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Manipulation of the Microbiome and Its Impact on Functional Recovery Following Ischemic Stroke

Michal Jandzinski

University of Connecticut, 2015

We are all covered from head to toe, internally and externally, by trillions of microbes without even realizing it. Who are these microscopic neighbors of ours? How did they come to inhabit every square inch of our bodies? What purpose do they serve? These are all questions that this paper will seek to explore and answer in the background section. Following the discussion, the background knowledge will be applied to a completely novel area of research: how the microbiome impacts ischemic stroke.

Background

Trends exist all over the world in all sorts of things. As of late, modern scientific literature has recently been seduced and infatuated with the microbiome, the bacteria that live inside all organisms. As this paper will demonstrate, though, this microbial fascination isn't without good reason. The aim of this background section is to give a thorough overview on the basic ideas behind the microbiome: what is it? Where does it come from? What does it do? And what does it do in diseases? At the end there will be a brief discussion on the "frontier" of microbiome research, so to speak, which is a growing body of evidence pertaining to the microbiome's influence on the brain. At the end of this section it will hopefully become clear why the microbiome is a legitimate area of concern for all concerned with health and wellness and why the microbiome should not be ignored as a potential source of insight and treatment opportunities as it pertains to the various diseases that humans are faced with today.

So what's the big deal?

For every single human cell in the body, there are roughly 10 microbial cells^[1]. These microbes inhabit nearly all parts of the body, residing all over the skin, throughout the entire gastrointestinal tract, in the oral and nasal cavities, and just about everywhere else. In many ways it would be naive to argue that this microbiome, "the collective genomes and gene products of resident microbes living inside and on humans", has no impact on a person's development and overall health^[1]. In modern scientific literature, there has been a massive wave of research showing the fundamental contributions of the microbiome to organisms' daily maintenance of homeostasis and development of diseased states. These microbial communities commonly form commensal relationships with hosts, carrying out functions that are vital to host survival such as food digestion, prevention of pathogen proliferation, and even assist proper development of the innate immune system^{[1][8]}. In other instances, specific groups of microbes or deregulation of normal microbiome populations can cause homeostatic dysfunction or disease^{[1-3][8-14]}.

Even though these microbes can have a wide range of impacts on health and even though there is a wide range of them, it has been found that all these microbes can actually be sorted into a very limited number of groups. More specifically only four main bacterial phyla, groups consisting of hundreds of bacterial genera and species, really constitute human microbiome populations: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria^[15] (Table 1). Despite the dominance by four bacterial phyla, there is still an impressive level of diversity in the relative microbial compositions at different body locations^{[1][16]}. Human skin, for example, contains

nearly insignificant relative abundances of Bacteroidetes relative to other body locations. Gastrointestinal tract studies have shown that, in both children and adults, the microbiome populations can be broken down to basically 2 groups; mainly composed of Bacteroidetes and Firmicutes, with the two phyla accounting for roughly 98% of 16s RNA sequences detected in the gut of mammals^{[1-3][17][19]}. The 16s RNA (rRNA) gene is used as a small genetic marker (about 1.5kb in size) that has been highly conserved amongst bacterial species and phyla to allow for species to be distinguished from one another along with being able to classify groups of microbes from the same or different phyla^[30].

Phylum	Main Classes	Commonly examples	General traits of microbes
Actinobacteria	Actinobacteria	<i>Bifidobacterium</i> , <i>Streptomyces</i> , <i>Nocardia</i>	Gram-positive with high content of G+C in DNA. Diverse morphologies, physiological/metabolic properties. Phyla includes pathogens(ie. <i>Nocardia</i>), GI commensals (ie. <i>Bifidobacterium</i>), and even microbes adapted to living in soils (ie. <i>Streptomyces</i>) ^{[1][18]}
Bacteroidetes	Bacteroidetes	<i>Bacteroides</i> , <i>Prevotella</i> , <i>Flavobacteria</i>	Gram-negative with 4 broad classes with microbes adapted to live in soils, seawater, and guts of animals. Display varied morphologies and range from strict aerobes to strict anaerobes. Often regarded as specialists of degradation of large organic substrates such as proteins and carbohydrates ^{[1][9]}
Firmicutes	Bacilli, Clostridia	<i>Clostridium</i> , <i>Staphylococcus</i> , <i>Enterococcus</i>	Gram-positive with cocci or bacillus morphologies. Typically display low G+C content in DNA. Play roles in energy resorption in the gut an have been implicated in many diseased states including obesity ^[1] .
Proteobacteria	Gammaproteobacteria, Betaproteobacteria	<i>Escherichia</i> , <i>Pseudomonas</i> , <i>Helicobacter</i>	Gram-negative bacteria that are typically pathogenic in nature ^[20] .

Table 1: The four predominant bacterial phyla that compose the human microbiome.

Out of all of the bodily locations that host the commensals, the gastrointestinal tract has proven to be the most significant to human health. By most estimates, the GI tract is the clear favorite area of residence for our microbial neighbors, hosting over 70% of the microorganisms in the body^[28]. While that may seem like an shocking amount, it makes sense; if a person took their own gut and flattened out all the villi and microvilli the structure would have a surface area of about 32 square meters, the floor size of a small studio apartment or about half a badminton

court^[29]. But the GI tract doesn't just offer the commensals a lot of real estate, it offers them lots of real estate that's full of nutrients and minerals that they can use to grow, survive, and proliferate, making it the clear choice for microbes that are deciding what portion of the human body to colonize. After establishing their community in a person's body, this overwhelming majority that resides in the gut plays an incredibly important role in a person's health. All the previously mentioned impacts of the microbiome stem directly from this gut community in one way or another.

Starting from day 1

This begs the question of where do these microorganisms come from? Are people born with them? Or do they infect hosts after birth? As it turns out, the bacteria that are found inside every person's gut are mainly descendants from an initial wave of microbes that colonize sterile newborns in the immediate moments following birth^[1]. During the seconds following birth, hundreds, thousands, and even millions of microbes begin attempting to colonize newborn's body. Studies comparing the microbiome compositions of vaginal and cesarean delivered newborns have shown dramatic differences between the two groups microbial populations, suggesting that a person's mode of birth delivery can play a dramatic role in determining the constituents of their microbiomes. As Figure 1 from Dominguez-Bello(2010) shows, microbiomes of vaginally delivered newborns were strikingly representative of the maternal vaginal and intestinal microbial populations while cesarean delivered newborns displayed microbiome compositions similar to those of their mothers' skin^[11]. Other studies have also shown a markedly reduced proportion of *Bifidobacterium* and *Bacteroidetes* ("good" bacteria) in the gastrointestinal tracts of cesarean delivered newborns when compared to vaginally delivered newborns^[9]. Regardless of mode of delivery, though, this initial microbial population that colonizes newborns has been found to distribute itself uniformly throughout the body of the infant, meaning that the mode of delivery of an individual will lay the foundation of all the body's future microbial communities^[11, 12].

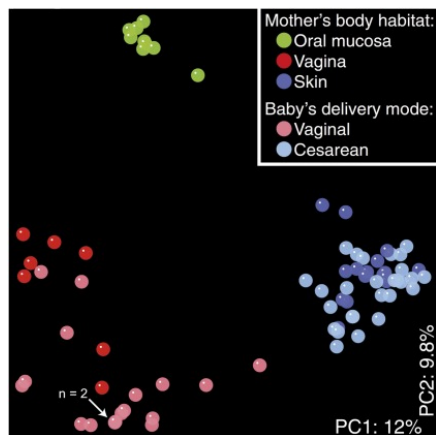


Figure 1: 16S bacterial RNA UniFrac analysis conducted by Dominguez-Bello(2010). Unifrac analysis uses phylogenetic information to calculate and display the genomic differences amongst microbial communities^[21]. Significant differences can be seen in newborns' microbiome compositions depending on the baby's mode of delivery at birth^[11].

So what happens after birth and the initial invasion of microbes? After an individual's microbiome begins forming after birth, the individual's environment and lifestyle for the rest of their life will shape the development of the microbiome. An example of a person's environmental factor that will play a role in microbiome development is the type of food that an infant will first be exposed to. Many mothers are presented with the decision of whether their children will be breast-fed or formula-fed, but what many are unaware of while making the decision is that their choice will determine how their child's microbiome will develop from the moment they feed them for the first time. Recent developments have led to the general belief in populations that being breast-fed is generally healthier for children, but not many are actually aware of what specific impact breast milk has on the health of a child. For starters, breast milk provides infants with a booster-pack of protective and beneficial goods like cytokines, growth factors, immunoglobulin's, lactotransferrin, lysozymes, and human milk oligosaccharides (HMO's)^[13, 14]. While all the components assist in proper immune development in the infant, HMO's play a particularly important role in protecting infants from disease and infection by acting as decoys for invading pathogens thereby preventing the infectious microbes from gaining access to epithelial cells^[23]. What has been most surprising, however, has been the finding that shows that the carbohydrate-rich component essentially functions as a prebiotic in the microbiome by stimulating and promoting the colonization and growth by *Bifidobacterium* sp^[23]. As such, multiple studies have shown that breast-fed infant actually present with much higher relative abundances of both *Bifidobacterium* and *Lactobacillus* (commonly considered beneficial bacteria) microbes in their gut microbiomes compared to formula-fed infants, who actually show a significantly increased rate of colonization by *Clostridium* and other pathogenic microbes (especially *C difficile*) which is probably attributed to the lack of protective HMO's in their diets^[23-25]. Not surprisingly, infants with proportions of beneficial microbes in their microbiomes have been shown to be better protected from allergies, diarrhea, necrotizing enterocolitis,

obesity, and even type II diabetes. It's truly amazing to think how such a seemingly small decision about a baby's first food can have such a significant impact on the development of their microbiome. What's more impressive, though, is how big of an impact the resulting microbiome can have on the baby's development and health later on.

But do environmental factors have a significant impact on the microbiome only during the initial stages of a person's life while the microbiome is in its early immature stages of development? Are microbial populations less affected later on in life as their diversity and population size increases? As it turns out, the microbiome's sensitivity is not lost with age. In fact, the microbial composition can be, and is, constantly affected by various environmental factors such as a person's diet. In 2006, Turnbaugh was able to rapidly and significantly alter the microbial compositions in mice containing human microbiomes^[26]. David(2013) showed that even short-term dietary changes can trigger dramatic compositional changes in the human microbiome by tracking microbial response when subjects shifted to entirely plant-based or animal-based diet. David(2013) showed animal-based diets significantly increased bile-tolerant microorganisms while decreasing the abundances of bacteria responsible for metabolizing plant polysaccharides (the opposite held true for microbiomes introduced to plant-based diets)^[26]. The changes that the microbiomes experience in response to dietary changes don't simply regress overtime, in fact, after initial changes occur in the microbiome, the microbiomes stabilize in their new states and remain stable indefinitely until a new environmental factor is introduced and they must change again^[27].

