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THE EFFECTS OF INTESTINAL SULFATE-REDUCING BACTERIA ON COGNITIVE BEHAVIOR AND INTESTINAL TRANSIT IN MICE

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by

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ABSTRACT

This research reports the effects of two metabolic groups of bacteria on physiological activity and cognitive function in C57BL/6 mice. In one experiment, the mice on a diet supplemented with red kidney bean for 24 hr were able to complete an eight-arm radial learning maze in a shorter time than mice on a diet without red kidney beans. The median time required for mice to complete the maze when fed red kidney beans was 118 sec while the median time for mice on standard diet to finish the maze was 270 sec. Based on the cycle threshold values when using universal bacterial primers on mid-small intestine contents, the mice fed the red kidney bean diet had a greater number of gene copies of bacteria as compared to the intestinal contents of mice receiving the standard diet. The introduction of 1×10^9 live cells of *Desulfovibrio vulgaris* into the stomach of mice by gavage resulted in reducing the rate at which contents in the small intestine traveled and impaired cognitive function as assessed using the eight arm radial learning maze. The impaired performance of mice in the learning maze occurred only with

actively metabolizing cells of *D. vulgaris* and not with heat-killed cells of *D. vulgaris*, or substituting saline or fermentable sugars for live cells of *D. vulgaris*. The amount of time spent in the center of the maze for mice receiving live *D. vulgaris* cells was 34 sec as compared to mice receiving heat-killed cells of *D. vulgaris* was 15 sec, saline was 13.5 sec and lactulose plus mannitol instead of live cells of *D. vulgaris* was 15.5 sec. Mice receiving live cells of *D. vulgaris* had almost ten times as many errors in completion of the maze than mice in the control groups. A contributing factor for impaired maze performance may be the high level of H_2S in the mouse intestine.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Introduction

Recently, there has been considerable interest in the contribution of intestinal bacteria to irritable bowel syndrome, Crohn's disease, and chronic gastroenteritis associated with Gulf War illness for which the etiological agent is not defined. Because of the difficulties in studying these intestinal diseases in humans, it is highly desirable to use mice as model systems. While this research is a mouse study, it is important to provide a review of features relating to intestinal diseases of humans because it sets the focus for this mouse study.

Microbiome of the human gut

The human microbiota consists of one-hundred trillion microbes that live in and on us with the vast majority maintaining residence within the large intestine. Comparatively, the microbiome includes tenfold more cells than the human body and over one-hundred fold more genes (Gill et al., 2006, Turnbaugh et al., 2007). The contribution of the microbiota is so large that it has been proposed to have a collective metabolic activity equal to a virtual organ inside the human body (O'Hara and Shanahan, 2006). Multiple levels of symbiotic mutualism take place between host and microbes and within the microbial community itself with far-reaching effects on the human host and vice versa.

H_2 is an important energy currency within the gut microbiota. H_2 is generated by fermentative (hydrogenogenic) bacteria (such as *Bacteroides* spp.) from food constituents that make their way through the intestines. If not for hydrogenotrophic microbes consuming the gas, H_2 would accumulate until the reaction became kinetically

unfavorable which would cause transit speed to increase and absorption from digestion to dwindle (Jahng et al., 2012).

There are two major groups of hydrogenotrophic microbes, sulfate-reducing bacteria (SRB) and archaeal methanogens (Nakamura et al., 2010). SRB are more common, found in ~60% of the population while methanogens makeup the remaining ~40% (Macfarlane et al., 2007, Rey et al., 2013). Individuals may have either or both microbial groups but typically only one will be predominant in the large intestine of the host (Strocchi 1994). Which group predominates depends on a multitude of factors including host diet, age, gender, geographic region, and demographics; as well as intestinal availability of substrates, pH and thermodynamic favorability (Levitt et al., 2006, McKay et al., 1985, Hudson et al., 1993, O'Keefe et al., 1991, Bjorneklett and Jenssen, 1982, Melcher et al., 1991, Sahakian et al., 2010). There have been numerous studies linking the overproduction of methane (CH_4) by methanogens to constipation predominant irritable bowel syndrome (C-IBS) as well as other gastrointestinal diseases (Pimentel et al., 2004, Othman et al., 2008, Basseri et al., 2011), but very little on the plausible role of SRB in disease phenotypes.

Relevant activities of sulfate-reducing bacteria

Sulfate-reducing bacteria (SRB) are anaerobic gram-negative bacteria that metabolically use sulfate as their terminal electron acceptor and convert hydrogen (H_2) to hydrogen sulfide (H_2S), a highly toxic gas. Survivors of accidental H_2S exposure, whether from industry or environment, report a variety of symptoms including depression, impaired concentration, impaired memory, and altered bowel habits (Kilburn, 1993, Fenga et al., 2002). H_2S is also generated endogenously in minute concentrations

by mammalian cells and functions as the third gaseous transmitter alongside carbon monoxide (CO) and nitric oxide (NO). H_2S serves as a vasodilator, a smooth muscle relaxant, and is involved in the process of long-term potentiation (the conversion of short to long term memory) in the hippocampus (Reiffenstein et al., 1992, Wang, 2010, Gadalla and Snyder, 2010). Since H_2S has many functions on different tissues, and it is typically maintained at very low concentrations, excess SRB growth may have a physiological impact.

Brain-gut axis

The term "Brain-gut axis" describes the crosstalk of the multiple branches of neural pathways of the central, peripheral, enteric nervous systems and the intestinal microbiota (Romijn et al., 2008, Indrio et al., 2013). The brain and the gut are connected by a complex bidirectional network that plays an imperative role in maintaining homeostasis of both the host and microbiota (Collins and Bercik, 2009). The gut microbiota communicates with the brain through three principal routes; microbial interaction with intestinal mucosal cells, immune cells, and neural endings. The brain in turn can also influence the microbiota through the release of signaling molecules from the lamina propria that can modulate intestinal secretion, permeability, and motility (Rhee et al., 2009). As expected this connection scheme is expanding with new research and clues on how to optimize the interaction between the brain and gut microbiota are becoming more defined.

An earlier study showed that introducing pathogenic bacteria into the gut could change host behavior (Lyte et al., 2006). Probiotic strains of bacteria have shown potential to modulate behavior and mood with communication between brain and gut

mediated via the vagus nerve (Brava et al., 2011, Bercik et al., 2010, Bercik et al., 2011). However, it is not known whether SRB or a change in resident microbe abundance would have the same effect.

