections rather than Swiss-rolled tissue sections. The use of transverse sections greatly limits the ability to generalize damage to a whole segment of the intestine especially in studies that assess tissue sections taken from regions ligated to assess permeability changes (Bansal et al., 2009; Lang et al., 2015). As mentioned above, ligation of GI tract alone in the absence of TBI is sufficient to create GI tract pathology, confounding the interpretation of TBI-induced gut damage in ligated tissue preparations. Beyond this specific shortcoming, there are also several caveats that need to be accounted for when assessing GI morphology using histological sections. One potential problem with using a transverse section is that there is simply less tissue to gather information from, so an error in cutting the tissue could lead to misinterpretation of the tissue. Orientation of the tissue when cutting can lead to over- or underestimation of the crypt-villi distance or crypt depth, and it can cause adjacent villi to appear fused or as if crypts are bifurcating (Ravelli & Villanacci, 2012). Swiss-rolled tissue provides a major benefit in this regard by allowing the researcher to assess segments of potential damage in the context of the directly adjacent proximal and distal segments. In the data presented in Chapter 2, only crypt-villi structures and crypt structures that were "well oriented" were included for measurement. Poorly oriented structures were attributed to artifacts from the sectioning process, but this may have also led us to skip certain segments that actually contained damage. This would especially be true if damage occurred in patches rather than manifesting uniformly throughout the tract. Future assessments of intestinal morphology should consider utilizing

both transverse sections and Swiss-rolled tissue when determining if there is shortening or lengthening of intestinal structures. This could be achieved by taking a transverse segment from both the proximal and distal ends of the region of interest and then Swiss-rolling the middle portion.

As is stated in the methods section of Chapter 2, the mice utilized for ileal crypt-villi distance and colonic crypt depth measurements were not all generated and randomized within one experimental cohort. This may explain some of the variation in our data. As can be seen in Figure 2.3, the time-matched sham and CCI mice are often more similar to each other than they are to their respective injury group at another timepoint. We believe that this is due to natural variation in the mice, rather than an actual effect of time and that this could have been prevented if all of the mice were injured at the same time with staggered euthanasia. Minimizing this variation could provide additional power for us to identify injury differences that may have only presented at a single timepoint.

3.4. Effects of TBI on the gut microbiome

Based on earlier TBI studies examining the gut microbiome that described an induction of dysbiosis after TBI, we originally hypothesized that TBI would lead to detrimental changes to the microbiome. After analyzing our data, we found that the phylum *Verrucomicrobiota* was differentially abundant in CCI mice at 1 d, 2 d, and 3 d after injury. We confirmed that the phylum level change we observed is due to *A. muciniphila* using qPCR. *A. muciniphila* is almost always considered to be a beneficial microbe, leading us to reframe our working hypothesis. Interestingly, Sgro et al. (2022) observed increases in taxa *Faecalibaculum* and *Lachnospiraceae,* which comprise potentially beneficial bacteria, in adolescent rats 1 d after a single rotational mTBI, and remark that the increase in these

two taxa may be part of a compensatory response. While most TBI studies have reported changes in the gut microbiome that are characterized as dysbiosis, there is little consensus on which bacteria are altered by TBI (see Tables 1.3 and 1.4). Further, it is difficult to determine whether changes in diversity or abundance of specific taxa are pathological, neutral, or beneficial without additional tests. Dysbiosis is frequently described as a change from a normal microbiome, yet defining a "normal microbiome" is incredibly difficult because the microbiomes of healthy individuals tend to vary greatly (reviewed in Brüssow, 2020). At a minimum, the gut microbiome is likely a modifying aspect of TBI outcomes but may not play into every brain injury. Additional research is required to identify changes consistent across species and injury models which may have clinical relevance and to come to a mechanistic understanding of what changes definitively contribute to or minimize enterogenic secondary brain injury before the microbiome can be considered a viable therapeutic target.

In looking for shared findings between studies, we found that several other studies have identified elevated levels of *Verrucomicrobiota* and/or *A. muciniphila* following experimental TBI (Baskin et al., 2023; Hou et al., 2021; Nicholson et al., 2019; Opeyemi et al., 2021). Beyond TBI, elevated *A. muciniphila* has also been observed at acute (1-3 d) timepoints after experimental stroke (Ge, Zadeh, Yang, Candelario-Jalil, & Mohamadzadeh, 2022; Stanley, Moore, & Wong, 2018) suggesting that elevation of *A. muciniphila* may be a broad response to CNS injury. Administration of live and pasteurized *A. muciniphila* has been suggested as a potential treatment for various conditions after being tested in mouse models of diet-induced diabetes (Everard et al., 2013), obesity (Plovier et al., 2017), ulcerative colitis (DSS induced; Bian et al., 2019), Alzheimer's