While only a handful were mentioned here, there are a plethora of environmental and lifestyle factors that can influence the development and composition of the microbiome. However, it was previously mentioned that when it comes to the gut, roughly 98% of resident microbes fall into just two main phyla of bacteria regardless of environmental changes. How can that be? Well, as it turns out, it's not a mere coincidence that the invading microbes were allowed to colonize and reside in the gut after birth, as the following sections will shed light on, host-microbiome interactions play critical roles in shaping how the immune system will develop and react to pathogens, in prevention of the propagation of these invading microbes, and carry out processes so vital to human digestion and metabolism that without them malnutrition would be unavoidable.

With Great Microbes Comes Great Immunity

Instead of triggering an outburst of protective measures by the immune system, the microbial

residents of the gut are a critical step in proper development to the system that will ultimately determine the overall health of the host. The role of the microbiome in the development of the systemic immune system is most readily seen in studies that have focused on germ-free (GF) animals that lack any form of colonizing bacteria. These mice that lack the normal endogenous microbes have been found to contain hypoplastic Peyer's patches, the organized lymphoid nodules responsible for intestinal immune surveillance and generation of the intestinal immune response^[30]. The spleens and other lymph node structures in the GF mice have also been found to be defectively formed or entirely functionally absent in mice lacking a proper microbiome^[30]. If the structures that are responsible for the immune response are defective then what about their products and immune cells? As it turns out, those too are harmed by the lack of a microbiome with GF mice presenting with inadequate levels of immune cells and their products, such as IgA-producing plasma cells and their immunoglobulins and major irregularities in circulating cytokine levels and profiles^[31-33].

Is it really the microbiome, or lack thereof, responsible for the immunodeficiency that occurs in the GF animals or is it a mere coincidence? This is the same question that was asked by researchers when the initial results were reported about the GF mice and to the dismay of many skeptics of the significance of the microbiome, specific microbial populations were found to play very specific roles in the modulation of immune development. Along with the previously mentioned deficiencies that have been reported in GF mice that lack the proper endogenous microbiome, the mice are also completely devoid of proper CD4+ T cell expansion^[34]. These T cells, are mature T helper cells that are absolutely necessary for a host's immune response to invading pathogens^[35]. Without any intervention, the GF mice would exhibit this deficiency their entire lives. This deficient phenotype was able to be entirely reversed through the administration of "good" microbes to the mice from the *Bacteroidetes* phyla, more specifically *Bacteroides fragilis*^[34]. More strikingly, it was found that the phenotype was reversed by specific interactions between the administered microbes and the immune system: dendritic cells recognized specific epitopes present on the *Bacteroidetes* phyla, presented the antigens to immature T lymphocytes in the mesenteric lymph nodes (a required step to T cell activation and expansion), and this presentation ultimately caused the development and proliferation of CD4+ T cells along with the lymphocyte-containing compartments in the spleen^[34]. The exposure and presence of just one specific group of microbes not only supports proper immune development, but is actually vital to the immune system's maturation at both a cellular and organ level.

Many would still attempt to argue that perhaps the immune system development can be triggered by any form of microbes, and there is no actual causal relationship between the microbes that are reported as "good" versus those that are considered harmful. A study comparing GF mice colonized by altered Schaedler flora (ASF) and GF mice colonized by microbial populations consisting of mainly *Bacteroidetes* phyla members showed just how important specific microbial populations and proportions are for proper immune development. ASF is a bacterial mix containing 8 bacterial species that are considered the "normal" or archetypal microbial species that are expected to be present in mice^[36]. The mix often contains relatively equal portions of each group and is commonly used in studies involving the GI tract since it gives researchers an easily controllable and reproducible microbiome population in their studies. The mix of microbes in the microbiomes of ASF colonized mice, however, isn't representative of the endogenous microbiomes of normal organisms. If the immune system required merely the exposure to microbes to trigger proper development as many microbiome skeptics would believe, then the ASF contains more than enough of a variety and number of microbes to trigger some sort of response. What was actually seen, however, was that ASF colonization wasn't sufficient to promote differentiation of Th17 cells, T cells necessary for immune functions such as recruitment, activation, and migration of neutrophils, interleukin-21/22 secretion, and anti-microbial immunity at various epithelial barriers^[37]. The GF mice colonized by microbial mixes found in normal endogenous microbiomes, more importantly, mixes containing high proportions of *Bacteroidetes* microbes, normal differentiation and proliferation of Th17 cells was seen^[38]. The findings point out the importance of the specific endogenous mixes of microbes that commonly reside in the endogenous microbiome. The immune system has clear requirements for exposure to specific antigens provided by the specific microbes present in the microbiome. Without the proper microbial compositions, the necessary and complex interactions between host-microbes cannot occur and the normal development of the immune system will be severely hindered.

The Great Wall of Bacteria

The most obvious yet commonly overlooked benefit that the commensals confer to hosts is the physical barrier that they provide against antigens from the external environment at the intestinal mucosa. The most intuitive way that the endogenous microbes do this is by physically excluding foreign invaders. While the exact mechanisms of action have yet to be elucidated, there is a clear exclusion of pathogens that occurs when normal microbes colonize the GI tract. Corr(2007) was able to show that many bacterial strains that are commonly used in probiotic blends such as

Bifdobacterium and *Lactobacillus* physically prevent the attachment and invasion of the pathogenic *Listeria* through the elaboration of their processes and secretion of compounds that induced epithelial cell immune/structural responses such as in recent reports that have described increased expression of tight junction proteins responsible for maintaining the integrity of multiple vital endothelial barriers like the blood-brain barrier following normal host-microbiome interactions that occur during development^[39, 82]. *Lactobacillus* has even been shown to decrease the ability of pathogens *E. coli* and *S. enteritidis* to bind their attachment sites on the ileal mucosa^[39- 41].

But the bacterial residents don't simply buy out all the real estate in the gut to keep pathogens out; they're armed with weapons and are more than willing to use them to keep out unwanted guests. Microbes from the *Lactobacillus* genus are an example of microbes that take it upon themselves to produce substances to directly prevent pathogenic colonization. So what do they do? The genus members all produce lactic acid, which creates low pH environments that greatly deter the growth of bacteria that are not adapted to surviving in the acidic environments^[30]. Many other strains of bacteria normally present in the endogenous microbiome also produce substances that function to prevent pathogen invasion, such as the *Rumminocus gnavus* bacteria that produce lantibiotics (peptide antibiotics produced by many Gram-positive bacteria to attack other Gram-positive invaders)^{[42][43]}. Many of the anti-microbial substances released by endogenous microbiome members often either target other bacteria that are similar to themselves, a simple yet effective mechanism to eliminate any potential competition for niches from invading pathogens. Other times the production or activation of the products depends on host mechanisms like enzymes which further demonstrates the incredible level of co-adaptation of the microbiome to the host^{[30][53]}.

Many of the microbes also help bolster host protective measures by carrying out important steps to induce expression of antimicrobial substances by the host or to activate anti-microbial precursors that the host secretes. One incredible example of this is the induction of defensin expression in the GI tract. Defensins are one type of anti-microbial peptides produced by the body to defend itself against bacterial, fungal, and viral infections^[44]. Defensins, and multiple other similar substances, are produced by secretory epithelial cells (Paneth cells) that are present at the base of crypts in the intestinal lumen and secretion has been shown to be controlled by the normal endogenous microbiome^{[30][45]}. Vaishnava(2008) showed that in order for a host to express full levels of antimicrobial compounds from the Paneth cells, the entire normal microbial

community was necessary, with reduced levels of expression in organisms lacking microbial phyla^[47]. Looking at the same relationship from a different angle, other studies have shown that administration of certain bacterial species on top of the already present normal microbiome can even induce expression of anti-microbial substances from the GI tract cells^[46, 47]. As mentioned earlier, some anti-microbial compounds are secreted in an inactive form by the host, many defensins are an example of this as they are secreted as prodefensins. These prodefensins aren't able to confer any protective benefits to the host until they undergo proteolytic cleavage by the enzyme matrilysin^{[30][48]}. Using GF mice, Lopez-Boado(2000) was able to identify that one of the major factors controlling production and secretion of the enzyme is the presence and colonization of the GI tract by *B. thetaiotaomicron* bacteria, yet another member of the "good" *Bacteroidetes* phyla, which were shown to induce enzyme expression by Paneth cells^[48]. Not only do the endogenous microbes play a critical role in causing the secretion of necessary protective molecules by the body to protect itself against invading pathogens, but the lack of a microbiome (or the lack of the proper microbiome composition) can severely hinder host defenses by not activating protective compounds or not activating the production of supporting molecules necessary for host defense.

The Microbe Diet

Food is something that people of all ages, genders, nationalities understand. Everyone has to eat, and in most cases, everyone enjoys it. But not many take the time to appreciate all the complex and interconnected processes that must occur in the body to absorb nutrients and convert the ingested organic substrates into useful energy. Studies of biochemistry and other scientific courses devote significant portions of time studying metabolic processes such as glycolysis, the citric acid cycle, and oxidative phosphorylation, making it seem as though humans possess incredible amounts of metabolic abilities. Without taking anything away from the complexity and engineering of these internal processes, the metabolic capabilities of the human body are actually quite pathetic without the microbiome. The genetic information that the microbiome contains for metabolism is far greater and far more versatile than the information encoded in the human genome^[49]. As a result, a significant portion of metabolism and metabolic homeostasis is under the control of the microbiome. The importance of the microbiome to metabolism is very intuitive, though, since digestion and absorption occurs primarily in the area where the GI microbiome resides. The significance of the microbes to the processes becomes even clearer through the numerous studies that have shown that GF mice require a markedly increased caloric intake on a daily basis compared to mice containing normal, unaltered microbiomes in order to

maintain the same body weight^[30]. So what exactly are these bacteria doing for the host? Well, they are vital to proper metabolism by carrying out two main functions.

The first of the functions that the microbiome carries out for the host in terms of digestion is by allowing the host to have access to calories that they would normally be unable to access without the aid of the microbes. One mechanism in which microbes unlock otherwise indigestible sources of nutrients is by metabolizing large portions of dietary fiber to short-chain fatty acids and usable monosaccharides^[50]. Not having bacteria present in the GI tract to metabolize the fiber would cause humans to miss out on a major energy source, but it would promote the production and accumulation of potentially toxic metabolic by-products by the body as a direct consequence of saprophytism, the process of obtaining nourishment from dissolved decaying organic matter, as shown by Vella(1999)^[50, 51].

Along with giving access to new energy sources, the microbiome also plays a role in promoting the efficient absorption of ingested nutrients. Not only have studies shown that the presence of the endogenous microbiome helps promote and support the activity of lipoprotein lipase in adipose tissue resulting in increased tissue fatty acid uptake, but administration of specific bacterial strands from the normal microbiome into GF mice has significantly improved nutrient absorption^[52]. Administration of *B. thetaiotaomicron* to GF mice resulted in increased colipase expression, an important cofactor used by pancreatic lipase, which led to increases in efficiency of hydrolysis and absorption of ingested lipids^[53]. Not only that, but an increased expression of the Na⁺/glucose cotransporter was seen in the intestine following the microbe administration and colonization of GF mice, also causing an increased glucose uptake by the organisms^[53].