Bacterial overgrowth in the small intestine

Many gastrointestinal disease phenotypes have been correlated with a change in bacterial composition and regional containment. In cases of small intestine bacterial overgrowth (SIBO) there is a loss of containment of colonic microbes and colonization of the small intestine occurs (Lin, 2004, Van Citters and Lin, 2005). Whether or not this increase of bacterial load has an effect on cognitive behavior is unknown. Raw red kidney bean (RRKB) causes bacterial overgrowth of the small intestine due to the lectin phytohaemagglutinin (PHA) (Andrews and Jayne-Williams 1974, Banwell et al., 1983, Ramadass et al., 2010).

Hypotheses

The goal of this investigation is to conduct research with mice to obtain information that is relevant to human intestinal diseases. The following hypotheses will be tested:

1) The rate of intestinal transit in mice will be influenced by hydrogen sulfide produced by metabolically active sulfate-reducing bacteria.
2) Cognitive behavior in mice will be influenced by hydrogen sulfide produced by metabolically active sulfate-reducing bacteria.
3) Cognitive behavior in mice will be influenced by bacterial overgrowth in the small intestine as a result of using red kidney bean as a dietary supplement.

Methodology

Animals

Five week old, female C57BL/6 mice (20-25g) were purchased from the Charles River Lab (Wilmington, DE). Upon arrival, the mice were housed in groups of 4 in polypropylene cages, placed on a 12hr light/dark cycle and kept on a standard rodent diet of Harlan Teklad Laboratory Diets. The procedures were approved by the Institutional Animal Care and Use Committee at the New Mexico VA Health Care System following guidelines provided by the Guide for the Care and Use of Animals of the National Research Council of the National Academies (National Research Council 2011).

Sulfate-reducing bacteria

The sulfate-reducing bacterium, *Desulfovibrio vulgaris* Hildenborough NCIB 8303, was grown on a lactate-sulfate medium previously published (Biswas et al. 2009) and contained 4ml of 60% Na lactate syrup, 2mg $FeSO_4 \cdot 7H_2O$, 2g NH_4Cl, 4g Na_2SO_4, 2g $MgSO_4$, 0.5g K_2HPO_4, and 1g yeast extract, per 1000ml H_2O. The medium was adjusted to pH 7.4 with 20% KOH and 10 ml was placed in 13 x 100 mm screw top test tubes fitted with caps containing a rubber septum, autoclaved, and flushed with nitrogen gas to create an anaerobic environment. Growth was initiated with a 1% inoculum and incubation was at 36^{o}C for three days. Bacteria were pelleted by centrifugation and re-suspended in 0.01M phosphate-buffered saline (PBS), pH 7.4, for gavage. Bacteria were enumerated by counting with Petroff-Hausser counting chamber (Hausser Scientific) and approximately 10^9 bacteria were gavaged into the stomach via the mouth per mouse for each experiment. Administration of liquid contents is by oral gavage, a technique that

uses a bulb tipped gastric gavage needle attached to a syringe to deliver compounds into the stomach following the IACUC standard procedure.

D. vulgaris and intestinal transit

Ten mice (five per group) were gavaged with a 400µl solution of a live suspension of *D. vulgaris* (100µl), mixed with 100µl (25 mg/ml) Rhodamine B isothiocyanate-Dextran (Sigma-Aldrich) (excitation 570nm /emission 590nm) and 200µl of PBS. Control mice were gavaged with PBS and Rhodamine B. Forty-five min post gavage mice were euthanized and the small intestine was excised by laparotomy and placed on a 12.5cm grid. Fluorescence was measured using the IVIS platform, which allows for large high-sensitivity imaging of fluorescent markers. Rhodamine B transit was analyzed using ImajeJ software (University of Ghent) and geometric center (GC) was calculated using a protocol introduced by Sallam et al., 2007. In this procedure, GC =sum of n x Pn for n = 1, 2, . . . 10. Where "n" was the number of the intestinal segment and "Pn" was the percentage of Rhodamine B detected from the corresponding segment. Data was expressed as average transit on a scale of 0-100 (0 being the most proximal point of the small intestine and 100 the most distal).

Maze learning and working memory analysis

Mice were tested in an eight-arm radial learning maze with a length of 30cm and a central chamber diameter of 22cm (TSE System, Chesterfield, MO). A model of the maze is given in Figure 1. The radial arm maze tests working memory function of the mice to recover food pellets (0.001g) from the ends of the eight arms while recording the

time, path, and entries of the mice. Working memory actively holds multiple pieces of transitory information in the mind where they can be manipulated and used for decision making (Brown and Cook, 1986, Tarragon et al., 2012). Sensors at the beginning and end of each arm record mouse position and bait acquisition. The bait is held in a raised food dish at the end of the arm so that the mice cannot see it. Additional baits were scattered about the outside of the maze to deter the mice from being able to smell specific baits in individual arms; this setup allows working memory to be tested rather than sight or smell. After a one-week acclimatization period, the mice were trained daily until consecutive perfect maze runs (without error) were recorded. Once training was completed, the mice were tested under different experimental treatments and euthanized. Each animal was randomized to only one treatment. A perfect run was recorded when the mouse entered each arm once and recovered the bait at the end of the arm before going on to the next arm. All mice were fasted for 4hr before maze trials to increase motivation for bait. Mice were trained daily until all mice completed the maze without a mistake. At that point, they are used for experimentation. The data were analyzed using 1-way ANOVA to test for significant difference between groups. Data are presented as 'median; interquartile range (Q1-Q3)'.

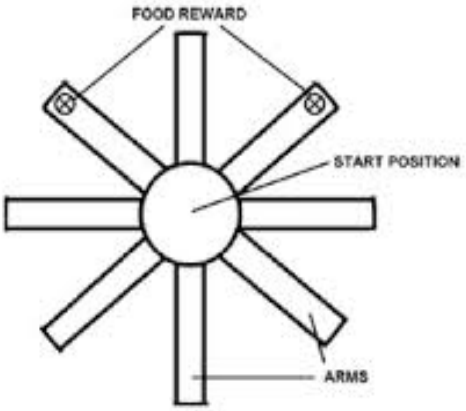

Figure 1. Eight-arm radial maze used in mouse studies

D. vulgaris and maze performance

There were four groups in the first maze experiment: 1) *D. vulgaris* mixed with lactulose and mannitol (SRB plus L/M), 2) Lactulose and mannitol (L/M), 3) killed *D.vulgaris* (via autoclaving) and lactulose mannitol (killed SRB plus L/M), and 4) Control (saline). Each animal was administered one treatment, tested in the maze, and then euthanized for tissue harvesting, measurement of relative SRB abundance, and H_2S concentration of small intestine and cecum.

In the second maze experiment, the specificity of SRB and the role of fermentation by-products were tested. Thus, SRB was compared to fecal slurry and treatments were tested without and with lactulose and mannitol as additional fermentable substrates. Six treatments were administered before testing maze performance using a repeated measure design where each animal was tested across each treatment in

randomized order: 1) Control (saline), 2) SRB, 3) FS 4) Control plus L/M, 5) SRB plus L/M, and 6) FS plus L/M. Each treatment was administered on day 1 with maze testing done on days 1, 2, 3, and 4 and separated by the next randomized treatment by a "washout" period of 1 week.