disease (APP/PS1; Ou et al., 2020), and amyotrophic lateral sclerosis (*Sod1-Tg*; Blacher et al., 2019). Even the administration of one of its surface proteins, Amuc_1100, was able to recapitulate many of the effects associated with the bacterium (Ottman et al., 2017; Plovier et al., 2017). However, in people with multiple sclerosis (MS), A. muciniphila is elevated and administration of this microbe in a mouse model of MS (experimental autoimmune encephalitis; EAE) induced a pro-inflammatory response (Cekanaviciute et al., 2017). However, in the same EAE model, another group found that administering the microRNA miR-30d led to an elevation in *A. muciniphila* that actually ameliorated EAE (Liu et al., 2019). It's possible that the pro-inflammatory response associated with *A. muciniphila* in individuals with MS may be more closely tied to their altered immune response, as elevated levels of antibodies against *A. muciniphila* have been found within their cerebrospinal fluid (Vallino et al., 2020). These studies exemplify the need for a better understanding of how *A. muciniphila* acts on the body, and the need to develop appropriate screens to identify individuals who may not respond well to the bacteria. Fortunately, for most people, administration of *A. muciniphila* is safe (Depommier et al., 2019; Turck et al., 2021). Prolonging the elevation of *A. muciniphila* or administration of its surface protein represents an interesting potential therapeutic that is yet to be explored in the context of TBI.

3.5. Experimental design considerations related to the microbiome and GI physiology

Within this dissertation, as detailed in Chapter 2's methodology, we utilized an established PEG clearance paradigm followed by reinoculation with a common microbiota (Wrzosek et al., 2018) followed by a mixed-bedding approach (Miyoshi et al., 2018) in

order to minimize between cage differences in the microbiome prior to injury. PEG clearance paradigms are a common part of colonoscopy preparation that leads to an immediate reduction in bacterial load (Drago, Toscano, De Grandi, Casini, & Pace, 2016). In humans, no microbiota are replaced leaving those microbes that remain to rebound, leading to long term changes to the gut microbiome (Drago et al., 2016).

Prior research conducted by Tropini et al. (2018) suggests that PEG-induced osmotic diarrhea disrupts the mucus layer, promotes the regrowth of pathogenic bacteria, and results in immune responses against commensal bacteria. In the same study, they showed that some commensal taxa (S24-7) are entirely lost without artificial reintroduction. Taking this into account, the introduction of new microbiota from healthy animals, as was done in our study, may mitigate some of the negative effects of PEG clearance, but additional tests would be needed to assess this.

Fortunately, PEG clearance is not associated with an increased likelihood of bacterial translocation (Kale et al., 1998). It is possible that our microbiome results are skewed from what they would be had we started with a non-manipulated microbiome. This may also play a role in the minimal changes that we saw in our study compared to the studies presented in Table 1.3. This is an important consideration and additional studies should be conducted to assess the generalizability of our findings.

Another common means of knocking down the microbiome for reinoculation is through the use of antibiotic cocktails (Manichanh et al., 2010). This was specifically avoided in our studies based on findings that showed that antibiotic-mediated microbiome depletion was associated with increased mortality and more severe neurological damage after experimental stroke (Winek et al., 2016). More recently, Celorrio et al. (2021) found

that antibiotic depletion of the microbiome exacerbates CCI pathology and the neuroimmune response (Celorrio et al., 2023). While the use of antibiotics is common and often necessary following TBI in humans, these studies suggest that inappropriate antibiotic usage could worsen outcomes.

Other fields have utilized germ-free mice to assess various conditions/disease states outside of the influence of the microbiome. This allows researchers to ascribe specific phenotypes as being "microbiota dependent." These mice are important tools for assessing the role of the gut microbiome on development. Germ-free mice vary substantially from conventionally raised mice, and they have altered behavior (Neufeld, Kang, Bienenstock, & Foster, 2011), immune systems (Macpherson & Harris, 2004; Round & Mazmanian, 2009), GI tract physiology (Husebye, Hellström, Sundler, Chen, & Midtvedt, 2001), and CNS development (Thion et al., 2018). Germ-free mice lack a detectable level of microorganisms because they are bred and live within microisolators. However, due to the differences in development of these mice it will be difficult to parse out what differences in injury responses are due to the absence of a microbiome versus altered brain organization and immune responses. A better model would require an immunocompetent mouse with a conventional microbiome from birth that could have its microbiome depleted at will without the use of antibiotics. Currently this is not possible.

A study by Le Roy et al. (2018) compared the above three microbiota transplant methods (PEG-clearance, antibiotics, and germ-free) and their impact on successful engraftment of a cecal microbiota transplant from genetically obese mice (B6.V-Lep*ob* /JRj) to 3 wk or 8 wk old, male, specific pathogen free (SPF), C57Bl/6J mice. Their findings suggest that depletion of the microbiome by PEG-clearance and antibiotic

treatment provide similar levels of donor microbiota engraftment at 3 and 9 wks after microbiota transplant, suggesting that the microbiome had stabilized by 3 wks. Germ-free mice had the least similar compositions to the donor material at 3 wks, but at 9 wks, the formerly germ-free mice had the most similar microbiome to the donor material. These findings suggest that the best means of transplanting in a foreign microbiome would likely be through the use of germ-free mice with the caveat that their physiology differs from that of conventional mice, and that it takes 9 wks for the microbiome to normalize. Thus, the authors suggest that the use of PEG-clearance or antibiotic depletion are viable alternatives to colonization of a germ-free mouse. Their data show that none of the available means of microbiota transplant are perfect, and as such researchers should weigh their options based on the needs of their experiment.