Without the presence of the microbiome, human hosts would miss out on enormous energy stores stemming from the lack of genetic capability to metabolize many forms indigestible oligosaccharides and polysaccharides. Additionally, the absorption of nutrients that the human body is equipped to deal with would be greatly reduced due to the lack of assistance that is normally provided by the microbiome. Proper nourishment and metabolism hinges on the presence of the microbiome, the human diet is truly a microbe diet; without them, inefficient digestion and malnourishment is inevitable.

Although this is nowhere near a full comprehensive outline of the benefits that the microbiome confers to the human host, it should be quite clear as to why the microbes are allowed to remain in the body after their initial colonization after birth. It's incredible to think of just how big of an

impact bacterial communities, and even simple specific bacterial antigens, have on the proper development and maturation of the immune system, a system which has the power to determine the entire course of a person's life. Along with supporting host defense development, the microbes even work to develop their own weapons against invading pathogens. Finally, many vital life-processes that an organism must carry out to survive are heavily influenced by the endogenous microbiome, which is best shown in the critical roles the microbial populations play in metabolism.

It's not you... It's the microbes

It's obvious now that the microbiome plays a major role in development and homeostasis maintenance. But the microbial populations playing such a major role in maintaining overall health and wellbeing in humans exposes humans to a whole new danger. The massive roles that the microbiome plays in various aspects of immune system development and control means that a loss of regulation of the microbiome itself could prove disastrous for the functions and processes that it regulates in the body.

As modern science has come to realize the importance of the microbiome to the everyday health of a person, there has been a great deal of data that has been published comparing the microbiomes of healthy and diseased individuals showing that microbial compositions greatly vary between the two groups in numerous diseases. To date, over 25 different diseases and/or syndromes have been associated with specific alterations in the microbiome^[54]. The implicated diseases range from obvious candidates such as obesity to seemingly unrelated neurological diseases like multiple sclerosis. In the following sections, the major health implications of the microbiome will be further explored in the context of a few diseases that have been shown to have their roots in the endogenous microbes residing in people's guts.

Fat Bacteria?

Based on the metabolic roles that the microbiome plays in the human body, it doesn't come as much of a surprise that one of the first diseases related to altered microbial compositions was obesity. Initial tests sought to compare the microbes that were present in normal "lean" mice and genetically obese mice by analyzing the 16s rRNA sequences in samples from the two microbiomes. The results showed a strikingly clear difference between the two groups in regards to the ratio of *Bacteroidetes* and *Firmicutes* (which make up well over 90% of the gut microbiome) with a dramatic decrease of about 50% in *Bacteroidetes* and a major proportional

increase in *Firmicutes* in obese mice when compared to lean mice^[55]. Further studies using other murine models for obesity and even human models found the persistence of the high *Firmicutes* to *Bacteroidetes* ratio in organisms suffering from obesity^[56].

The clear divide between the two groups suggested that the drastically skewed ratio must signify some sort of molecular change in the obese mice, and Turnbaugh(2007) demonstrated the molecular explanation by delving deeper into the genomic differences between the “lean” microbiome and obesity-associated one. Compared to “lean”-microbiome containing mice, obese mice showed a marked elevation in genes for enzymes involved in polysaccharide digestion, suggesting the obese mice are simply ultra-efficient at squeezing out every last bit of energy from ingested food^[57]. Fecal analysis confirmed the theory by demonstrating a greatly reduced amount of energy remaining in the feces of obese mice^[57].

The final test for the obesity-associated microbiome came in the form of microbiome transplants from obese mice to GF mice. After colonization of the gut of GF mice by microbiomes from either obese or lean donors, it was found that the obese phenotype was transmissible, meaning that the microbiome itself was, at least in part, directly responsible for the diseased phenotype^[57, 58]. GF mice receiving the “obese” microbiome showed a 60% increase in fat storage and developed insulin-resistance after just 14 days compared to the GF mice receiving the “lean” microbiome^[54, 57]. When the colonized GF mice were fed an obesity-inducing diet, both groups showed weight gain, but more impressively, the obesity-associated microbiome mice showed increased weight gain compared to “lean” microbiome mice and the microbiomes of “lean” mice actually began to shift to resemble the obesity-associated microbiome as obesity began to set in^{[57][58]}. When plain GF mice lacking a microbiome of any kind were fed the same obesity-inducing diet, however, they failed to experience any significant weight gain and never presented with an obese phenotype, giving further support for the microbiome-caused obesity condition^[59].

Burning Gut

While microbes can’t literally set a person’s gut on fire, they can (and commonly do) cause the painful set of conditions referred to as Inflammatory Bowel Diseases (IBD). The role of the microbiome in IBD’s or more specifically in Crohn’s disease and ulcerative colitis, was elucidated in much the same way as it was in obesity: by initially comparing the microbiome of healthy individuals compared to those suffering from the disease. Three main common trends have been reported by studies seeking to explain the role of the microbiome in IBD’s: IBD patients have a decreased diversity and stability in their gut microbiomes, they have markedly

decreased amounts of microbes that commonly confer anti-inflammatory properties to the host, and the microbiome functions in altered or defective ways in IBD patients when compared to healthy individuals^[54, 60].

Many studies have attributed the initial triggering of IBD to defective mucosal barriers that allows for infection and colonization of portions of the gut by pathogenic bacteria like the *Proteobacterium Mycobacterium* and *Klebsiella* or the *Actinobacteria* phyla member, *Campylobacter*^[61]. After invasion by the opportunistic pathogens, they begin to quickly propagate and severely diminish microbial diversity in the microbiome by essentially kicking out endogenous microbes and out-proliferating other microorganisms. Others have also reported elevated levels and proportions of pathogenic *E. coli* strains tightly bound to apical intestinal epithelium's of Crohn's disease patients^[63]. IBD patients also showed greatly decreased amounts of *Faecalibacterium prausnitzii*, a bacterium that has been shown to be associated with anti-inflammatory functions in the body by reducing the secretion of proinflammatory cytokines while increasing that of interleukin-10, a molecule that functions as a regulator of the immune system that prevents hyperactive immune responses^[63, 64]. While many relationships are still being explored between the microbiome and IBD, there is already evidence of a causal relationship similar to obesity as the dysbiosis seen in the microbiome and it's subsequent loss of immune regulation that occurs in IBD has been greatly reduced and improved in models of IBD via administration and gut colonization by anti-inflammatory microbes like *Faecalibacterium prausnitzii*^[64].

Aging as a symptom?

While obesity and IBD are only two small examples of diseases that have been related to microbiome dysregulation, they point to very important points regarding the microbiome and health. While the environment influences and shapes the microbiome throughout life, especially early in life, later on the microbiome is capable of directly influencing the host and its health; the relationship is a two-way street, it's bidirectional. This is shown by how various environmental aspects can influence the composition of the microbiome, but more importantly by how specific disease phenotypes can be transmitted to healthy organisms by simply transplanting and colonizing the healthy organism's gut with the disease-associated microbiomes.

What has been one of the most interesting findings in microbiome research has been the way an individual's age impacts the microbiome, which is bringing in results that make age look like a negative symptom of a changing microbiome. What many researchers have found is that

microbial compositions are far from being considered static, they're constantly changing and adjusting after the initial colonization with extremely dramatic changes occurring during infancy through adulthood^[17]. When Agans(2011) sought to compare the gut microbiomes of adolescents to adults, for example, his results showed clear age-related microbiome differences between the (Figure 2)^[21].

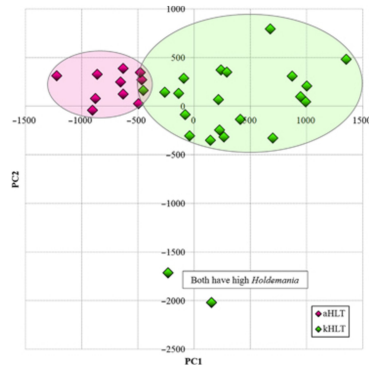


Figure 2: Principle component analysis of microarray data collected by Agans(2011) comparing healthy pre-adolescent/adolescent children (kHLT) and healthy adult (aHLT). The gut microbiomes were measured and compared by detecting microbial 16s rRNA genes ^[21].

Comparing samples from healthy adults and children, they found that both groups had microbiomes dominated by the two major phyla (*Bacteroidetes/Firmicutes*) as expected, but the microbiome of young individuals had a statistically significant increased abundance of beneficial *Bacteroidetes* bacteria and *Bifidobacterium*^[21]. Other studies have reported similar differences in microbial compositions between age while showing nearly-perfect correlations between the aged microbiome compositions and poorer scores on frailty indices used to evaluate age-related clinical signs of deterioration in mice^[65]. Van Tongeren(2005) was even able to correlate increases in frailty in older individuals with decreases in bacterial groups that were previously discussed as playing important beneficial roles in host health such as *Lactobacillus* and *F. prausnitzii* bacteria^[66].

While many of the reported data sets have been reported as correlations, it's impressive to see such tight correlations between aging and specific compositional changes in the microbiome. What's more interesting to think about is how aging has been shown to bring about microbiome changes that have been associated with disease and negative health effects, such as increases in "harmful" *Firmicutes* in proportion to *Bacteroidetes*, decreases in microbial diversity, and decreases in "beneficial" microbes associated with immune regulation and homeostatic maintenance^[66-68]

The New Frontier: The Brain

Many of the discussed diseases and health impacts related to the microbiome make sense, the conditions all have some sort of mechanistic and intuitive link to the gut area of the body. But how could the gut microbiota possibly influence other peripheral systems in neurological disorders? How does it influence the nervous system? While the link connecting the microbiome and brain has long evaded researchers, recent findings are making breakthroughs in this new frontier of microbiome research.

To date, reports have been maintaining evidence that the microbiome and the metabolites of its microbes play significant roles in influencing behavioral and brain processes such as stress responses, emotion-driven behavior, and various biochemical pathways in the brain^[69]. While much of the evidence is still fairly new and not many causal relationships have been established, spending some time to review the current preclinical evidence of the gut-brain relationship is worthwhile due to the consistent trends found in reports and possible clinical implications of the findings.

Using GF mice and microbiome transplantation, Sudo(2004) was able to show the microbiome plays a clear role in adult stress-responsiveness by monitoring the hypothalamic-pituitary-adrenal reaction to stress of GF mice to mice colonized by normal microbiomes. Not only were plasma levels of stress-response molecules (such as ACTH and corticosterone) significantly elevated in GF mice lacking microbiome compared to mice with normal microbiomes, but the GF mice actually presented with decreased brain-derived neurotrophic factors in the cortex and hippocampus^[69, 70]. To follow up on the findings the research group administered “beneficial” bacteria in the form of *Bifidobacterium* to some of the GF mice and compared them to GF mice that were administered pathogenic *E. coli* finding that the GF mice receiving beneficial microbes demonstrated a reversal of their previously elevated response to stress while mice receiving *E. coli* presented with an even further elevated response to stress^[70]. While rapid responsiveness to stress is essential to survival, the exaggerated and prolonged stress molecule elevation in GF mice is purely detrimental to the host’s health.