Fecal slurry

Fecal slurry (FS) was prepared from fresh mouse fecal matter the day of the experiment. The fecal matter was converted into slurry by mixing in PBS (0.2g/1ml) followed by vortexing. The slurry was then filtered through glass wool to remove undigested materials that were too large to pass through the pore of the needle used for gavage.

Isolation of DNA and qPCR from sulfate-reducing bacteria

Following the completion of maze testing mice were additionally tested for relative bacterial load. DNA from the twenty-four mice of the first maze experiment was extracted from the proximal, mid, distal-regions of small intestine, cecum, colon, and liver using the DNeasy Blood & Tissue Kit 69506 (QIAGEN, Valencia, CA) manufacturer protocol. Isolated DNA concentrations were normalized across all samples and then subjected to qPCR using targeted primers to assess the relative concentrations of two specific dissimilatory sulfite reductase genes, *dsrA* and *dsrB* that are used by SRB to convert hydrogen to H_2S (Deplancke et al., 2000). Mouse actin primers were used for tissue size normalization against the *dsrA* and *dsrB* primers to assess relative amounts of SRB genes in the tissues. Data were represented as cycling threshold (CT) units where a lower value represents greater number of gene copies in the tissue sample.

Table 1. Oligonucleotide DNA primers used for PCR amplification of genes used in this study.

Primer	Gene target	Sequence (5' to 3')[a]	T_m[b](°C)
DSR1F	*dsrA*	ACSCACTGGAAGCACG	54
DSR1334R	*dsrA*	TYTTCCATCCACCARTCC	54
DSRp2060F	*dsrB*	CAACATCGTYCAYACCCAGGG	54
DSR4R	*dsrB*	GTGTAGCAGTTACCGCA	54
VMPS96F	MouseActin	AAGAGCTATGAGCTGCCTGA	51.8
VMPS96R	Mouse Actin	TACGGATGTCAACGTCACAC	51.8
1048F	16S rRNA	GTGSTGCAYGGYTGTCGTCA	53.8-57.9
1194R	16S rRNA	ACGTCRTCCMCACCTTCCTC	53.8-57.9

a. Wobble positions are shown as follows: S= G or C, Y = C or T, R= A or G, and K = G or T.
b. T_m, melting temperature.

Maze Experiment I: use of live and dead bacteria

Twenty-four mice were trained until all had completed the maze without making an error (4 experimental sets, 6 per group). On the day of the test, mice were fasted for a total of 4 hr and gavaged with treatment 2 hr before maze testing. The groups tested were the sulfate-reducing bacteria *D. vulgaris* with lactulose and mannitol (SRB plus L/M), L/M alone, killed *D. vulgaris* (via autoclaving) and L/M, and Control (saline). Following maze testing, mice were euthanized and tissues harvested (proximal, mid, distal small intestine, cecum, colon, and liver) for relative *dsrA* and *dsrB* gene concentrations. H_2S concentration in cecum was measured by aspirating cecal gas with a 1-ml tuberculin syringe, wrapped in parafilm to ensure an air tight seal, and stored until tested. The sample was then analyzed for hydrogen sulfide using a gas chromatograph (CHM-1) with sensitivity in parts per billion (ppb) (OralChroma, Japan).

Maze Experiment II: additions made along with live bacteria

Six mice were trained until all completed the maze without making an error. They were then tested daily for four days and allowed a one week washout period to return to normal maze behavior before being gavaged with the next randomized treatment and tested again for maze performance. There were six different treatments: SRB, fecal slurry (FS), and controls without and with lactulose mannitol (SRB, FS, Control, SRB plus L/M, FS plus L/M, Control plus L/M). All mice were continually rotated through testing with each experimental group until each mouse was tested with every treatment.

Data analysis and statistical approaches

For maze experiments, repeated measures one-way ANOVA using a linear mixed model approach was used to compare groups against each other and pre-treatment baseline results. For intestinal gas samples one-way ANOVA on cube root transformed data (to correct for distributional problems) was used for group post hoc Tukey comparisons.

Bacterial overgrowth and maze performance

There were two groups of mice administered different diets, standard chow (Control), and chow supplemented with raw red kidney bean (RRKB) purchased from a commercial provider. RRKB was used to induce small intestinal bacterial overgrowth (Banwell et al., 1983). After one day of the supplemented RRKB diet, animals were tested in the maze and then euthanized for bacterial concentration measurement of tissues. Control mice (n = 6) continued to be fed standard rodent chow while RRKB mice (n = 6) were fed the standard rodent chow supplemented with 24% powderized raw red kidney bean.

Isolation of DNA and qPCR of mice induced with bacterial overgrowth

Following the completion of maze testing mice were additionally tested for relative bacterial load. DNA from the proximal, mid, distal-regions of the small intestine, cecum, colon, and liver was extracted by DNeasy Blood & Tissue Kit 69506 (QIAGEN, Valencia, CA) using manufacturer protocol. Isolated DNA concentrations were normalized across all samples and then subjected to qPCR using targeted primers to assess the relative bacterial concentrations. Mouse actin primers were used for

normalization against the 16S universal primers to assess relative amounts of bacteria genes in the tissues. Information on primers used is given in Table 1. Data were represented as cycling threshold (CT) units where a lower value represents greater number of gene copies in the tissue sample.

Results

Intestinal transit impaired by live *D. vulgaris*

It was observed that SRB slowed transit of Rhodamine B with the fluorophore localized primarily to the first one-third of small intestine, when compared to control mice which had the strongest fluorescent signal in the last one-third of small intestine (Figure 2 and Figure 3). The fluorescent marker was also observed to be less well dispersed through the small intestine in SRB group when compared to Control mice.

The geometric center (GC) of the fluorescence was calculated (lower GC value represents slowed transit). SRB mice showed slower transit (GC Median=35.2; IQR= 31.1-42.1) compared to controls (61; 51.5-75.75) ($p<0.01$) (Figure 4). The geometric center reflecting the average midpoint position of the fluorophore was located between proximal third and mid-third of the small intestine for the SRB mice while in Controls, the fluorophore was located between the mid- third and distal third of the small intestine.

Testing is underway to understand whether the delayed intestinal transit following SRB gavage is due to the presence of H_2S generated by this bacteria as H_2S has been reported to inhibit normal peristaltic function of the small intestine in *in-vitro* experimental preparations (Gallego et al., 2008, Nagao et al., 2011 and 2012).