Model animal species and even strain should also be considered when comparing findings across studies that assess changes to the GI tract and microbiome after experimental TBI. Although rats and mice have similar GI anatomy and physiology, there are differences in small intestinal villus shape, quantity of gut-associated lymphoid tissue, water retention, and colonic motility (McConnell, Basit, & Murdan, 2008; Yip, Balasuriya, Spencer, & Hill-Yardin, 2022). Within species, Balb/C mice, as utilized in Bansal et al. (2009), have been shown to have differences in GI contractility and motility as compared to the C57BL/6J mouse strain used within this dissertation (Gama et al., 2020). Even substrain differences based on vendor have been found to alter behavior (Bryant et al., 2008) and susceptibility to epilepsy (Löscher, Ferland, & Ferraro, 2017), obesity (Siersbæk et al., 2020), viral infection (Villarino et al., 2016) and T-cell response (Ivanov et al., 2008).

To mitigate vendor-based microbiome differences, some studies have suggested the use of cohousing mice from different vendors to normalize the microbiome through horizontal bacterial transfer (Caruso, Ono, Bunker, Núñez, & Inohara, 2019). Cohousing of mice across treatment conditions is a potential way to rule out the role of the microbiota in a given phenotype or even genotype (Lipinski et al., 2021) and to minimize the effects of "starting" microbiomes on experimental outcomes. However, cohousing cannot be utilized when working with male mice due to home-cage aggression and fighting (Theil et al., 2020). To circumvent this, we and others have utilized a mixed-bedding approach, to normalize the microbiome prior to experimental manipulation (Miyoshi et al., 2018). Minimizing differences in the microbiome prior to injury increases the likelihood of observing changes to the microbiome due to a study's experimental manipulation. However, if the goal of a study is to observe how the microbiome changes in response to experimentation, then cohousing may minimize or completely abrogate microbiota differences between groups. As such, the approach utilized is dependent upon the research question. Nonetheless, the overall need for these controls is an important consideration for all animal research. More broadly, the sensitivity of the gut microbiome to a host of factors such as genetics, diet and environmental conditions, necessitates multiple labs replicating critical aspects of prior experiments to gauge the robustness of the microbiome findings before assessing new experimental paradigms.

Sex is another important consideration that has not been addressed regarding the effects of TBI on the microbiome and GI tract. Despite studies that clearly show that the brain's response to TBI can vary depending upon sex (Gupte, Brooks, Vukas, Pierce, & Harris, 2019), to our knowledge all but Sgro et al. (2022) and Baskin et al. (2023) have

been conducted using male animals. Additionally, there are sex-dependent differences in microbiome (Markle et al., 2013), so it is possible that the sex-based differences in TBI may be in part due to differences in the microbiome. This possibility warrants the use of both male and female animals in future studies.

Outside of the differences between animals and injury models there is a major hurdle that is still present, yet fixable, when trying to compare studies that assess changes to the gut microbiome after TBI: 16s sequencing data availability. Most TBI studies have not made their microbiome data publicly available. There are many steps to analyzing microbiome data all of which contain choices that must be made by the researcher. Many of these choices can drastically alter diversity and composition metrics as well as differential abundance profiles. Very strong differences between groups are likely to be identified regardless of analysis technique, but this is far from acceptable. Beyond differences in microbiome analysis, there are also differences in reporting that make it difficult to compare studies. For example, "Study A" may report findings at a species level while "Study B" may report findings at a genus level. This decision could be due to differences in sequencing depth or based simply on statistical significance, but it makes comparing these findings extremely difficult. While some inferences can be made about the genera from Study A's findings, they would be far from concrete, as a species within a genus can be differentially abundant, but that genus, as a whole, may not be. Because of the quantity of data that is generated through 16s sequencing, it is not surprising that these differences in reporting occur, but it is far from ideal. In many fields, microbiome data tends to be readily available for download, but this is not the case in TBI research. The number of studies that have been published in the TBI field is now large enough that a

metanalysis using the 16s sequencing data from multiple studies could be very informative. This would allow researchers to identify any commonalities between microbiome outcomes and provide potential targets for therapeutic intervention.

3.6. Goblet cell assessment

The mucus layer is supplied by the goblet cells that are interspersed amongst the rest of the GI tract's epithelial cells. The mucus layer provides a protective physical and chemical barrier between the luminal contents and the epithelial cells, yet little is known regarding the role of the mucus layer in preventing bacterial translocation after TBI (Dupont, Heinbockel, Brandenburg, & Hornef, 2014). To our knowledge, only one other study has assessed changes to goblet cells after experimental TBI. Houlden et al. (2016) counted goblet cells in the cecum 3 d following experimental stroke or lateral WDI. In the stroke animals there was a significant reduction in cecal goblet cells, but the TBI injured animals showed no difference compared to sham mice. While we did not assess the cecum, we did assess the other portions of the colon, and we found that CCI increased goblet cell density at 1 d post-injury in the medial colon. Increased density could reflect either increased numbers of goblet cells or increased goblet cell size. Based on the estimated colonic epithelial cell turnover time of 5-7 d under normal conditions (Barker, 2014), it is unlikely the increased density is due to more goblet cells, but it is possible that cell numbers could increase due to expedited differentiation of cells rather than turnover. If numbers were unchanged, this would indicate an increase in the amount of mucin held within the goblet cells' theca. Either of these options could be indicative of transient goblet cell hyperplasia. Several inflammatory GI conditions are associated with goblet cell hyperplasia, which appears to be an immune-mediated response that is also modulated by

bacterial metabolites (Birchenough, Johansson, Gustafsson, Bergström, & Hansson, 2015). Further investigation is required to determine how this is occurring in the context of TBI. Our ability to make conclusions about the mucus layer proper is limited in our current study because the mucus layer itself was not assessed. Mucus layer assessment requires specific tissue preparation so as not to disturb the mucus layer, and the flushing of luminal contents prior to Swiss rolling prevents the possibility of proper mucus layer examination. As such, additional steps would need to be taken in future studies to preserve the mucus layer for assessment.

