An Investigation of the Gut Microbiota, Microglia, and Peripheral Immunity in the Sod1 Animal Model of ALS

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Accessibility
An Investigation of the gut microbiota, microglia, and peripheral immunity in the Sod1 animal model of ALS

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A Thesis in the Field of Biology
for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Amyotrophic lateral sclerosis is a devastating and debilitating disease as patients maintain cognitive abilities while losing motor function before dying, often from respiratory muscle paralysis within three to five years of symptomatic onset. With 10% of cases acquired from genetic mutations (familial), an environmental cause is implicated in 90% of cases of ALS. The gut microbiome is an environmental factor that has been associated with processes in neurologic diseases and is a prime target to investigate sporadic causes of ALS because of how it may affect protein aggregates and inflammatory responses that contribute to motor neuron death. The gut microbiome can communicate to the brain by a few pathways including immune activation and neuroactive metabolite secretion. Microglia, the innate immune cell of the central nervous system, has been implicated in ALS and other neurodegenerative diseases and can be affected by the microbiome. This research demonstrates that the gut microbiome of the Sod1 ALS animal model upregulates microglia genes associated with RNA processing, lysosomal function, and protein degradation in naïve mice, which is linked with decreased Akkermansia. Furthermore, treatment of Sod1 mice with Akkermansia muciniphila or butyrate producing bacteria, Agathobacter rectale and Roseburia intestinalis, delayed disease progression and reversed Sod1-related effects in genes involved with the unfolded protein response and microglial activation. These findings provide evidence that specific microbes can ameliorate and delay disease progression in the Sod1 murine model of ALS and this demonstration of protection is linked with modulating genes implicated in ALS.
Acknowledgments

In honor and remembrance of the wisdom, drive, and heart of Aaron Lawal Abiola Andu.

I would like to acknowledge the encouragement, instruction, and support from Harvard University and Dr. Howard Weiner who offered an opportunity and resources to meet my goal of contributing to the conversation of neurodegenerative diseases during this Golden Age of neurologic investigation.

My final acknowledgment is to Dolapo and Marvala who have championed me throughout this learning experience with steadfast confidence and curated an environment that continues to support my success.
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Amyotrophic lateral sclerosis background and pathology

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that specifically affects the upper and lower motor neurons of the brain and spinal cord (Hickman, Sen, and Morsett, 2018). Clinical presentations of ALS include muscle weakness, disability, and death from respiratory failure, with a median survival of three to five years after symptomatic onset. Motor neuron death, considered a pathologic signature in ALS, causes different symptoms based on the neurons impacted. Lower motor neuron death, demonstrated earlier in disease, results in fasciculations, and hyporeflexia while upper motor neuron death causes muscle atrophy and lesions (Gordon, 2011). A damaged motor neuron junction leads to progressive weakness and paralysis, muscle atrophy, and spasticity.

Beyond clinical presentation, ALS is a genetically and clinically heterogeneous disease in which the interaction between genomic background and environmental factors are thought to play a major role (Hickman, Sen, and Morsett, 2018). Most ALS cases are sporadic but approximately 10 % are familial and acquired from mutations in specific genes including superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (TARDBP),
chromosome 9 open reading frame 72 (C9orf72), and fused in sarcoma (FUS) (Hickman, Sen, and Morsett, 2018). While these genes are predominantly mutated in ALS cases, there are more risk loci implicated in disease pathogenesis. In the normal state, The SOD1 gene typically reduces oxidative stress however, mutated SOD1 typically via gain of function activity, results in motor neuron death, though the exact mechanism is not fully understood (Gordon 2011). The remaining 90% of cases are classified as sporadic, which suggests there is an environmental influence of disease.

Gut microbiome contents modulate ALS symptoms and progression

The gut microbiota, which has a major role in several neurodegenerative diseases, may be one environmental factor that triggers or affects the progression of ALS. Patients with neurodegenerative disease have alterations in their gut microbiome, which may contribute to the disease (Wright et al, 2018). The microbiota secretes bioactive metabolites that modulate metabolism, immunity, and epigenetic processes.

Antibiotic treatment in mice can provide valuable insight on whether the microbiome plays a role in the progression of neurodegenerative disease. Antibiotics administered to Multiple sclerosis and Alzheimer’s models ameliorates disease (Mestre et al., 2019). However, in diametric contrast, disease is exacerbated in both antibiotic and germ-free Sod1 mice indicating the role of the gut microbiome in pathogenesis. Consistent with this, Blacher et al. found an increase of motor neuron death after depleting the microbiota with antibiotics (2019). This evidence indicates that an altered microbiota can have significant consequences on motor function and survival in Sod1 mice prompting the
investigation: How does the microbiota influence disease progression in amyotrophic lateral sclerosis?

Prior to the onset of symptoms, Sod1 mice display changes in gut function including damage to the structure of the intestinal tight junctions and increased levels of pro-inflammatory cytokines measured from serum and the intestines (Wright et al, 2018). Thus, whether the microbiota drive symptoms in ALS or the changes in the microbiome manifest as a result of changes in gut structure and function is currently unknown.

*Akkermansia* and butyrate producing bacteria attenuate disease

*Akkermansia muciniphila* (*A. muc*) is a species that may have a protective effect in disease progression in an animal model of ALS. Sod1 mice exhibit a gradual decline in the abundance of *A. muc* (Blacher et al., 2019). When *A. muc* was administered to antibiotic treated Sod1 mice, the bacteria improved motor function and neurologic scores (Blacher et al., 2019). This is noteworthy because 10 other strains failed to have a protective effect. Beyond changes in neurologic score, treatments with *A. muc* increased spinal cord cells, presumed to be neurons, and decreased brain atrophy. Moreover, *A. muc* colonization substantially prolonged the lifespan of Sod1 mice comparable to controls and the other 10 bacterial strains. *A. muc* secretes metabolites including nictoinamide (NAM) that might contribute to the microbe’s beneficial effect. Sod1 mice were then treated with NAM instead of *A. muc* which increased neurological motor test performance, though the increase was not robust, which the authors attribute to dosing (Blacher et al., 2019).
Therefore it is possible that NAM secreted from gut microbiota may be able to affect motor neuron function in the murine ALS models. The authors conducted an observational investigation in ALS patients that indicate that the global gene content in the microbiome is significantly different and the genes involved in NAM metabolism are decreased. Based on their findings, increasing *A. muc* may be an approach to treat ALS, yet the potential mechanisms are not fully characterized.