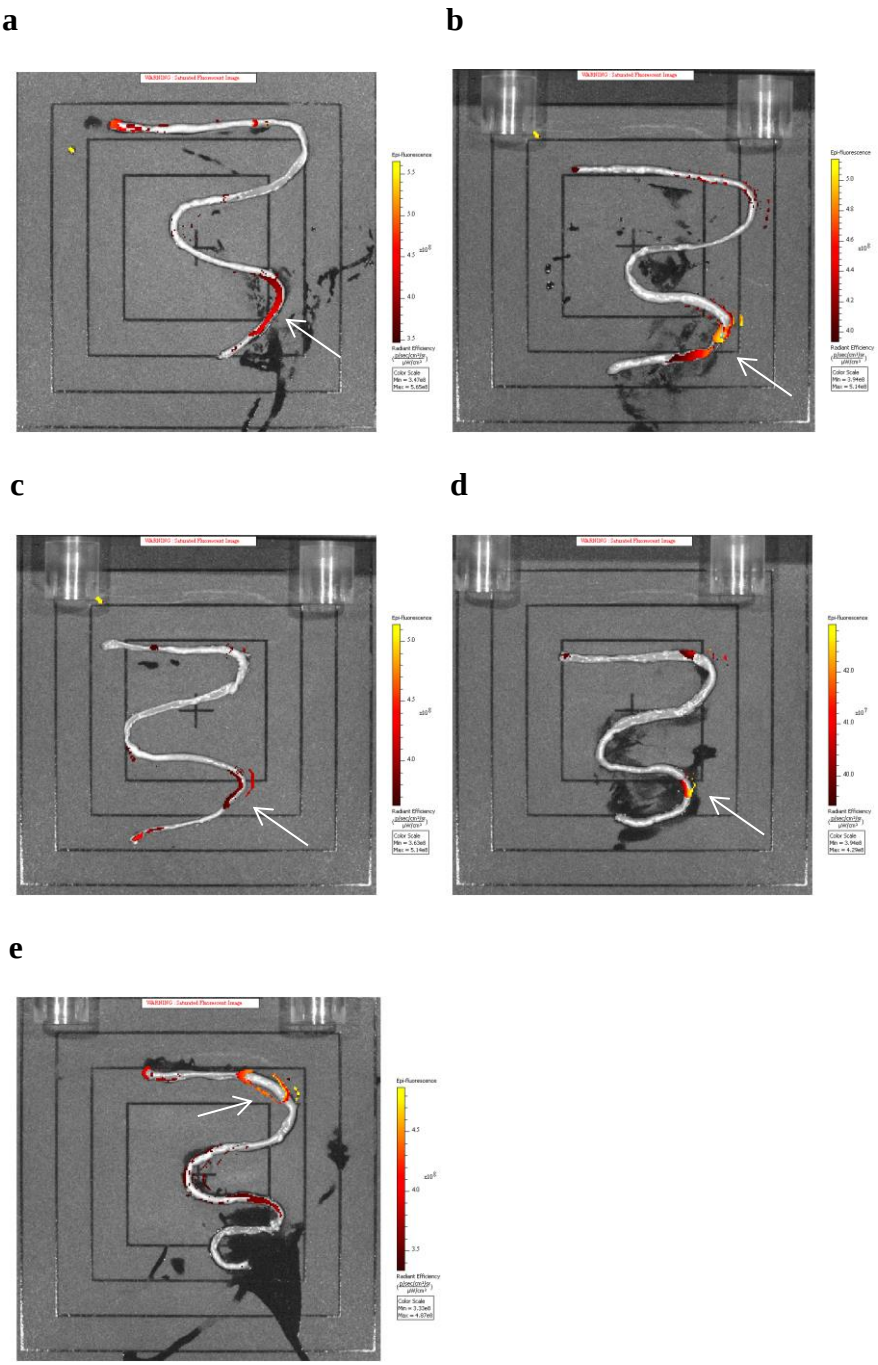

Figure 2. Images of fluoresence within *ex-vivo* small intestine in the five mice used as controls. Location of highest fluorescence concentration is marked with an arrow on each image.