3.7. Intestinal hypoxia after TBI

Beginning on a technical note, pimonidazole-HCl (hypoxyprobe-1; HP1) has been utilized to assess hypoxia in the GI tract in colitis models as a qualitative assessment to determine if tissue hypoxia is present and/or increased within the GI tract. This assay is not often quantified. Our method of quantifying colon hypoxia is a modest improvement over a previous method where multiple stepwise measurements were taken (Kelly et al., 2015) because our method provides continuous intensity information and is not influenced by variations in mucosa layer thickness. Our approach utilizes free and readily available software to measure the depth and intensity of HP-1 labeling and serves as an improved tool for assessing intestinal hypoxia that adds a level of nuance in quantification that was not previously available.

GI tissue hypoxia may be a potential link between changes to the microbiome and to the intestinal epithelium after TBI because intestinal hypoxia is a major driver of both intestinal stem cell differentiation and microbiome changes. While researchers have shown that blood flow to the GI tract is reduced as part of the sympathetic response to brain injury

(Olsen et al., 2013; Wang et al., 2011), tissue oxygenation has not been assessed at a tissue level. Under healthy conditions, the GI tract functions in a state known as physiologic hypoxia (Zheng, Kelly, & Colgan, 2015). Physiologic hypoxia refers to the gradient of increasing hypoxia along the basal to luminal axis of the GI tract, and it is characterized by decreased oxygen availability near the luminal interface. We found that the depth and intensity of this hypoxia is elevated acutely at 3 d post-CCI. Because hypoxia appeared to be increasing between 1 d and 3 d post-injury, future studies should examine longer survival timepoints to determine whether hypoxia persists or even worsens.

We utilized a moderate-TBI model where we did not see overt intestinal pathology. However, prior studies have identified pathology, so it is worth investigating if intestinal hypoxia is increased under conditions of severe TBI and the spatiotemporal relationships of any changes to tissue damage.

Additionally, further experiments are needed to determine the mechanism behind intestinal hypoxia after TBI. Future studies could work toward determining if this is due to metabolic/ mitochondrial changes, the presence of clotting in the GI tract, alterations in blood flow to the GI tract, or through infiltration of polymorphonuclear leukocytes (i.e. neutrophils, eosinophils, and basophils) that require rapid metabolism of oxygen. Increased colon hypoxia in response to acute inflammation is believed to be a driver of inflammatory resolution (Cartwright & Colgan, 2023). This idea supports our current working hypothesis that the GI tract attempts to compensate for acute intestinal barrier dysfunction that arises after TBI, but it is also possible that elevated colon hypoxia may be detrimental or part of the post-TBI gut pathology.

3.8. Potential mechanisms of GI tract and microbiome changes after TBI

It was not until relatively recently, that TBI researchers began investigating the effects of TBI on the GI tract and the role of the gut microbiome in secondary injury progression. Preclinical efforts to understand the effect of isolated TBI on the GI tract did not begin until the late 1990s and early 2000s (Hang et al., 2003; Shohami, Gati, Beit-Yannai, Trembovler, & Kohen, 1999), and the microbiome was not experimentally implicated in TBI until the mid-2010s (Houlden et al., 2016; Ma et al., 2017). As a relatively young field, the focus has been on describing changes to the GI tract and gut microbiome after TBI. These studies have been largely observational. To design therapeutics that target GI pathology to improve TBI outcomes, the systems, cellular, and molecular mechanisms underlying disruption of the gut-brain axis need to be studied. Foundational work has provided proof-of-concept that the injured brain can be influenced by changes to the GI tract, albeit negatively (Celorrio et al., 2021; Hanscom et al., 2021a; Ma et al., 2017). These observational findings are important in that they help to narrow down potential mechanisms that culminate in broader TBI-related GI dysfunction. The following section will detail potential means by which brain injury may alter the GI tract and gut microbiome (Figure 3.1). It is important to note that TBI has some effect on nearly every organ system, so given the complexity of regulation of the gut-brain axis, there are many avenues for investigation.