Other microbial metabolites may also be protective in ALS. Butyrate is a short chain fatty acid (SCFA) bacterial product, a primary energy source for intestinal epithelial cells (Zhang et al., 2017). This SCFA also increases gut integrity by increasing tight junctions and has anti-inflammatory effects on the host by promoting regulatory T-cells (Furusawa et al., 2013). In research conduct by Zhang et al., Sod1 mice received intestinal bacteria metabolites for 2.5 months assessing survival and metabolism (2017). The Sod1 experimental group received 2% butyrate via drinking water which promotes increased intestinal barrier function and preserved neuromuscular function. These results suggest that short-chain fatty acids produced by the gut microbiota could affect ALS progression, and further suggests that butyrate is a potential therapeutic agent. While the Zhang et al. (2017) study administered butyrate, it could be beneficial to administer butyrate-producing microbiota because microbes continuously produce the metabolite in the lower GI tract, instead of the rapid absorption of butyrate in the stomach when administered orally. Our collaborators have detected a decrease in butyrate producing bacteria in patients with ALS (unpublished data, personal communication Katharine Nicholson MGH), including *Agathobacter rectale* and *Roseburia intestinalis (ArRi)*, suggesting these bacteria may have a protective role in ALS.
Butyrate also has properties that promote post-translational modifications, modulate energy metabolism, and moderate the inflammatory process in the gut (Bauer et al., 2019). Even further, butyrate can alter microglia, the major immune cell in the brain (Sampson et al., 2016). Butyrate levels have been implicated in ALS, but much of the butyrate and microglia interface has yet to be explored. A study conducted by Matt et al. (2018) increased the butyrate producing bacteria through a high soluble fiber diet to modify microglial activation in male Balb/c mice. Results indicate that peripheral immune systems can shift microglia from homeostatic to a reactive, pro-inflammatory phenotype, a state associated with decreased IL-1β promoter gene methylation (Matt et al, 2018). IL-1β surplus is associated with decreased cognitive function in rodent models and Alzheimer’s patients, providing evidence that immune activation can modulate microglia expression and neurologic function. Aged mice that receive an intraperitoneal injection of sodium butyrate exhibit a decreased release of microglial pro-inflammatory cytokines and elongation of microglial processes (Matt et al, 2018). After lipopolysaccharide mediated immune activation, there was no significant difference in the microglial genomic profile, but the sodium butyrate group showed decreased IL-1β expression in induced inflammatory conditions. This provides a solid foundation for the investigation of whether butyrate producing bacteria can alter neuroinflammation via microglia.
Proteinopathies in ALS

A pathological hallmark of Alzheimer’s Disease, Parkinson’s disease, Huntington’s disease, and ALS includes aggregation of misfolded proteins. In ALS, cytoplasmic aggregates in affected motor neurons and neighboring cells are sourced from improper folding and/or the mislocalization of proteins; the mechanism that results in these inclusions is unknown though protein homeostasis pathway dysregulation is indicated (Ramesh & Pandey, 2017). Protein misfolding develops into a pathology as a consequence of the dysregulation of the cellular quality control mechanisms that degrade aggregated proteins, including the ubiquitin proteasome system or autophagy-lysosome pathway protein degradation pathways. The autophagic pathway targets cytoplasmic proteins, soluble and insoluble misfolded proteins, and organelles of the cell (Ramesh & Pandey, 2017). Regulation of the synthesis, folding, and degradation is maintained by a proteostatic network and dysfunction of this network may contribute to the cytotoxicity that ultimately damages motor neurons. In patients suffering from both familial and sporadic ALS, SOD1 inclusions in aggregated proteins flag degenerating motor neurons in the spinal cord and cortices of the brain (Forsberg et al., 2010). Further understanding of protein aggregate formation and maintenance in ALS can provide insight of the broad cytoarchitecture and cellular mechanisms involved in ALS as well as other allied neurodegenerative diseases.
Non-cell autonomy and microglia in ALS

While motor neurons are the primary cell type implicated in the pathophysiology of ALS, this disease can develop, in part, through non-cell autonomous mechanisms that contribute to motor neuron damage as the gene mutations responsible for familial cases are expressed in many cell types. Overexpression of mutant SOD1 exclusively in motor neurons is not sufficient to drive early disease onset, nor is reduction in neuronal SOD1 sufficient to slow the rate of disease progression (Boilée et al., 2006). However, when mutant SOD1 is selectively silenced in microglia or when wild type (WT) Sod1 non-neuronal cells are transferred into chimeric mutant Sod1 mice (Clement et al., 2003), motor neuron degeneration is delayed, and survival is significantly extended. These findings highlight the non-cell autonomous nature of motor neurotoxicity in this disease and the importance of glial cells in disease progression and motor neuron degeneration in ALS.

Microglia are implicated in neuroinflammation in ALS

ALS patients exhibit increased proliferation and activation of microglia and increased levels of pro-inflammatory cytokines (Butovsky et al., 2012). Immune cell dysfunction underwrites the hallmark neuroinflammatory phenotype observed in both human disease and animal models of ALS. Neuroinflammation in ALS is distinguished by T lymphocyte infiltration, high inflammatory cytokine production, and microglial activation (Liu & Wang, 2017). Even in the pre-symptomatic phase of ALS, increased
inflammation is observed. Immune responses from microglia and other cells can have effects on motor neurons. Thus, additional work is warranted to identify the mechanisms by which these cells contribute to motor neuron death.

In homeostasis, microglia have a neuroprotective function and work to maintain a healthy CNS (Hickman, Sen, and Morsett, 2018). Microglia have an essential role in clearing debris and defending against invading pathogens. However, microglia can become activated and contribute to neural toxicity. In several neurodegenerative diseases, microglia become chronically inflammatory, a state with a unique molecular and transcriptional signature (Zravy et al., 2019). It has been suggested that microbiota dysbiosis may initiate chronic microglial activation. In ALS, peripheral monocytes are functionally altered and invade the CNS, disrupting microglial function and potentially contributing to pathogenesis of ALS. Understanding microglia-mediated neuronal toxicity can provide insight into the pathogenesis of the disease. Recent evidence demonstrates that the microbiota can regulate microglia function and is hypothesized to play a role in neurologic diseases. Additionally, it is not known whether, Akkermansia modulates microglial function in any model; therefore, this study will examine the relationship between A. muc and microglial activation. The extent to which the microbiome modulates microglia in ALS and the application of this novel therapeutic approach of oral microbiota administration remains elusive.
Animal models for ALS

While no ALS murine model fully recapitulates the human disease, the Sod1-G93A (Sod1 hereafter) transgenic mouse was the first ALS murine model developed and is still used extensively in current research efforts (Lutz, 2018). The Sod1 mouse model closely mimics the disease phenotype of ALS, such as massive motor neuronal loss in the ventral horns of the spinal cord and reactive gliosis. SOD1 mutations account for about 20% of the familial cases and 1% of the sporadic cases (Lutz, 2018). This model also presents with SOD1 inclusions that have been observed in other genetic forms of ALS, including C9orf72 and FUS subtypes, in addition to sporadic forms.

Research Aims, Goals, and Hypothesis

The primary goal of this research is to investigate how specific microbial organisms protect the Sod1 model from disease pathogenesis. Based on previous scientific research, my central hypothesis is that specific microbes can modulate disease progression in ALS murine models by influencing peripheral or CNS immunity. We will administer either *Akkermansia muciniphila* or butyrate producing bacteria *Agaothobacterium rectalis* and *Roseburia intestinalis*, evaluate disease progression, weight, and motor coordination in Sod1 mice, and characterize changes in peripheral immunity and transcriptional profiles of central nervous tissues.
This research may promote the development of ALS therapeutics to decrease neuronal insult, ameliorate muscular impairments, and ultimately increase survival in ALS patients that suffer from the debilitating disease of ALS. There is a demand for a pharmacological intervention that could help attenuates neuronal injury and neuroinflammation in ALS. The relationship between the microbiome and peripheral immune mediated-microglia activation is largely uncharted therefore, these set of experiments will help uncover the underlying mechanisms that may help combat the high morbidity of this disease.