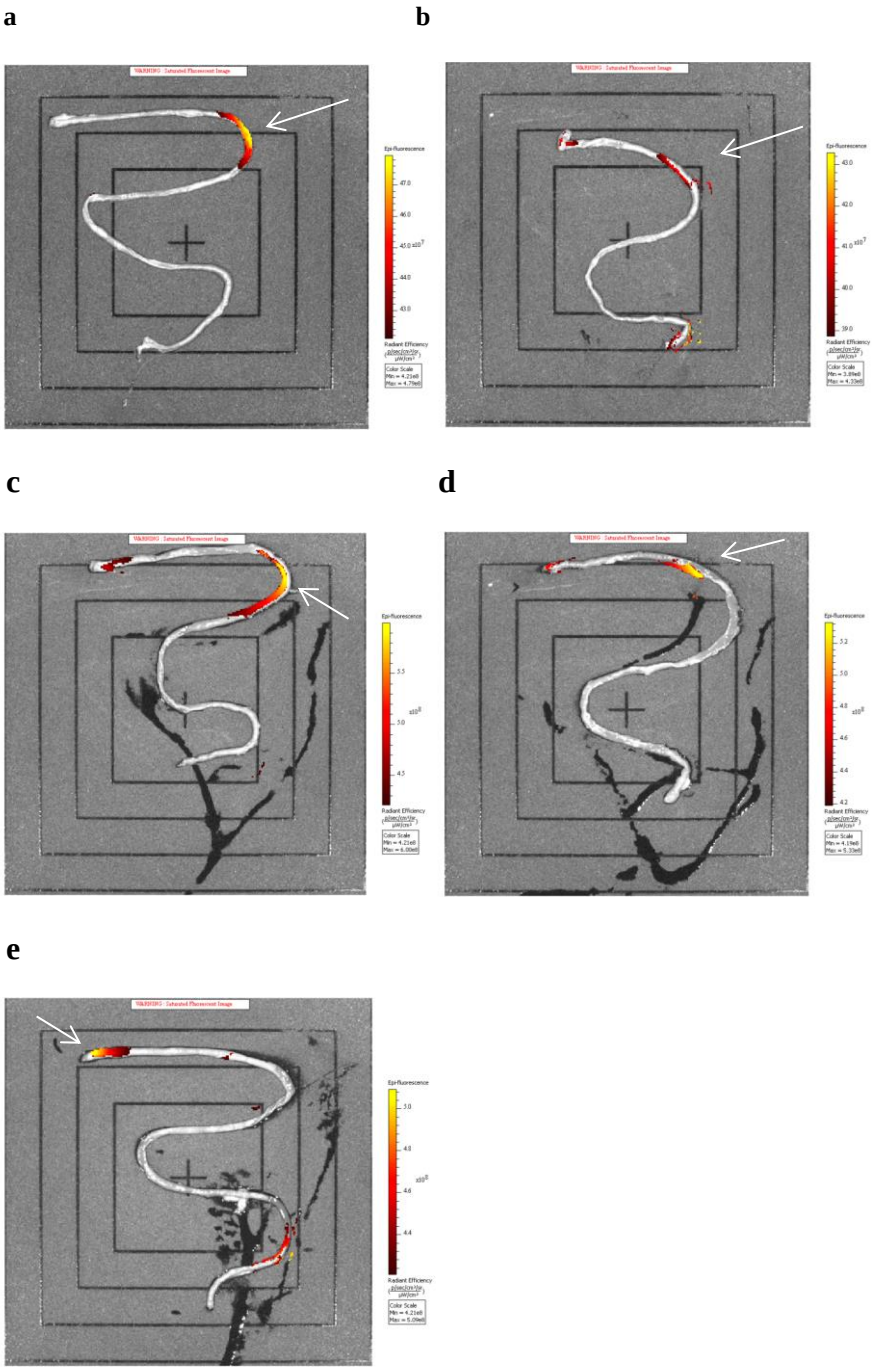

Figure 3. Images of fluoresence within *ex-vivo* small intestine in the five test mice. Location of highest fluorescence concentration is marked with an arrow on each image.

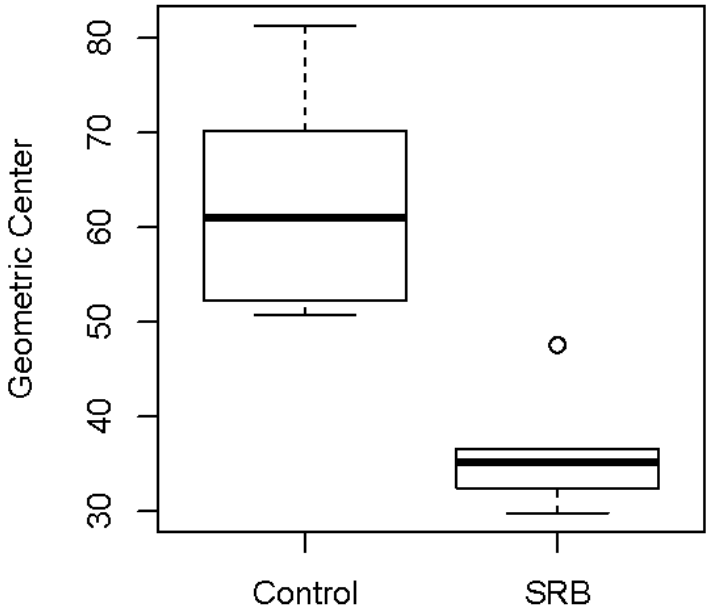

Figure 4. Geometric center of fluorophore distance traveled through small intestine.

Live *D. vulgaris* impairs learning maze performance

Mice treated with live SRB required more arm entries (Number of entries) (Median =16.5; IQR= 11.75-23.5 arm entries) to collect eight baits than controls (8; 8-12), L/M (8; 8-8), or mice treated with killed SRB (8; 8-9.5) ($P<0.05$) (Figure 5). Live SRB generated the greatest number of errors (9.5; 5.25-15.5 errors) compared to control (0; 0-4), mice treated with lactulose/mannitol (L/M) (0; 0-0.5), or killed SRB (0; 0-2.25) ($P<0.05$) (Figure 6). There was no significant difference in the total time to complete the maze across the different test groups (live SRB 145; 120.5-159 s, control 133; 113.75-141.75 s, L/M 98.5; 75.75-140 s, killed SRB 98; 85.75-112.25 s).

Since there were more errors in the live SRB group than the others but the similar total time across groups, the mice must have made more entries within the same time span indicating no reduction in physical activity. Live SRB spent more time in the center of the maze (34; 22.5-52.75 s), than control (13.5; 8.75-38.75 s), L/M (15.5; 10.25-24.75 s), and killed SRB (15; 9.75-30.5 s) ($P<0.05$) (Figure 7). Greater time in the center of the maze indicated greater time spent by the animal going through its choices for the next arm entry.

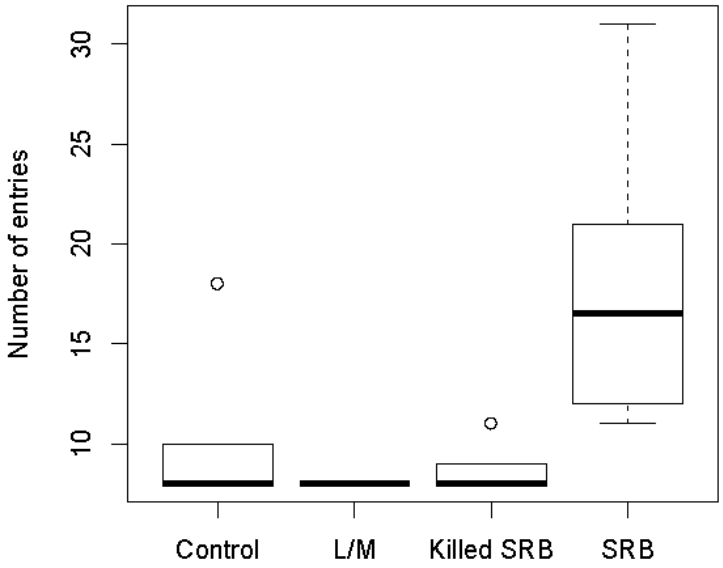

Figure 5. Number of times mice entered maze arms to retrieve bait. Entries for mice administered saline (Control), lactulose plus mannitol (L/M), killed sulfate-reducing bacteria (killed SRB) and live sulfate-reducing bacteria (SRB). Data are plotted as median with interquartile range.

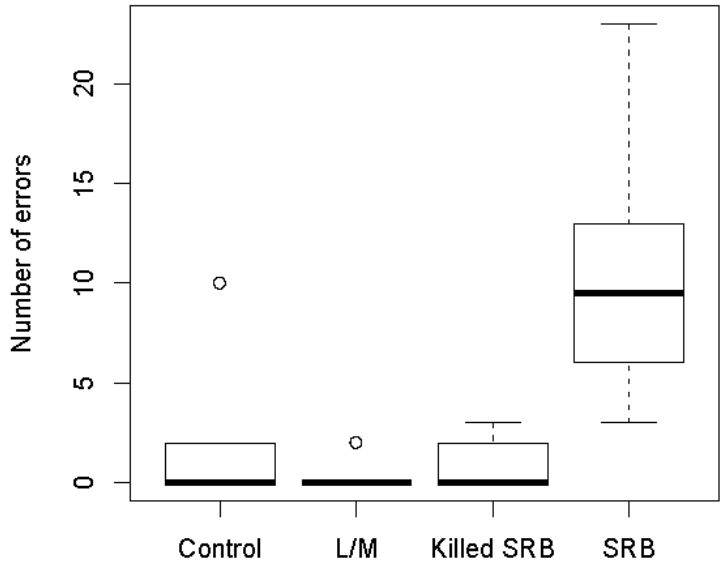

Figure 6. Number of errors mice made in the maze. Results are for mice administered saline (Control), lactulose plus mannitol (L/M), killed sulfate-reducing bacteria (killed SRB) and live sulfate-reducing bacteria (SRB). Data are plotted as median with interquartile range.