But stress responsiveness wasn’t the only area of the human psyche that has been implicated in being impacted by the microbiome. Many other reports have shown that a link exists between the microbiome and depression, anxiety-linked behavior, learning and memory, and even social behavior^[69, 71, 82]. Pathogenic bacteria and high proportions of *Firmicutes* have been linked to increased emotional behavior, increased incidences of anxiety-like phenotypes, impaired

learning and memory functions, and reduced social interactions and ASD-like behaviors^[81-86]. Phenotypes such as depression and anxiety have been reversed through the use of probiotics containing “beneficial” and normal microbial populations, seeking to push hosts’ microbiomes towards compositions that are normal^[84, 86]. A fascinating find has been made using ASD model mice, however, use of probiotics containing *Bacteroides fragilis* to behavioral and social improvements in mice^[87].

Other intriguing evidence has emerged from research looking at these specific phenotypes, with multiple studies linking the effects of diet on the microbiome to the depressed phenotype^[72]. Meta-analyses suggested that healthy diets (more specifically Mediterranean-style diets) and the beneficial effects on the microbiomes, markedly reduce the incidence of depression in individuals and seem to confer protection against cognitive decline when compared to diets rich in sugars and fat, which are tightly correlated to increased psychological symptomology^[73]. But while these results are impressive to see, are there truly any causal relationships between the microbiome and the depressed and anxious phenotypes? Or is it just a coincidence? Studies led by Bested(2013) and others sought to solve this question by seeing if psychological phenotypes, like other disease phenotypes, could be transmitted via microbiome transplantation and the results were nothing short of stunning, showing that not only could the anxious phenotype be transferred via gut microbiome transplantation, but manipulation of the gut microbes via probiotics and antibiotics can alter and reverse depressed phenotypes^[74-76].

So if these psychological phenotypes can be transferred by the microbiome then what is really going on a molecular level, what specific microbes are responsible? As it turns out, all the microbial populations that have been previously discussed as part of the normal healthy microbiome and those as part of the pathogenic, disease-causing group retain their respective roles when influencing the nervous system.

Many of the microbes that have been described as harmful, especially elevated proportions of *Firmicutes* to *Bacteroidetes*, commonly compromise the tight junctions that are meant to form an epithelial barrier to the outside environment^[77]. This disruption, commonly referred to as “leaky gut,” is caused by lack of proper host-microbiome interactions that have been previously discussed as vital to immune development and the lack of needed beneficial microbes to confer protective benefits against invading pathogens. When the intestinal defenses are compromised, bacteria-derived lipopolysaccharides (LPS) can access to system circulation and levels rise throughout the body triggering immune and inflammatory responses that can be tracked by rising

levels of pro-inflammatory cytokines throughout the body^[78]. This sort of inflammation has been implicated as one of the causative factors of depressive symptoms, as demonstrated by studies that have found high levels of immunoglobulins against bacterial LPS circulating in the plasma in patients suffering from chronic depression^[79]. In animal models researchers have even been able to induce the depressed phenotype by prolonged administration of LPS, which can be reversed through administration of anti-depressants^[79]. Along with sending molecules into circulation, those suffering from leaky gut are also prone to experiencing microbial translocation across epithelial boundaries that would normally be impermeable to bacteria, which has been shown to trigger autoimmune responses by immune systems that target intestinal serotonin and its receptors, culminating in increased feelings of fatigue and illness behavior in patients^[80].

Another impressive recent development in the search for the link between the gut and the brain has been the work of Brainiste(2014) which has shown that the lack of the normal endogenous microbiome actually functions to increase permeability of the blood-brain barrier, thereby giving potentially harmful metabolites of immune responses and pathogenic microbes access to the brain itself^[82]. There have even been links made between brain development and the microbiome such as GF mouse models displaying decreased brain-derived neurotrophic factor in the hippocampus, altered expression of GABA A and B subunits, and altered expression of NMDA receptor subunits^[88, 90]. Other studies have found that abnormal microbial compositions can actually decrease the abundance of proteins needed for proper brain development such as PSD-95 and synaptophysin in the brain^[90].

While this discussion won't be able to give a full explanation and run-down of the discussed studies and the exact molecular mechanisms responsible for the microbiome induced effects on the brain one point should be clear: they definitely exist. While many precise causal relationships are still yet to be found, the tight correlations are starting to show the growing importance of the microbiome to all aspects of health. Not only are the microbes affecting systems in the gut, but abnormal and pathogenic microbes can, and do, have very negative effects on peripheral systems throughout the body, going as far as the brain to affect the host's psyche and neural development.

The microbiome and its effects on the human body are both varied and complex. But it cannot be denied that a relationship between the two exists. Not only does it play a vital role in development throughout life, it can ultimately affect the host's health and wellbeing. While

many are often quick to write-off studies relating the microbiome to various diseases, modern scientific literature shows that it's undeniable that the endogenous commensals are involved on some level in nearly all human diseases. This basic belief was what prompted the study that will be discussed in later sections.

Introduction

Each year, nearly 800,000 individuals residing in the United States will have a stroke. Of these, about 130,000 cases will prove fatal while many of the survivors will be forced to live with disability for the remainder of their lives. Out of all strokes over 87% are ischemic strokes. An ischemic stroke, in the most basic sense, is the initiation of the ischemic cascade in response to a loss of blood supply to a part of the brain. The lack of oxygen due to a lack of blood to neurons severely hinder the cells' abilities to synthesize ATP through normal processes which forces cells to switch to anaerobic metabolism. While anaerobic metabolism can generate sufficient levels of ATP for basal levels of survival for very brief periods of time, it is in no way prepared to sustain cellular life for the time frames that are involved in strokes, which can last from minutes to several hours. This energy failure will ultimately result in the slowing and failure of ATP-dependent ion pumps in neurons that are absolutely vital for survival. Pump failure will lead to cell depolarization and excess neurotransmitter release. At the cellular level, this cascade will culminate in the production of harmful free radicals, reactive oxygen species, and the over activation and production of ion-dependent enzymes resulting in the breakdown of the cell's components and cell death. If, or when, the brain is re-perfused, the tissue will experience further injury on the macro scale due to a sweeping inflammatory response to cell damage, damage to the blood-brain barrier, and rampant edema. The widespread incidence of this debilitating condition costs the United States an estimated \$36.5 billion dollars every single year. Despite this, clinicians are armed with very little to combat the disease.

Recent research developments have brought about the rise in awareness about the importance of the microbiome, the various gut flora present in all organisms, in relation to disease prevention, management, and treatment. Many of the risk factors for stroke including high blood pressure, obesity, and diabetes have been found to be not only affected by the microbiome, but can also be changed, altered, and even reversed by changing the microbiome itself. Alterations in microbial diversity can increase the risk of many diseases ranging from obesity to multiple sclerosis to aging, but changes in the microbiome after ischemic stroke have not yet been examined. In addition, it is unknown if changes in the microbiome can influence either stroke severity or stroke recovery. This paper will seek to find the answer to these questions.

This paper will demonstrate the success of microbiome transplantation between recipient and donor mice, will explore the trends seen in the composition of the microbiome after stroke, and will seek to find a link between the microbiome and stroke outcomes. Being able to successfully

and selectively alter the microbiome in an organism to reduce the harmful effects of stroke and improve recovery would not only be a completely novel potential direction in stroke research, but could offer a plethora of innovative treatment options that could be readily implemented through techniques already used for the treatment of *Clostridium difficile* infection or through simple probiotic formulas. This study's findings could lead to invaluable insights into the relationship between the gut, the immune system and the brain and may be able to identify new targets to enhance functional recovery after stroke.

Purpose

This project sought to examine the effect of the microbiome on stroke outcomes after experimental stroke in mice. Initial experiments were conducted to confirm reports that marked changes can be observed in the microbiota with age and results demonstrated that marked differences occur in the microbiome of young vs. aged mice. It is now widely recognized that that as organisms' age, there is a corresponding increase in circulating inflammatory markers. This pro-inflammatory milieu makes organisms less able to cope with a variety of stressors, including stroke. Aged animals (and humans) have increased mortality and poorer recovery after ischemic stroke compared to their young counterparts. Clear differences in the ratio of the two major phyla of microbes present in murine microbiomes (*Bacteroidetes*/*Firmicutes*) in young vs. aged mice were observed, with aged mice presenting microbiome compositions associated with harmful health effects. Young animals have a high ratio of *Bacteroidetes* to *Firmicutes* while aged animals have the opposite. A similar relationship has also been seen in the human microbiome. Additionally, it was found that after an experimental stroke the microbiome composition of young animals' changes to resemble the composition of that seen in aged non-stroke mice. These findings could prove to be of substantial value to stroke research since it has already been confirmed by many other studies that high relative abundances of *Firmicutes* to *Bacteroidetes* (ratios resembling aged animal microbiomes) are tightly correlated to hypertension, cardiovascular complications, and obesity; all of which are direct risk factors that can both increase the incidence of ischemic stroke as well as dramatically reduce recovery after an ischemic event^[50-56, 91, 92].

The goal of these experiments was to examine the effect of transferring a young microbiome into an aged animal, to determine if restoring a more "youthful" microbiome will reduce morbidity and mortality after stroke. I hypothesized that transplantation of theoretically beneficial

microbiome from young mice into aged mice can improve functional recovery in aged recipients post-stroke when compared to aged mice that receive aged donor transplants. Using the same logic, a group of young mice received aged fecal transplants, which I hypothesized would experience worsened stroke outcomes when compared to the young controls.

Materials/Methods

Mice

The experiment used a total of 48 male mice. 24 young mice housed at the laboratory following birth and were housed two mice per cage for a period of two months prior to any experimental procedures for habituation purposes. 24 aged mice born 4/2013 ordered from The Jackson Laboratory were also used in the experiment and were also held in the lab for a 2-month habituation period after arrival. At time of the procedure young adult mice were approximately two months of age (considered to be equivalent to 18 year old humans) while aged mice were 16 months of age making them equivalents to 55-60 year old humans. Mice were housed in standard mouse cages with suspended wire mesh bottoms to prevent cannibalism. This study was conducted in accordance with the National Institute of Health guidelines for the care and use of animals in research and under protocols approved by the Center for Lab Animal Care at the University of Connecticut Health Center.

Fecal Transplantation

3 aged mice and 3 young mice were chosen as donor mice whose feces would be used for microbiome transplants into recipient mice. Fecal pellets from the donor mice were collected and placed in 1.0mL of iced phosphate buffered saline solution (PBS). Final volumes were adjusted to ensure a concentration of 120 mg donor feces per mL PBS. Sterile wooden toothpicks were used to mash fecal pellets in the PBS until a paste-like consistency was reached followed by vortexing the solution at maximum speed for a total of 1 minute. The solutions were then centrifuged at $800 \times g$ for 3 minutes. The supernatant from the centrifuged solution was then removed and used for the transplants into the recipient mice. Protocol was similar to those described in previous studies^[94, 95].