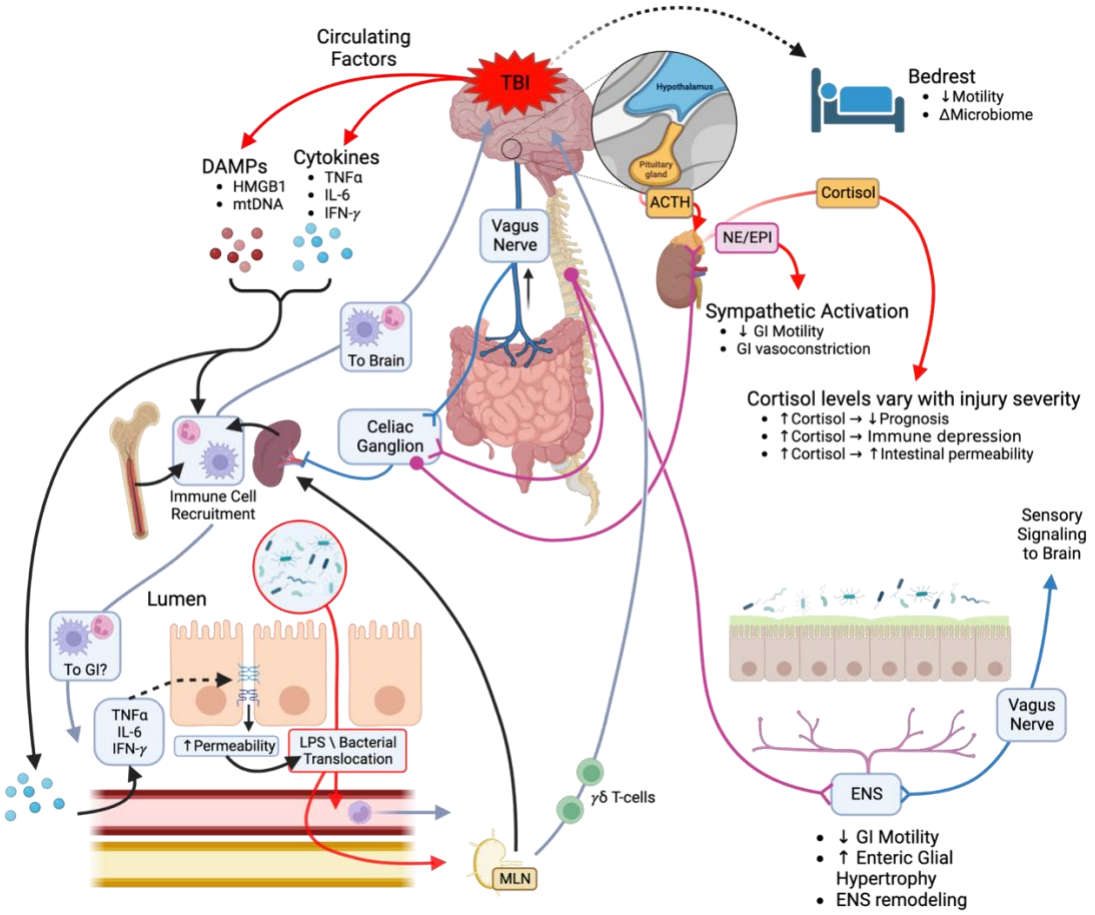


Figure 3.1: Potential mechanisms of GI and microbiome alterations after TBI

TBI initiates a complex series of events that span beyond the brain and can lead to GI dysfunction and changes in the gut microbiota. Despite GI dysfunction after TBI being observed both in the clinic and in preclinical models, our understanding of how brain injury leads to changes in the GI tract is limited. This figure illustrates a wide variety of potential mechanisms of TBI-induced GI and microbiome changes, and these mechanisms run the gamut from changes in activity associated with bedrest to complex multi-system signaling cascades. Up (↑) and down (↓) arrows represent an increase and decrease respectively, Δ corresponds with "change in." Abbreviations not found elsewhere in the text: Norepinephrine (NE); Epinephrine (Epi); High mobility Group box 1 (HMGB1); mitochondrial DNA (mtDNA); Tumor necrosis factor-α (TNFα); Interleukin-6 (IL-6); Interferon-γ (IFN-γ)

From a symptomatic perspective, gut dysmotility is one of the most common GI-related symptoms in brain injured individuals (Expósito-Tirado et al., 2003). Reduced motility is most pronounced in individuals with severe TBI likely due in part to prolonged bedrest and inactivity (Iovino et al., 2013; Šket et al., 2017). Similarly, mice with moderate TBI have reduced spontaneous motor movement early after injury (i.e. mimicking bedrest; Qu et al., 2016), have increased intestinal transit time (Cannon et al., 2023) and reduced intestinal contractility (Olsen et al., 2013). Outside of the context of TBI, reduced GI motility alters the composition of the microbiome (Khalif, Quigley, Konovitch, & Maximova, 2005; Zhu et al., 2014). Individuals with constipation were found to have altered microbiome profiles and elevated intestinal permeability, which were normalized following pharmacologic intervention to relieve constipation, implicating reduced motility as a causative factor (Khalif et al., 2005). These findings suggest that changes to the microbiome after injury may be due, in part, to changes in motor activity after TBI.

Observations of motility changes following TBI point towards a change to the cells responsible for coordinating motility, the ENS. Enteric neurons are highly plastic, but they are also sensitive to inflammatory stimuli. During intestinal inflammation, it has been shown that glial activation can lead to purinergic signaling cascades that result in enteric neuron death (Brown, McClain, Watson, Patel, & Gulbransen, 2016; Gulbransen et al., 2012). While enteric neurons themselves have not been assessed after TBI, there is evidence of enteric glial hypertrophy at chronic timepoints after TBI suggesting the ENS is responding in some way after TBI (Ma et al., 2017). Additionally, loss of enteric neurons has been observed in other preclinical models of CNS trauma such as stroke (Cheng et al., 2016; Cheng et al., 2018) and spinal cord injury (Lefèvre et al., 2020).