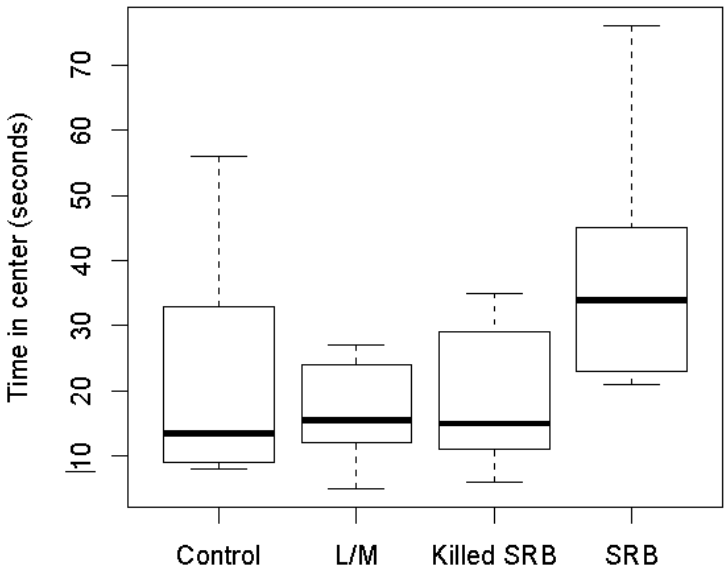

Figure 7. Time spent in the center of the eight arm radial maze. Mice were administered saline (Control), lactulose plus mannitol (L/M), killed sulfate-reducing bacteria (killed SRB) or live sulfate-reducing bacteria (SRB). Data are plotted as median with interquartile range.

Levels of hydrogen sulfide in cecum of mice

It was also observed that greater cecal concentration of H_2S was associated with worse maze performance: Concentration of hydrogen sulfide in live SRB median= 1,102; IQR=700-1457.5 ppb, control 31.5; 0-36 ppb, L/M 104.5; 71.25-119.75 ppb, and killed SRB 110; 0-223.5 ppb (Figure 8). Hydrogen sulfide concentrations in the small intestine were significantly lower in control (0; 0-17.75 ppb), than all other groups: live SRB (0; 0-97.5 ppb), L/M (72; 45-86 ppb), and killed SRB (0; 0- 90.5 ppb) ($P<0.0005$).

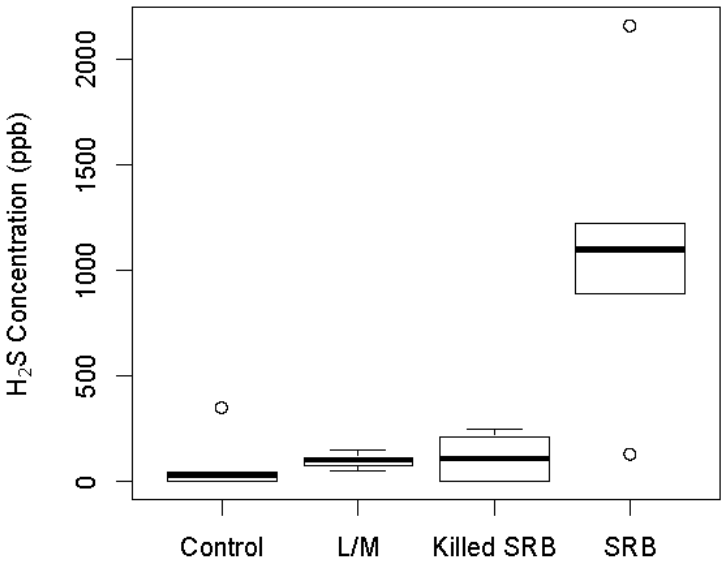

Figure 8. Concentration of hydrogen sulfide in the cecum of mice. Animal received saline (Control), lactulose plus mannitol (L/M), killed sulfate-reducing bacteria (killed SRB) or live sulfate-reducing bacteria (SRB). Data are plotted as median with interquartile range.

Detection of dsrA and dsrB genes by qPCR

Hydrogen is converted to H_2S by live SRB via the enzymatic products of two specific dissimilatory sulfite reductase genes, *dsrA* and *dsrB*. Expression levels of *dsrA* and *dsrB* were measured by qPCR. In the small intestine, colon, cecum, and liver, cDNA was synthesized from isolated mRNA. *dsrB* gene expression was higher in live SRB (CT=32) than killed SRB (CT=37) in the cecum. Number of copies of *dsrB* gene was higher for the live DSV group in the cecum (CT=32) than small intestine (CT=37). There was no difference in the number of copies of *dsrA* gene in cecum between the live (CT=32) and killed SRB (CT=32) groups.

Effect of fecal slurry on maze performance

The median time spent in the center of the learning maze (Median time in center) was significantly greater for mice gavaged with live sulfate-reducing bacteria (SRB) (18.5; IQR= 9.25-36.5 sec) than controls (15; 11-22 sec) or mice gavaged with fecal slurry (FS) (15; 10-24.5 sec) ($P<0.05$) (Figure 9). Total time was not significantly different between SRB (189.5; 125.75-266.75 sec), control (168.5; 120.25-258.5 sec), and FS (159; 121.5-244.5 sec) ($P=0.41$). Number of arm entries was not significantly different between SRB (8; IQR= 8-12 arm entries), control (8; 8-9.75), and FS (8; 8-10) ($P=0.06$); hence there was no significant difference in number of errors between SRB (1; IQR= 0-4 errors), control (0; 0-1.75), and FS (0; 0-2) ($P=0.07$).