**Primary objective:** Delay the onset of paresis and extend survival in the Sod1 mouse model with microbial colonization and investigate related mechanisms by characterizing changes in peripheral immune activation and transcriptional changes in the brain and spinal cord. Three specific aims are the tools used to approach the primary objective.

**Specific Aim 1:** Investigate whether microbiota from Sod1 mice modulates microglia expression

**Methods:** Colonize wild-type (WT) C57Bl6J mice with microbiota from non-carrier control mice or Sod1 mice. Following four weeks of colonization, microglia will be sorted from the spinal cord and sequenced using the SmartSeq2 methodology. Transcriptional changes will be determined using DESeq2. We will also investigate which bacteria differ between nontransgenic controls (NTC) and Sod1 microbiota treated mice by sequencing
the V4 region of the microbial 16S rRNA gene from microbiota samples collected over the course of the experiment.

**Expected results:** If the microbiota contributes to ALS pathogenesis, we expect that microglia genes involved in inflammation and neurodegenerative disease will be increased in mice colonized with Sod1 microbiota. We predict that the microbiome is altered in mice colonized with Sod1 vs NTC control microbiota and that these bacteria will be associated with changes in microglia.

**Specific Aim 2:** Investigate whether specific microbes can improve ALS disease pathogenesis in the Sod1 mouse model

**Methods:** Treat non-transgenic littermate controls (NTC) and Sod1 mice with specific bacteria, *Akkermansia muciniphila* (*Akkermansia*, hereafter), and butyrate producing microbes, *Agathobacter rectale* and *Roseburia intestinalis* (*ArRi*, hereafter): measure changes in weight, perform behavioral testing for motor function, track neurological score progression, collect transcription profile of spinal cord, and quantitation for IFN-γ, IL-4, IL-10 and IL-17 levels from FACS ICC analysis of spleen and mesenteric lymph nodes of mice.

**Expected results:** There will be a delay in disease progression in the *Akkermansia* and butyrate treated groups demonstrated by reduced weight loss, retention of rotarod performance, and deferment of neurological score advancement.
Specific Aim 3: Investigate whether specific microbes alter Sod1 disease progression by modulating peripheral immunity or modulating biologic processes related to ALS pathogenesis.

Methods: At day 120, corresponding to disease onset, we profiled peripheral immune populations in the spleen and mesenteric lymph nodes by measuring the activating of T cells and the secretion of cytokines IFN-γ, IL-4, IL-10 and IL-17 levels by flow cytometry. At day 120, we also collected spinal cord and brain tissue and characterize gene transcriptional profiles using the Nanostring Neurodegenerative Disease panel of over 700 genes.

Expected Results: ALS patients have decreased T-regulatory cells; butyrate producing bacteria and Akkermansia can increase T-regs. Thus, we predict that our bacterial treatments will increase regulatory cell markers (IL-10) and decrease inflammatory cell markers (IFN-γ, IL-17, and GM-CSF). Also, Sod1 mice have elevated expression of microglia genes involved in neurodegenerative diseases (Krasemann et al., 2017).
Chapter II.

Materials and Methods

To investigate the role of the microbiome in an animal model of ALS, two in vivo experiments were conducted. Study 1 investigated the pathogenic potential of the Sod1 microbiota. We transferred microbiota from Sod1 mice and their non-transgenic litter mate controls to WT mice, then characterized gene expression in sorted microglia, and detected altered bacteria by 16s rRNA high-throughput sequencing (Figure 1, Aim 1 study design). Study 2 investigated how specific microbes influences motor coordination and disease progression mechanisms in ALS murine model (Figure 2, Aim 2, 3 study design). Sod1 and NTC mice were colonized with specific bacteria, and underwent behavioral testing for motor coordination and balance, neurologic scoring to monitor disease progression, and tracking changes in weight in Aim 2. Using the same mice, Aim 3 involved understanding mechanism, including identifying altered gene expression in the cellular transcriptome of central nervous tissue by Nanostring and investigating changes in immunity using fluorescence-activated cell sorting (FACS) analysis of mesenteric lymph nodes and the spleen.
Animals

The mice in Study 1 and Study 2 were female and provided by The Jackson Laboratory (Bar Harbor, ME). All mice were kept on a 24-hour reverse light/dark cycle with lights on from 7 am to 7 pm. All protocols were carried out in accordance with approved IACUC protocols.

Study 1: Does the Sod1 microbiota regulate microglial pathways involved in ALS? C57Bl/6J (n=20) were used in this study for microbiome colonization and microglia analysis. At four weeks of age, 20 mice received QUAD antibiotics (described below) for three days, antibiotics were stopped for one day, then WT microbiota or Sod1 microbiota was transferred via oral gavage. Mice received a gavage of 100 μl twice a week for four weeks. Mice were humanely euthanized at 8 weeks for FACS and for microglia transcriptional analysis.

Figure 1: Study design for Aim 1. WT mice received a 3-day course of QUAD antibiotics to eradicate the microbiome, followed by a single day of no treatment to allow antibiotics to be eliminated from circulation. To reconstitute the microbiome, mice received an oral gavage twice a week for four weeks of either Sod1 or non-carrier control microbiota. Microglia transcriptional profiles were characterized by RNA sequencing. 16S rRNA sequencing was used to characterize the bacteria altered in colonized mice.
Antibiotic preparation

Quad antibiotics (Neomycin 1.0 mg/ml, Ampicillin 1.0 mg/ml, Metronidazole 1.0 mg/ml, Vancomycin 0.5 mg/ml) were administered over 3 days by means of a light sensitive water bottle to the wild type mice prior to microbiota colonization.

Administration of microbiome inoculum

In an anaerobic chamber, three H2O treated, non-carrier control murine ceca were diluted in Pre-reduced Anaerobically Sterilized (PRAS) saline at a concentration of 1g contents/10 mL saline. Also, three H2O treated Sod1 carrier murine ceca prepared in PRAS saline and administered at the same dose. Inoculum was stored in -80°C.

Microglia isolation

Wild type mice were perfused transcardially with ice-cold Hanks' Balanced Salt Solution and mononuclear cells were isolated from the spinal cords using a 37%/70% discontinuous Percoll gradient. Anti-LyC6 antibody and a monoclonal antibody that recognizes FCRLS was used to distinguish resident microglia from recruited myeloid cells. Isolated cells were stained with FCRLS (indicated as APC) [clone 4G11, 3 μg ml⁻¹],
CD11b (PeCy7) [clone M1/70, BD Biosciences, 2 μg ml⁻¹] and Ly6C antibody [clone HK1.4, Biolegend, 2 μg ml⁻¹] to isolate CNS-resident microglia.