Endogenous gut microbiomes of the recipient mice were suppressed as much as possible prior to transplantation via administration of 2 concentrated antibiotic doses. Streptomycin HCl

suspended in sterile water at a concentration of 500 mg per mL was used. Each mouse received 50 μ L per dose administered directly into their oral cavities. 24 hours following the initial antibiotic dose, the mice received a second dose of the antibiotic to attempt to suppress endogenous microbial populations as much as possible.

Fecal transplants of the supernatants collected from donor feces were administered to recipient mice at 24 and 48 hours following the second antibiotic dose. (Figure 3)

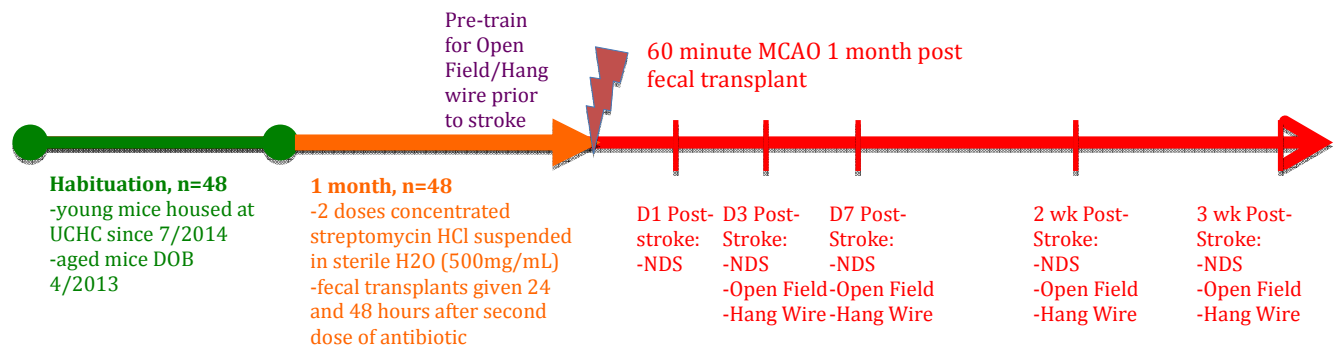


Figure 3: Experimental design. Young and aged mice were both held unaltered for a 2 month habituation period prior to any experimentation (green section of timeline). After habituation, antibiotics and fecal transplants were administered to mice as described. Following transplantation, mice were held in cages for 1 month to allow for newly transplanted microbiomes to colonize and stabilize in the gut of the recipient animals (orange section). At the end of the 1 month post-transplant, mice were pre-trained on behavioral tests and at exactly 1 month post-transplant, stroke mice were given experimental 60 minute middle cerebral artery occlusions and sham mice were given sham surgeries. Mice were scored for neurological deficit at days 1, 3, 7, 14, and 21 post-stroke and were tested via hang wire and open field behavioral tests at days 3, 7, 14, and 21. At 3 weeks post-stroke, mice were sacrificed using a 2% Avertin solution at a dose of 0.1mL/10g body weight and perfused with heparinized PBS, followed by 4% paraformaldehyde. Once fixed, the brain tissue will be harvested, placed in 30% sucrose until dehydrated. The brains will be sectioned into 30 μ m-thick slices on a microtome and the sections will then be stained using cresyl violet to measure and analyze infarct size to compare tissue damage.

Fecal Collection

Fecal samples from mice were collected both prior to experimental procedures and following transplantation. To collect samples, mice were removed from cages and individually placed on sterile surfaces until they defecated. Stool samples were then collected and placed in sterile tubes to be stored at -80° Celsius. Sterile protocol was followed during collection. 70% ethanol was used on all surfaces prior to collection and in between mice during stool collections. Gloves were changed between each mouse to avoid all potential sources of cross-contamination of samples.

Experimental Stroke

Focal transient cerebral ischemia was induced in the experimental mice via a 60 minute

reversible right middle cerebral artery occlusion under isoflurane anesthesia followed by reperfusion as previously described^[93]. Silicon coated sutures, 0.21 and 0.23 mm in dimension, were used to occlude the middle cerebral arteries of young and aged animals, respectively. Throughout the surgery and ischemia mouse rectal muscle temperature was measured and monitored using a Monotherm system maintaining body temperature at 37 degrees Celsius via an automated feedback mechanism. Sham mice underwent the same procedure except sutures were not advanced into the middle cerebral arteries for occlusion. The model provided for a consistent model of ischemia in the murine brain that was uniform throughout the cohort. Following ischemic stroke, all animals were given 0.2 mL saline injections subcutaneously for the first week following the procedure along with free access to wet mashed food to ensure survival

Behavioral Testing

Prior to all behavioral testing, all mice were acclimated for 1 hour in the testing rooms in their home cages. Sterile protocol was followed with the testing rooms and equipment. All equipment was cleaned with 70% ethanol in between trials and in between mice. Tests were conducted at the same time of day each time they were administered for consistency. Animals were pre-trained on all tests prior to stroke to assess baseline scores for all the mice.

Neurological Deficit Scoring

Neurological deficit scores (NDS) were collected before and after stroke. Score was taken immediately following stroke at reperfusion, 1 day post-stroke, and at days 3, 7, 14, and 21 post-stroke as shown in the experimental design in Figure 3. The score is determined using a 5-point scale: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; 4, no spontaneous locomotor activity or barrel rolling.

Open Field Test

The open field test is administered to mice to assess general locomotor activity. The starting placement of each mouse's trial was in the front right corner of a clear, acrylic box (16 inches by 16 inches). Once placed in the starting spot, each mouse was allotted a 10 minute interval, during which it was completely free to roam and explore the enclosed area. Activity was quantified using a computer operated PAS open field system computer program (San Diego Instruments, San Diego, CA). Locomotor activity was measured as the total number of laser beam breaks within the area as the mouse moved about. The test was administered to the mice prior to stroke

to assess basal levels of movement and then at days 3, 7, 14, and 21 post-stroke.

Hang Wire Test

The hang wire test assesses the sensorimotor functions in mice. A wire cage top of dimensions 18 inches by 9 inches was used for the procedure. Mice were placed on top of the cage in the center, and the wire lid was then slowly inverted and placed at a height of 9 inches above an empty cage bottom containing regular bedding. The time between inversion to the moment the mouse fell from the inverted cage was measured. A total of three trials was used per mouse, with a 45 minute gap in between each trial. The average of the three trials was then compared to the latency to fall measured at baseline for each mouse.

Cresyl Violet Staining

After sacrifice, brains of each mouse were harvested, placed in 30% sucrose until dehydrated. The brains will be sectioned into 30µm-thick slices on a microtome and the sections will then be stained using cresyl violet to measure and analyze infarct size to compare tissue damage.

Statistics

All values are expressed as mean±SEM and analyzed with a t-test for two groups. All assessments were performed by a blinded investigator. The criterion for statistical significance was $P < 0.05$.

Results

Aged vs. Young Microbiome Comparison

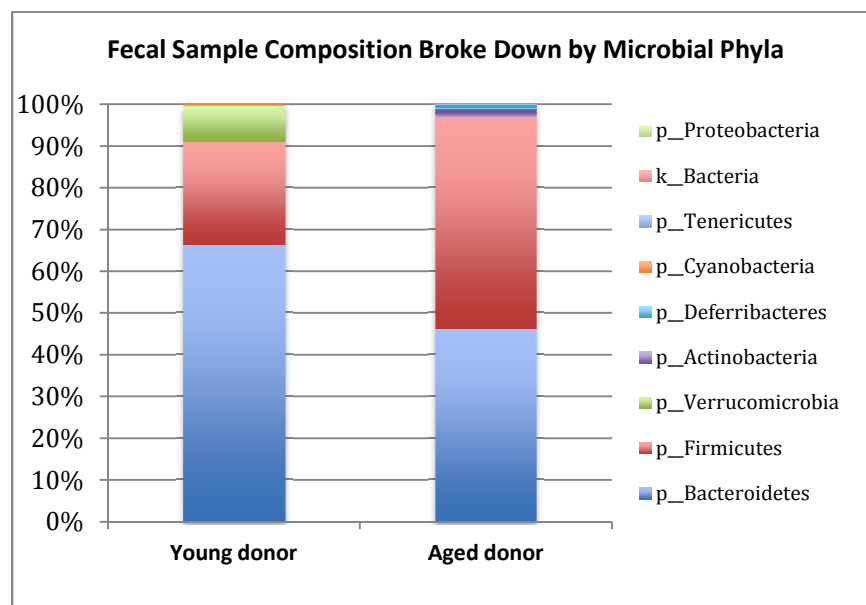


Figure 4: Sequencing data from samples collected from Aged and Young mice. Breakdown of fecal sample composition at the phylum level. Samples collected prior to any experimental treatment during habituation periods to assess basal levels of microbial compositions between the groups of mice. Data confirmed previous reports stating that >90% of the gut microbiome is composed of Firmicutes and Bacteroidetes phyla. Trends of an increasing Firmicutes to Bacteroidetes ratio was also seen with increasing age as previously reported by other studies.

Prior to any experimental procedures, fecal samples were collected from aged donor mice and young donor mice for purposes of confirming previous reports on microbial compositions. Sequencing data confirmed reports of gut microbiomes being >90% composed of two phyla (*Bacteroidetes/Firmicutes*). As shown in Figure 4, young microbiome samples displayed high *Bacteroidetes/Firmicutes* ratios, with *Bacteroidetes*(in blue) composing roughly 65% of the microbiome on average. Aged mice, demonstrated the opposite ratio with much greater abundances of *Firmicutes*(in red). Interestingly enough, young samples contained larger portions of *Verrucomicrobia* within their microbiomes, with the phyla varying in abundances between 8-9% in the animals while the phyla was virtually unseen in aged samples at abundances of nearly zero in most animals while the aged animals showing the highest abundances never contained more than 4% of the phyla. Many of the aged animals also contained a presence of *Deferribacteres* while nearly all young samples contained no detectable presence of the phyla.

Microbiome Transplants

Aged Mice	Stroke	Sham
Young fecal transplant	n=8	n=4
Aged fecal transplant (control)	n=8	n=4
<i>total</i>	<i>n=16</i>	<i>n=8</i>
Young Mice		
Young fecal transplant (control)	n=8	n=4
Aged fecal transplant	n=8	n=4
<i>total</i>	<i>n=16</i>	<i>n=8</i>
Total		
n=48 mice (24 young/24 aged)	32 strokes	16 shams

4 Types of Fecal Transplants carried out:

1. Young donor→Young recipients (control)
2. Young donor→Aged recipient
3. Aged donor→Aged recipient (control)
4. Aged donor→Young recipient

Table 2: (on left) the breakdown of the basic groups used in experimental design. A total of 8 separate groups were used to give a total n of 48 mice. Different transplant groups were divided into stroke and sham groups, sham surgeries as controls. (on right) the 4 types of microbiome transplants that were used in the experimental design. 2 controls groups were used to control for antibiotic treatment and the transplant procedures themselves.