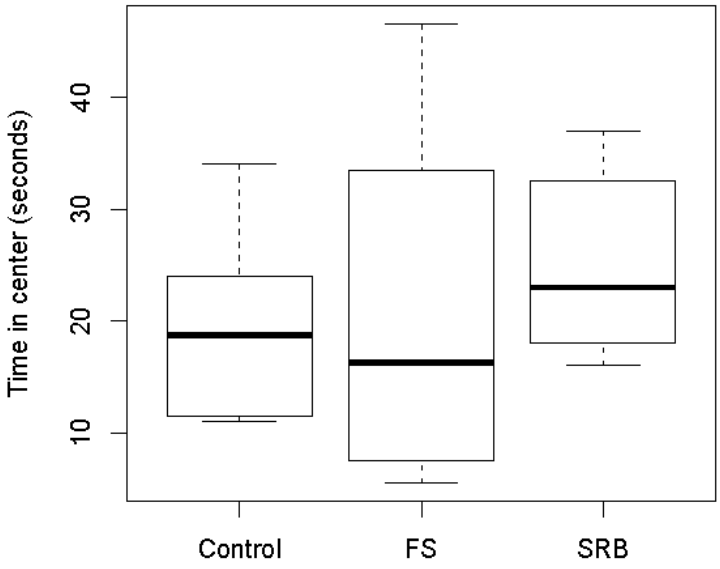

Figure 9. Time spent in the center of the maze. Mice received saline (control), live sulfate-reducing bacteria (SRB) or fecal slurry (FS) in a repeated measures experiment. Data are plotted as median with interquartile range.

Learning maze performance with raw red kidney beans

The total maze run time (Total time) was shorter for RRKB (Median= 117: IQR 102.4-153.9 sec) than Controls (270.5: 167.3-314 sec) ($p<0.05$) (Figure 10). The RRKB group had less time spent in arms (88.5: 53.5-124.5 sec) than Controls (209: 164.5-270.3 sec) ($p<0.01$). There was no significant difference in center time between RRKB (22: 6-25.5 sec) and controls (30.5: 12.25-64) ($p=0.08$). There was no significant difference in total errors between RRKB (2: 0-6.5 errors) and controls (6.5: 1-14.75) ($p=0.21$).

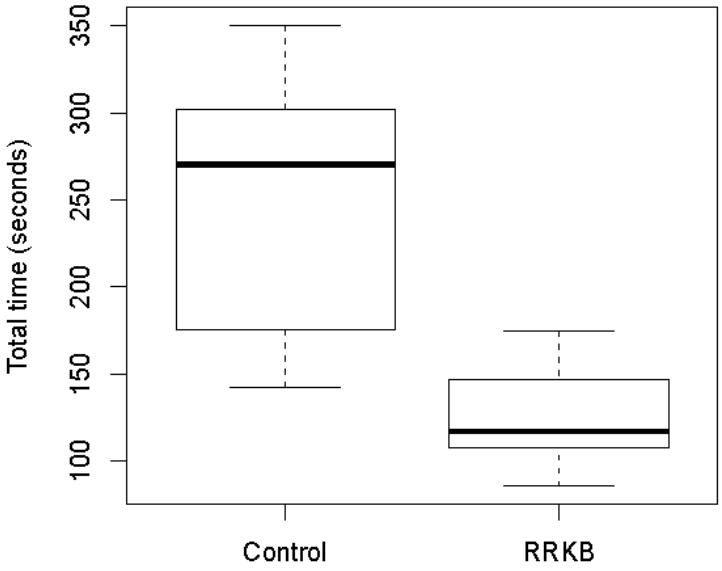

Figure 10. Total time for mice to complete maze. Mice were fed a regular diet (Control), or raw red kidney bean diet (RRKB). Data are plotted as median with interquartile range.

Detection of bacterial concentration by qPCR

The cycle threshold group average for mid-small intestine control mice was 26.981±1.46 whereas the RRKB supplemented mice were shown to have 22.688±1.45 ($P<0.01$). This is a substantial increase in bacteria in the mid third of the small intestine, confirming bacterial overgrowth of the RRKB group in this region.

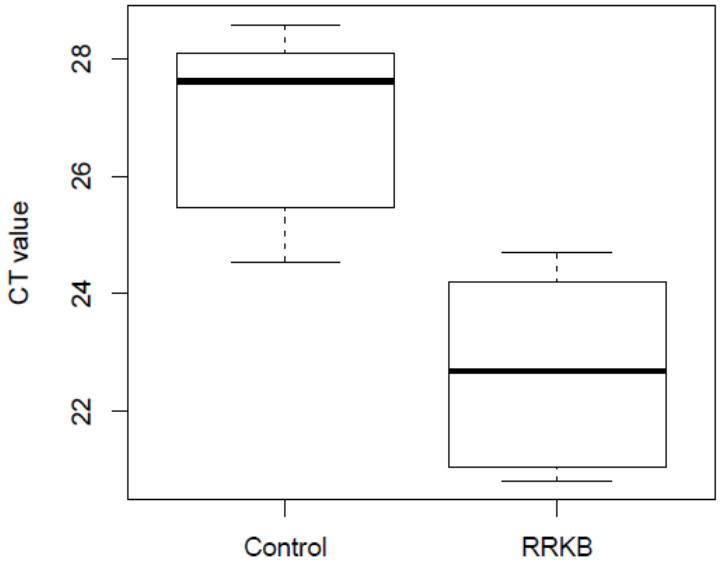

Figure 11. Cycle threshold values for 16S universal bacterial primer for mid-small intestine of control and mice receiving raw red kidney beans. Lower cycle threshold value represents higher number of gene copies. RKB refers to mice receiving the red kidney bean diet.

Discussion

In this project, the hypothesis was tested whether resident microbes could affect host physiology by investigation of the effect of administering SRB into the gut on intestinal transit and learning maze performance in mice. The following was found: 1) SRB slowed intestinal transit, 2) Live but not killed SRB impaired learning maze performance and this effect was not reproduced by fecal slurry demonstrating its species specificity, 3) Inducing expansion of the resident gut microbial community (bacterial overgrowth) using raw red kidney beans altered learning maze performance. Collectively, these findings support the hypothesis that resident gut microbes affect the physiology of mice.