Intestinal barrier impairment is a consistent finding in preclinical TBI model although the timing and duration vary (see Table 1.1 and 1.2). Assessments of intestinal barrier function are most often utilized to suggest the potential passage of bacteria, or their products/components, from the intestinal lumen into circulation. However, to our knowledge, circulating levels of LPS or bacterial translocation have not been assessed in humans after TBI. Other forms of neurotrauma such as stroke (Stanley et al., 2016) and spinal cord injury (Kigerl et al., 2016) have shown evidence of bacterial translocation out of the GI tract to lymphatic tissue and non-lymphoid organs in preclinical models. Increases in GI permeability are believed to be due to dysregulation of tight junction proteins. TBI could elevate pro-inflammatory cytokines that increase GI permeability through tight junction breakdown or induction of epithelial cell apoptosis (Goretsky et al., 2012). Loss of proper tight junction function between epithelial cells is known to occur with exposure to the inflammatory cytokines TNFα, IFN-γ, and IL-6, all of which are elevated in the circulation following TBI (Morganti-Kossman et al., 1997; Ross, Halliday, Campbell, Byrnes, & Rowlands, 1994; Woiciechowsky et al., 2002). It is unknown if the levels of inflammatory cytokines after TBI are high enough to induce tight junction breakdown, but TNFα and IL-6 are elevated in the small intestine after WDI in rats as early as 3 h and out to at least 1 wk post-injury (Hang, Shi, Li, Li, & Wu, 2005). Systemic (intraperitoneal) administration of TNFα leads to barrier loss between small intestinal epithelial cells via occludin internalization within 90 minutes after injection (Marchiando et al., 2010), and there are several anti-TNFα treatments available on the market that restore gut barrier function in individuals with Crohn's disease (Cassinotti et al., 2022; Suenaert et al., 2002). The role of circulating TNFα on GI permeability after TBI could be

investigated through the use of an enterocyte-specific TNF membrane receptor 1 knockout mouse. An enterocyte-specific knockout would avoid altering TNFα-mediated endothelial BBB breakdown from interfering with expected TBI initiated BBB breakdown. Similar enterocyte specific knockout strategies could be utilized to assess the role of other circulating inflammatory cytokines in gut-barrier dysfunction after TBI.

Circulating cortisol levels are commonly elevated at chronic timepoints after TBI. This is believed to be a major contributing factor to long-term immunosuppression after TBI (Dong, Zhi, Bhayana, & Wu, 2016). In rat models of chronic stress, it has been shown that elevated glucocorticoids play a role in downregulation of the gene for tight junction protein Claudin-1 (Zheng et al., 2017). This indicates that circulating cortisol levels may also play a role in intestinal barrier dysfunction after TBI.

Additionally, elevated post-TBI hypertension may be a contributing aspect to intestinal permeability following TBI. Blood pressure is commonly elevated early after severe TBI (reviewed in Krishnamoorthy, 2017) and is believed to be due to excessive sympathetic signaling in response to injury. In a study utilizing spontaneously hypertensive rats, onset of hypertension led to substantial intestinal permeability and loss of tight junction proteins (Santisteban et al., 2017).

It is hypothesized that all of these factors work in conjunction to produce GI dysfunction after TBI. Signaling between the immune system, CNS, PNS, and GI tract occurs through numerous interconnected mechanisms making it difficult to pinpoint a single target to minimize GI dysfunction following TBI. Minimizing GI dysfunction is an important goal for quality of life of brain injured individuals, and traditionally

investigated, brain-centric therapeutics may also help to minimize GI dysfunction after TBI. Ultimately though, the goal of investigating GI dysfunction after TBI is to identify novel, GI-related targets that can help mitigate or improve outcomes within the brain.

The gut-brain axis is bidirectional, and there are many potential mechanisms by which the GI tract and microbiome may be signaling back to the brain. While this has not been the focus of this dissertation, we will briefly touch on several mechanisms of gut-to-brain signaling that may play a role in brain outcomes after TBI.

The bidirectionality of the gut-brain axis is perhaps best exemplified by the vagus nerve which directly links the brain to the GI tract. Vagal afferents provide sensory information from the gut to the brain whereas vagal efferents are largely parasympathetic. In response to inflammatory stimuli, vagal efferents initiate the cholinergic anti-inflammatory pathway (Pavlov, Wang, Czura, Friedman, & Tracey, 2003).

Vagal nerve stimulation (VNS) is a highly active area of research that is already utilized clinically. Depending upon the site of electrical stimulation, VNS can be used to modulate activity of both afferent and efferent fibers. Despite an incomplete understanding of the mechanism of action, stimulation of vagal afferents is approved for use by the United States Food and Drug Administration (FDA) in treatment of drug-resistant epilepsy (Morris & Mueller, 1999), treatment resistant depression (Kamel, Xiong, Gott, Kumar, & Conway, 2022), and as a rehabilitative device for stroke (Liu et al., 2022). VNS that targets parasympathetic efferents is also being investigated to modulate neuroimmune interactions in disease states for various organs that are innervated directly and indirectly by the vagus nerve. Following TBI, it is hypothesized that the anti-inflammatory signaling of the vagus

nerve to the periphery may be insufficient due to a sympathetic dominance that outweighs parasympathetic signaling (Blanke, Holmes, & Besecker, 2021). VNS at 6 hr following WDI led to a reduction in circulating TNFα (Bansal et al., 2012), reduced intestinal permeability, and prevented ileal villus blunting compared to TBI only controls (Bansal et al., 2010a). VNS is currently in clinical trials for treatment of mild TBI, and is a likely candidate for the first FDA approved therapy for TBI (Bremner & Woodbury, 2023).