16S rRNA sequencing

DNA was extracted using the Qiagen DNeasy PowerLyzer kit and sequencing libraries were prepared with barcoded-fusion primers for 104 samples. Microbiota from WT mice (n=20) colonized with Sod1 and Non transgenic control for Sod1 (NTC) were collected over the course of the experiment, with ileum, cecum, colon samples collected at sacrifice. Quantitative insights for microbial ecology (QIIME2) was used to demultiplex and quality filter sequences, assign taxonomy, calculate β-diversity between samples to examine the magnitude of group wise differences based on UniFrac distance calculate relative abundance, and visualize the data. Significant differences in taxa will be determined by linear discriminant analysis effect size (LEfSe).
Study 2: Can the microbiota play a protective role in an animal model of ALS? Non-carrier transgenic controls for the Sod1-G93A strain (NTC) (n=18) and Sod1-G93A (n=36) were used to study how colonization of microbes impact motor function and disease progression. Starting from 9 weeks of age, mice received a bacterial treatment of either *Akkermansia*, *Agathobacter rectale* and *Roseburia intestinalis*, or vehicle control at 200 μl weekly. At week 17, the NTC mice (n=18) and six Sod1 mice from each treatment group (n= 18) were sacrificed for FACS and spinal cord RNA sequencing. The remainder of the Sod1 mice from this cohort continue until survival (n=18). All mice from this cohort were weighed, assessed on the rotarod, and the Sod1 mice were neurologically scored.
Administration Bacterial Inoculum

*Akkermansia muciniphila* was cultured for four days under anaerobic conditions on Brain Heart Infusion (BHI) agar. *Agathobacter rectale* (*A. rec*) was cultured on YCFAs, *Roseburia intestinalis* (*R. int*) was cultured on BHI plates, both in anaerobic conditions for 24 hours. Bacterial inoculum was prepared in 8% glycerol and stored in -80°C. Sod1 and NTC mice received a gavage of 200 μl once a week until end point was reached.

Behavioral testing of motor coordination

Starting on day 73, the Sod1 and NTC mice were trained twice a week for two weeks on rotarod performance. This involved placing the mice on the rotarod until they were able to successfully balance for greater than 200 seconds. To assess motor coordination, Rotarod (Ugo Basile Mouse Rotarod NG) performance was assessed weekly in acceleration mode, increasing from 4 rotations per minute to 44 rotations per minute over 300 seconds. Each mouse ran three trials per day of testing, with a rest period between each trial, and trials were averaged in analysis. The rotarod device records fall time.
Mice were neurologically scored by a system developed by ALS Therapy Development Institute (ALS TDI) (Hatzipetros et al., 2015): Score of 0: Full extension of hind legs away from lateral midline when mouse is suspended by its tail, and mouse can hold this for two seconds, suspended two to three times. Score of 1 (symptomatic onset): Collapse or partial collapse of leg extension towards lateral midline (weakness) or trembling of hind legs during tail suspension. Score of 2 (paresis onset): Toes curl under at least twice during walking of 12 inches, or any part of foot is dragging along cage bottom/table. Score of 3: Rigid paralysis or minimal joint movement, foot not being used for generating forward motion. Score of 4: Mouse cannot right itself within 30 sec after being placed on either side (Table 1, Sod1 neurological score algorithm).

Sod1 mice received neurologic scoring twice a week starting from day 75. Symptomatic disease onset is defined as the age in which animals present tremor and/or defective hind limb coordination for two consecutive days. The neurologic scoring ranges from values 0 to 4. Pathological endpoint, score of 4, is defined by the inability of a mouse to right itself in 30 seconds after being placed on the side.
Table 1

<table>
<thead>
<tr>
<th>Score</th>
<th>Signs</th>
<th>Required Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal—no signs of neurological disease present</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Abnormal motor capabilities/coordination/movement. Hyperflexia, crossed spread of spinal reflexes, and shaking of the limbs when suspended in the air. No significant gait abnormalities.</td>
<td>Removes mouse houses from cage if present. Other enrichment will remain.</td>
</tr>
<tr>
<td>2</td>
<td>Bilateral hindlimb paresis. Mouse drags hindlimbs over flat surface. Exhibits incontinence. Hindlimb paralysis - develop a progressive worsening paresis involving primarily the hind limbs with atrophy of the skeletal musculature.</td>
<td>Same as above PLUS, that mouse, along with at least one additional mouse, will be moved into a cage with alpha dri bedding and standard enrichment + provide food pellet and hydra gel at cage level</td>
</tr>
<tr>
<td>3</td>
<td>Hind and forelimb paralysis. Mouse barely moves around.</td>
<td>As above PLUS provide diet gel 76A and hydro-gel at the cage level</td>
</tr>
<tr>
<td>4</td>
<td>Severe paralysis and inability to forage for food or water. Moribund; Mouse unable to right itself after being placed on side for 30 seconds.</td>
<td>Humane Euthanasia</td>
</tr>
</tbody>
</table>

FACS analysis

Mesenteric lymph nodes and spleens were collected from Sod1 and NTC mice at sacrifice. Tissues were homogenized in complete medium (non-essential amino acids, 10% fetal calf serum, penicillin/streptomycin, beta-mercaptoethanol, isocove’s modified dulbecco’s medium), and a single cell suspension was created via a filter. For the spleen, red blood cells were lysed with ACK. T-cell populations were characterized by staining with fluorescently labeled antibodies for cell surface markers CD3 (stained BV605), CD4 (BV785), LAP (BV421), CD44 (PE) and CD62L (APC), and for the intracellular transcriptional factor FoxP3-FITC. To assess cytokine secretion, cells were stimulated with
PMA + ionomycin and stained for GM-CSF (stained PE), IFN-γ (BV421), IL-4 (APC), IL-10 (FITC), and IL-17 (PeCy7). Cells were analyzed on a 12-Laser BD Fortessa instrument (Brigham and Women’s, Building for Transformative Medicine, Boston, Ma). FlowJo software was used to analyze data and create representative plots. T cells and cytokines were calculated as a percentage of CD4+ T-cells. FMOs and compensation controls for the markers were used to develop gating strategy.
Chapter III.

Results

RNA sequencing of microglia from microbiota colonized mice

To investigate whether the Sod1 microbiota could contribute to pathogenesis, the microbiota was depleted in WT mice with three days of high dose antibiotics, then control or Sod1 microbiota was transferred twice a week by oral gavage. We isolated microglia by FACS sorting and sequenced microglia gene expression. The Sod1 microbiota upregulated genes involved in RNA processing, including *Fus* and poly-adenylate binding protein (*Pabpc1*), as well as increased sequestosome-1 (*Sqstm1*), the autophagy receptor that interacts with Fus (Figure 3). Sod1 microbiota also modulated genes involved in the unfolded protein response and involved in lysosomal degradation, both important pathways in ALS (Butovsky et al., 2012). Transferring Sod1 microbiota to WT mice modulates microglia pathways involved in ALS including genes involved in RNA processing (*Fus*), protein degradation (*HSPA1b* and *USP2*), lysosomal function (*CD68* and *Lyz2*). While these observations were not consistent with our hypothesis that microbiota may worsen disease by modulating CNS immunity, however, they highlight protein degradation and RNA processing as key pathways regulated by the gut microbiome in Sod1 pathology.
We sequenced the V4 region of the 16S rRNA gene in 104 samples. Qiime2 was used for analysis and quality filtering was performed by DADA2. Unique bacterial sequences (features) with less than 100 counts were removed. 250 bases were sequenced from the forward read and 61 bases were sequenced from the reverse read. The average quality score was above 30 for the first read from base position 1 until 249. Q30 corresponds to a 0.1% error rate. Read 2 was above Q30 for all bases. In total, after filtering, there were 2,364,313 reads, with a median depth of coverage of 22,978 (Figure 4). We investigate changes by performing a principal coordinate analysis of unweighted UniFrac.
distances. As shown in Figure 5, samples cluster by treatment, and are significant for differential clustering based on PERMANOVA testing (Table2).