Hydrogen sulfide delays intestinal transit

Previously, H_2S was reported to inhibit intestinal smooth muscle contractile activity in an *in vitro* preparation in humans, rats, and mice (Gallego et al., 2008). Both longitudinal and circular muscle contractile activity of the small intestine have been reported to be impaired by the presence of H_2S in rats (Nagao et al., 2011; 2012). Additionally, H_2S is known to modulate the activity of interstitial cells of Cajal (ICC) that generate the pacesetter activity responsible for organizing the contractions and relaxation of the intestinal wall in mice (Parajuli et al., 2010, Yoon et al., 2011). However, the effect of this gas on intestinal transit in the whole animal is not known. H_2S is a neurotransmitter typically maintained in low concentrations for the purpose of regulating bowel motility in rats (Gil et al., 2011). Stress-induced colonic hypermotility has been associated with decreased production of endogenous H_2S in rats (Liu et al., 2013). Its inhibitory role is further supported by the finding that endogenously produced

H_2S acts on presynaptic terminals of splanchnic nerves to decrease fast cholinergic synaptic input which modulates CNS control of gastrointestinal motility in mice (Sha et al., 2013). Past studies showed that endogenously produced H_2S inhibits smooth muscle contraction. The data reported here extends the published observations by showing that intestinal transit is also affected by H_2S generating SRB. The most probable mechanism for the slowing of intestinal transit would be the reported inhibitory effect of H_2S on intestinal smooth muscle contractions by inhibition of fast synaptic input. Since bacterial overgrowth is commonly found in disorders such as irritable bowel syndrome and inflammatory bowel disease where altered bowel habits are common (Pimentel et al., 2000, Weinstock et al., 2008), the slowing effect on intestinal transit by H_2S-generating SRB may provide a novel explanation for the patient's complaint and in turn, a target for developing new diagnostic and therapeutic approaches. While methane gas excreting methanogens are well connected to constipation-predominant IBS (Lin and Pimentel, 2005, Pimentel et al., 2006, de Lacy Costello et al., 2013), our finding points to the possibility that H_2S excreting SRB may also be a contributing factor in IBS. Our finding of slowed transit of the fluorescent marker after administration of SRB would suggest that any condition associated with increased abundance of SRB would slow transit leading to symptoms such as constipation. This slowed intestinal transit could be due to H_2S gas generated by SRB.

Bacteria impair cognitive performance

In the next study, it is reported that following administration of SRB into the gut of mice there is impairment of spatial memory and learning behavior as demonstrated by altered learning maze performance. Increased time spent in center of the maze indicates

that live SRB had a negative influence on spatial working memory since increasing the time needed to identify the next correct arm for bait acquisition correlates with learning and memory impairment (Brown and Cook, 1986). This evidence links for the first time resident gut bacteria with cognitive function. The role of pathogenic gut microbes on cognitive function has been reported (Lyte et al. 2006). However, the idea that a single resident microbe that is a common member of the gut microbiota of healthy individuals could also impact brain function is remarkable. Since SRB are frequent but not a universal member of an individual's colonic microbiota (Macfarlane et al., 2007), our study shows that the specific composition of the gut microbial community may determine host cognitive function. Under normal circumstances, H_2S generated by SRB may not be a threat to the host as the gut microbiota is usually compartmentalized to the large intestine where a highly efficient detoxification mechanism is available to handle this toxic gas. In contrast, in the event of bacterial overgrowth or other forms of dysbiosis where even a normal resident microbe such as SRB may proliferate and extend their colonization from the large to the small intestine where the capacity to detoxify H_2S is significantly limited, exposure to H_2S may cause greater symptoms.

Administration of live cells of *D. vulgaris* showed no reduction in the level of physical activity. In most illness, it is expected that the level of physical activity is reduced because of an elevated release of cytokines resulting from the activation of host immune response. The administration of SRB caused no decline in the physical activity of mice as demonstrated by similar total time to complete the maze. Instead, there was an increased rate of error within similar total time for maze completion suggesting a different mechanism than that of illness behavior response.

Impaired maze performance was associated with greater cecal concentration of H_2S and greater number of copies of the SRB functional gene, *dsrB*. Since SRB converts H_2 to H_2S, this accumulation of H_2S is likely the result of increased load of SRB. It was found that all test groups including L/M group had greater concentration of H_2S in the small intestine than controls but only the live SRB group had greater concentration of H_2S in the cecum. These findings suggest that in the small intestine where the removal of H_2S is less efficient, even increasing the amount of fermentable substrates could result in a detectable increase in the availability of this toxic gas. However, to show an increase in H_2S in the large intestine where the detoxification system for H_2S is highly efficient, it takes the arrival of more live SRB.

Since the physiologic concentration of H_2S in the body is low and can be generated endogenously by the host, it is plausible that a build-up of this gas caused by overgrowth of SRB could have deleterious effects on the host. Since endogenous H_2S contributes to smooth muscle function, vascular tone and long term potentiation (LTP) in the hippocampus of the brain it is possible that exposure to abnormal amounts of H_2S can disrupt smooth muscle function such as intestinal transit and learning and memory. LTP is of critical importance to cognitive function because it is the process by which memories are laid down by forming neuronal connections. Because the radial arm maze requires past learned behaviors (knowing that there is one bait at the end of each arm to acquire) and current working memory (knowing which arms have already had the bait removed), it is likely that the increased time spent in the center of the maze is a result of H_2S impairing hippocampal LTP. It would be useful in future studies to correlate the

impaired maze performance with neuronal long term potentiation measurements to test the mechanism by which H_2S-generating SRB impacts cognition.

Bacterial overgrowth improves cognitive response

In the final study, it is reported that feeding mice a diet containing raw red kidney bean (RRKB) decreased total time to complete the maze while causing small intestine bacterial overgrowth (SIBO). The increased speed that the RRKB mice ran the maze is likely a hyper-vigilance behavioral response due to the acute effect of bacterial overgrowth in the intestine. At the first sign of increased bacterial load, the body may ramp up physiological processes as a means of countering the increased probability of bacterial translocation following loss of intestinal bacterial containment and intestinal barrier function. This finding is consistent with a previous study reporting that mice fed a 50% diet of lean ground beef over three months increased microbial diversity to improve working and reference memory (Li et al., 2009). Other studies reported that humans administered lipopolysaccharide had significantly increased response to emotional visual stimuli and improved reaction time (Kullmann et al., 2013, Grigoleit et al., 2011). The influence of dietary manipulation on learning and behavior is well documented, but accumulating evidence point to the role of the gut microbial community as an outcome of a changed diet. Gut bacterial effects on host behavior may be dependent on activation of vagal pathways in the enteric nervous system as shown by the anxiolytic effects of *Bifidobacterium longum* in a chronic colitis mouse model (Bercik et al., 2011).

Conclusion

It has been found that the resident gut microbiota indeed have profound effects on host physiology. Sulfate-reducing bacteria delay intestinal transit and impair cognitive working memory of mice. Sulfate-reducing bacteria are a necessary group of bacteria in the human colon, but in cases of their overgrowth, the host's cognitive function and intestinal transit may be impaired. It has also been found that raw red kidney bean induced bacterial overgrowth improves maze run time in mice, showing that the body's immediate reaction to an increased bacterial load could be to heighten physiological functions. Since we live in a world constantly shaping our gut microbiota whether by diet, pathogens, antibiotics, probiotics, etc., it is important for people to be aware of the possible physiologic effects that different microbes and their relative abundance can have on multiple aspects of cognitive and digestive health. It is clear that when considering the overall health of an individual, the health of the individual's microbiota is a key factor that should also be taken into account.

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