The experimental procedure required various control groups to control for antibiotics, the transplant procedures, and then stroke surgeries. As shown by Table 2, the aged and young mice were broken down into 4 main transplant groups: 1, young mice receiving young microbiomes; 2, young mice receiving aged microbiomes; 3, aged mice receiving aged microbiomes; 4, aged mice receiving young microbiomes. The aged and young mice that received microbiome transplants from their own respective age group were the control groups for any differences that could have arisen from the antibiotics or transplant procedures themselves. The 4 main transplant

groups were then further broken down into mice that would receive stroke surgeries and mice that would only receive sham surgeries to control for the surgical procedure.

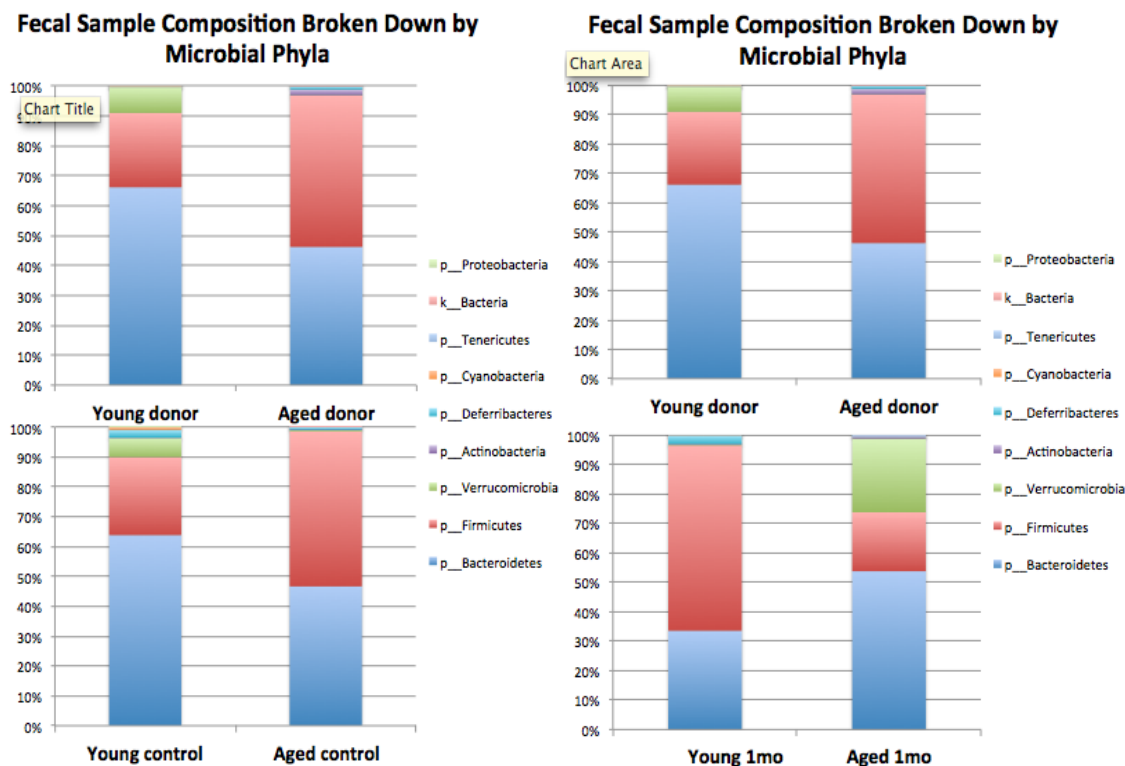


Figure 5: (on left) on top is the sequencing data of fecal samples from donor mice that were used, as shown on a larger scale in Figure 4. Below is the sequencing data of fecal samples collected from young and aged recipient mice that received microbiomes from their own respective age group. (on right) donor data depicted on top for comparison purposes again, below is sequencing data of fecal samples from young mice receiving aged microbiome transplants and aged mice receiving young microbiome transplants. In all cases, successful microbiome transplantation could be seen in clear transmission of the *Bacteroidetes/Firmicutes* ratio from donor mice to recipients 1 month following transplant.

Figure 5 depicts sequencing data at a phylum level of fecal samples collected from young and aged recipient mice 1 month after microbiome transplantation. Young controls showed successful transplantation from young donor mice, showing clear similarities in microbial composition to donors as well as successful transmission of high the high *Bacteroidetes/Firmicutes* ratio. Aged mouse controls showed the same results except with the opposite microbial ratio, displaying low *Bacteroidetes/Firmicutes* ratios as would be expected in aged microbial populations. Sequencing data of fecal samples from successful young and aged recipient mice 1 month following transplantation showed successful transmission of the *Bacteroidetes/Firmicutes* ratio of interest from donors to recipients.

Sequencing data of fecal samples taken from Young mice that underwent successful fecal transplants from Aged mice showed a reversal of the *Bacteroidetes/Firmicutes* ratio, resembling

the low ratio that was commonly seen in Aged mice. An elevated proportion of the *Deferribacteres* phyla could also be observed that was greater than in any young controls and donors. Data from fecal samples taken from Aged mice than underwent successful transplants from Young mice also showed a common trend of reversal in the *Bacteroidetes/Firmicutes* ratio. The resulting high ratio resembled that of young donors and greatly exceeded any of the ratios seen in Aged donors/controls. An extraordinary elevation in the *Verrucomicrobia* phyla was also commonly seen in the Aged mice receiving young fecal transplants but not in any of the aged controls/donors (Figure 6).

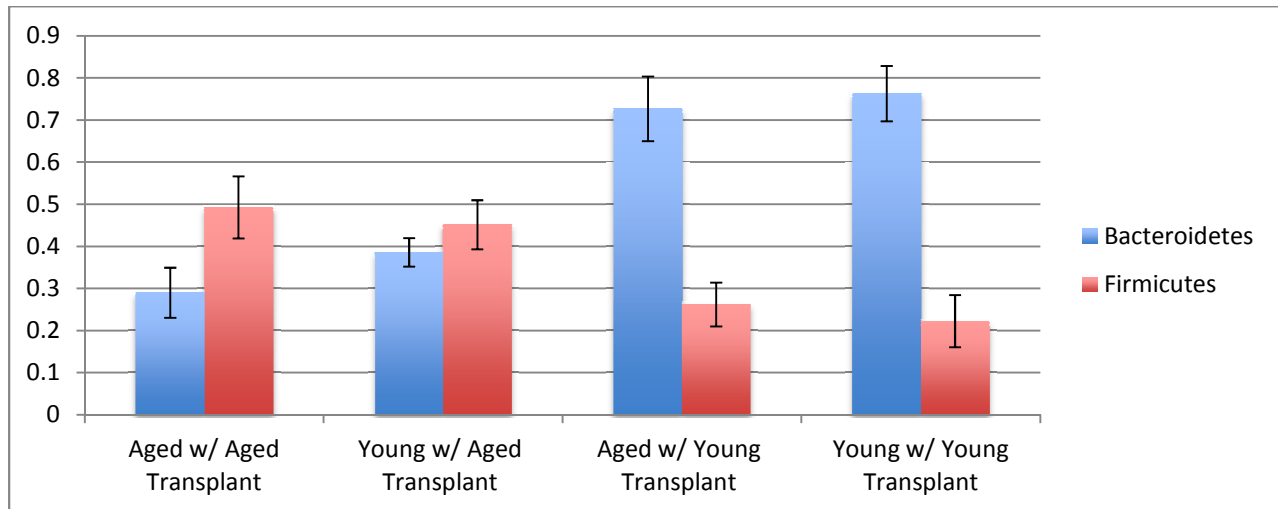


Figure 6: Relative abundances of *Bacteroidetes/Firmicutes* in mice following fecal transplantation. Marked elevation of *Bacteroidetes* in mice receiving young microbiomes while the opposite is true in mice receiving aged microbiome transplant. Previously reported aged dependent ratios of *Bacteroidetes/Firmicutes* microbes were observed in mice receiving transplants.

NDS

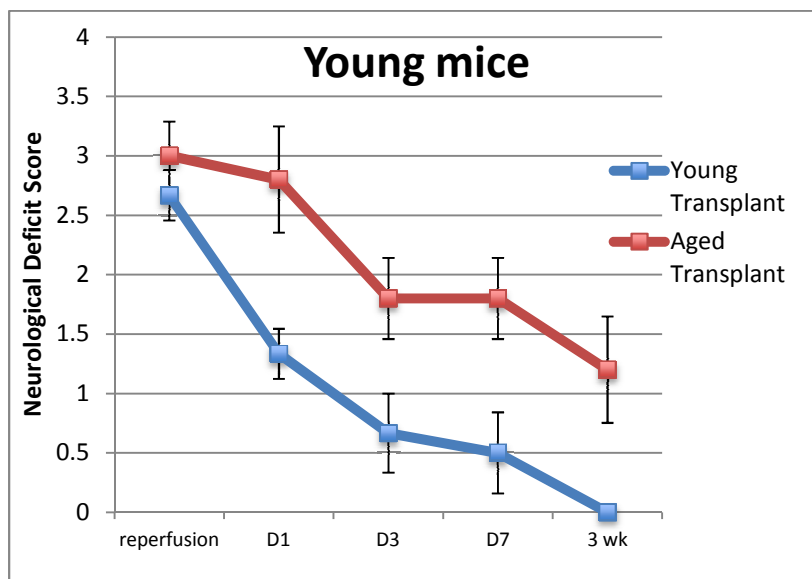


Figure 7a: Neurological deficit scores of young mice post-stroke. Prior to reperfusion all animals scored 0 on the NDS scale, indicating no basal levels of neurological deficit. Following stroke, significantly improved recovery was seen in young mice with gut microbiomes of young donors recovering after 3 weeks. Young mice that received the microbiomes of aged donors demonstrated markedly reduced recovery and never fully returned to basal levels of no neurological deficit.

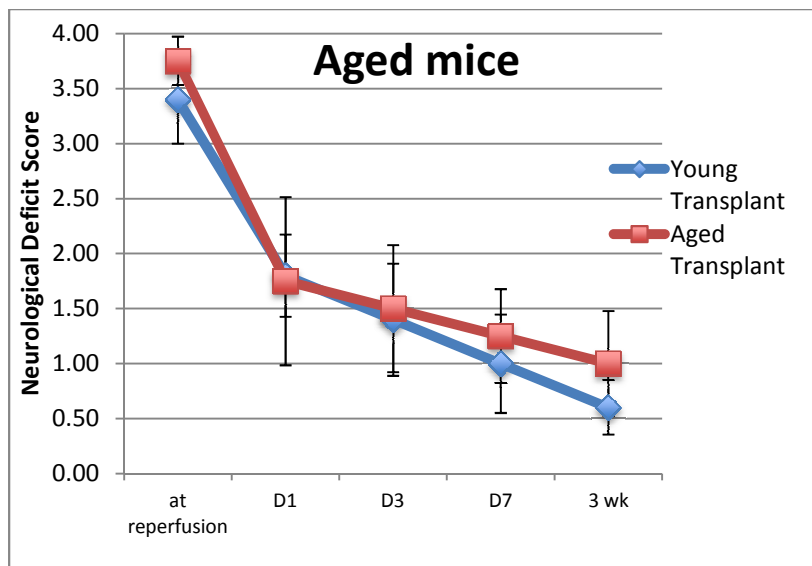


Figure 7b: Neurological deficit scores of aged mice obtained following stroke. All animals had basal scores of 0 prior to stroke indicating no neurological deficit prior to ischemia. No significant differences between aged mice receiving aged and young microbiomes were observed. No full recovery was seen at 3 weeks as it was with young mice, however. The final overall trend of recovery suggested full functional recovery of aged mice that received young microbiomes while aged mice containing aged microbiomes appeared to be trending to never fully functionally recover.