Figure 4. Histogram of depth of coverage
Table 2. Permanova testing for clustering differences between groups.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Sample size</th>
<th>Permutations</th>
<th>pseudo-F</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTC</td>
<td>d36fecal</td>
<td>20</td>
<td>999</td>
<td>18.30</td>
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Figure 5. Principal Coordinates Analysis of colonized mice. PCoA of all gut microbiota samples, fecal and intestinal, from WT mice colonized with Sod1 microbiota or non-carrier controls (NTC).
Figure 6. The microbiota of wild type mice colonized with Sod1 (SOD) and nontransgenic controls (NTC) A) relative abundance of bacteria in fecal and intestinal samples. Color key indicates bacterial names, colors repeat every 12 colors and arrows indicate taxa that correspond to first three series of 12. B) relative abundance of select bacteria that differ, * p < .05, ** p < .02, *** p < 0.001, LEfSe (Segata et al., 2011).
We characterized the microbiota composition over time and observed that after four days, after the first antibiotic treatment (Figure 6A) at 36 days of life led to a limited number of bacteria composing much of the microbiota, which is consistent with early niche colonization. Two weeks into the microbial colonization, at 43 days of life, the microbiota began to stabilize, while the samples taken at experimental endpoint, 8 weeks of life, provide insight into the biodiversity of microbiota colonized wild type mice.

The microbiota takes time to stabilize, thus we focus on samples taken 4-week post-colonization (day 56 of life). There were three genera that were decreased in the wild type mice treated with Sod1 microbiota: Akkermansia, Bacteroides, and Olsenella (Figure 6B). Faecalibaculum rodentium, Pseudoflavonifractor, and Acutalibacter muris were elevated in Sod1 colonized mice suggesting that these bacteria could be detrimental in ALS. It is possible that gut bacteria may have important disease specific roles supported by the fact that antibiotics ameliorated disease in other models of neurodegenerative disease, but worsened disease in ALS models.

Treatment with Akkermansia and butyrate-producers (ArRi) delay Sod1 weight loss

Weight was collected twice per week throughout the course of the study. We found a strong genetic effect in the weight. The Sod1 mice weighed less than the WT mice. Between the Sod1 mice colonized with bacteria, the Akkermansia treated mice had the greatest preservation of weight compared to vehicle treated mice across the duration of the experiment, though butyrate producing microbe treatment was also demonstrated increased
weight (Figure 7), with weight between treatment groups and vehicle diverging around day 140. The higher weight in the treated Sod1 mice suggests that these bacteria slow disease progression as muscle atrophy, and therefore decreased mass occur as disease progresses.

*Akkermansia* and butyrate-producers (*ArRi*) delay progression of motor symptoms

Starting at week 11, prior to symptomatic onset, Sod1 and NTC mice were trained twice a week for two weeks on the rotarod, a behavioral assessment of motor coordination. The purpose of this test is to assess and compare the impact of bacterial colonization on the motor function of Sod1 mice. The mice were then assessed weekly starting from day 90 until experimental endpoint. We found that *Akkermansia*-treated mice were able to maintain balance on the accelerating rotarod for the longest period of time as measured by latency to fall compared to the vehicle control group, though both *Akkermansia* and *ArRi* groups had better performance than the vehicle group (Figure 7:B-C). We detected improved motor performance at approximately weeks 18 of age when latency to fall times diverged between vehicle and treatment groups. Though not statistically significant the increase of fall to latency time in treatment versus vehicle groups suggests an increase in motor coordination from *Akkermansia* and *ArRi* treatment.
Figure 7: Bacterial modulation of behavioral phenotypes in the Sod1 model. Nontransgenic controls (NTC) and Sod1 mice were colonized with either vehicle, Akkermansia muciniphila (SOD-A) or Butyrate producing microbes: E. rectale + R. intestinalis (SOD-B). A) Weight was significantly higher in A and B treated Sod1 mice, 1-way ANOVA. B-C) Rotarod behavioral testing assessed weekly. D-I) Neurological Score (NS) was assessed. Though there was not a significant difference in the time of onset between V, A, and B treated Sod1 mice (E), there is a significant delay in the regression of neurological condition as assessed by neurologic score (D). A score of 2, indicative of paresis onset in mice, was significantly delayed by A and B treated mice, shown for all treatments together and separately (F-H). Vehicle treated mice progress to a NS of 3 earlier than other treatment groups (I).
Starting at week 19 of age, we performed neurological scoring of Sod1 mice twice a week, which includes a tail suspension test to assess hindlimb function and a walking test that assesses the gait of the mouse. A score of 0 indicates the pre-symptomatic stage. A score of 1 is given for hindleg clasping or hyperflexia and marks symptomatic onset. A score of 2 is given when the mouse begins to drag its feet and indicates the onset of paresis or muscle weakness in the hindlimbs of the mouse. A score of 3 is given when motor impairments are observed in both hind and forelimbs, which indicates rigid paralysis of the hindlimbs and decline of function of the forelimbs. A score of 4 indicates a mouse that cannot right itself within 30 seconds when placed on its side, and this meets humane euthanasia criteria and marks the survival endpoint.

As the mice aged, the bacterial treatments delayed the progression of neurologic scores compared to vehicle treated Sod1 mice (Figure 7: D). While we found that our bacterial treatments
did not alter time to symptomatic onset (score of 1, Figure 7: E), mice treated with butyrate producing microbes had the slowest progression to paresis onset (score of 2, Figure 7: F-H), and butyrate producers and Akkermansia treated groups were significant in delay of progression to paresis (score of 3, Figure 7: I). In addition, vehicle Sod1 mice progressed to rigid paralysis (score 3) and endpoint euthanasia criteria (score of 4) prior to any of the other treatment groups, which indicates that Akkermansia and Butyrate producing bacteria slow disease progression in Sod1 from symptomatic onset to survival.

The effect of protective bacteria on peripheral T-cell responses

To investigate the effect of Akkermansia and butyrate producing bacteria on peripheral immunity, markers for CD4+, Foxp3+, CD44+, CD62+, LAP+ markers were used to characterize the T cell populations in mesenteric lymph nodes (MLN) and splenic tissue at sacrifice using fluorescent antibodies and flow cytometry (Figure 8). The proportion of CD4+ T-helper cells was increased significantly in the Akkermansia treatment group in both MLN and spleen. FoxP3+ and LAP+ T cells have a regulatory function that can lessen inflammation, and ALS patients have decreased regulatory T cells (Tregs). We found that treatment with Akkermansia significantly decreased the proportion of LAP+ and Foxp3+ cell populations in total CD4+ T-cells.

Naïve T cells are identified as CD62L- CD44+, T effector memory cells are identified as CD62L- CD44+, T central memory cells are identified as CD62L+ CD44+. Comparing Sod1 mice to non-transgenic controls (WT), we found that Sod1 mice had higher naïve T cells and lower central memory cells. Next, when we examined the effect
of treatment, we found that our bacterial treatments lowered naïve T-cells and increased central memory T cells, which is the opposite effect as we observed for Sod1 vs. WT.