As discussed in Chapter 1, immune factors such as DAMPs and cytokines are released into circulation following a TBI and trafficking of immune cells to the brain. Circulating cytokines such as TNFα, IL-6, and IFNγ are elevated following TBI as a means to recruit immune cells to the site of injury. It is likely that immune cells outside of those that have historically been assessed after TBI also play a role in TBI pathogenesis. Benakis et al. (2016) showed that in a mouse model of stroke there is trafficking of $\gamma\delta$ T-cells from the GI tract to the meninges that are believed to contribute to further inflammation. Recent work from Abou-El-Hassan et al. (2023), utilizing a $\gamma\delta$ T-cell deficient mouse, shows that $\gamma\delta$ T-cells contribute to behavioral deficits after CCI, act as a driver of neuroinflammation. In conventional CCI injured C57BL6/J mice they showed that administration of antibodies against a subset of $\gamma\delta$ T-cells reduced neuroinflammation, DAMP release, blood brain barrier breakdown, and mitigated behavioral deficits.

3.9. Moving forward

TBI has long been recognized as a whole-body condition within the clinic and by those living with a brain injury. Nonetheless, preclinical TBI research, as a field, has

historically focused on elucidating mechanisms of secondary damage and therapeutics within the brain parenchyma proper. Even TBI studies that administer a therapeutic agent systemically frequently only assess how the agent affects brain and behavioral related outcomes despite exposing many other organ systems to the therapy. More recently, researchers have gained an appreciation for the peripheral effects of TBI (detailed further in Chapter 1). With a growing interest in the potential of the microbiome to modulate everything from weight gain (Bäckhed et al., 2004) to psychological conditions (Kelly et al., 2016), many neurotrauma researchers began looking towards the gut as a potential means of improving outcomes after TBI. However, changes to the microbiome after TBI have generally been inconsistent between studies (see Tables 1.3 and 1.4). This is not limited to the neurotrauma field and is indicative of a larger issue associated with consistency of microbiome findings across labs. Above, we touched on some of the potential reasons for differences in findings between labs, but another factor is the general reliance on marker gene studies (i.e. 16s rRNA gene sequencing). The human gut microbiome consists of some consistent mainstay taxa but ultimately differs between individuals. Despite these differences, in most healthy people, the microbiome is capable of carrying out the necessary functions to maintain a symbiotic relationship with the host (Shanahan, Ghosh, & O'Toole, 2021). This is, in part, due to a high level of functional redundancy between microbial species that reside within mammalian intestinal tracts (Blakeley-Ruiz et al., 2019; Eng & Borenstein, 2018). There is also a high level of horizontal gene transfer between microbiota, meaning that the same species in two different people may have different functional potentials (Borodovich, Shkoporov, Ross, & Hill, 2022). Such complexities support a need to move toward metagenomics and metabolomics

analysis of the gut microbiome (Bishop et al., 2022; Maccaferri, Biagi, & Brigidi, 2011). These technologies offer a substantial improvement in the ability to interpret and resolve what the microbiome is doing in response to TBI. They also inform us of potentially druggable processes associated within functional changes to the microbiome. Another important benefit of metabolomic and metagenomic analyses for TBI is reduced reliance on identifying the pre-injury microbiota composition. If whole microbiome processes or metabolic changes are more consistent than changes in microbiota composition in response to neurotrauma, the likelihood of identifying potential microbiome-based prognostic biomarkers improves. If we hope to modulate the microbiome as a means of improving outcomes after TBI, or at least minimizing secondary damage, this is an important next step.

Microbiota transplant is both a mechanistic tool and a potential therapeutic. As a tool, researchers have transplanted fecal matter from individuals with diet-induced obesity (Turnbaugh et al., 2009), non-alcoholic fatty liver disease (Le Roy et al., 2013), and major depression (Kelly et al., 2016) to germ-free mice and successfully recapitulated many of the aspects associated with each condition suggesting a causative role for the microbiome in these conditions. As a treatment, fecal matter transplant involves transplantation from a healthy individual to a "sick" individual. Clinically, fecal transplants have been used to successfully overcome recurrent *C. difficile* infections (Ianiro et al., 2018), but there has been recent interest in the possibility of using microbiota transplants to treat other conditions (Ianiro et al., 2018). Parker et al. (2022) recently showed that gut and neuropathology associated with aging can be transferred from an aged mouse to a young mouse via fecal matter transplant. Transplant recipient, young mice exhibited intestinal

permeability, microglial activation, retinal inflammation, and increased circulating inflammatory factors. Additionally, when the reverse experiment was performed and aged mice received a microbiota transfer from young mice, aged mice showed a significant reduction in these same symptoms. While our study showed rather limited and potentially beneficial changes to the microbiome, others have shown more extensive changes to the microbiome after TBI. A microbiota transplant from injured mice that showed more extensive changes to uninjured mice, may provide insight into the sufficiency of the post-TBI microbiome to induce neuroinflammation or recapitulate other aspects of TBI pathology in the absence of physical brain trauma. This same style of experiment could also be conducted to determine if microbiota transplant from healthy mice to injured mice is capable of minimizing common histopathology and behavioral impairments associated with TBI. Taking this idea a step further, instead of utilizing microbiota transplant samples collected from TBI injured mice, future research could collect fecal samples from brain injured people at both acute and chronic timepoints after their injury as a means to assess the post-TBI microbiome's role in neuroinflammation.