Microbiome composition seemed to have a significant impact on recovery from neurological deficit in young mice (Figure 7a). Young mice that received young microbiota had markedly improved recovery times after stroke according to their NDS scores compared to the mice that received aged transplants. The mice that received young microbial compositions also demonstrated full functional recovery at 3 weeks whereas the animals that contained aged microbiome never fully recovered from ischemia.

A significant difference in functional recovery wasn't seen in aged mice regardless of microbiome composition (Figure 7b). 1 week post-stroke, however, a difference in trends began to form as aged mice containing aged microbiomes seemed to be on track to fully recover while recipients of aged microbiota appeared to plateau in their recovery suggesting a lack of full functional recovery. The study would need to be repeated, however, with increased survival times prior to sacrifice to confirm this observation.

Hang Wire Test

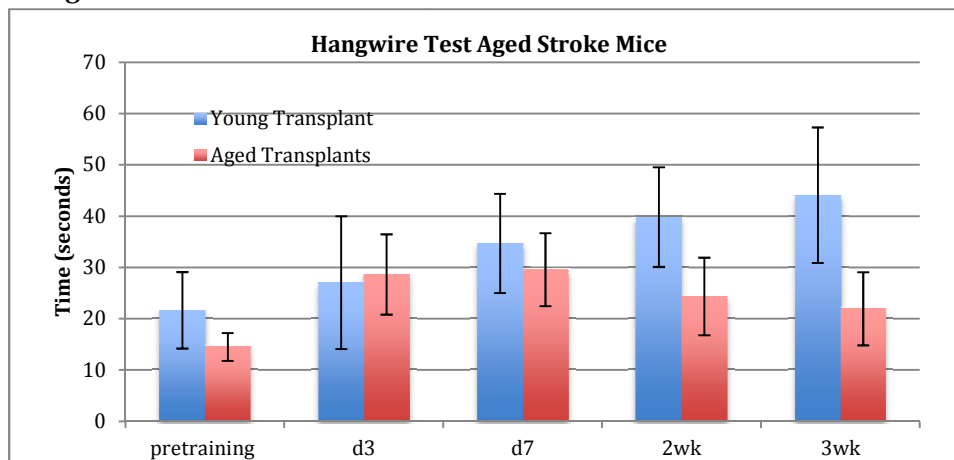


Figure 8a: Latency to fall during hang wire test for aged mice post-stroke. Mice tested pre-stroke then 3, 7, 14, and 21 days after stroke. Aged mice that received young microbiome transplants outperformed the aged mice that received microbiome transplants from aged donors. Differences in performance were dramatically amplified by stroke.

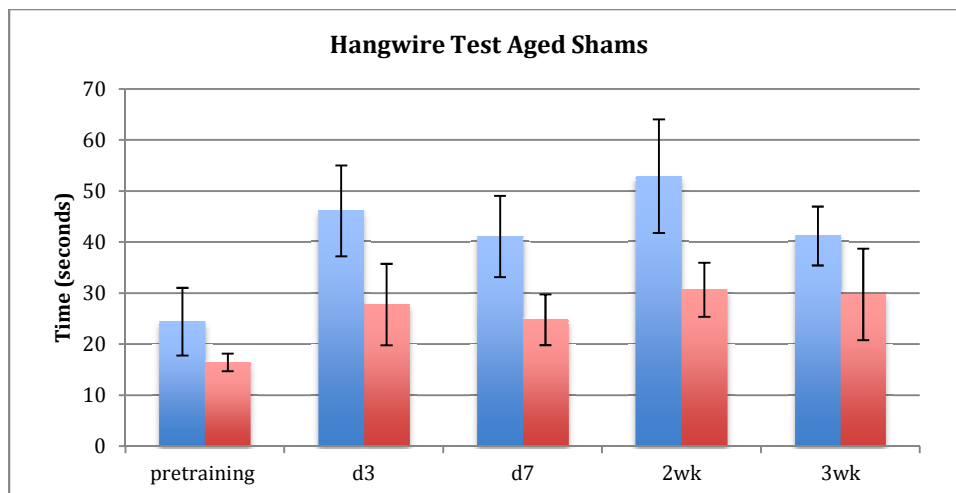


Figure 8b: Latency to fall during the hang wire test for aged mice that received sham surgeries instead of middle cerebral artery occlusions. Mice with young microbiomes outperformed those with aged microbiomes but at 3 weeks post-surgery the differences were roughly the same as they were prior to surgery.

Figures 8a and 8b depict the data collected from aged mice that underwent stroke surgery (Figure 8a) and those that underwent sham surgery (Figure 8b). In both groups, aged mice that received young microbiome transplants performed better on the hang wire test as depicted by their increased latencies to fall. Aged mice that received transplants from young donors had improved performances on the hang wire test as compared to those that received transplants from aged donors at basal levels during pre-training and the differences were found to be further amplified following stroke when measured at 3 weeks whereas the differences in performance stayed relatively stable in sham surgery mice.

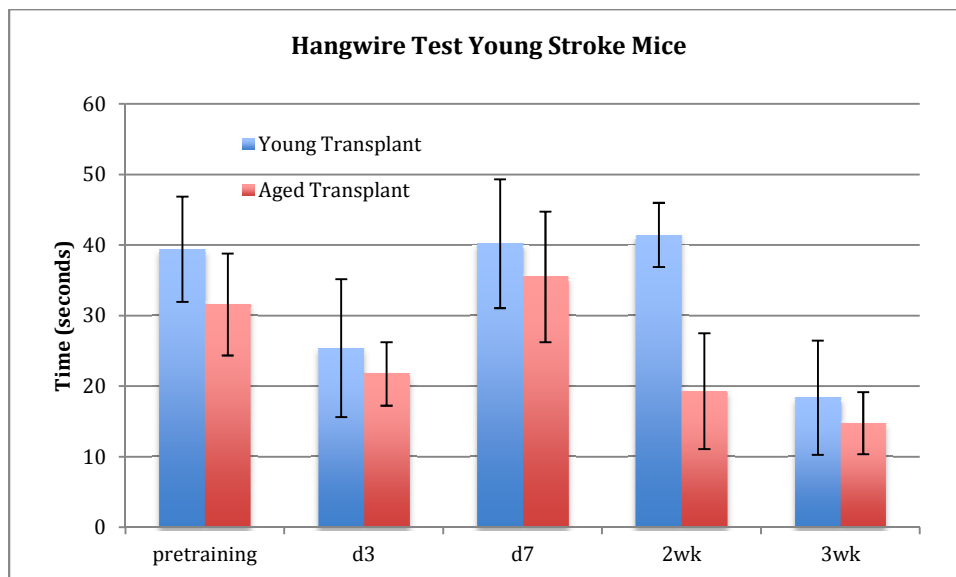


Figure 9a: Latency to fall during hang wire test for young mice that underwent ischemic stroke. Mice with young microbiomes outperformed those with aged microbiomes. Stroke appeared to amplify the differences at 2 weeks post stroke but the two groups were closer in performance by 3 weeks post-stroke.

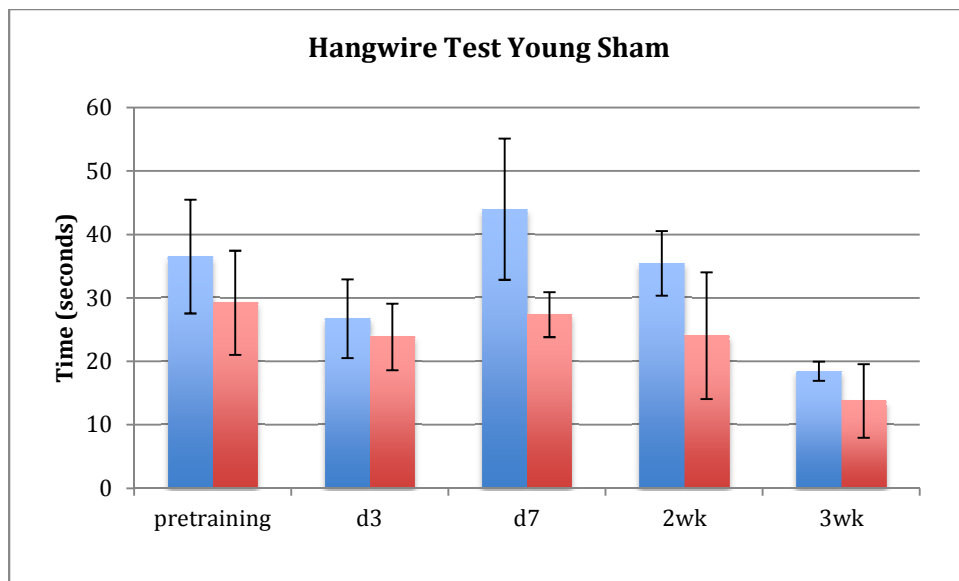


Figure 9b: Latency to fall on the hang wire test following sham surgeries. Similar differences in performance as in other groups, young microbiomes outperforming aged.

Trends noticed in aged animals were also seen in young mice except on a reduced scale. Young animals that received microbiota of aged animals had a markedly decreased latency to fall as compared to mice that received transplants from young donors during initial phases of recovery as seen by the marked differences in fall latency at weeks 1 and 2 post-stroke. At 3 weeks post-stroke, however, all young animals had nearly the same performance on the hang wire test, mirroring the recovery of young mice as seen in the NDS data.