T cell subsets can be defined by the cytokines they secrete, IFN-γ is a cytokine indicative of a proinflammatory Th1 response, IL-4 is indicative of a Th2 response, IL-17 is secreted by Th17 cells and can have an important function in repair, but if GM-CSF is also increased, these cells can lead to pathogenic inflammation. IL-10 is secreted by T regs and can dampen inflammation. These cytokines in stimulated T cells and found that Akkermansia treated Sod1 mice had a decrease of the proinflammatory cytokine IL-17 compared to the vehicle control mesenteric lymph node tissue (Figure 9). Other changes were modest and in cytokines that had low production, suggesting that there is not overt inflammation in these animals at onset.
Figure 8. Immune responses in T cell populations. Upper panel: gating strategy for T cells activation and regulator markers with FMO controls in red. Lower panel, T cell populations in non-carrier controls (WT) and Sod1 mice colonized with Akkermansia and butyrate producing bacteria. * p < 0.05, ** p < 0.01, *** p < 0.001. * notes genotype effect, * notes bacteria effect.
Figure 9. T cell cytokine secretion. A) Gating strategy for cytokine activation with FMO controls shown in red. B) Cytokine populations in nontransgenic controls (NTC) and Sod1 mice colonized with *Akkermansia* and butyrate producing bacteria. *°* notes genotype effect, *°°* notes bacteria effect.
To understand the disease process in Sod1 mice, we isolated RNA from the spinal cord at disease onset, day 120, and compared Sod1 to non-carrier controls (WT). Many genes were differentially expressed, but there was a significant increase in genes that indicate microglial activation, including *Trem2* (Triggering receptor expressed on myeloid cells 2), *Apoe* (Apolipoprotein E), and *CD44* (Figure 10). *Trem2* is an *Apoe* activator, and this pathway has been indicated as a regulator of the MGnD phenotype in ALS, Multiple sclerosis, and Alzheimer’s disease (Krasemann et al., 2017). Additionally, increased *CD44* expression in microglia parallels ALS disease progression and might indicate neuroinflammation (Winkler et al., 2012).
Akkermansia and butyrate producers (ArRi) reverse Sod1 effects on transcriptional profiles

Sod1 and NTC mice aged 9 weeks received either Akkermansia, butyrate producing microbes (ArRi), or vehicle weekly. At four months of age, the Nanostring Neurodegeneration Panel helped to elucidate changes in expression of the cellular
transcriptome of the lumbar region of the spinal cord. Differential testing was performed using the NSolver Advanced Analysis Module in which the distribution of each gene is used to select the optimal model for differential expression. Sod1 mice exhibited significant differences in expression in genes involved in microglial activation, unfolded protein response (UPR), and interactive genes with biological relevance to the neuropathophysiology of the Sod1 model. Interestingly, both bacterial treatment groups reversed Sod1 associated changes. In Sod1 mice, we found that both bacterial treatments reversed Sod1 associated changes, including decreased expression of Fus, and increase of two interacting genes oxidative response 1 (Oxr1) and survival motor neuron 1 (Smn1) (Tosolini & Sleig, 2017). Despite Sod1 mice not having mutations in Fus, Sod1 mice accumulate Fus inclusions, which may contribute to neuronal toxicity (Li et al., 2016). Furthermore, these bacteria alter the expression of genes involved in protein degradation, including presenilin-binding protein ubiquilin 1 (Ubqln1) (Kim et al., 2009). The effect of gene expression on specific microbe colonization has unveiled critical pathways modulated by the microbiota at the time of onset of neurologic symptoms in mice.
Figure 11: Bacterial modulation of spinal cord transcriptional profiles in Sod1 mice. A) WT and Sod1 mice were treated with vehicle (V), Akkermansia (A), or butyrate-producing (B) bacteria: E. rectale + R. intestinalis. Spinal cord RNA was measured by the Nanostring neuropathology panel at onset, day 120. B) Global pathways affected by bacteria in Sos1 mice. C-D) Genes modulated by Akkermansia and butyrate-producing bacteria in Sod1 mice. E) Specific genes altered in Sod1 mice that are reversed by treatment with Akkermansia or butyrate-producing bacteria. * p < 0.05, ** p < 0.01.
Chapter VI

Discussion

Significance of results

ALS is a neurodegenerative disease, which impacts motor neurons in the spinal cord and brain and is typically fatal within three-five years of diagnosis. Both genetic and environmental factors contribute to disease, but no single gene accounts for most sporadic cases of ALS prompting the investigation into the role of various environmental factors on disease pathogenesis. Research suggests that neurons die through apoptotic or inflammatory pathways (Gordon, 2011); the intestinal microbiome is an environmental factor that is essential in modulating both central and peripheral immunity (Wright et al., 2018), which we hypothesized to play a role in ALS pathogenesis.

The microbiota shapes the host immune system to protect against invading microbes and promotes the induction of inflammatory mediators (Wright et al., 2018). Not only does the microbiome change gastrointestinal and peripheral immunity, but it can also affect immune responses in the brain. The gut-brain axis is a bidirectional communication and an integral intermediary between external and internal environmental factors via immunological, endocrine, or neural pathways (Wright et al., 2018).

Patients with neurodegenerative disease have alterations in their gut microbiome, which may contribute to the disease. Studies have shown that the transfer of microbiota from patients with Multiple sclerosis (Berer et al., 2017) or from patients with Parkinson’s disease (Sampson et al., 2016) worsens disease severity when transferred to animal models.
The microbiota may affect neurologic disease because they secrete bioactive metabolites that modulate metabolism, immunity, and epigenetic processes (Wright et al, 2018).

In ALS animal models, the relationship between the gut microbiome and neurological health is currently under investigation as human and murine microbiota show significant modifications. The bacterial population is altered in the gut of ALS patients (Brenner et al., 2018), and altering the gut microbiome in animal models can change disease progression (Blacher et al., 2019). In order to investigate whether the microbiota from a model of ALS could contribute to disease, we directly transferred microbiota from Sod1 mice to WT mice and sorted microglia.

ALS patients exhibit increased proliferation and activation of microglia and increased levels of pro-inflammatory cytokines (Butovsky et al., 2012). Immune cell dysfunction is integral to neuroinflammation observed in both human disease and animal models of ALS. Neuroinflammation in ALS is distinguished by T lymphocyte infiltration, high inflammatory cytokine production, and microglial activation (Liu & Wang, 2017). Even in the pre-symptomatic phase of ALS, increased inflammation is observed. Immune responses from brain-resident microglia and other cells can have effects on motor neurons. In ALS, peripheral monocytes are functionally altered and invade the CNS, disrupting microglial function and potentially contributing to pathogenesis. The extent to which the microbiome modifies microglia in ALS and the application of this novel therapeutic approach of oral microbiota administration is still unclear, but our data indicates that the microbiota can regulate genes associated with ALS.

In order to investigate whether microbiota from a model of ALS could contribute to disease, we directly transferred bacteria from Sod1 mice to WT mice, and sorted
microglia. We found that the Sod1 microbiota upregulated genes involved in RNA processing, lysosomal function, and protein degradation.