Due to the inherent complexity associated with both TBI and the gut-brain-axis, there are numerous experimental directions which still warrant exploration. There is a need to better understand how brain injury leads to GI dysfunction and microbiome changes as well as a need for a better understanding of what aspects of the microbiome may modulate the systemic response to brain injury.

3.10. Summary

Our findings add to the growing body of literature assessing changes to the GI tract and microbiome after TBI. Within this dissertation, we have assessed the effects of TBI on

intestinal barrier dysfunction, small and large intestinal morphology, and goblet cells abundance, as well as the fecal microbiome. We hypothesize that moderate TBI induces a brief gut leakiness that triggers a compensatory response involving both the GI tract and the gut microbiota to reaffirm the gut barrier. This is based on our findings that CCI in male C57BL/6J mice leads to a transient intestinal barrier dysfunction, that results in increased goblet cells density in the proximal colon and elevated colonic hypoxia. We believe that these conditions then promote the expansion of the beneficial bacteria *A. muciniphila*. These findings point towards several promising future directions by which the GI tract may be modulated after TBI to minimize intestinal dysfunction.

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Vita

Anthony J. DeSana

EDUCATIONAL EXPERIENCE

2016 – 2023	**University of Kentucky** – Lexington, KY Ph.D. in Physiology (*upon acceptance by the graduate school*) Dissertation: Effects of Traumatic brain injury on the intestinal tract and gut microbiome Cumulative GPA: 3.74
2011 – 2015	**Seton Hill University** – Greensburg, PA B.A. in Honors in Psychology, summa cum laude Minor in Biology Cumulative GPA: 3.96

PROFESSIONAL AND RESEARCH EXPERIENCE

2016 – 2023	**Graduate Research Assistant** University of Kentucky – Lexington, KY Ph.D. training under Kathryn E. Saatman, Ph.D.
2023	**Teaching Assistant** University of Kentucky – Lexington, KY Course Director: Tim McClintock, Ph.D.
2016	**Student Temporary Employee (STEPs)** University of Kentucky – Lexington, KY Lab of Kathryn E. Saatman, Ph.D.
2015	**Temporary Laboratory Technician** University of Kentucky – Lexington, KY Lab of Kathryn E. Saatman, Ph.D.
2014 – 2015	**Institutional Research Intern & Honors Program Capstone** Seton Hill University – Greensburg, PA Survey-based research project under Edith L. Cook, Ph.D.
2014	**CNUP Summer Undergraduate Research Fellow** University of Pittsburgh – Pittsburgh, PA Undergraduate research training under C. Edward Dixon, Ph.D.
2014	**Supplemental Instruction Leader** Seton Hill University – Greensburg, PA
2013	**Student Housing Focus Group Facilitator** Seton Hill University – Greensburg, PA

SELECTED SCHOLASTIC AND PROFESIONAL HONORS

May 2023	Trainee Presenter, Kentucky Spinal Cord and Head Injury Research Trust Symposium – Lexington, KY
March 2023	Trainee Presenter, University of Kentucky 18th Annual Center for Clinical and Translational Science Spring Conference – Lexington, KY
March 2023	Trainee Presenter, American Society of Neurochemistry Annual Meeting – Lexington, KY
June 2022	Poster Presentation Honorable Mention, National Neurotrauma Symposium – Atlanta, GA
November 2022	Alumni Presenter, Presentation for Seton Hill University Freshmen – Virtual Presentation
July 2018 – June 2020	T32 Trainee, "Neurobiology of CNS Injury & Repair" Training Grant (5T32 NS077889)

PROFESSIONAL PUBLICATIONS

DeSana, A.J., Barrett, T.A. & Saatman, K.E. (2022). P291 Traumatic brain injury induces acute increase in intestinal permeability and subacute changes to the gut microbiome of mice. *Journal of Neurotrauma*. Jun 2022. A-1-A-128. http://doi.org/10.1089/neu.2022.29126.abstracts

DeSana, A.J., Barrett, T.A. & Saatman, K.E. (2021). PSA14-093 Traumatic brain injury induces acute increase in intestinal permeability and subacute changes to the gut microbiome of mice. *Journal of Neurotrauma*. Jul 2021. A-1-A-132. https://doi.org/10.1089/neu.2021.29111.abstracts

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Osier, N.D., Carlson, S.W., **DeSana, A**., & Dixon, C.E. "Chronic Histopathopathological and Behavioral Outcomes of Experimental Traumatic Brain Injury in Adult Male Animals." *Journal of Neurotrauma.* December 2015, 32(23): 1861-1882. https://doi.org/10.1089/neu.2014.3680