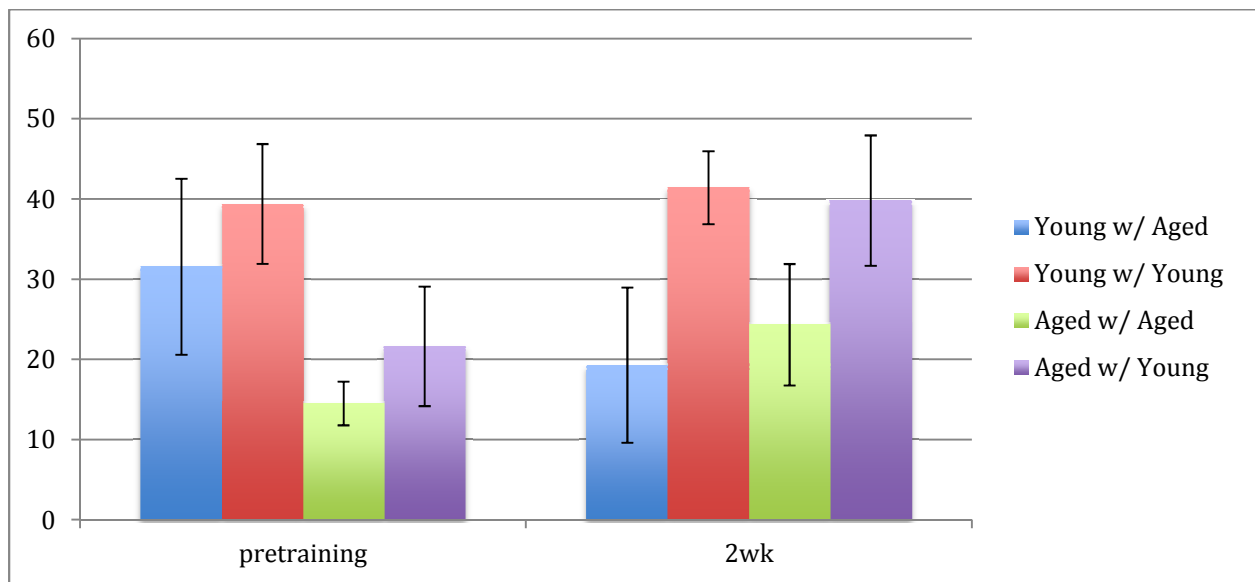


Figure 11: Latency to fall on the hang wire test of young and aged mice at pre-training pre-stroke and 2 weeks post-stroke. Pre-stroke young mice outperformed aged mice, however the young mice that were the recipients of microbiome transplants from young donors outperformed young mice that received transplants from aged donors. A similar trend within the aged mice was seen with the young microbiome recipients outperformed aged microbiome recipients. At 2 weeks post-stroke, the differences within age groups were significantly amplified.

Open Field Test/CV Analysis

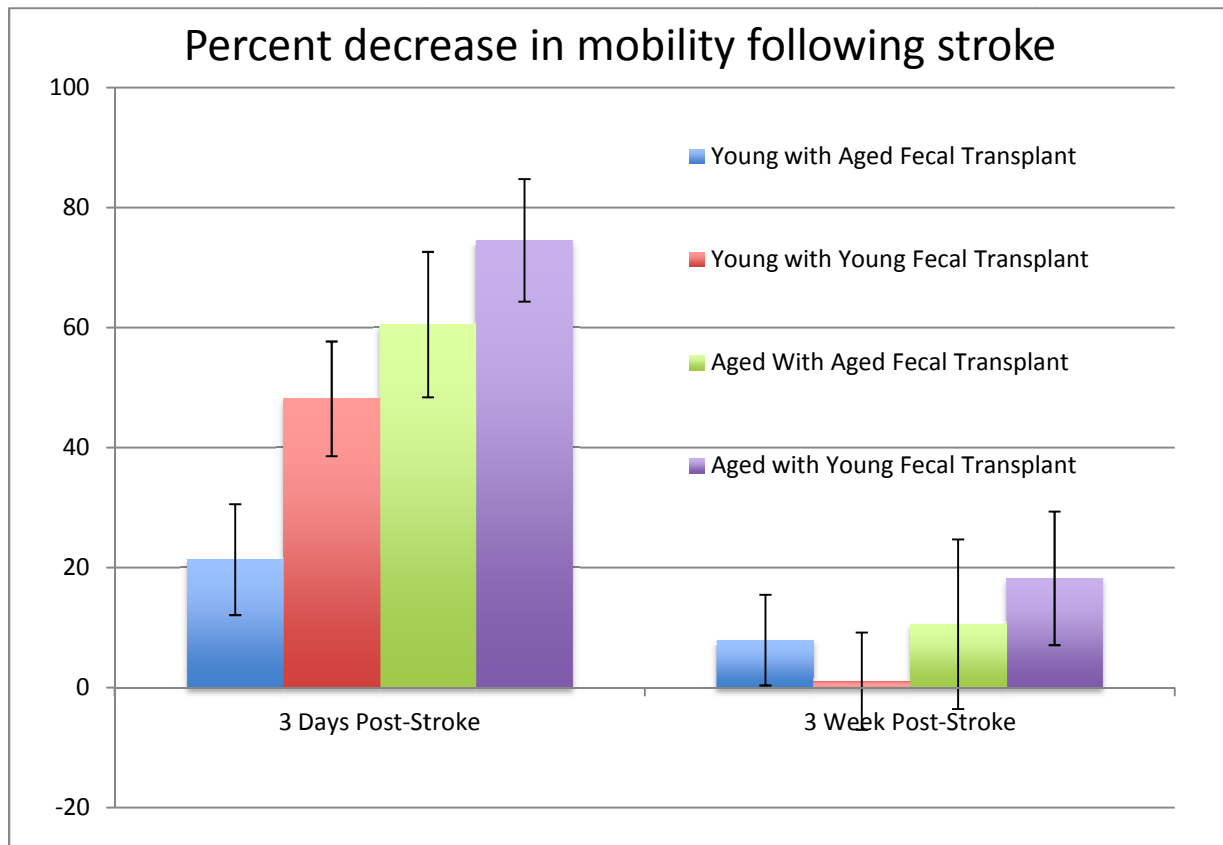


Figure 12: Open field data collected from mice following stroke. No significant differences were found between any of the groups during trials. As shown in the figure, large variation was seen in the cohort and no clear trends were observed.

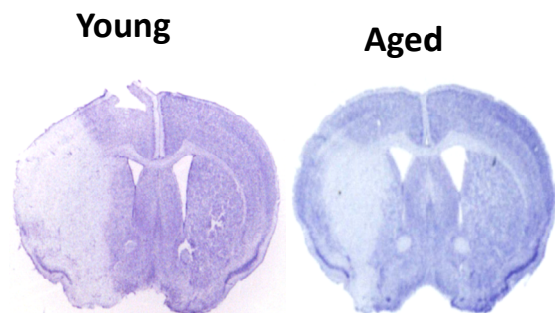


Figure 13: Cresyl violet staining of harvested mouse brains 3 weeks post-stroke. Infarct volume did not appear to be dependent on microbiome composition. Infarct volume of young mice was significantly larger than that of aged mice, as has been reported in the literature.

No significance was found in the results of the open field test or during CV staining/analysis of the harvested brains following stroke. Perhaps a larger n is required to see trends or perhaps the microbiome has no impact on infarct volume. Open field test would need to be repeated again with a larger n since the results seem to contradict the collected data from the hang wire tests and NDS.

Discussion

The experimental results clearly showed encouraging trends linking the microbiome to functional recovery following ischemic stroke. As it has been reported in the literature, microbiome samples collected via the feces confirmed the phylum-level differences between aged and young organisms. Young microbiomes seem to contain a high *Bacteroidetes/Firmicutes* ratio while the opposite is true for aged mice (Figure 6). The low ratio, as present in aged animals, has been linked to various risk factors for stroke and diseased conditions in the literature, which formed the basis of the hypothesis that this “harmful” population of microbes could potentially hinder functional recovery in young animals following stroke while giving them “beneficial” microbiome of young animals to aged animals could help positively impact functional recovery following stroke^[8-17].

The NDS and hang wire test results helped support this hypothesis by suggesting the young microbiome can be protective or encouraging of functional recovery in both young and aged animals. Both young and aged mice that received aged microbiome transplants demonstrated decreased recovery based on the NDS and hang wire test results. The differences in the animals were present pre-stroke, but were dramatically amplified and obvious following ischemia, suggesting that perhaps the aging microbiome doesn’t play a major role until some sort of stressor is present, at which point young microbiomes can confer protective benefits while the aged microbiome lacks the capacity to do so.

The low *Bacteroidetes/Firmicutes* ratio has been linked to increased circulation of inflammatory markers, cardiovascular risk, tendency for obesity; all risk factors for stroke^[30-68, 91, 92]. This has proven harmful to multiple inflammatory diseases, so it is not much of a stretch to expect the ratio to have a negative impact on a cardiovascular disease like ischemic stroke. Stroke has been also linked to cause an increase in gut permeability, and “leaky gut” syndrome has commonly been linked to disease and harmful effects on host health following gut microbe translocation and the transmission of microbe metabolic products into systemic circulation^[77-80]. The combination of leaky gut post-stroke and harmful metabolic products resulting from the relatively high *Firmicute* abundance in the aged microbiome could be the explanation for worsened recovery in animals containing the aged microbiome post-stroke. It could also help explain the reason for the nearly non-existent differences between groups prior to stroke, whereas following ischemia there

are dramatic behavioral differences. This could also explain why animals containing a high *Bacteroidetes/Firmicutes* ratio seem to be more protected from stroke and demonstrated improved recovery since they have been reported to confer protective benefits to the host and promote up-regulation for proteins in tight endothelial junction (which could help protect from leaky gut or blood-brain barrier breaches), promote improved responses to stress in terms of a less overactive stress response, and help prevent large inflammatory responses by the innate immune system^[50-56, 70, 90-92].

It was disappointing to not observe any of the same trends in the open field test. Unfortunately, no significant results were collected from the test, which failed to further support the hypothesis of the young microbiome being conducive of functional recovery post-stroke while the aged microbiome is harmful. Aged and young organisms are known to have different infarct volumes following ischemic attack. While young animals typically experience greatly improved functional recovery after stroke than their aged counterparts, the young mice typically have much larger infarct volumes in their brains. CV staining and analysis did not demonstrate any positive or negative impacts of microbiome composition in the experimental mice.

While these results are very promising and inspiring, since the trends are very clear in the NDS and hang wire results and are theoretically supported by the literature, much more work will need to be done in the future to elucidate the exact link between the microbiome and ischemic stroke. While the n of 48 was a fairly large number, there was still a fair amount of variation observed in the data so the n will have to be greatly increased and further tests will need to be conducted. The open field test should be repeated on a larger scale to find trends that could potentially exist that were overlooked. A longer survival time of 4-6 weeks should be used for following cohorts to allow for a greater window of time to see the development of the trends that were seen during these experiments. While the tests that were used with this cohort gave glimpses into the motor impacts of the microbiome on stroke recovery, it would be interesting to expand the pool of behavioral tests to more cognitive-based tests such as the novel object recognition test which tests the learning and memory or elevated maze tests that test the animals for anxiety. All these thoughts will be put into consideration for future experimentation to create a full test of the microbiome's impact on an organisms motor and cognitive recovery following ischemic stroke. The results reported here, though, give invaluable insight into this novel area of research offering support that the microbiome does, in fact, play some role in functional recovery of mice.

Using the data reported here, the hope will be to see similar trends in future data. If successful,

these results could truly prove to be monumental, offering totally new potential avenues for stroke treatment and prevention.

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