RNA processing resulting in mis-spliced transcript isoforms and translation of proteins that misfold has biologically relevant implication in ALS as protein aggregates are found in the motor neurons and muscles of patients and ALS animal models. This thesis work demonstrates that the fused in sarcoma gene (*Fus*), is a gene upregulated in spinal cord microglia in wild-type mice that received an oral gavage of the Sod1 microbiota 100 ul twice a week for four weeks. This gene produces Fus proteins that bind to DNA and RNA, and these proteins have been implicated in cellular processes that includes cell proliferation, DNA repair, transcription regulation, and RNA and microRNA processing. In a study conducted by Mitchell et al (2013), *Fus* overexpression in wild-type mice resulted in motor neuron degeneration, early onset tremors, and hind limb paralysis. Beyond *Fus*, we found that Sod1 microbiota upregulated genes involved in RNA processing including poly-adenylate binding protein (*Pabpc1*), as well as increased sequestosome-1 (*Sqstm1*), the autophagy receptor that may interact with Fus. Upregulation of this network is the first evidence that the gut microbiota may activate pathways that eventually lead to protein aggregates that contribute to motor neuron death in ALS.

Skeletal muscle is an abundant and dynamic tissue in the body and contains copious amounts of sarcoplasmic reticulum. In ALS, muscle weakness and atrophy are common clinical manifestations for this disease. MyoD, a protein integral for muscle differentiation, is a transcription factor involved in myogenesis, a process that is required for skeletal muscle repair after insult (Bohnert, McMillan, & Kumar, 2018). MyoD and other myogenin transcription factors directly target Xbp1 linking this gene with myogenesis.
*Xbp1* effects a subset of genes in myoblasts and myotubes integral in the function of the endoplasmic reticulum, growth, and DNA damage and repair pathways. Overexpression of the spliced Xbp1 proteins acts as an inhibitor of the expressions of myogenesis markers and yields smaller myotubes in culture. Further work must be done to elucidate the relationship between the *Xbp1* gene and muscle formation and maintenance, but this suggests that *Xbp1* can act as a regulator in myoblast differentiation to multinucleated myotube (Bohnert, McMillan, & Kumar, 2018).

Unfolded or misfolded proteins that accumulate in the lumen of the ER trigger a physiological canonical cellular stress pathway known as the unfolded protein response (UPR) (Pirog et al., 2019) and *Xbp1* signaling is integral in alleviating that stress. We found that the Sod1 microbiota upregulated *Xbp1*. Upregulation of this gene in the microglia of the spinal cord may indicate stress due to aggregates, possibly early inclusion formation, in the cells of naïve wild type mice that received a gavage of Sod1 microbiota. The ER triggered *Xbp1* branch of UPR has been associated with neurodegenerative diseases including Huntington’s disease, Alzheimer’s and many different musculoskeletal phenotypes.

CD68 is a glycoprotein component of the lysosomal vesical membrane which we found was upregulated in WT mice treated with Sod1 microbiota (Brettschneider et al., 2012). Though this marker indicates macrophages, including microglia often a marker for activated microglia, in patients with increased upper motor neuron score found increased CD68 and microglia pathology in cervical anterior horns. This study also suggests that Sod1 microbiota treatment to wild type mice was sufficient to drive this gene expression in microglia.
Ctsz encodes for the protein cathepsin Z, expressed primarily on antigen presenting cells (Allan et al., 2017) and its expression was upregulated in microglia from mice colonized with Sod1 microbiota. This particular locus has previously been under inspection for its role in increasing susceptibility of Multiple Sclerosis. In experimental autoimmune encephalomyelitis (EAE) murine models, Cathepsin Z deficient mice developed lower levels of neuroinflammation and had decreased TH17 responses. Cathespsin Z, considered an epigenetic risk factor for MS via hypomethylation, promotes neuroinflammation in this mouse model. Our findings that ctsz is upregulated following Sod1 microbiota administration could possibly indicate neuroinflammation in the spinal cord tissue of WT mice (Allan et al., 2017).

To identify which bacteria from Sod1 mice were associated with these responses, or identify control microbiota that could be protective, we sequenced the microbiota in Sod1 and NTC colonized mice. We found that Sod1 colonized mice had lower Akkermansia, which is consistent with previous studies for its protective role in Sod1 mice (Blacher et al., 2019). In the ALS microbiome, Akkermansia muciniphila is altered in disease progression. Sod1 mice exhibit a gradual decline in the abundance of Akkermansia.

Bacteroides is another genus that was decreased in the WT colonized with Sod1 microbiota. Bacteriodes can induce T-regs and protect in animal models of Multiple sclerosis; this appears to happen through an IL-10 mediated pathway (Ochoa-Repáraz et al., 2010). Lastly, Olsenella is a newly described bacteria and little is known about its role in neurologic disease, but a study conducted by Reynders et al. (2020) indicates various levels of Olsenella and Roseburia in immunologic diseases though the direction of the trend is not indicated in the literature. Akkermansia, Bacteriodes, and Olsenella were
decreased in the Sod1 microbiota treated WT mice, so I hypothesized that they could have beneficial role in ALS and neurologic disease.

In the same group of mice, *Faecalibaculum rodentium*, *Pseudofalvonifractor*, and *Acutalibacter muris* were increased in the gut microbiome of WT mice treated with Sod1 microbiota suggesting a potentially detrimental bacteria in ALS. *Faecalibaculum* has previously been associated with protection from amyloid plaque deposition in a model of Alzheimer’s disease (Cox et al., 2019). It is likely that gut bacteria may have important disease specific roles supported by the fact that antibiotics ameliorate disease in models of Alzheimer’s and worsen in models of ALS. Not much is known about the roles of *Pseudoflavonifractor* or *Acutalibacter muris*, which provides an opportunity for thorough exploration of the individual effects of these bacteria to move from correlative investigation to causative investigation.

This work confirms that *Akkermansia* improves disease progression and showed for the first time that *Agathobacter rectale* and *Roseburia intestinalis (ArRi)* can also improve ALS disease pathogenesis in the Sod1 mouse model. Weight was significantly higher in *Akkermansia* and *ArRi* treated Sod1 mice compared to vehicle treated Sod1 mice. Motor function impairment was delayed in bacteria treated Sod1 mice as assessed in rotarod tests, as vehicle treated mic did not plateau in latency to fall over time, instead these mice steadily decreased.

Even further, neurological condition decreased most rapidly in the vehicle treated mice. While the treatment did not alter time to onset, the progression from symptomatic to paresis onset was delayed in *Akkermansia* treated mice, and further delayed in the mice treated with butyrate producing bacteria. Thus, our novel treatment regimen of two-
butyrate producing bacteria may have an even greater protective effect than *Akkermansia* on survival. In ALS, neurodegeneration can occur in the upper motor neurons or the lower motor neurons (spinal cord to muscle). With paresis indicating lower motor neuron degeneration essential for spinal cord to muscle communication, an assessment on the interaction between butyrate and these specific neurons would do much to elucidate the mechanisms for disease progression. Lastly, vehicle treated Sod1 mice progressed, though some mice in this cohort are yet to reach endpoint, so full survival data is not yet available for the bacteria colonized Sod1 mice. This data supports the hypothesis that specific microbes modulate behavioral phenotypes and disease progression in the Sod1 model.

To understand the potential biologic mechanism, we investigated how our bacterial treatments altered gene expression in the spinal cord using the Nanostring Neurodegeneration panel, which represents many pathways important for ALS. We found gene expression in biological processes related to autophagy, unfolded protein response, oxidative stress, and microglia activation. There was downregulation in genes involved in matrix remodeling. It is known that Sod1 mice have activated microglia and altered protein processing, thus this data suggests that our bacterial treatments can modulate CNS pathways important for ALS.

In both ALS patients and animal models, CD4+ lymphocytes serve a neuroprotective function in slowing the rate of disease progression through mediation of glial homeostasis (Lewis, Manning, Rossi, & Krieger, 2012). Evidence suggests that CD4+ T-cells slow disease progression and increase survival. CD4+ T-helper cells are increased in the blood of Sod1 mice from age 16 weeks until end point (Beers et al., 2011). Consistent with this, we found that at 17 weeks of age, prior to paresis onset, greater quantities of
CD4+ were deployed in mesenteric lymph nodes (MLN) and splenic tissue in the *Akkermansia* treated group compared to both the vehicle and butyrate microbe treatment Sod1 groups, suggesting that *Akkermansia* upregulates CD4+ production, and might contribute to the delay in neurological score progression in Sod1 mice.

FoxP3+ T regs have the function of dampening the proinflammatory response (Lewis, Manning, Rossi, & Krieger, 2012). T regs were increased in the slower progressing stages of disease, including times prior to paresis onset and decreased during the rapidly progressing phase. Sod1 mice that receive a transfer of endogenous Foxp3+ T regs in the early stages of disease had prolonged survival, suggesting that suppressing inflammation by Foxp3+ T-regs is beneficial. In contrast to the reported literature, we found that in MLN and splenic tissue, Foxp3+ T reg levels were significantly decreased in the *Akkermansia* treated Sod1 mice compared to the glycerol group, and an even more significant decrease compared to the butyrate mice. The relationship between Foxp3+ levels and progression rates are inverse in ALS. A limitation is that we did not evaluate the levels of T regs in the intestinal lamina propria, which has closer proximity to the gut microbiota, or in the central nervous system, where they can traffic in times of inflammation. Thus, these discordant results prompt further investigation. Of note, while the reduction was significant from 10% FoxP3 T regs in glycerol treated Sod1 mice, these cells decreased to only 8% in *Akkermansia* treated Sod1 mice. The fact that we observed improvement in these animals suggests that the decrease in T regs was not large enough to contribute to disease progression.

At day 120, the *Akkermansia* colonized Sod1 mice have significantly less latency associated peptide (LAP+) T cells in the mesenteric lymph nodes than both the vehicle
ArRi treated mice. There is a genotypic decrease effect across all treatment groups. Butyrate microbiota treatment groups increased frequency while the Akkermansia treatment effect yields a decrease of LAP+ percentage comparable to the NTC. There has not been much research investigating the relationship between LAP+ T regs and ALS, but because these cells can also dampen inflammation, we would hypothesize a similar beneficial role in ALS.

It is known that the gut microbiota can induce T cells to differentiate from naïve T cells to effector memory T cells and central memory T cells. Central memory T cells can self-renew and provide long-lasting immunologic memory to a stimulus, whereas effector memory cells directly target the stimulus and are non-self-renewing. We examined the populations of these cells and found that our bacterial treatments increased central memory T cells, thus lowering population levels of effector and naïve cells. This indicates that these bacteria may play a role in T cell differentiation. While we found decreased naïve T cells and increased central memory cells in Sod1 mice, this was partially reversed when we treated with our bacteria. However, the effect was modest, suggesting that other mechanisms may play an important role in protection.

At 17 weeks of age, prior to paresis onset, the Akkermansia treated mice Sod1 mice had a decrease of the proinflammatory cytokine IL-17 compared to the vehicle control mesenteric lymph node tissue. IL-17 dependent pathways can contribute to disease progression in ALS. In a study conducted by Fiala et al. (2010), serum collected in patients with sporadic and familial ALS found increased levels of this cytokine in the serum of patients. There is much to be understood concerning cytokine levels in ALS patients, but IL-17A is a chemokine that trends significantly higher in in those suffering from the
devastating disease compared to non-autoimmune effected healthy controls in early disease stages. This trend might decrease with Functional Rating Scale or Neurologic Score. Though we saw a subtle, but significant change in IL-17, future directions might measure the cytokines that are known to induce IL-17, including IL-1β, IL-6, and IL-23.

Initially, we hypothesized that the gut microbiome may influence the disease progression of ALS modulating peripheral immunity, decreasing proinflammatory markers, and increasing regulatory markers. However, our data that our bacterial treatments modestly lowered Tregs does not support this hypothesis. Though there are a few statistically significant findings in the cytokine data, these may not be biologically relevant as the cell counts are low. This suggests that the protective effect that we observe is linked to alternative mechanisms. Our data on gene expression in the spine indicates instead that the gut microbiome may modulate disease through upregulating of genes involved in microglia activation, RNA splicing, unfolded protein response mechanisms, and autophagy. It is possible that the microbiota could have a direct effect via the secretion of metabolites that enter systemic circulation and cross the blood brain barrier or pass into the CSF, which would be a physiologic route not dependent on the peripheral immune system.

Study Limitations

While the study was designed to elucidate the impact of the gut microbiome in disease progression, not all the animals in this cohort have reached end point, therefore
complete survival data have not been collected. At the time of submission, the mice were 155 days old, with an expected potential survival time of 200 days. Furthermore, our CD8+ marker used in the FACS analysis panel did not stain successfully as apparent in FACS analysis and the lack of FMO staining. This incomplete panel excludes information about cytotoxic T cell activation, which has been implied in ALS pathogenesis (Jansson et al., 1995). Lastly, though there were significant observations highlighting the role of microbiota composition on genetic expression of tissues implicated in ALS, there was no direct investigation of the gut microbiome secreted metabolites to the central nervous system to confirm these observations. These finding have provided insight towards understanding the mechanisms of how specific microbes of the gut microbiome influences animal models in ALS.

Future Research Directions

Lower motor neurons of the spinal cord become pathologic prior to the upper motor neurons of the brain. Lower motor neurons travel from the spinal cord to the muscle; degeneration of these neurons produce weakness, atrophy, and cramps (Gordon, 2011). Further along in disease progression upper motor neurons degeneration leads to paralysis. With findings indicating that butyrate producing microbes and Akkermansia delay paresis onset, as observed through a delay of neurological score of 2 in bacteria colonized mice, it would be beneficial to make the biological association between the bacterial metabolites of these specific microbes and lower motor neuron staining for inclusions.
Also, with data suggesting that specific microbes can delay disease progression and possibly extend survival, the investigation of the biological mechanisms involved in the Sod1 mouse paresis onset and the potential impact of nicotinamide and butyrate, the microbe products of *Akkermansia* and *ArRi* respectively, is paramount for future research (Blacher et al., 2019). This would require metabolomics to investigate and quantify the active metabolites involved in the protective effect observed by *Akkermansia* and butyrate producers, *ArRi*, in ALS.

**Conclusion**

We sought to investigate whether the gut microbiota could either contribute to or protect against pathogenesis in an animal model of ALS. Our hypothesis was supported by identifying ALS-related pathways in microglia by transferring Sod1 microbiota to naïve mice leading to slowing disease progression by transferring beneficial bacteria to Sod1 mice. Though we hypothesized that the gut microbiota influences ALS disease progression through mediation of peripheral immune activation, data suggests that the gut microbiome impacts disease progression through influencing genes involved in protein misfolding and aggregate formation, which are common broad pathologies observed in neurodegeneration. The ability of specific microbes to reverse the Sod1 effect in mice has been integral into the investigation between role of microbiota and disease progression in an ALS animal model. Together this data suggests that specific microbes may have a protective effect in delaying disease progression.


